Metabolic Engineering von *Methylobacterium extorquens* zur *de novo* Synthese von organischen Säuren und Isoprenoiden aus Methanol

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Inhaltsverzeichnis

Abkürzungsverzeichnis

Abb.	Abbildung
BTM	Biotrockenmasse
CBB	Calvin-Benson-Bassham Zyklus
CoA	Coenzym A
DMAPP	Dimethylallylpyrophosphat
DNA	Desoxyribonukleinsäure (-acid)
DTNB	5,5'-Dithiobis-(2-Nitrobenzoesäure), Ellmanns Reagenz
EMCP	Ethylmalonyl-CoA-Weg
FAD	Flavinadenindinukleotid
FPP	Farnesylpyrophosphat
FPPS	Farnesylpyrophosphatsynthase
GlcNAc	N-Acetylglucosamin
GGPP	Geranylgeranylpyrophosphat
GGPPS	Geranylgeranylpyrophosphatsynthase
GPP	Geranylpyrophosphat
GPPS	Geranylpyrophosphatsynthase
H_4F	Tetrahydrofolat
H_4PMT	Tetrahydromethanopterin
ICL	Isocitrat-Lyase
IDI	Isopentenylpyrophosphatisomerase
IPP	Isopentenylpyrophosphat

MEP	Methylerythritolphosphat-Weg (auch unter DXP-Weg bekannt)
MO	Mikroorganismus/-men
mRNA	Messenger Ribonukleinsäure (-acid)
snRNA	Small Nuclear Ribonukleinsäure (-acid)
tRNA	Transfer Ribonukleinsäure (-acid)
MTBE	Methyl-tert-butylether
MVA	Mevalonatweg
NYMEX	New York Mercantile Exchange
PEP	Phosphoenolpyruvat
PHA	Polyhydroxyalkanoid
PHB	Polyhydroxybutyrat
PQQ	Pyrrolochinolinchinon
RBS	ribosomale Bindestelle
RuMP	Ribulosemonophosphat-Weg
Tab.	Tabelle
TCA	Tricarbonsäure
TIR	Translationsinitiationsrate
ZSSI	α-Humulen-Synthase aus *Zingiber zerumbet* (Shampoo Ingwer)

1. Zusammenfassung

Die Substitution von klassischen, mit der Nahrungsmittelproduktion in Konkurrenz stehenden, Substraten wie Glukose durch alternative Kohlenstoffquellen in der Biotechnologie ist sowohl aus ethischer, als auch aus ökonomischer Sicht erstrebenswert. Diese Arbeit beschreibt die Synthese von Bulkchemikalien in Form zweier Dicarboxylsäuren und einer Feinchemikalie in Form eines Sesquiterpens aus dem alternativen Substrat Methanol mit Hilfe genetisch veränderter Stämme des methylotrophen α-Proteobakteriums *Methylobacterium extorquens*.

Mesacon- und (2*S*)-Methylsuccinsäure sind Dicarboxylsäurederivate der CoA-Ester Mesaconyl- und (2*S*)-Methylsuccinyl-CoA, die als Intermediate im Ethylmalonyl-CoA-Weg (EMCP) vorkommen. *M. extorquens* nutzt den EMCP für die Regeneration von Glyoxylat, das für das Wachstum auf C1-Substraten wie Methanol obligatorisch ist. In dieser Arbeit konnte erstmals Mesacon- und (2*S*)-Methylsuccinsäure *de novo* durch die Expression einer für die Vorstufen Mesaconyl- und (2*S*)-Methylsuccinyl-CoA aktiven Thioesterase produziert werden. Ein kobaltlimitiertes Wachstum von *M. extorquens* führte aufgrund mangelnder Cofaktorversorgung zweier Vitamin-B_{12}-abhäniger Mutasen im EMCP zu einer Akkumulation der beiden CoA-Ester-Vorstufen, womit eine Produktion von 0.65 g/l Mesacon- und (2*S*)-Methylsuccinsäure erreicht wurde. Weitergehende Untersuchungen belegten außerdem einen positiven Effekt eines ausgeschalteten PHB-Zyklusses auf die Produktion der beiden EMCP-Dicarboxylsäurederivate.

Diese Arbeit beinhaltet zusätzlich grundlagenwissenschaftliche Untersuchungen zur Substitution der EMCP-katalysierten Glyoxylatregeneration durch einen heterologen Glyoxylatzyklus in EMCP-negativen *M. extorquens*-Stämmen. Dabei konnte erstmals ein methanolverwertendes, methylotrophes Bakterium identifiziert werden, das einen Serin-Zyklus in Kombination mit dem Glyoxylat-Zyklus zur Kohlenstoffassimilation verwendet, ohne dabei zusätzliche Stoffwechselwege zur CO_2-Fixierung wie den EMCP, RuMP oder CBB-Zyklus zu verwenden.

Die Präsenz einer nativen C30-Carotinoidbiosynthese, ausgehend von der Vorstufe Farnesylpyrophosphat (FPP), empfiehlt *M. extorquens* als Produktionsorganismus für (Sesqui-)Terpene. In dieser Arbeit wurde mit Hilfe einer induzierbar gesteuerten Expression einer Terpensynthase in Form einer α-Humulen-Synthase, einer FPP-Synthase und eines prokaryontischen Mevalonatweges, erstmals die *de novo* Synthese eines Terpens aus Methanol am Beispiel des α-Humulens etabliert. Durch optimierte Expressionen der Terpensynthase, FPPS und einzelner MVA-Gene mit Hilfe angepasster Translationsinitiationsraten der jeweiligen ribosomalen Bindestellen und der Verwendung eines in der nativen Carotinoidbiosynthese inhibierten *M. extorquens*-Stammes wurden finale Produkttiter von bis zu 1.65 g/l α-Humulen in Fed-Batch-Fermentationen erreicht.

Diese kumulative Dissertation beinhaltet außerdem einen Reviewartikel, in dem der verwendete Mikroorganismus *M. extorquens* in mikrobiologischer, genetischer, biochemischer und auch biotechnologischer Hinsicht ausführlich beschrieben wird. Zudem gibt ein Buchkapitel eine Übersicht über die Verwendung von Methanol in der Biotechnologie.

2. Einleitung

2.1 Kohlenstoffquellen in der weißen Biotechnologie

Die weiße oder industrielle Biotechnologie wird als Bereich der Biotechnologie definiert, der die Herstellung von Chemikalien unter Einsatz von Mikroorganismen oder Enzymen als Katalysatoren in technischen Verfahren umfasst (www.biotechnologie.de). Als Energie- und Kohlenstoffquelle bzw. als Substrat können dabei unterschiedliche organische Verbindungen dienen, wobei eine vielfältige Kategorisierung, z.B. nach Ursprung, Anzahl der Kohlenstoffatome, Energiedichte usw., möglich ist. „Klassische“ Substrate der Biotechnologie sind Einfachzucker (z.B. Glukose), Proteinhydrolysate oder Fette und Öle (Fuchs 2014). Diese Kohlenstoffquellen werden von nahezu allen in der Biotechnologie verwendeten Mikroorganismen verwertet und haben eine hohe Energiedichte, was in der Regel zu schnellen Wachstums- und Produktionsraten führt. Jedoch vereint diese „klassischen“ Substrate ein Nachteil: die Herstellung basiert auf der Kultivierung von Agrarpflanzen, wodurch sie in direkter Konkurrenz zur Nahrungsmittelproduktion stehen.

Obwohl biotechnologische Produktionssysteme im Allgemeinen als ressourcensparend und umweltfreundlich gelten (Hatti-Kaul 2007; Marshall 2013; Soetaert und Vandamme 2006), so ist doch die Produktion von (Bio-)Kraftstoffen oder Chemikalien aus Nahrungsmitteln oder Ressourcen, die in direkter Konkurrenz zu deren Produktion stehen, ethisch und auch ökonomisch bedenklich (Zinoviev 2010). Durch die steigende Weltbevölkerung werden in Zukunft mehr Ackerflächen zur Nahrungsmittelversorgung benötigt werden, insbesondere dann, wenn die ressourcenintensive Fleischproduktion in den Schwellenländern sich prozentual der der Industrieländer annähert (Trostle 2010). Für die Nachhaltigkeit und gesellschaftliche Akzeptanz biotechnologischer Prozesse ist daher die Substitution „klassischer“ Substrate der ersten Generation durch Substrate der nächsten Generation oder auch alternativer Kohlenstoffquellen, wie z.B. Lignocellulose, CO_2 oder Methanol, von entscheidender Bedeutung (Tuck 2012). Alternative Kohlenstoffquelle sind dadurch definiert, dass sie selbst kein

Nahrungsmittel sind und auch nicht mit der Produktion von Nahrungsmitteln konkurrieren (Zinoviev 2010).

Alternative Kohlenstoffquellen bzw. Substrate der nächsten Generation für die Biotechnologie können in zwei Hauptkategorien eingeteilt werden: biologische Abfälle und Substratmoleküle mit nur einem Kohlenstoffatom (C1-Quellen). Bei biologischen Abfälle kann es sich um nicht weiter verwertbare Reste der Nahrungsmittelproduktion handeln, z.B. Molke als Beiprodukt der Milch- und Käseproduktion oder Chitin bzw. dessen Hauptmonomer N-Acetylglucosamin als Abfallprodukt der Shrimpsproduktion oder um allgemeine Holz- und Pflanzenreste aus Lignocellulose, die bei der Ernte von Agrarpflanzen oder der Holzindustrie anfallen. Bei der Produktion von Biodiesel entstehendes Glycerin ist ein weiteres biologisches Abfallprodukt, das als Substrat in der Biotechnologie eingesetzt werden kann (Li 2013). Die zweite Hauptkategorie der alternativen Substrate umfasst C1-Kohlenstoffquellen. Dazu zählen unter anderem Kohlenstoffdioxid (CO_2), Methan (CH_4), Methanol (CH_3OH) und auch Synthesegas, eine Mischung aus Kohlenstoffmonoxid (CO) und Wasserstoff (H_2). Diese Substrate können aus petrochemischen und biologischen Ressourcen gewonnen werden, wobei aus der jeweiligen Methodik erheblich unterschiedliche Preise und Treibhausgasemissionen resultieren. Tabelle 1 gibt einen Überblick über Kohlenstoffquellen in der Biotechnologie, deren Kosten, verwertende Mikroorganismen und deren molaren Sauerstoffbedarf für die Oxidation.

Die Auswahl des Substrates für einen biotechnologischen Prozess hängt von einer Vielzahl von Faktoren ab, darunter das zu produzierende Produkt, der verwendete Mikroorganismus (Substratspektrum, Handhabbarkeit usw.), verfahrenstechnische Aspekte (Löslichkeit, Wärmeentwicklung, Begasung, Sicherheitsaspekte usw.) und die Verfügbarkeit des Rohstoffes, die sich aus geographischen, saisonalen und nicht zuletzt auch politischen Bedingungen ergibt. So hat der Fracking-Boom in den USA zu einer hohen Verfügbarkeit und zu einem stark gesunkenem Preis von Erd- und Synthesegas und damit auch von Methan geführt (siehe Tabelle 1, Preis EU ←→ USA), was sich auch in der Förderung von Unternehmen und öffentlichen Forschungsprojekten widerspiegelt (Williams 2009). Ein weiteres Beispiel ist Rohglycerin als Abfallprodukt der Biodieselproduktion aus Substraten der ersten Generation, dessen sehr günstiger

Preis (siehe Tab. 1) maßgeblich von der Biodieselsubvention abhängt (Knothe 2005; Li 2013). Weiterhin kann auch die Patentsituation für ein Unternehmen von entscheidender Bedeutung für die Wahl eines Substrates für einen bestimmten Produktionsprozess sein.

Ein generell am besten geeignetes Substrat existiert aufgrund der Vielzahl der genannten Faktoren nicht. Eine Kohlenstoffquelle kann nur für einen bestimmten Prozess und für ein an einem bestimmten Standort hergestelltes Produkt am geeignetsten sein. „Geeignet“ kann sich dabei neben der Wirtschaftlichkeit auch auf den Faktor Nachhaltigkeit beziehen.

So ist für ein hochpreisiges Produkt die Wahl des Substrates aus wirtschaftlicher Sicht weniger bedeutend, da der Einfluss auf die Gewinnmarge geringer ausfällt. Sie kann jedoch bedeutend sein, wenn dadurch erhöhte Ausbeuten aufgrund eines anderen Stoffwechsels bzw. Mikroorganismus möglich werden oder Patente von Konkurrenten umgangen werden können. Auch aus Sicht des Marketings kann die Verwendung einer alternativen Kohlenstoffquelle vorteilhaft sein. Ganz erheblichen Einfluss auf die Wirtschaftlichkeit hat hingegen die Auswahl des Substrates bei der Produktion von Bulkchemikalien, also niedrigpreisigen Produkten wie z.B. Biokraftstoffen oder Monomeren für die Polymersynthese. Hier ist die Verwendung eines Substrates, das preiswert und in hohen Mengen verfügbar ist, sowie hohe Ausbeuten zulässt, essentiell für einen wirtschaftlichen Prozess.

Ein Schwerpunkt aktueller biotechnologischer Forschung liegt daher auf der Nutzbarmachung alternativer Kohlenstoffquellen. Die Verwendung von Holz- und Pflanzenabfällen ist aufgrund der relativen standortunabhängigen und hohen Verfügbarkeit von weltweit mehr als 2^10 t (Pagliaro 2007) attraktiv für biotechnologische Prozesse. Der Hauptbestandteil pflanzlicher Trockenmasse ist Lignocellulose, die wiederum zu 35-50 % aus Cellulose (lineare Polymerkette aus β-1,4-D-Anhydroglukose), 20-35 % aus Hemicellulose (amorphe Polysaccharide aus Xylose, Arabinose, D-Glukose, D-Mannose, D-Galaktose und Glukuronsäure) und zu 5-30 % aus Lignin (verzweigte polyaromatische Verbindung aus Paracoumaryl-, Coniferyl- und Sinapylalkoholen) besteht (Jäger und Büchs 2012). Die Herausforderung für die Nutzung von Lignocellulose als Substrat ist die komplexe Struktur der einzelnen

Komponenten. Lignin ist aufgrund der starken kovalenten Bindungen der aromatischen Alkohole und deren Toxizität als Monomere kaum als Substrat nutzbar (Ruiz-Duenas und Martinez 2009). Der Aufschluss von Cellulose und Hemicellulose erfordert aufgrund der semi-amorph-kristallinen Mikrofibrillenstruktur mehrere enzymatische und/oder chemische Schritte, um die Monomere als Substrat verfügbar zu machen. Biotechnische Produktionen aus Lignocellulose basieren daher meist auf mehrstufigen Prozessen inklusive chemo-enzymatischer Vorbehandlung und Hydrolyse, gefolgt von der eigentlichen mikrobiellen Produktion und der anschließenden Produktaufarbeitung (Jäger und Büchs 2012). Die hohen Kosten eines solchen mehrstufigen Prozesses und die nicht mögliche Verwertbarkeit von Lignin führen dazu, dass wettbewerbsfähige biotechnologische Industrieprozesse basierend auf Lignocellulose bis dato kaum etabliert sind (Hasunuma 2013; Jäger und Büchs 2012).

CO_2 ist eines der bedeutendsten natürlichen und anthropogenen Treibhausgase, wodurch die Verwendung als Kohlenstoffquelle, insbesondere wenn CO_2 aus Abgasen von z.B. Kraftwerken verwendet wird, nachhaltige Prozesse mit negativer CO_2-Bilanz ermöglichen würde (Lopez 2013). Die größten Herausforderungen stellen dabei die genetische Handhabbarkeit und die großtechnische Fermentation CO_2-fixierender MO, wie z.B. photoautotropher Mikroalgen oder Cyanobakterien dar (Cheah 2014; Varshney 2014). Auch das Einbringen eines CO_2-Fixierungsstoffwechselweges in etablierte MO wie *Escherichia coli* konnte bisher nicht erreicht werden, wodurch CO_2-basierte biotechnologische Produktionsprozesse heute noch nicht im Industriemaßstab etabliert sind.

Hinsichtlich der Flexibilität bieten Methan, Methanol und auch Ethanol den Vorteil, dass deren Produktion sowohl aus petrochemischen als auch aus nachwachsenden Rohstoffen möglich ist. Etablierte Prozesse können je nach wirtschaftlicher Verfügbarkeit ohne Mehraufwand auf das aus nachwachsenden Rohstoffen (z.B. aus Biogas) gewonnene Substrat umgestellt werden. Solche Vorgehensweisen begünstigen die Etablierung industrieller Prozesse auf Basis nachwachsender Rohstoffe (Bertau 2014; Ge 2014; Schrader 2009). Des Weiteren sind Methanol, Methan oder Ethanol keine komplexen Substrate, wodurch Vorbehandlungen zur mikrobiologischen Verwertbarkeit wie bei Lignocellulose entfallen. Außerdem können Minimalmedien mit definierten Bestandteilen verwendet werden, was im Regelfall mit reduzierten

Tabelle 1: In der Biotechnologie verwendete Kohlenstoffquellen inkl. molarer Masse und Sauerstoffbedarf ($\beta_{O2/X}$), Marktpreis (p: petrochemisch, r: aus erneuerbaren Rohstoffen; Stand 01/2015) und Mikroorganismen, die diese C-Quelle als Substrat verwenden können.

Kohlenstoffquelle	C-Atome	M [g/mol]	$\beta_{O2/X}$ [mol O_2 / mol BTM][a]	Preis [U$/kg]	Verwertende Mikroorganismen	Referenzen
Glukose/ Industriezucker aus Zuckerrohrmelasse	6	180,16	0,82	0,22 (US) 0,49 (EU)	Diverse, z.B. *Escherichia*, Hefen, *Bacilli*, *Corynebacteriaceae*	Peralta-Yahya (2012), Fuchs (2014), FNR (2014)
Maissilage	-	-	c	0,05 (US)	Diverse	Tong (2012)
Käsemolke	-	-	c	1,05 (EU)	Diverse, z.B. *Lactobacteriaceae*	Panesar und Kennedy (2012), Käse-Börse (2014)
N-Acetylglucosamin (Chitin)	8	221,21	0,82	~1 (CHN)	*Rhodotorula glutinis*, einige Archeen, rekombinante *Corynebacterium glutamicum*	Chen (2010), Sun und Liu (2006), Matano (2014), german.alibaba.com (2014)
Xylose (Hemicellulose-Hydrolysat)	5	150,13	1,86	0,07 (US)	Diverse, z.B. *Escherichia*, manche Hefen (z.B. *P. stipitis*)	Hinman (1989), Fleisch (2006)
Glycerin	3	92,09	1,02	0,31 (EU)	Diverse, z.B. *Escherichia*, *Clostridia*, *Citrobacter*	Li (2013), FNR (2014)
Ethanol	2	46,07	1,88	0,69 (US) 0,77 (EU)	Diverse, z.B. *Acetobacteriaceae*, *Saccharomycetaceae*	NYMEX (www.tradingeconomics.com/commodity/ethanol);agrarheute.com (2014); Fuchs (2014)
Methan	1	16,04	2,46	0,24 (US)[b] p: 0,41 (D) r: 0,97 (D)	Methanotrophe, z.B. *Methylococcus capsulatus*	Schrumm (2010), Hanson und Hanson (1996)
Methanol	1	32,04	1,69	p: 0,45(EU) r: 0,9 (EU)	Methylotrophe, z.B. *Methylobacterium extorquens*, *Bacillus methanolicus*	Bertau (2014), Schrader (2009), Chistoserdova (2011)
Synthesegas (CO + H_2)	1	28,01	d	N/A	Clostridien, z.B. *Clostridium ljungdahlii*	Fleisch (2006), Henstra (2007)
CO_2	1	44,01	d	N/A	Photo- und Chemoautotrophe, z.B. Chloroflexi, *Ralstonia eutropha*	Sheehan (2009), Fuchs (2014)

a: Berechnung nach Helm (2002) unter der Annahme einer allgemeinen Summenformel für mikrobielle Biomasse von $CH_{1,8}O_{0,5}N_{0,2}$ (Roels 1980)
b: Berechnet anhand des durchschnittlichen Erdgaspreises 2013 an der NYMEX; Annahmen: Erdgas besteht zu 80 % aus CH_4 und 1 m^3 Erdgas entspricht 10 kWh (Helm 2002)
c: nicht genau bestimmbar, da Komplexsubstrat mit unterschiedlicher Zusammensetzung
d: CO und CO_2 sind nur Kohlenstoff-, nicht jedoch Energie- und Kohlenstoffquellen gleichzeitig, daher ist $\beta_{O2/X}$ nur in Kombination mit der jeweiligen Energiequelle bestimmbar

Produktaufarbeitungskosten einhergeht (Schrader 2009). Etablierte biotechnologischen Produktionsorganismen wie *E. coli* oder *Saccharomyces cerevisiae* können C1-Substrate wie Methanol oder Methan natürlicherweise nicht verstoffwechseln. Für die Nutzung dieser C1-Quellen spielen daher natürliche methan- und methanolverwertende MO (siehe Tab. 1) eine entscheidende Rolle, insbesondere deren genetische und mikrobiologische Handhabbarkeit. Obwohl das verfügbare Wissen und die Anzahl der Werkzeuge nicht denen der zuckerverwertender Bakterien oder Hefen entspricht, so ist die Handhabbarkeit von Methylotrophen jedoch deutlich einfacher, als die synthesegasverwertender *Clostridien* oder autotropher MO.

2.1.1 Methanol als alternative Kohlenstoffquelle

Methanol (CH_3OH) ist ein bedeutender Rohstoff der chemischen Industrie mit einer Produktion von mehr als 50 Mio. Tonnen im Jahr 2011 (Bertau 2014). Mehr als 80 % des Methanols werden als Grundbaustein für weiteren Produktsynthesen verwendet, woran Formaldehyd (ca. 30 %) und MTBE (ca. 20 %) den größten Anteil haben (MMSA 2010). Die Verwendung von Methanol als Kraftstoff nahm von 2005 bis 2010 um mehr als 55 % zu, was vor allem an der zunehmenden Nutzung von Methanol als Benzinbeimischung in China liegt (Bertau 2014; MMSA 2010). Methanol wird heute nahezu ausschließlich im Nieder- oder Mitteldruckverfahren aus Synthesegas hergestellt, wobei Synthesegas aus petrochemischen (zumeist aus Kohle oder Erdgas) oder auch aus nachwachsenden Rohstoffen, wie z.B. Biogas, hergestellt werden kann (Olah 2005). In einer ersten Pilotanlage auf Island konnte zudem die direkte Produktion von Methanol aus CO_2-Abgasen und elektrolytisch hergestellten Wasserstoff gezeigt werden, wobei für die Wirtschaftlichkeit dieses Verfahrens die zukünftige (Kosten-)Effizienz der Wasserstoffproduktion ausschlaggebend sein wird (Bertau 2014). Ob Methanol in Zukunft vermehrt aus erneuerbaren Rohstoffen gewonnen wird, hängt maßgeblich von der Entwicklung des Öl- und Kohlepreises und den damit verbundenen Anreizen für die Industrie zur Weiterentwicklung alternativer Syntheseverfahren ab. Die verschiedenen Möglichkeiten der Methanolproduktion aus

petrochemischen und erneuerbaren/ alternativen Ressourcen sind in Abbildung 1 dargestellt.

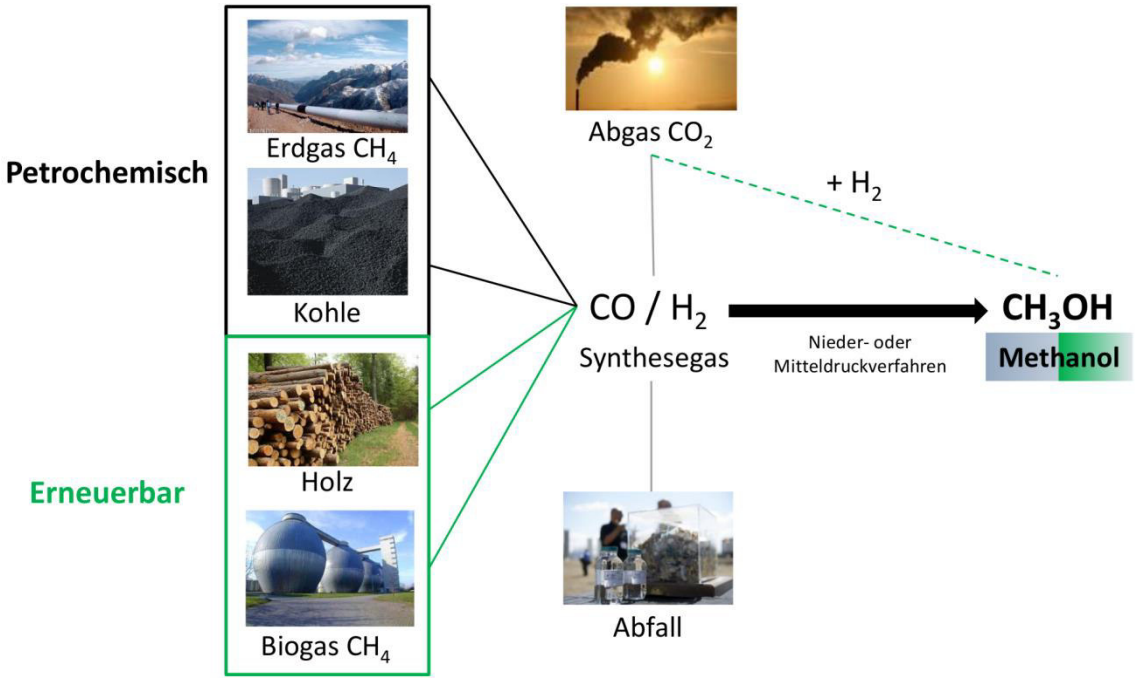


Abbildung 1: Rohstoffe für die Methanolproduktion aus Synthesegas.

Weltweit wird die Produktionskapazität für Methanol aufgrund hoher Nachfrage stark erweitert. Vor allem in China sind mehrere Großproduktionsanlagen geplant oder im Bau (Fleisch 2006). Es wird daher angenommen, dass der Marktpreis von Methanol (siehe Tab. 1) weiter sinkt, was die Attraktivität des Rohstoffes weiter erhöhen wird.

Die beschriebene flexible und quasi standortunabhängige Produktion von Methanol, die saisonal unabhängige Verfügbarkeit und der wettbewerbsfähige und vermutlich weiter sinkende Preis, machen Methanol zu einen interessanten Substrat für die Biotechnologie. Bereits in den 70er-Jahren wurden methanolverwertende MO in großtechnischen Anlagen von bis zu 1000 m^3 für die Produktion von Single Cell Protein verwendet (Faust 1977; Mac Lennan 1973; Solomons und Litchfield 1983). Neben den genannten Vorteilen ist die hohe Reinheit des verfügbaren Methanols und die mögliche Verwendung von Minimalmedien, in denen nur definierte Mengen an Salzen und Methanol enthalten sind, ein wichtiger Aspekt für die Verwendung von Methanol als Substrat. Dadurch werden Aufwand und Kosten für die Produktaufarbeitung erheblich gesenkt, da keine Komplexbestandteile aus dem Medium vom finalen Produkt abgetrennt werden müssen.

Im Vergleich mit anderen alternativen Kohlenstoffquellen wie Ethanol, Methan oder Synthesegas sind zudem folgende Vorteile zu nennen: i) Methanol ist im Gegensatz zu Methan und Synthesegas nicht gasförmig, sondern flüssig, was Transport, Lagerung, aber auch die Handhabbarkeit in Fermentationen stark vereinfacht (gute Löslichkeit, keine Gasdiffusionslimitierung); ii) Methanolverwertende MO sind im Gegensatz zu Methanverwertern (Methanotrophe) oder synthesegasverwertenden MO im Regelfall mikrobiologisch und genetisch einfacher handhabbar und haben zudem höhere Wachstumsraten (siehe auch Abschnitt 2.2); iii) der Marktpreis von Methanol liegt mit 0,45 U$/kg im Bereich anderer alternativer Substrate (siehe Tab. 1); iv) der molare Sauerstoffbedarf von 1,69 mol O_2/ mol BTM für die Oxidation von Methanol (siehe Tab. 1) ist hoch und geht mit einer hohen Wärmeentwicklung von 37 J/l in einer Fermentation einher (Snedecor und Cooney 1974), was eine hohe Kühlleistung erfordert und allgemein als zentraler Nachteil von Methanol als Fermentationssubstrat angesehen wird - allerdings ist der Sauerstoffbedarf für andere alternative C-Quellen, wie Ethanol oder Methan noch höher (siehe Tab. 1), was den Nachteil für Methanol relativiert.

Zusammengefasst ist Methanol ein attraktives Substrat für die Biotechnologie, dessen Produktion nicht in Konkurrenz zur Herstellung von Nahrungsmitteln steht, was ubiquitär verfügbar ist und im Gegensatz zu anderen möglichen alternativen C-Quellen bedeutende Vorteile bietet. Entscheidend für wirtschaftliche und nachhaltige methanolbasierte Prozesse wird die weitere Verbesserung und Kostenoptimierung der Methanolsynthese aus nachwachsenden Rohstoffen sein, sowie Prozesse mit hohen Ausbeuten durch maximal effiziente mikrobiologische Produktionsorganismen.

2.2 Methylobacterium extorquens

Methylobacterium ist eine weit verbreitete Bakteriengattung, die aus unterschiedlichen Habitaten wie Abwasser, Boden oder der Phyllosphäre isoliert werden konnte (Doronina 1996; Kohler-staub 1986; Vorholt 2012). Die am meisten untersuchte Art dieser Gattung ist *Methylobacterium extorquens*, wobei der Stamm

AM1 seit mehr als 50 Jahren als Modellorganismus für die Erforschung der Methylotrophie, also der Verwertung von Kohlenstoffquellen ohne C-C Bindung als einzige Kohlenstoff- und Energiequelle, fungiert (Anthony 1982; Anthony 2011).

M. extorquens ist ein pink-pigmentierter, fakultativ methylotropher (PPFM, siehe Abb. 2 und 3), obligat aerober Mikroorganismus, d.h. in der Lage, neben den C1-Quellen Methanol und Methylamin, auch nicht-methylotrophe Substrate mit C-C Bindungen aus zwei, drei oder vier Kohlenstoffatomen wie Acetat, Ethanol, Pyruvat oder Succinat unter Anwesenheit von Sauerstoff zu verwenden (Ochsner 2014). Isoliert wurde der durch Carotinoide pink gefärbte Stamm AM1 als Kontaminant in Methylaminmedium (Peel und Quayle 1961). Bis heute sind insgesamt 5 verschiedene *M. extorquens*-Stämme isoliert worden.

Durch die Rolle als Modellorganismus sind umfangreiche Kenntnisse zum Stoffwechsel (inkl. stöchiometrischem metabolischen Modell und Flussanalysen), dem Proteom und Transkriptom, sowie ein vollständig sequenziertes und annotiertes Genom von *M. extorquens* vorhanden. Die dazukommende Verfügbarkeit einer Vielzahl genetischer Werkzeuge zur Gendeletion, (induzierbaren) Expression, genomischen Integration usw., machen *M. extorquens* zu einem attraktiven Produktionsorganismus zur Verwertung der alternativen C-Quelle Methanol. Weitere umfangreiche Informationen zu *M. extorquens* und seiner Rolle in der Biotechnologie sind in dem Review „*Methylobacterium extorquens*: methylotrophy and biotechnological applications“ (siehe 4.3) zusammengefasst.

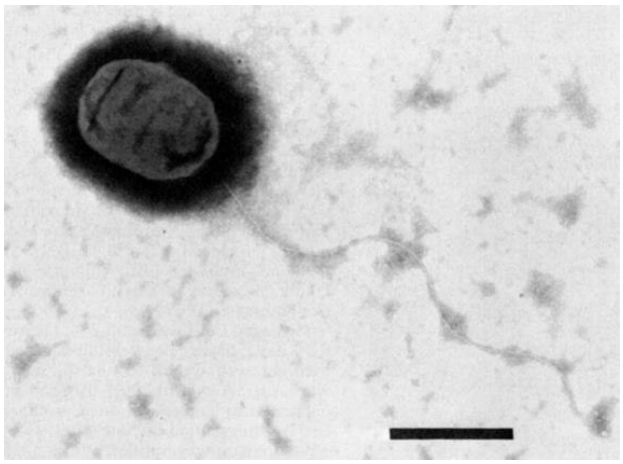


Abbildung 2: Elektronenmikroskopische Aufnahme von *M. extorquens*. Balken = 1 µM (Urakami und Komagata 1984)

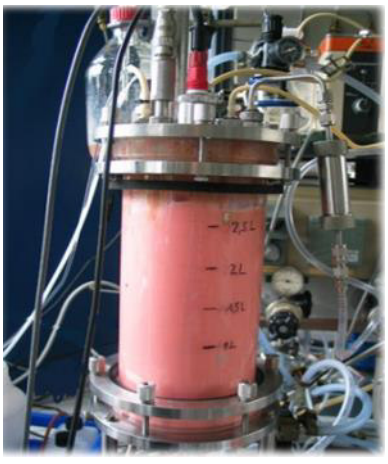


Abbildung 3: *M. extorquens* AM1 in einem 2,5 l-Bioreaktor bei hoher Zelldichte

2.2.1 Methylotrophie in *M. extorquens*

Die Nutzung von Molekülen ohne C-C Bindung als einzige Kohlenstoff- und Energiequelle erfordert einen speziellen Stoffwechsel, der prinzipiell modular aufgebaut ist, d.h. auf verschiedenen Stoffwechselwegen basiert, die in unterschiedlichen Kombinationen vorkommen können. Vereinfachend kann die Methylotrophie in drei Hauptteile gegliedert werden: i) die Oxidation (evtl. inkl. Demethylierung und/oder Dehalogenierung) eines methylierten Substrates zu Formaldehyd oder einem Methyl- bzw. Methylenradikal (meist assoziert mit H_4F); ii) die Oxidation von Formaldehyd oder Methyl-/ Methylen-H_4F zu CO_2 und iii) die Assimilation eines Kohlenstoffatoms von Formaldehyd mit dem RuMP oder CBB-Zyklus oder von Methylen-H_4F und CO_2 über den Serin-Zyklus (Chistoserdova 2011).

M. extorquens AM1 oxidiert Methanol mit Hilfe einer PQQ-abhängigen Methanol-Dehydrogenase mit hetero-tetramerischen $\alpha_2\beta_2$-Struktur (codiert von *mxaFI*) zu Formaldehyd (Anthony 1986). Alternativ kann Methylamin von einer Methylamin-Dehydrogenase zu Formaldehyd oxidiert werden (Chistoserdov 1994). Formaldehyd wird über einen H_4PMT-abhängigen Stoffwechselweg weiter zu Formiat und durch mehrere Formiat-Oxidasen abschließend zu CO_2 oxidiert (Chistoserdova 2007; Chistoserdova 1998). Schließlich wird ein Kohlenstoffatom von Methylen-H_4F über den Serin-Zyklus assimiliert, wobei das Methylen-H_4F durch die spontane Kondensation von H_4F und Formiat oder durch deren Ligation, unter ATP-Verbrauch, mittels einer Formiat-Tetrahydrofolat-Ligase (Ftl) gebildet werden kann (Marx 2003). Die Entnahme von Metaboliten, z.B. PEP, aus dem Serin-Zyklus macht eine Regeneration von Glyoxylat notwendig, um den Stoffwechselweg am Laufen zu halten. *M. extorquens* verwendet dazu den Ethylmalonyl-CoA Weg (Erb 2007; Peyraud 2009), der im folgenden Kapitel 2.2.2 genauer beschrieben wird. Der Primärstoffwechsel von *M. extorquens* ist in Abbildung 4 dargestellt. Detailliertere Informationen zum gesamten Stoffwechsel befinden sich in Publikation 4.3 (Ochnser 2014).

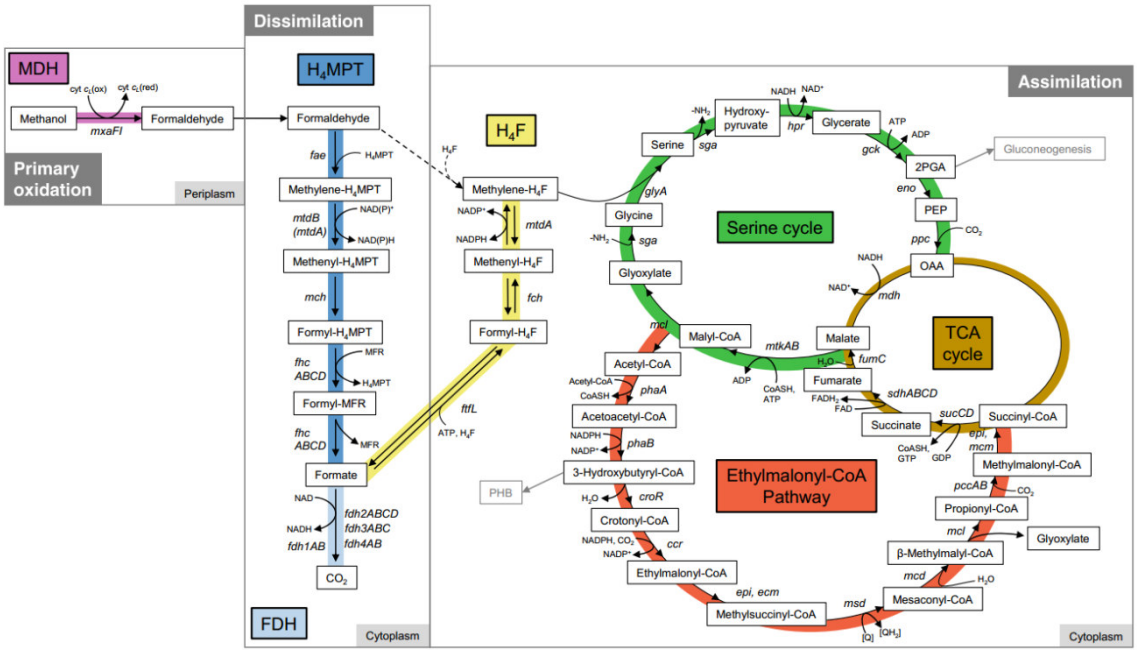


Abbildung 4: Der Primärstoffwechsel von *Methylobacterium extorquens* AM1. Nach Ochsner (2014)

2.2.2 Der Ethylmalonyl-CoA Weg (EMCP)

Wie in 2.2.1 beschrieben, wird in methylotrophen Organismen, die den Serin-Zyklus zur Kohlenstoffassimilation verwenden, ein Stoffwechselweg zur Regeneration eines Glyoxylatmoleküls nach drei Durchläufen des Serin-Zyklus und der Entnahme eines dessen Metaboliten benötigt. Das kann entweder durch den klassischen Glyoxylat-Zyklus mit Hilfe einer Isocitrat-Lyase (ICL) katalysierten Spaltung von Isocitrat zu Succinat und Glyoxylat erfolgen oder in ICL-negativen Organismen durch den EMCP (Anthony 1982; Erb 2007). Der EMCP umfasst 12 Reaktionen und 11 Intermediate in Form von CoA-Estern (siehe Abb. 5), wobei netto 2 NADPH, 1 FAD und 2 CO_2 als Co-Substrate/-Faktoren benötigt werden. Wie in Abbildung 4 dargestellt, überlappt der EMCP mit dem Serin-, dem TCA- und dem PHB-Zyklus. Spezifische Intermediate des EMCP sind das namensgebende Ethylmalonyl-CoA, das als *R*- und *S*-Enantiomer vorkommt und das (2*S*)-Methylsuccinyl-CoA. Ein weiteres Merkmal ist die Präsenz von zwei Carboxylasen (Crotonyl-CoA- und Propionyl-CoA-Carboxylase) und zwei Cobalamin-abhängigen Mutasen (Ethyl- und Methylmalonyl-CoA-Mutase). Eine detaillierte Übersicht des EMCP inklusive Cofaktoren und den Strukturformeln aller Intermediate gibt Abbildung 5.

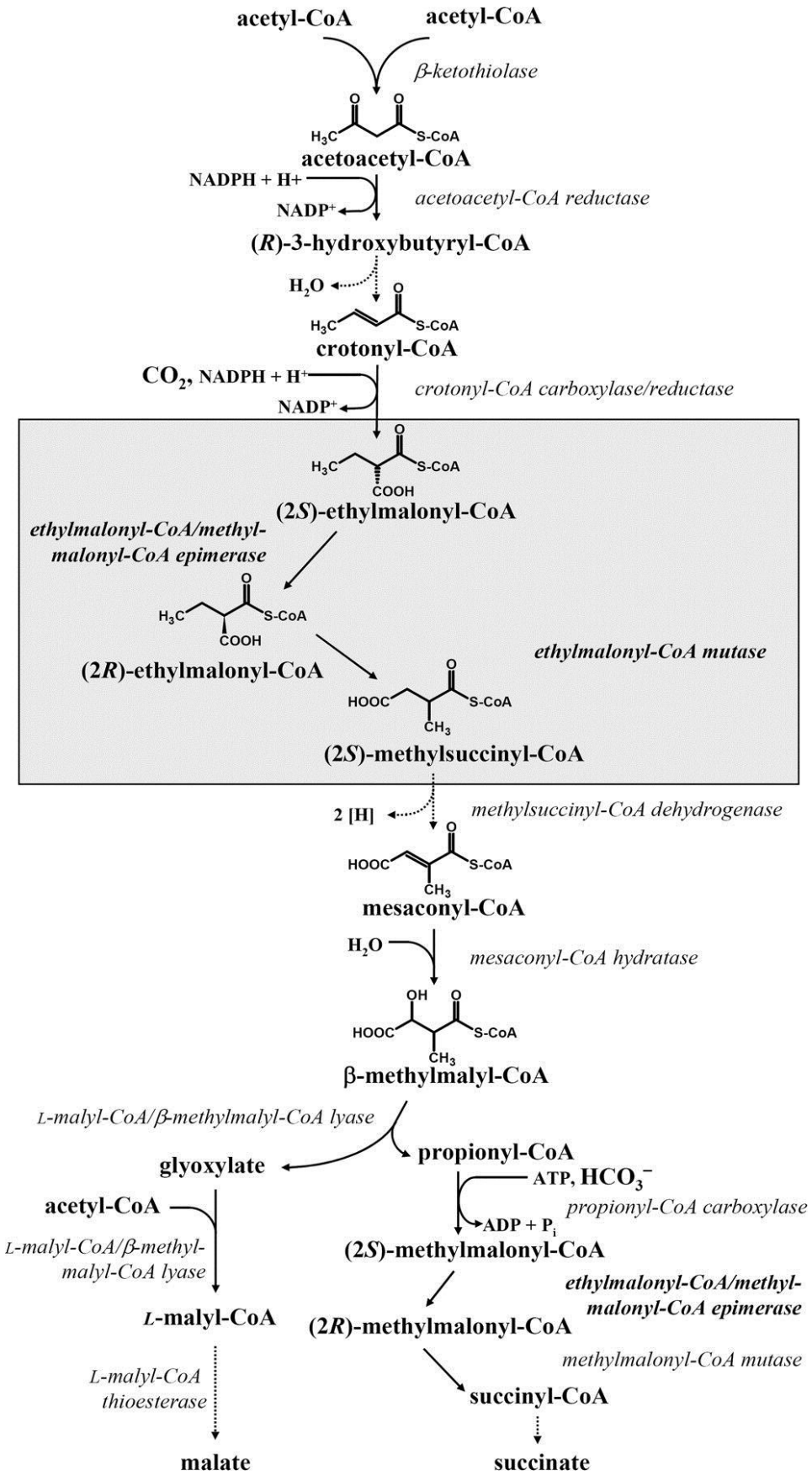

acetyl-CoA
acetyl-CoA
β-ketothiolase
acetoacetyl-CoA
NADPH + H+
NADP+
acetoacetyl-CoA reductase
(R)-3-hydroxybutyryl-CoA
H2O
crotonyl-CoA
CO2, NADPH + H+
NADP+
crotonyl-CoA carboxylase/reductase
(2S)-ethylmalonyl-CoA
ethylmalonyl-CoA/methyl-malonyl-CoA epimerase
(2R)-ethylmalonyl-CoA
ethylmalonyl-CoA mutase
(2S)-methylsuccinyl-CoA
2 [H]
methylsuccinyl-CoA dehydrogenase
mesaconyl-CoA
H2O
mesaconyl-CoA hydratase
β-methylmalyl-CoA
L-malyl-CoA/β-methylmalyl-CoA lyase
glyoxylate
propionyl-CoA
ATP, HCO3−
ADP + Pi
propionyl-CoA carboxylase
acetyl-CoA
L-malyl-CoA/β-methyl-malyl-CoA lyase
(2S)-methylmalonyl-CoA
ethylmalonyl-CoA/methyl-malonyl-CoA epimerase
L-malyl-CoA
(2R)-methylmalonyl-CoA
methylmalonyl-CoA mutase
L-malyl-CoA thioesterase
succinyl-CoA
malate
succinate

Abbildung 5: Der Ethylmalonyl-CoA-Weg (EMCP) inklusive beteiligter Enzyme und Cofaktoren. Die Reaktionen der Ethymalonyl-CoA-Epimerase und –Mutase sind grau hinterlegt. Nach Erb (2008)

2.2.3 *M. extorquens* als biotechnologischer Produktionsorganismus

Das tiefgehende mikrobiologische, metabolische und genetische Verständnis von *M. extorquens* macht ihn als Produktionsorganismus für die nachhaltige Synthese unterschiedlicher chemischer Substanzen aus der Kohlenstoffquelle Methanol interessant. Wie bereits in 2.1 und 2.2 diskutiert, empfiehlt sich die Nutzung des günstigen und im großen Maßstab verfügbaren Methanols vor allem für die Produktion von Bulkchemikalien.

M. extorquens synthetisiert natürlicherweise Poly-3-hydroxybutyrat (PHB) als Speicherstoff und wichtigen Abfluss für Redoxäquivalente (Föllner 1997). Die Synthese wird von der Polyhydroxyalkanoat-Synthase PhaC, ausgehend von (3*R*)-Hydroxybutyryl-CoA, katalysiert (Korotkova und Lidstrom 2001). PHAs haben vielfältige Anwendungen als Verpackungs-, Textil-, Medizin- oder Haushaltsmaterialien (Keshavarz und Roy 2010). Bereits 1986 wurde durch Suzuki und Kollegen in einer stickstofflimitierten Fermentation mit *M. extorquens* ein bemerkenswerter Produkttiter von 149 g/l PHB bei einer Ausbeute von 0.2 g/g Methanol erreicht. Bis heute wurde die Produktion einer Vielzahl weiterer PHAs mit *M. extorquens* etabliert, worunter auch PHB-co- und -ter-Polymere mit gegenüber reinem PHB stark verbesserten Eigenschaften sind (Ochsner 2014).

Die ebenso als Bulkchemikalie geltende Aminosäure Serin mit einer Jahresproduktion von mehr als 3 Millionen Tonnen (Sanchez und Demain 2008) wird heute im industriellen Maßstab aus Methanol und Glycin mit Methylobakterien produziert (Hagishita 1996; Ikeda 2003). Dabei wird die Reaktion der Serin-hydroxymethyltransferase des Serin-Zyklus ausgenutzt (siehe Abb. 4).

Der spezielle Stoffwechsel von *M. extorquens*, insbesondere der EMCP (siehe Abb. 5), bietet darüber hinaus Vorstufen für die Synthese weiterer interessanter Produkte. Die Dicarboxylsäurederivate der EMCP-CoA-Ester sind interessante Synthons und Monomere für die Produktion neuartiger Polymere (Alber 2011). Die Enantiomere des Ethylmalonyl-CoAs und (2*S*)-Methylsuccinyl-CoA kommen zudem exklusiv im EMCP vor. Weiterhin könnten die CoA-Ester des EMCP auch als Bausteine für eine mögliche

Polyketidsynthese dienen. Bemerkenswert ist zudem die Präsenz von Acetoacetyl-CoA im EMCP, wodurch ein Intermediat des Mevalonat-Weges (Isoprenoidsynthese) im Primärstoffwechsel vom MEP nutzenden *M. extorquens* vorhanden ist (siehe 2.5). Für potenzielle Isoprenoidsynthesen ist das ein bedeutender Unterschied und Vorteil zu den klassischen biotechnologischen Produktionsorganismen *E. coli* und *S. cerevisiae*, bei denen Acetoacetyl-CoA nur im Sekundärstoffwechsel vorkommt und damit ein niedriger metabolischer Fluss zu diesem Intermediat existiert.

Zusammenfassend ist *M. extorquens* ein bereits etablierter biotechnologischer Produktionsorganismus. Er bietet aufgrund seines Metabolismus, des heute existierenden Wissens und verfügbarer genetischer Werkzeuge ein erhebliches Potenzial für die Verbesserung bereits etablierter Synthesen und die Produktion neuartiger Chemikalien, darunter Dicarboxylsäuren, Isoprenoide und Polyketide. Bereits etablierte biotechnologische Produktionen und potenzielle Synthesen mit *M. extorquens* werden in Publikation 4.3 ausführlich beschrieben. Einen generellen Überblickt über den Einsatz von Methanol in der Biotechnologie bietet zudem das Buchkapitel „Methanol in Biotechnology“ (siehe 4.4).

2.3 „Metabolic Engineering“ als Werkzeug der Biotechnologie

„Metabolic Engineering“ beschreibt die direkte Modifikation von Stoffwechselwegen. Dabei können einzelne biochemische Reaktionen oder auch komplette native und nicht-native Stoffwechselwege verändert, hinzugefügt oder entfernt werden (Stephanopoulos 1999; Stephanopoulos 2012). Entscheidend für die Etablierung des „Metabolic Engineerings“ vor etwa 20 Jahren war die Entwicklung gentechnischer Werkzeuge zur gezielten (Über-)Expression, Deletion und Modifikation bestimmter Gene und anderer DNA-Bereiche, um Stoffwechselwege gezielt verändern zu können (Woolston 2013). Diese gentechnischen Tools haben sich bis heute stark verbessert und vervielfacht, inklusive regulatorischer RNAs, justierbar induzierbare Promotoren und *in silico* designbare ribosomale Bindestellen (Borodina und Nielsen 2014; Chen und Nielsen 2013). Hinzu kommen die Möglichkeiten des direkten Designs von

Proteinen und Enzymen zur Veränderung derer Reaktionsprodukte (Fisher 2014; Marcheschi 2013). Weiterhin sind metabolische Flussanalysen zur Messung der Reaktionsabläufe ganzer Stoffwechselwege oder einzelner Reaktionen *in vivo* (Toya und Shimizu 2013) möglich und computergestützte Simulation von metabolischen Netzwerken, mit der die Wirkung der genannten Tools schon im Vorfeld designt und abgeschätzt werden kann (Copeland 2012; Moura 2013).

Beim Metabolic Engineering als Werkzeug für die Etablierung mikrobieller Synthesen chemischer Substanzen kommt der Bereitstellung von Enzymen in hohen bzw. ausbalancierten Mengen eine große Bedeutung zu. Ein hoher und konstanter Stofffluss durch einen Stoffwechselweg zum Zielprodukt ist nur bei hohen und ausbalancierten Enzymaktivitäten möglich. Deren spezifische Aktivität variiert, kann jedoch im Regelfall mit der Proteinquantität korreliert werden, so dass hohe Produktmengen meist auch hohe Proteinmengen der jeweiligen Stoffwechselenzyme erfordern. Regulationen auf Proteinebene und die Stabilität der Enzyme spielen ebenfalls eine wichtige Rolle für einen hohen Stofffluss.

Hohe Proteinmengen erfordern eine effiziente Transkription und Translation des entsprechenden Gens bzw. der jeweiligen mRNA. Die Transkription lässt sich u.a. durch die Stärke des Promotors, der Anzahl der Kopien des Gens, der Anzahl der Kopien des Plasmids (bei episomaler Expression) und snRNAs beeinflussen (Blazeck und Alper 2013; Kang 2014; Stephanopoulos 2012; Wang 2012). Einfluss auf die Translation hat z.B. die Art des Startcodons (ATG, GTG, CTG, TTG), die RBS (in Prokaryonten die Shine-Dalgarno-Sequenz), die Codonnutzung und etwaige, zusätzlich benötigte Proteine zur korrekten Faltung wie Chaperone oder zusätzliche Translationsfaktoren (Pirie 2013; Waegeman und Soetaert 2011). Auf alle der genannten Faktoren lässt sich mit modernen gentechnischen Tools und der heutigen Möglichkeit der kostengünstigen DNA-Synthese Einfluss nehmen. Zu beachten ist jedoch, dass die Mehrzahl der Tools nach wie vor auf die klassischen biotechnologischen Hosts beschränkt ist. Entscheidend ist auch ein umfangreiches biochemisches Wissen zu den einzelnen Enzymen und Regulationen, um überhaupt die notwendigen Modifikationen durchführen zu können. Manche Tools wie die Optimierung der Codonnutzung sind zudem nicht immer sinnvoll bzw. erfolgreich: so zeigten *M. extorquens*-Stämme mit codonoptimierten Proteinen einen bis zu 90 %-igen Fitnessverlust gegenüber Stämme

mit Proteinen ohne Codonoptimierung, vermutlich aufgrund mangelnder Verfügbarkeit der entsprechend beladenen tRNAs (Agashe 2013).

Mit Hilfe der genannten Technologien konnte durch das Metabolic Engineering die mikrobielle Synthese diverser chemischer Produkte, wie organischer Säuren (Chen und Nielsen 2013; Cheng 2013; Liu und Jarboe 2012), Aminosäuren (Becker und Wittmann 2012), Kraftstoffe und Basischemikalien (Cho 2014), sowie Isoprenoiden (Immethun 2013; Tippmann 2013; Vickers 2014) in hohen Titern und Ausbeuten erreicht werden. Dabei ist das Metabolic Engineering nicht mehr nur auf die biotechnologischen Standardorganismen wie *E. coli* (Chen 2013), *S. cerevisiae* (Borodina und Nielsen 2014; Krivoruchko und Nielsen 2014), *C. glutamicum* (Woo und Park 2014) oder Lactobacteriaceaen (Gaspar 2013) beschränkt, sondern ist dank neuer gentechnischer Werkzeuge und Methoden auch in Hyphenpilzen (Caspeta und Nielsen 2013), Pflanzen (Araguez und Valpuesta 2013; Staniek 2013) oder Zellkulturen (Datta 2013) möglich.

Trotz dieser rasanten Entwicklung ist die Bedeutung des Metabolic Engineerings bzw. die von gentechnisch veränderten MO in der industriellen Biotechnologie und der gesamten chemischen Industrie ausbaufähig. Das lässt sich gut am Beispiel organischer Säuren zeigen. So werden nach heutigem Stand der Technik lediglich drei von 17 industriell relevanten Dicarboxylsäuren fermentativ, 14 jedoch nach wie vor durch chemische Synthese hergestellt (Alonso 2014). Von den drei mikrobiologisch produzierten Säuren Glukonsäure, Itaconsäure und Succinat (Bernsteinsäure), wird lediglich Succinat industriell auf Basis rekombinanter Stämme produziert, z.B. von der Firma DSM mit Hilfe eines gentechnisch modifizierten *S. cerevisiae*-Stammes (DSM 2010). Die Produktion von Glukonsäure und Itaconsäure hingegen beruht auf der Fermentation der *Aspergillus*-Wildtypstämme *A. niger* und *A. terreus* (Alonso 2014). Für Bulkchemikalien wie Dicarboxylsäuren sind letztlich die Produkt- und Raum-Zeit-Ausbeuten, die Reinheit bzw. Aufarbeitung und die insgesamt daraus resultierenden Kosten, die entscheidenden Größen für die Wirtschaftlichkeit eines Prozesses. Trotz der bedeutenden Fortschritte des Metabolic Engineerings sind die genannten Parameter für viele Produkte nach wie vor nicht kompetitiv zu der entsprechenden chemischen Synthese. Auch die Stabilität genetisch veränderter MO ist oft unzureichend für einen langfristigen Prozess. Nur im Falle einer höheren Gewichtung der Nachhaltigkeit und der Akzeptanz höherer Preise, könnte die biotechnologische

Produktion hier kurz- bis mittelfristig industriell relevant werden. Das würde jedoch nur gelten, wenn die biotechnologischen Synthesen auf alternativen Kohlenstoffquellen wie z.B. Methanol (aus erneuerbaren Rohstoffen) basieren (siehe 2.1 und 2.1.1).

Bei anderen Substanzklassen, wie z.B. den Isoprenoiden, ist eine biotechnologische Produktion ohne Metabolic Engineering nicht in sinnvollen Ausbeuten und Produktkonzentrationen möglich. Aufgrund der komplexen chemischen Strukturen der Isoprenoide und deren geringen Konzentrationen in den jeweiligen natürlichen pflanzlichen Produzenten ist die biotechnologische Produktion vieler Isoprenoide mit Hilfe genetisch veränderter MO effektiver und nachhaltiger als die oftmals aufwendige und umweltschädliche chemische Synthese oder Extraktion (Immethun 2013). Der Einsatz mancher Isoprenoide, darunter auch Carotinoide, als Nahrungsmittelzusatz führt hier zu einem anderen Problem des Metabolic Engineerings und dem damit verbundenem Einsatz gentechnisch veränderter MO: Die Akzeptanz der Gentechnik ist in der Gesellschaft, vor allem in Europa, gering, insbesondere wenn es um die Verwendung in Lebensmitteln geht. Auch das Aufkommen neuartiger Begriffe wie „Synthetische Biologie“ als Erweiterung oder Neudefinition des Metabolic Engineerings werden zur allgemeinen Akzeptanz dieser Technologie kaum positiv beitragen, weshalb sie kritisch diskutiert werden (Stephanopoulos 2012).

Zusammengefasst ist das Metabolic Engineering ein bedeutendes Werkzeug und Forschungsfeld der industriellen Biotechnologie, ohne das die Synthese vieler Produkte in wettbewerbsfähigen Ausbeuten nicht möglich ist oder sein wird. Es bedarf jedoch weiterer Verbesserungen der Titer, Ausbeuten und genetischen Stabilität, um gegenüber chemischen Synthesen wettbewerbsfähiger zu werden.

2.4 Mikrobielle Synthese von Dicarboxylsäuren – Stand der Technik

Dicarboxylsäuren sind bedeutende Plattformchemikalien für die Synthese von Polyamiden, Diolen und Polyestern (siehe Abb. 6), deren jährliches Marktvolumen einem Wert von ca. 15 Milliarden U$ entspricht (Jang 2012; Lee 2011). Zusätzlich

werden Dicarboxylsäuren als Nahrungsmittelzusätze und Chelatoren eingesetzt (Lee 2002; Werpy und Petersen 2004). Mehrere Dicarboxylsäuren befinden sich in der Liste der „Top value added chemicals from Biomass“ des US-Ministeriums für Energie, bei denen aus Gründen der Nachhaltigkeit und Wirtschaftlichkeit eine biotechnologische Produktion aus alternativen Kohlenstoffquellen als besonders lohnenswert angesehen wird (Werpy und Petersen 2004).

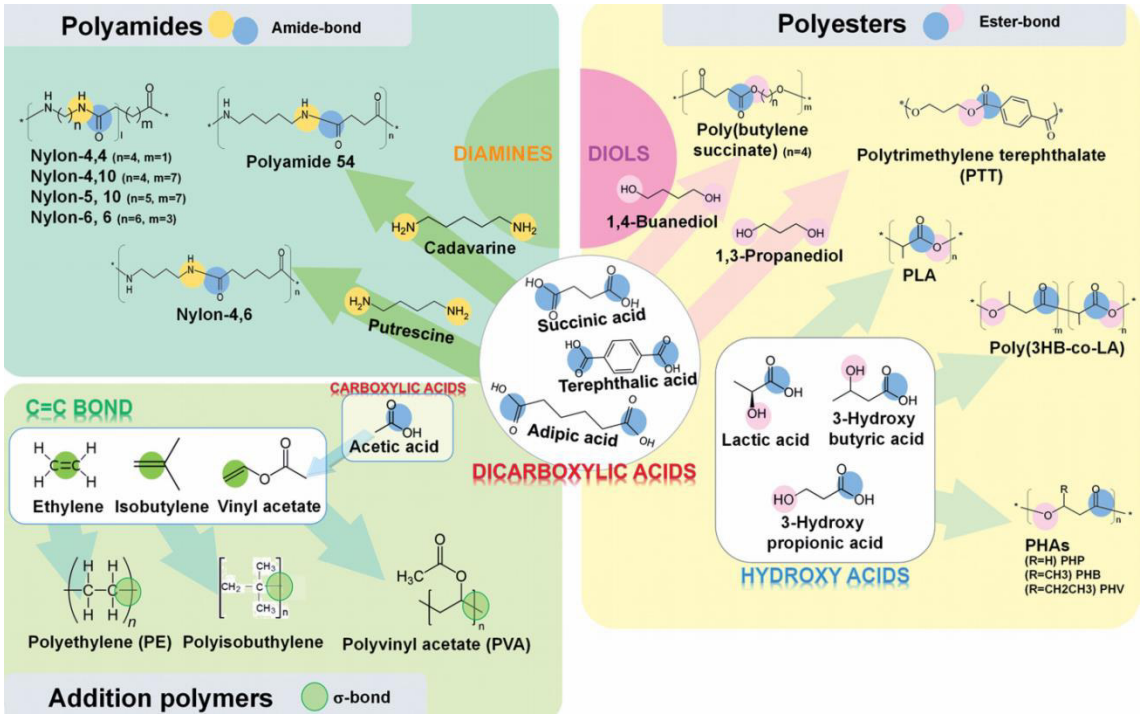


Abbildung 6: Einsatz von Dicarboxylsäuren als Monomere für die Synthese verschiedener Polymere. Nach Jang (2012)

In der biotechnologischen Produktion von Dicarboxylsäuren wurden in den letzten zwei Dekaden bedeutende Fortschritte erzielt, wobei der Schwerpunkt auf der Synthese von Succinat und Adipinsäure lag (Cheng 2013; Polen 2013; Sauer 2008). Als meist verwendete MO sind dabei rekombinante *E. coli* (Chen 2013; Yu 2011), *C. glutamicum* (Wieschalka 2013) und *S. cerevisiae* (Borodina und Nielsen 2014) zu nennen, wobei auch Wildtyporganismen wie *Actinobacillus succinogenes* für die Succinat- oder *Aspergillus terreus* für die Itaconatproduktion (Jang 2012) (siehe auch 2.3) zum Einsatz gekommen sind.

Obwohl für Dicarboxylsäuren wie Succinat, Malat oder Fumarat finale Produkttiter im hohen zwei- oder sogar dreistelligen g/l-Bereich bei Ausbeuten bis zu 1,1 g/g Substrat erreicht wurden (Alonso 2014; Cao 2011; Jang 2012), so ist doch die biotechnologische

Produktion vieler Säuren im industriellen Maßstab (noch) nicht etabliert (siehe 2.3). Eine Hauptursache dafür sind die niedrigen Preise von Dicarboxylsäuren als Bulkchemikalien, wodurch ein stark optimierter biotechnologischer Produktionsprozess erforderlich ist, um überhaupt in den Bereich der oft sehr niedrigen Produktionskosten etablierter chemischer Synthesen zu kommen.

Die Produktaufarbeitung von Dicarboxylsäuren (Downstream Processing) aus Fermentationsbrühen hat einen Gesamtkostenanteil von 50 – 70 % (Bechthold 2008) und ist damit der größter Kostenfaktor. Dabei spielen die Art und Anzahl der Nebenprodukte (in *E. coli* oder *S. cerevisiae* u.a. Acetat, Ethanol, Lactat, Fumarat) und auch das verwendete Substrat eine entscheidende Rolle. So verteuern komplexe Substrate wie Molke, Melasse oder Lignocellulose aufgrund der Vielzahl zusätzlich eingebrachter Substanzen und anderer nachteiliger Parameter wie einer erhöhten Viskosität der Kulturbrühe, die Produktaufarbeitung erheblich (Cheng 2012). Die Verwendung von *M. extorquens* und der alternativen C-Quelle Methanol könnte sich hier aufgrund mehrerer Faktoren als vorteilhaft erweisen: i) in großen Mengen benötigte Dicarboxylsäuren könnten aus einer günstigen und alternativen C-Quelle produziert werden; ii) es können Minimalmedien ohne Komplexbestandteile verwendet werden, was die Produktaufarbeitung erleichtert und iii) der Kulturüberstand von *M. extorquens* enthält bei der Kultivierung auf Methanol wenige Nebenprodukte (siehe 4.3), was ebenfalls die Kosten der Produktaufarbeitung reduziert. Entscheidend für einen kommerziell wettbewerbsfähigen Prozess bleibt aber auch hier eine optimierte Synthese mit hoher Ausbeute und Produkttitern.

2.5 Mikrobielle Synthese von Sesquiterpenen – Stand der Technik

Sesquiterpene bilden die größte Gruppe der Isoprenoide mit mehr als 7000 bekannten Vertretern (Fraga 2013). Sie bestehen aus 3 Isopreneinheiten, d.h. insgesamt 15 C-Atomen, wobei lineare, zyklische, bizyklische und verzweigte Strukturen bekannt sind. Natürlicherweise werden Sesquiterpene aus dem Vorläufermolekül Farnesylpyrophosphat (FPP) durch Sesquiterpensynthasen gebildet. FPP selbst wird durch die Kondensation zweier Moleküle IPP und einem Molekül DMAPP (Isomer von

IPP) gebildet (Stevens 1992). Für die Biosynthese des Isoprenoidgrundbausteins IPP sind wiederum zwei Stoffwechselwege bekannt: der Mevalonatweg (MVA), welcher in Eukaryoten, Archeen und Gram-positiven Kokken vorkommt und der Methylerythritolphosphatweg (MEP oder auch DXP), der in Gram-negativen Bakterien und eukaryotischen Organellen vorkommt (Kuzuyama 2002). Einen Überblick über die Isoprenoidbiosynthese gibt Abbildung 7.

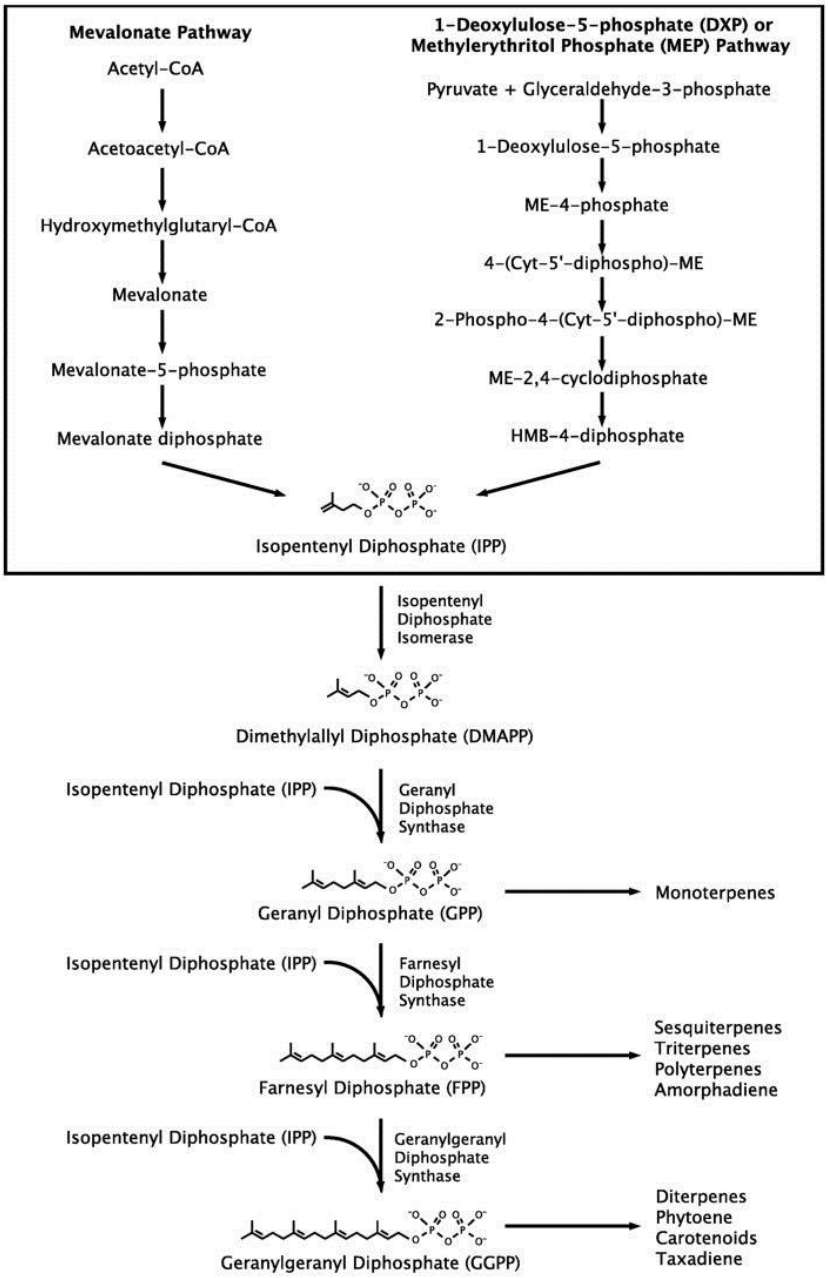


Abbildung 7: Darstellung der Biosynthese von IPP über den MVA und MEP-Weg mit anschließender Kondensation zu den Vorstufen GPP für Monoterpene (C10), FPP für Sesquiterpene (C15) und GGPP für Diterpene (C20). Nach Immethun (2013)

Sesquiterpene haben etablierte oder vielversprechende Anwendungsmöglichkeiten, z.B. als Medikamente, Biokraftstoffe oder Aroma- und Riechstoffe (Berger 2009; Renninger und McPhee 2008; White 2008). Da die chemische Synthese oder Extraktion aus den natürlichen Produzenten (zumeist Pflanzen) für viele Terpene nur in niedrigen Ausbeuten, gekoppelt mit hohen Preisen und dem Einsatz schädlicher Chemikalien bzw. Katalysatoren möglich ist, sind Sesquiterpene und allgemein Isoprenoide prädestiniert für die biotechnologische Produktion (Immethun 2013; Peralta-Yahya und Keasling 2010; Peralta-Yahya 2012). Am besten erforscht ist die Produktion von Amorphadien bzw. Artemisininsäure (beides Vorstufen des Anti-Malaria-Medikaments Artemisinin) und die des potenziellen Biokraftstoffes Farnesen (Asadollahi 2008; Martin 2003; Renninger und McPhee 2008). Deren Biosynthese wurde durch die heterologe Expression eines MVA-Weges in *E. coli* erreicht, der natürlicherweise den MEP besitzt (siehe Abb. 7), oder durch die Deregulation des nativen MVA und der Ergosterolbiosynthese in *S. cerevisiae*. Dabei wurden Titer von mehr als 1 g/l und Substratausbeuten von bis zu 50 % der maximalen theoretischen Ausbeute erreicht (Anthony 2009; Asadollahi 2008; Immethun 2013; Nowroozi 2014; Peralta-Yahya 2012; Renninger und McPhee 2008). Im Vergleich zum MVA ist der Wissensstand über den MEP geringer und bisherige Versuche von dessen Überexpression in nicht-MEP-enthaltenden MO wie *S. cerevisiae* blieben erfolglos. Daher ist die Bedeutung des MEP in der biotechnologischen Isoprenoidsynthese deutlich geringer als die des MVA (Zhao 2013).

Nahezu alle beschriebenen biotechnologischen Sesquiterpenproduktionen beruhen auf der Umsetzung von Glukose. Mit Ausnahme von Glycerin, Ethanol und CO_2 wurden bis dato keine alternativen Kohlenstoffquellen als Substrat verwendet (Chandran 2011; Yoon 2009). Für hochpreisige Feinchemikalien wie Sesquiterpene ist die Wahl des Substrates aus Kostenaspekten weniger bedeutend als für Bulkchemikalien (siehe 2.4). Für die wachstumsgekoppelte Produktion von Isoprenoiden sind zudem Substrate mit hoher Energiedichte einhergehend mit schnellem Wachstum der/des MO vorteilhaft. Die C-Quelle wird jedoch bedeutender, wenn Sesquiterpene wie Farnesen oder α-Bisabolen, bzw. deren Derivate Farnesan und α-Bisabolan, als Biokraftstoff verwendet werden sollen (Peralta-Yahya 2011; Wang 2011). Die Frage, ob biotechnologisch produzierte Sekundärmetabolite jemals wettbewerbsfähige Alternativen zu

petrochemischen Kraftstoffen darstellen werden, ist hier berechtigt. Die Diskussion läge jedoch außerhalb der Thematik dieses Kapitels.

Nichtsdestotrotz würde die Verwendung alternativer Kohlenstoffquellen wie Methanol die in Abschnitt 2.1 und 2.4 diskutierten Vorteile (Nachhaltigkeit, Minimalmedium usw.) mit sich bringen. Im spezifischen Fall von *M. extorquens* als methanolwertenden Produktionsorganismus ist zusätzlich die Präsenz von Acetoacetyl-CoA im Primärstoffwechsel (EMCP) als Vorteil zu nennen (siehe 2.2): Der hohe metabolische Fluss zu diesem Intermediat des MVA (siehe Abb. 7) geht vermutlich mit einer erhöhten IPP-Synthese eines heterolog exprimierten MVAs in *M. extorquens* einher. Dadurch könnten Raum-Zeit- und Substratausbeuten im Vergleich zu konventionellen Produktionsorganismen (*E. coli* oder *S. cerevisiae*) theoretisch gesteigert werden. Außerdem wäre die oft schwierige heterologe Expression einer Acetoacetyl-CoA-Synthase obsolet (Harada 2009).

3. Aufgabenstellung und Zielsetzung

Ziel dieser Arbeit war die Etablierung der *de novo* Synthese von Dicarboxylsäurederivaten des EMCPs und eines Terpens aus Methanol mit Hilfe des methylotrophen Modellorganismus *Methylobacterium extorquens*.

Für die Synthese der EMCP-Säurederivate sollte zunächst eine Akkumulation der Produktvorstufen in Form der jeweiligen CoA-Ester (siehe Abb. 5) erreicht werden. Dazu sollten die codierenden Gene der Enzyme, die den gewünschten CoA-Ester umsetzen, deletiert werden. Die verschiedenen Gendeletionen werden aufgrund der Essentialität des EMCP mit einem Verlust der Methylotrophie einhergehen. Diese sollte durch das Einbringen eines heterologen Glyoxylatzyklus, der die Funktion der Glyoxylatregeneration des EMCP übernimmt, wiederhergestellt werden.
Weiterhin sollten Thioesterasen identifiziert werden, die in der Lage sind, die Thioesterbindung der EMCP-CoA-Ester zu hydrolysieren und damit die Freisetzung der jeweiligen Dicarboxylsäurederivate zu erreichen. Bisher sind keine Thioesterasen mit einer entsprechenden Funktion in der Literatur beschrieben.

Die biotechnologische Produktion von Terpenen aus Methanol mit *M. extorquens* sollte anhand der *de novo* Synthese des Sesquiterpens α-Humulen demonstriert werden. Dazu sollte das Gen der α-Humulen-Synthase aus *Zingiber zerumbet* und eine FPPS für eine erhöhte Produktion der Vorstufe FPP episomal exprimiert werden. Ein zusätzlicher heterologer MVA sollte die α-Humulensynthese durch die erhöhte Bereitstellung von IPP weiter steigern. Um eine akzeptable Codonnutzung für *M. extorquens* zu gewährleisten, wurde als Spender des MVAs das Bakterium *Myxococcus xanthus* ausgewählt, dessen Codonnutzung der von *M. extorquens* ähnelt.

4. Überblick über die Manuskripte

4.1 Thioesterases for ethylmalonyl-CoA pathway derived dicarboxylic acid production in *Methylobacterium extorquens* AM1

Sonntag F., Buchhaupt M., Schrader J. (2014) Applied Microbiology and Biotechnology 98(10):4533-44

In dieser Publikation wird ein Screening von Thioesterasen, also Enzyme die in der Lage sind Thioesterbindungen zu hydrolysieren, hinsichtlich ihrer Aktivität für die CoA-Ester des Ethylmalonyl-CoA Weges beschrieben. Sechs potentiell aktive Kandidaten wurden dazu auf Basis einer umfassenden Literaturrecherche ausgewählt und deren Aktivität zunächst *in vitro* mit einem DTNB-Enzymaktivitätstest untersucht. Keiner der getesteten Thioesterasen zeigte dabei eine spezifische Aktivität gegenüber einem der 5 untersuchten EMCP-CoA-Ester. Jedoch konnte die bakterielle Thioesterase YciA mit einer durchschnittlichen spezifischen Aktivität von 8 U/mg eindeutig als insgesamt aktivstes Enzym identifiziert werden. Infolgedessen wurde das Gen *yciA* mit einer optimierten RBS konstitutiv in *Methylobacterium extorquens* AM1 exprimiert, woraufhin eine Freisetzung von bis zu 70 mg/l Mesacon- und 60 mg/l (2*S*)-Methylsuccinsäure im Kulturüberstand von auf Methanol gewachsenen Zellen gemessen werden konnte. Interessanterweise konnte eine Abnahme der Konzentration beider Säuren in der stationären Wachstumsphase der Kulturen festgestellt werden, was die Frage eines aktiven Säureaufnahme- und Verwertungsmechanismus von EMCP-Dicarboxylsäurederivaten in *M. extorquens* aufwarf. Wachstumsanalysen zeigten, das *M. extorquens* sowohl Mesacon- als auch 2-Methylsuccinsäure und weitere EMCP-Dicarboxylsäurederivate als alleinige Energie- und Kohlenstoffquelle nutzen kann. Zudem konnte mittels BLAST-Analyse das Gen des Dicarboxylsäuretransporters DctA und die dazugehöriger Regulatorproteine (DcuS, DcuR und DcuB) im Genom von *M. extorquens* AM1 identifiziert werden.

4.2 High-level production of ethylmalonyl-CoA pathway derived dicarboxylic acids by *Methylobacterium extorquens* under cobalt-deficient conditions and by polyhydroxybutyrate negative strains

Sonntag F., Müller J.E.N., Kiefer P., Vorholt J.A., Schrader J., Buchhaupt M. (2015). Applied Microbiology and Biotechnology DOI 10.1007/s00253-015-6418-3 (In press: available online)

Diese Publikation ist eine Weiterentwicklung der in 4.1 beschriebenen *de novo* Synthese von Mesacon- und (2*S*)-Methylsuccinsäure aus Methanol mit Hilfe eines *yciA* exprimierenden *M. extorquens* AM1. Für eine Steigerung der Produktion wurden drei Aspekte untersucht: i) Verhinderung der Wideraufnahme der produzierten EMCP-Säurederivate in der stationären Wachstumsphase, ii) Steigerung des metabolischen Flusses durch den EMCP zu den Produkten durch das Ausschalten der PHB-Synthese und iii) Erhöhung der Produktvorstufenverfügbarkeit, d.h. der Konzentration der EMCP-CoA-Ester. Die Wiederaufnahme der Säuren in der stationären Wachstumsphase konnte durch eine 30-fache Reduzierung der im Medium verfügbaren Natriumkonzentration verhindert werden, wobei gleichzeitig die Maximalkonzentrationen der produzierten Mesacon- und (2*S*)-Methylsuccinsäure verdoppelt wurde.

Die Deletion des PHA-Synthase Gens *phaC* führte zu einer 3-fach gesteigerten Synthese von Mesacon- und (2*S*)-Methylsuccinsäure, jedoch traten bereits nach kurzer Kultivierungszeit des Δ*phaC*-Stammes Suppressoren in hoher Frequenz auf, die die positive Eigenschaft der erhöhten Säureproduktion verloren und deren Produktsyntheseraten sogar unterhalb denen des Wildtypes lagen.

Durch 60-fach verringerte, wachstumslimitierende Kobaltkonzentrationen im Medium, konnte eine deutliche Akkumulation aller EMCP-CoA-Ester nachfolgend der Ethylmalonyl-CoA-Mutase gemessen werden, was auf die Hemmung der Aktivität der beiden Cobalamin-, d.h. der Kobalt abhängigen Mutasen im EMCP (siehe 2.2.2) zurückzuführen ist. Eine Kombination der limitierenden Kobaltverfügbarkeit und der Expression der RBS-optimierten Thioesterase *yciA* (siehe 4.1) führte schließlich zu einer Produktion von 0.65 g/l Mesacon- und (2*S*)-Methylsuccinsäure im Schüttelkolbenmaßstab.

4.3 *Methylobacterium extorquens*: methylotrophy and biotechnological applications

Ochsner A.M., Sonntag F.*, Buchhaupt M., Schrader J., Vorholt J.A. (2014) Applied Microbiology and Biotechnology 99(2):517-534 [*authors contributed equally]*

Dieses Review gibt einen Überblick über den methylotrophen Modellorganismus *Methylobacterium extorquens*. Darin werden verschiedene bekannte Stämme mit Fokus auf Stamm AM1, der Stoffwechsel und dessen Regulation auf C1- und anderen Kohlenstoffquellen, sowie bisher verfügbare genetische Werkzeuge beschrieben. Des Weiteren gibt dieses Review einen detaillierten Überblick über etablierte und potenzielle biotechnologische Produktsynthesen mit *M. extorquens*.

4.4 Methanol in Biotechnology

Hippmann S., Bertau M., Holtmann D., Sonntag F., Veith T., Schrader J. Methanol: The basic chemical and Energy Feedstock of the Future, Bertau M., Offermanns H., Plass L., Schmidt F. und Wernicke H-J., Springer-Verlag Berlin Heidelberg 2014, Chapter 6.5.3., pp. 561-575

Dieses Buchkapitel gibt einen Überblick über den Einsatz von Methanol als Kohlenstoffquelle in der Biotechnologie. Dabei werden sowohl pro- als auch eukaryontische Bioproduktionssysteme hinsichtlich ihres Stoffwechsels und spezifischer Prozesscharakteristiken beschrieben. Zusätzlich gibt es einen Überblick über die enzymatische Produktion von Methanol aus Formaldehyd und Methan, sowie den potenziellen Einsatz von Methanol als Kohlenstoffquelle in der Abwasseraufbereitung.

4.5 Engineering *Methylobacterium extorquens* for de novo synthesis of the sesquiterpenoid α-humulene from methanol

Sonntag F., Kroner C., Lubuta P., Peyraud R., Horst A., Buchhaupt M., Schrader J. Metabolic Engineering, accepted

In diesem Manuskript wird die *de novo* Synthese des Sesquiterpens α-Humulen aus Methanol mit Hilfe eines genetisch veränderten *M. extorquens* AM1-Stammes beschrieben. Dazu wurden die α-Humulen Synthase ZSSI aus *Zingiber zerumbet*, eine FPPS zur Bereitstellung der α-Humulen Vorstufe FPP aus *S. cerevisiae*, sowie ein prokaryontischer Mevalonatweg aus *Myxococcus xanthus* zur Synthese der

Isoprenoidvorstufe IPP heterolog in *M. extorquens* exprimiert. Mit Hilfe des Cumat-induzierbaren Plasmidsystems pQ2148 und der Optimierung der Translationsinitiation der ZSSI, FPPS und IDI durch die Anpassung deren ribosomaler Bindestellen konnte ein funktionelles, nicht-lethales Plasmid konstruiert werden, durch das *M. extorquens* AM1 bis zu 58 mg/l α-Humulen aus Methanol im Schüttelkolbenmaßstab synthetisiert. Eine weitere Steigerung des Produkttiters im Schüttelkolben um 30 % auf 75 mg/l konnte durch die Verwendung des in der Carotinoidbiosynthese inhibierten Stammes CM502 erreicht werden. Weiterhin wurde ein Up-Scaling des Prozesses in einem 2L-Fermenter demonstriert, wobei Biotrockenmassen von mehr als 30 g/l und finale α-Humulen-Konzentrationen von 1.65 g/l erreicht wurden. Dabei entsprach die Ausbeute von 0.031 $g/g_{Methanol}$ 12 % der maximal möglichen, theoretischen Ausbeute.

5. Diskussion

5.1 Produktion von Dicarboxylsäurederivaten des EMCPs aus Methanol

5.1.1 Identifikation von Thioesterasen mit hydrolytischer Aktivität für CoA-Ester des EMCPs

Für die *de novo* Produktion von Dicarboxylsäurederivaten des EMCP mussten zunächst Thioesterasen identifiziert werden, die in der Lage sind, die Thioesterbindungen der EMCP-CoA-Ester zu hydrolysieren (die allgemeine Reaktion einer Thioesterase ist in Abb.8 dargestellt). Wie in Publikation 4.1 beschrieben, zeigten 5 von 6 auf Basis einer Literaturrecherche ausgewählten Thioesterasen *in vitro* Aktivität hinsichtlich der Abspaltung von CoA für die EMCP-CoA-Ester Ethylmalonyl-, *rac*-Methylsuccinyl-, Mesaconyl-, beta-Methylmalyl- und Methylmalonyl-CoA. Dabei zeigte die bakterielle Thioesterase YciA aus *E. coli* die durchschnittlich höchste spezifische Aktivität, hatte jedoch genau wie die insgesamt weniger aktiven Thioesterasen TesB, Acot4, PaaI und Bch keine deutliche Spezifität für eines der 5 getesteten CoA-Ester Substrate (siehe Tab.2 in Publikation 4.1).

Es ist bekannt, dass Acyl-CoA-Thioesterasen entweder ein sehr breites Substratspektrum (sogenannte „broad-range“ Thioesterasen) oder, aufgrund einer sehr spezifischen Funktion innerhalb eines bestimmten Stoffwechselweges, eine hohe Substratspezifität aufweisen (Cantu 2010). Zur letzteren Gruppe gehören auch die in 4.1 untersuchten Thioesterasen Acot4 (Succinyl-CoA Hydrolyse in Peroxisomen von Säugetieren (Westin 2005)) und PaaI (4-Hydroxybenzoyl-CoA Hydrolyse im Benzoesäureabbau (Ismail 2008)). Trotz der strukturellen Ähnlichkeit der EMCP-CoA-Ester (C4 und C5-Acyl-CoAs) zum Hauptsubstrat der Acot4 (Succinyl-CoA), zeigte dieses Enzym nur eine sehr geringe spezifische Aktivität für die CoA-Ester des EMCPs, was die hohe Substratspezifität dieses Enzyms unterstreicht. Die analysierten „broad-range“ Acyl-CoA-Thioesterasen TesB und YciA sind zwar gegenüber allen EMCP-CoA-Estern aktiv, jedoch liegen die in 4.1 gemessenen spezifischen Aktivitäten teilweise deutlich

unter denen anderer Substrate, wie z.B. Palmitoyl-CoA für TesB. Die natürliche Funktion bakterieller „broad-range“ Acyl-CoA-Thioesterasen ist nach wie vor nicht eindeutig geklärt, da oft weder Deletions- noch Überexpressionsmutanten veränderte Phänotypen, Lipidzusammensetzungen o.ä. aufweisen (Cho und Cronan 1993; Naggert 1991; Zhuang 2008). Es wird daher vermutet, dass die ubiquitär verbreiteten „broad-range“ Acyl-CoA-Thioesterasen als eine Art Notfallmechanismus für den Abfluss ungewollt akkumulierender CoA-Ester dienen, womit auch eine Verarmung von CoA verhindert wird (Zheng 2004). Dahingehend ist auch die nicht vorhandene Spezifität von TesB und YciA hinsichtlich der EMCP-CoA-Ester nicht überraschend, wobei die im Vergleich zu linearen CoA-Estern geringeren Aktivitäten vermutlich an der Struktur der EMCP-CoA-Ester liegen. Diese sind im Gegensatz zu den Substraten mit hoher Aktivität, wie z.B. Palmitoyl-CoA, kurzkettiger, durch die zusätzliche Carboxylgruppe hydrophiler und weisen zudem verzweigte Kohlenstoffketten auf.

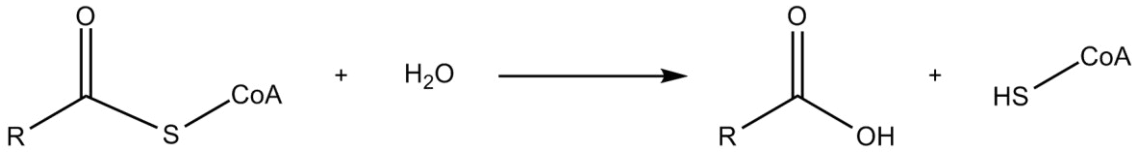


Abbildung 8: Reaktion der Hydrolyse einer Thioesterbindung eines Acyl-CoA-Esters von einer Acyl-CoA-Thioesterase

Die nicht vorhandene Substratspezifität der Thioesterase YciA zeigte sich auch bei der Expression des Gens *yciA* in *M. extorquens*. Dies führte zu der Freisetzung von Mesacon- und (2*S*)-Methylsuccinsäure in den Kulturüberstand, also einer CoA-Abspaltung von Mesaconyl- und (2*S*)-Methylsuccinyl-CoA *in vivo* (siehe Publikation 4.1). Das ist der erste Nachweis einer biotechnologischen *de novo* Synthese von Dicarboxylsäurederivaten des EMCPs, die zudem aus der alternativen C-Quelle Methanol gewonnen worden.

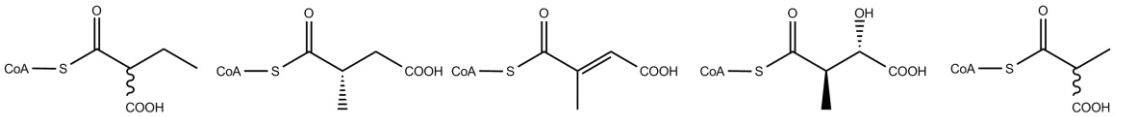
Die Produktion von Mesacon- und (2*S*)-Methylsuccinsäure konnte dabei durch eine 110-fache Erhöhung der Translationsinitiationsrate (TIR) der RBS der *yciA* insgesamt um den Faktor 8,3 gesteigert werden. Das Design der RBS erfolgte dabei *in silico* mit Hilfe des Ribosome Binding Site Calculators (Salis 2009). Dieses Modell basiert auf der Berechnung der freien Bindungsenthalpie des Ribosoms und der RBS, die durch die Sequenz und damit Struktur der umliegenden DNA-Bereiche (Anfangssequenz des

Gens, Bereich zwischen RBS und Gen und ein etwa 50 bp großer Abschnitt vor der RBS) vorgegeben wird. Eine minimierte Bindungsenthalpie bedeutet eine starke Bindung des Ribosoms an die RBS, wodurch die Initiation der Translation beschleunigt und damit die gesamte Proteinbildung erhöht wird (siehe auch 2.3). Auch im Falle der optimierten RBS der YciA ist es mit hoher Wahrscheinlichkeit zu einer erhöhten Proteinbildung und damit zu einer gesteigerten Hydrolyseaktivität der EMCP-CoA-Ester gekommen. Allerdings wurde ein direkter Nachweis der YciA-Proteinkonzentration nicht durchgeführt (siehe 4.1).

Eine weitere Erhöhung der Proteinbildung könnte u.a. durch eine Codonoptimierung der YciA oder durch die Expression mehrerer Kopien des Gens erreicht werden. Dies muss jedoch nicht zwangsweise mit einer erhöhten Produktbildung einhergehen. Nur im Falle einer ausreichenden Bereitstellung der beiden Produktvorstufen, Mesaconyl- und (2*S*)-Methylsuccinyl-CoA, könnte eine gesteigerte Hydrolyseaktivität als Resultat einer erhöhter Enzymkonzentration auch zu mehr Produkt führen.

Für einen wirtschaftlichen Prozess stellt die parallele Produktion zweier strukturell ähnlicher Dicarboxylsäuren eine Herausforderung dar. Beide Säuren müssten vermutlich bei der Produktaufarbeitung chromatographisch aufgetrennt werden, um sie einzeln und in hoher Reinheit zu erhalten, was mit hohen Kosten einhergehen würde (Cheng 2012). Eventuell wäre aufgrund der unterschiedlichen pK_S-Werte von 3,09 und 4,75 (Mesaconsäure) bzw. 4,13 und 5,64 (Methylsuccinsäure) aber auch eine nacheinander stattfindende Präzipitation durch CaO, $Ca(OH)_2$ oder NH_4 möglich.

Nichtsdestotrotz wäre die Identifikation einer Acyl-CoA-Thioesterase mit einer spezifischen Aktivität für nur einen der EMCP-CoA-Ester aus wissenschaftlicher Sicht und auch für einen möglichen industriellen Prozess interessant. Weitere potenzielle Kandidaten dafür wären z.B. die „broad-range“ Acyl-CoA-Thioesterasen Pte1p aus *S. cerevisiae* (Maeda 2006) oder die YciA aus *Haemophilus influenza* (Zhuang 2008), die für den EMCP-CoA-Estern strukturell ähnlichen Substanzen, u.a. dem verzweigten Isobutyryl-CoA, hohe Aktivitäten aufweisen. Damit könnten auch von der YciA nicht hydrolysierte EMCP-CoA-Ester, wie das (2*R*)- oder (2*S*)-Ethylmalonyl-CoA oder (2*R*/3*S*)-Methylmalyl-CoA (siehe Abb. 9) bzw. deren Dicarboxylsäurederivate als Produkte zugänglich gemacht werden.



(2*R*)-Ethylmalonyl-CoA (2*S*)-Ethylmalonyl-CoA | (2*S*)-Methylsuccinyl-CoA | Mesaconyl-CoA | (2*R*,3*S*)-methylmalyl-CoA | (2*S*)-methylmalonyl-CoA (2*R*)-methylmalonyl-CoA

Abbildung 9: Auswahl biotechnologisch interessanter CoA-Ester des EMCPs.

Für zukünftige Aktivitätsscreenings wäre ein direkter *in vivo* Ansatz aus Gründen der Zeitersparnis von Vorteil. Ähnlich dem von McMahon und Prather (2014) gezeigten Ansatz könnten potenzielle Thioesterasen in *M. extorquens* mit akkumulierten EMCP-CoA-Estern (siehe 5.1.2 bzw. Publikation 4.2) exprimiert werden und das Spektrum freigesetzter Dicarboxylsäuren im Produktüberstand gemessen werden.

Aufgrund des bereits diskutierten breiten Substratspektrums von vielen Acyl-CoA-Thioesterasen, ist die Identifikation eines Enzyms mit spezifischer (und gleichzeitig hoher) Aktivität für nur einen der EMCP-CoA-Ester aber unwahrscheinlich. Daher sollte auch die Möglichkeit des Enzym- oder Protein-Engineerings in Betracht gezogen werden. So konnten Lee und Kollegen (2009) die Substratspezifität und katalytische Aktivität der Thioesterase I (α/β-Faltung) aus *E. coli* durch die Modifikation des nicht-katalytischen Trp23 verändern. Auch für „Hot-Dog"-gefaltete Thioesterasen wie die YciA sind Strukturmodelle und katalytische Zentren bekannt (Labonte und Townsend 2012; Willis 2008), was eine Voraussetzung für ein mögliches, rationales Protein-Engineering darstellt. Eine Herausforderung für die Modifikation der YciA-Substratspezifität ist jedoch die hohe strukturelle Ähnlichkeit der EMCP-CoA-Ester. Präzise *in silico* Simulationen der Enzym-Ligand-Interaktionen (z.B. mit SwissDock (Grosdidier 2011)) und ein hoher Aufwand für das Screening entsprechender Mutanten *in vivo* wären vermutlich unvermeidbar.

5.1.2 Verbesserte Mesacon- und (2*S*)-Methylsuccinsäureproduktion durch Steigerung des metabolischen Flusses zu den Produkten und verstärkter Verfügbarkeit der Produktvorstufen

Die in Publikation 4.1 erreichten Produkttiter in Höhe von 70 mg/l Mesaconsäure und 60 mg/l (2*S*)-Methylsuccinsäure stellen einen ersten Nachweis für die Möglichkeit der *de novo* Synthese beider Säuren aus Methanol dar. Die Konzentrationen und

Produktivitäten sind jedoch für eine industriell wettbewerbsfähige Produktion von potenziellen Bulkchemikalien deutlich zu gering. Folglich werden weitere Verbesserungen erforderlich.
In Publikation 4.2 wurden dazu zwei Hauptansätze verfolgt: Zum einen wurde die PHB-Synthese durch die Deletion des PHA-Synthase codierenden Gens *phaC* unterbunden und zum anderen wurden die Konzentrationen von Mesaconyl- und (2*S*)-Methylsuccinyl-CoA als Produktvorstufen durch die Aktivitätsreduzierung der zwei Kobalt abhängigen Mutasen des EMCPs (siehe 2.2.2) erhöht.

Das Verhindern der PHB-Synthese durch die Deletion des *phaC*-Gens bewirkt eine dreifache Steigerung der Mesacon- und (2*S*)-Methylsuccinsäureproduktion bei gleichzeitiger Wachstumsverringerung eines *yciA* exprimierenden *M. extorquens*. Durch die auftretenden Suppressoren ist die Nutzung der Δ*phaC*-Mutante in einem stabilen Prozess jedoch nicht möglich (siehe 4.2). Die Identifikation der Ursache, z.B. eines möglichen Regulators, wäre für eine Stabilisierung der Mutante vorteilhaft. Durch Transcriptomics könnten unterschiedlich stark exprimierte Gene in der Δ*phaC*-Mutante und den Δ*phaC*-Suppressoren identifiziert werden, was unter Umständen Anhaltspunkte für mögliche Regulationsmechanismen liefert. Sollte das Auftreten der Δ*phaC*-Suppressoren, wie in 4.2 diskutiert, tatsächlich mit einem NADH-Anstau und der damit verbundenen Steigerung des TCA-Flusses verknüpft sein, so könnte die Expression einer NADH-Oxidase (Bao 2014; Park 2014) einen alternativen Abfluss für NADH bereitstellen und damit die Bildung der Suppressoren möglicherweise verlangsamen oder verhindern. Außerdem wäre es interessant zu überprüfen, ob das Phänomen der Δ*phaC*-Suppressoren auch mit dem Stamm *M. extorquens* PA1 auftritt, der aufgrund seiner höheren Wachstums- und Substrataufnahmerate (Nayak und Marx 2014a) eventuell einen besser geeigneten Stamm für die Produktion von EMCP-Dicarboxylsäurederivaten als Stamm AM1 darstellt.

Der Einsatz wachstumslimitierender Kobaltkonzentrationen führte zu einer 3 bzw. 15-fachen Akkumulation von Mesaconyl- und (2*S*)-Methylsuccinyl-CoA, was mit einer 6-fachen Steigerung der *de novo* Synthese von Mesacon- und (2*S*)-Methylsuccinsäure einherging (siehe Abb. 4 und 5 in Publikation 4.2). Auch die Produktausbeute pro Substrat konnte von 0,03 $g/g_{Methanol}$ auf 0,17 $g/g_{Methanol}$ für beide EMCP-

Dicarboxylsäurederivate gesteigert werden (siehe Tab.2 in 4.2). Diese Ausbeuten entsprechen 9 bzw. 12 % der maximalen theoretischen Ausbeute an Produkt pro Substrat für (2*S*)-Methylsuccinsäure (max. 0,79 $g/g_{Methanol}$) bzw. Mesaconsäure (max. 0,81 $g/g_{Methanol}$), was anhand eines stöchiometrischen metabolischen Modells von *M. extorquens* (Peyraud 2011) ermittelt wurde.

Insgesamt liegen die Ausbeuten und Titer für Mesacon- und (2*S*)-Methylsuccinsäure noch deutlich unter denen anderer biotechnologisch produzierter C4- und C5-Dicarboxylsäuren wie Succinat, Fumarat oder α-Ketoglutarat (Cao 2011; Jang 2012; Wieschalka 2013). Es ist allerdings zu beachten, dass nahezu jeder dieser Prozesse auf der Verwertung von nicht-alternativen Substraten (vor allem Glukose) basiert und die Ausbeuten und Produktkonzentrationen dieser Arbeit im Gegensatz zu denen der zitierten Referenzen im Schüttelkolbenmaßstab und nicht im Fermenter erreicht wurden. In einem zweistufigen Fermentationsprozess mit einer Wachstumsphase im Medium ohne Kobaltlimitierung und einer Produktionsphase mit Kobaltlimitierung könnten vermutlich mehr als 20- bis 30-fach höhere Produktkonzentrationen erreicht werden, da die bisherige Maximalkonzentration von 0.65 g/l bei einer OD_{600} von nur 2 gemessen wurden. Höhere Ausbeuten pro Substrat und auch pro Biomasse könnten mit der zuvor diskutierten Stabilisierung des Δ*phaC*-Stammes und einer für den (stabilisierten) Δ*phaC*-Stamm zur Produktion der EMCP-Dicarboxylsäurederivate optimierten Kobaltkonzentration erreicht werden. Auch durch die Überexpression von EMCP-Genen könnte der Fluss zu den Produktvorstufen und damit die Produktion der Säuren wahrscheinlich weiter erhöht werden. So führte im Falle der 1-Butanolsynthese mit *M. extorquens* über die Vorstufen Butyryl- und Crotonyl-CoA (letzteres als Intermediat im EMCP, siehe Abb. 5) die Überexpression der Crotonase (*croR*) zu einer 50 %-ig gesteigerten Butanolproduktion (Hu und Lidstrom 2014).

5.2 Substitution des EMCP in *M. extorquens* durch einen heterologen Glyoxylatweg

Die in Publikation 4.2 verwendete Methode des kobaltlimitierten Wachstums von *M. extorquens* und der damit verbundenen Akkumulation aller EMCP-CoA-Ester nachfolgend der Reaktion der Ethylmalonyl-CoA-Mutase, ist eine Möglichkeit der

erhöhten Bereitstellung von EMCP-Intermediaten als Vorstufen für weitere Produktsynthesen. Die spezifische Anhäufung eines CoA-Esters ist mit dieser Methode jedoch nicht möglich. Dies wäre aber besonders für substratunspezifische „broad-range“ Acyl-CoA-Thioesterasen (siehe 5.1.1) oder für eine mögliche Polyketidsynthese mit *M. extorquens* (Betrachtungen dazu in Publikation 4.3) von großem Vorteil.

Eine Möglichkeit für die spezifische Akkumulation eines EMCP-CoA-Esters ist die Deletion des codierenden Gens des Enzyms, das die Umsetzung des gewünschten Intermediates katalysiert, z.B. Δ*ecm* zum Anstau von (2*R*)-Ethylmalonyl-CoA (siehe Abb.5). Dabei wird jedoch der Fluss durch den EMCP und damit die für die Methylotrophie obligatorische Glyoxylatregeneration unterbunden (siehe auch Abschnitt 2.2.1 und 2.2.2). Eine alternative Möglichkeit der Glyoxylatregeneration in Methylotrophen mit Serin-Zyklus (siehe 2.2.1) ist der Glyoxylatzyklus in Form der Isocitrat-Lyase und Malat-Synthase, wobei nur die Reaktion der ICL (siehe Abb. 10) in *M. extorquens* natürlicherweise nicht vorhanden ist.

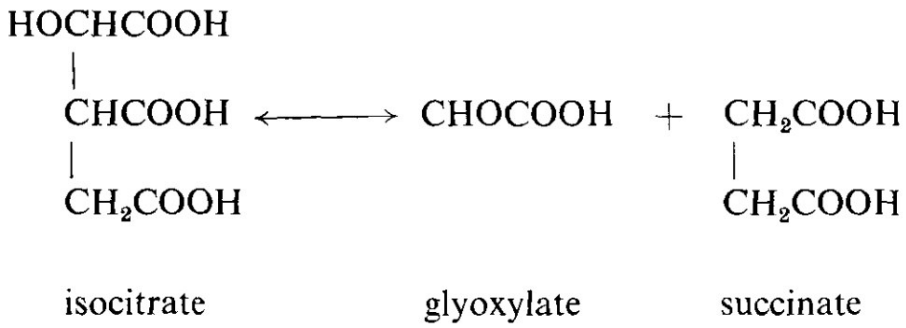


Abbildung 10: Katalysierte Reaktionen der Isocitrat-Lyase. Nach Anthony (1982).

In weiterführenden Forschungsarbeiten (siehe 7.2.1) wurde der Ansatz der Glyoxylatregeneration mittels eines heterologen Glyoxylatzyklus in unterschiedlichen EMCP-negativen *M. extorquens*-Stämmen untersucht. Wie erwartet verloren 6 von 7 Stämmen mit unterschiedlichen deletierten EMCP-Genen die Fähigkeit zur Methylotrophie (Ausnahme: Stamm Δ*mclA1*, siehe Abb.2 in 7.2.1). Das Wachstum dieser Stämme auf Methanol konnte jedoch trotz nachweislich (ausreichend) aktiver Expression einer ICL nicht wiederhergestellt werden. Eine Ursache dafür konnte nicht identifiziert werden: Metabolomics und Konzentrationsmessungen von Reduktionsäquivalenten zeigten, dass mit hoher Wahrscheinlichkeit weder die Verarmung noch starke Akkumulation eines Zentralstoffwechselintermediates bzw.

von NAD(P)(H) der Grund für die nicht wiederherstellbare Methylotrophie ist. Entsprechend der Reaktionsbilanzen der beiden Stoffwechselwegkombinationen (Serin$^+$ EMCP$^+$ ICL$^-$ und Serin$^+$ EMCP$^-$ ICL$^+$, siehe 7.2.1) und der Simulationen mit Hilfe eines metabolischen Models (Peyraud 2011) sollte methylotrophes Wachstum eines EMCP$^-$ und ICL$^+$ *M. extorquens*-Stammes aber möglich sein. Andere metabolische Ursachen als die in 7.2.1 untersuchten, sind jedoch nicht offensichtlich. Vermutlich spielen regulatorische Faktoren eine Rolle, die durch rationale Herangehensweise nur schwer zu identifizieren sein werden. Beispiele aus der Literatur für einen äquivalenten Austausch eines kompletten primären Stoffwechselzyklusses sind, soweit bekannt, nicht vorhanden, so dass eine Übertragung möglicher Ursachen nicht möglich ist.
Eine Möglichkeit für die Methylotrophiewiederherstellung wäre das Evolutionary Engineering (Vanee 2012): Durch langsames Entfernen einer für EMCP-negative Stämme nutzbaren C-Quelle wie Succinat und schrittweises Erhöhen der Methanolkonzentration in einem Chemostaten, könnten sich nach vielen Generationen wachstumsfähige Zellen durchsetzen. Deren Analyse auf genomischer und/oder metabolischer Ebene im Vergleich zu den nicht-wachstumsfähigen Deletionsstämmen könnte letztlich zur Ursache des Problems führen.

Weiterhin wird in den weiterführenden Arbeiten (siehe 7.2.1) die Identifikation des Serin-Zyklus$^+$, EMCP$^-$, Glyoxylat-Zyklus (ICL)$^+$, RuMP$^-$ und CBB-Zyklus$^-$ Methylotrophen *Granulibacter bethesdensis* beschrieben. Im Gegensatz zu bisher beschriebenen Serin-Zyklus$^+$ und ICL$^+$ Methylotrophen ist dieser Organismus nachweislich in der Lage Methanol als alleinige Kohlenstoff- und Energiequelle zu nutzen, während die von Anthony (1982) beschriebenen Bakterien nur Methylamin, jedoch nicht Methanol verwerten können. Aufgrund nicht vorhandener Genomsequenzen dieser Organismen kann jedoch die Existenz eines EMCPs in diesen MO nicht ausgeschlossen werden.
G. bethesdensis könnte zukünftig als Modellorganismus für die Erforschung von Serin-Zyklus$^+$ und ICL$^+$ Methylotrophen dienen. Durch biochemische und OMICS-Analysen seines Metabolismus sind eventuell auch Unterschiede zu den ICL$^+$ und EMCP$^-$ *M. extorquens*-Stämmen festzustellen und damit Rückschlüsse auf die Probleme bei deren Methylotrophiewiederherstellung.

5.3 *De novo* Synthese von Terpenen aus Methanol mit *M. extorquens* am Beispiel des Sesquiterpens α-Humulen

Wie in Abschnitt 2.2 beschrieben, bietet sich *M. extorquens* aufgrund seiner natürlichen Carotinoidbiosynthese und der Präsens des MVA-Intermediates Acetoacetyl-CoA im Primärstoffwechsel als MO für die Produktion von Isoprenoiden aus der alternativen C-Quelle Methanol an. In Manuskript 4.5 wird die erfolgreiche Etablierung der *de novo* Synthese des Sesquiterpens α-Humulen beschrieben, basierend auf der heterologen Expression der α-Humulen-Synthase ZSSI, einer FPPS aus *S. cerevisiae* und eines prokaryontischen MVA.

Die Tatsache, dass die Expression der α-Humulen-Synthase ZSSI ohne zusätzliche Expression einer FPPS oder eines MVAs bereits zu Produktkonzentrationen von 10 mg/l führen zeigt, dass *M. extorquens* einen hohen natürlichen FPP-Pool bzw. einen hohen metabolischen Fluss zu diesem Prenyldiphosphat hat. Die Expression einer Geraniol-Synthase und GPP-Synthase führte außerdem zur Synthese von bis zu 1 mg/l des Monoterpens Geraniol (Daten in 4.5 nicht gezeigt). *M. extorquens* scheint sich daher nicht nur für die Produktion von Sesquiterpenen zu eignen, sondern auch für die Produktion von Mono- und höchstwahrscheinlich auch Triterpenen (C30-Grundgerüst). Ob *M. extorquens* natürlicherweise auch GGPP bildet ist unklar, da theoretisch für die Biosynthese von Menaquinon, Hopanen und seinen C30-Carotinoiden (Sandmann, unveröffentlicht) lediglich FPP als Vorstufe benötigt wird. Dennoch ist u.a. für Stamm AM1 eine GGPPS im Genom (MexAM1_META1p2924 (Vuilleumier 2009)) annotiert. Mit Hilfe der nachweislich aktiven Expression einer Diterpensynthase und entsprechender Produktanalytik könnte überprüft werden, ob die GGPPS wirklich exprimiert wird bzw. GGPP von *M. extorquens* gebildet wird. Dabei ist jedoch zu beachten, dass im Falle sehr niedriger GGPP-Konzentrationen und schlechter Expression der Diterpensynthase kein Produkt analysierbar sein könnte, obwohl GGPP vorhanden ist. Eine LCMS-basierte Analyse der Prenyldiphosphate könnte in diesem Fall helfen.

In Manuskript 4.5 wird die erstmalige biotechnologische Synthese eines Terpens aus der alternativen Kohlenstoffquelle Methanol beschrieben. Wie bereits in Abschnitt 2.5 diskutiert, beruht ein Großteil bisher veröffentlichter biotechnologischer Terpenproduktionen auf der Umsetzung von Glukose als Substrat. Bisher beschriebene biotechnologische Isoprenoidsynthesen aus alternativen C-Quellen (siehe Tab. 1) sind in Tabelle 2 aufgeführt.

Tabelle 2: Vergleich von Prozessparametern unterschiedlicher biotechnologischer Terpensynthesen aus alternativen Kohlenstoffquellen. Die maximalen Titer wurden, wenn nicht anders gekennzeichnet, im Fermenter erreicht. $Y_{P/S}$: Produktausbeute pro Substrat; $Y_{P/S}/Y_{P/S}max$: Prozentualer Anteil der erreichten Ausbeute an der maximal möglichen theoretischen Ausbeute; k.A.: keine Angaben

Produkt	Substrat	MO	max. Titer [g/l]	$Y_{P/S}$ [g/g] ([mol/mol])	$Y_{P/S}/Y_{P/S}max$ [%]	Referenz
α-Humulen (C15)	Methanol	*M. extorquens*	1,65	0,031 (0,005)	12	Diese Arbeit
Amorphadien (C15)	Ethanol	*S. cerevisiae*	37	0,053 (0,012)	28[a]	Westfall (2012)
Farnesol (C15)	Glycerin	*E. coli*	0,135*	0,01 (0,044)	k.A.	Wang (2010)
Taxadien (C20)	Glycerin	*E. coli*	1	0,025 (0,085)	7,6[a]	Ajikumar (2010)
β-Carotin (C40)	Glycerin	*E. coli*	0,476*	0,019 (0,003)	k.A.	Yoon (2009)
Limonen (C10)	CO_2	*Synechococcus* sp. PCC7002	0,004*	k.A.	k.A.	Davies (2014)
Limonen (C10)	CO_2	*Anabeana* sp. PCC7020	0,003*	k.A.	k.A.	Halfmann (2014b)
α-Bisabolen (C15)	CO_2	*Synechococcus* sp. PCC7002	0,0006*	k.A.	k.A.	Davies (2014)
Farnesen (C10)	CO_2	*Anabeana* sp. PCC7020	0,003*	k.A.	k.A.	Halfmann (2014a)

*im Schüttelkolben

[a] umgerechneter Anteil der IPP-Ausbeute an der maximal möglichen IPP-Ausbeute. Bestimmt anhand einer *in silico* Analyse nach Gruchattka (2013)

Den Daten aus Tabelle 2 ist zu entnehmen, dass der erreichte Produkttiter und die Ausbeute der α-Humulenproduktion aus Methanol mit *M. extorquens* die meisten

bisher publizierten Werte für Isoprenoidsynthesen aus alternativen C-Quellen übertreffen bzw. in einer ähnlichen Größenordnung liegen (Ausnahme: Amorphadien aus Ethanol). Das ist vor allem im Vergleich zu Glycerin-basierten Prozessen bemerkenswert, dessen Nutzung als alternatives Substrat durchaus kritisch zu betrachten ist (Abfallprodukt der subventionierten Biodieselproduktion, die mit der Nahrungsmittelproduktion konkurriert, siehe Abschnitt 2.1). Des Weiteren sind die Titer und Ausbeuten der auf der Umsetzung von CO_2 basierten Terpensynthesen mit Cyanobakterien um mehr als das 100-fache geringer, als die in dieser Arbeit etablierte α-Humulenproduktion aus Methanol. Auch wenn die Verwertung von CO_2 als der wohl nachhaltigste Prozess angesehen werden kann (siehe 2.1), so machen doch die äußerst geringen Ausbeuten und finalen Produktkonzentrationen einen solchen Prozess derzeit aus wirtschaftlicher Sicht uninteressant. Lediglich die von Westfall (2012) beschriebene Ausbeute und Produktkonzentration der Amorphadiensynthese aus Ethanol mit *S. cerevisiae* übertrifft die Werte dieser Arbeit deutlich.

5.3.1 Optimierung des Mevalonatweges für eine erhöhte Bereitstellung des Terpengrundbausteins IPP

Für die weitere Verbesserung der Produktivität der Terpensynthese mit *M. extorquens* ist mit hoher Wahrscheinlichkeit eine Optimierung des MVAs bzw. des metabolischen Flusses durch diesen Stoffwechselweg nötig. Einen Hinweis darauf gibt die Tatsache, dass die Humulenproduktion durch den Einsatz des in der Carotinoidbiosynthese inhibierten Stammes CM502 um 30 % gesteigert werden konnte. Durch die verringerte Synthese der natürlichen Carotinoide aus FPP in diesem Stamm steht für die Humulensynthese vermutlich mehr von dieser direkten Vorstufe bereit (siehe 4.5). Es ist daher anzunehmen, dass durch eine erhöhte Produktion von IPP bzw. FPP auch höhere α-Humulenkonzentrationen bzw. Ausbeuten erreicht werden können.

Der MVA besteht aus 7 Reaktionen (siehe Abb. 7), wobei die Kondensation zweier Acetyl-CoA Moleküle zu Acetoacetyl-CoA in *M. extorquens* bereits im EMCP vorhanden ist (siehe 2.2.2). Dementsprechend wurden nur die 6 Gene *hmgs*, *hmgr*, *mvaK*, *mvaD*,

mvaK2, *mvaD* und *fni* heterolog mit dem Plasmidsystem pQ2148 (Kaczmarczyk 2013) exprimiert (siehe Abb. 11).

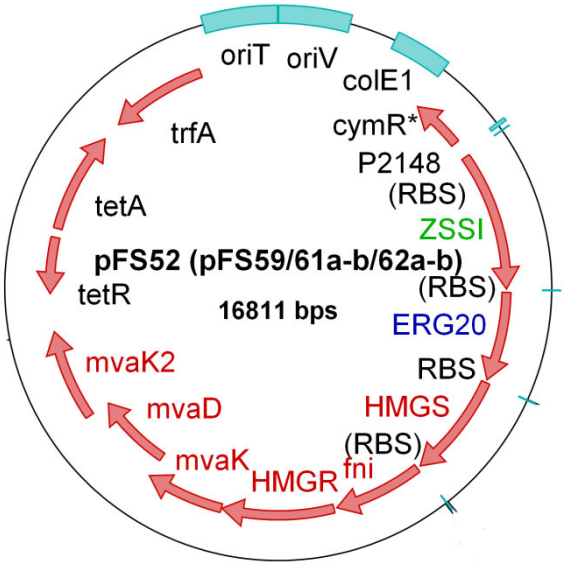


Abbildung 11: Plasmidkarte von pFS52, 59, 61a/b und pFS62a/b aus Fig. S1 des Manuskriptes 4.5 (Plasmide unterscheiden sich nur durch verschiedene TIR der RBS der *zssI*, ERG20, *hmgs* und *fni*). Dargestellt ist die Anordnung der heterolog exprimierten Gene α-Humulen-Synthase (ZSSI), FPPS (ERG20) und die des MVA aus *Myxococcus xanthus* (HMGS: 3-Hydroxymethylglutaryl-CoA-Synthase, HMGR: 3-Hydroxymethylglutaryl-CoA-Reduktase, fni: IPP-Isomerase, mvaK: Mevalonat-Kinase, mvaD: Pyrophosphomevalonat-Decarboxylase, mvaK2: Phosphomevalonat-Kinase).

Durch die externe Zugabe des MVA-Intermediates Mevalonat konnte keine weitere Steigerung der Humulensynthese erreicht werden (siehe 4.5), was ein starkes Indiz dafür ist, dass die Mevalonatsynthese durch die HMGS und HMGR nicht limitierend für die Terpenproduktion ist. Folglich liegt die Limitierung in der Synthese des IPPs und damit auch des α-Humulens vermutlich in der Reaktion vom Mevalonat zum IPP, d.h. im oberen MVA, katalysiert durch die MvaK, MvaD und MvaK2 (siehe Abb. 11). Eine Limitierung des MVA-Flusses durch die beiden Kinasen MvaK und MvaK2 wurde auch in vergleichbaren Arbeiten mit *E. coli* bei der heterologen Expression eines MVAs aus *S. cerevisiae* festgestellt (Anthony 2009; Redding-Johanson 2011).

Mit Hilfe von Metabolomics, d.h. Konzentrationsmessungen der MVA-Intermediate, müsste zunächst untersucht werden, ob und welche der vermuteten Zwischenprodukte akkumulieren. Im Falle einer Akkumulation könnte durch Transkriptomics, Proteomics und Aktivitätsmessungen des entsprechenden katalysierenden Enzyms festgestellt werden, ob dessen Aktivität im Vergleich zu den

anderen Schritten zu gering ist und ob dies eine Folge geringer Transkription oder Translation ist. Je nach Ergebnis könnte die Transkription durch das Einbringen eines zusätzlichen Promotors, z.B. vor der *mvaK* (siehe Abb. 11), oder die Translation durch optimierte RBS vor den einzelnen Gene optimiert werden. Durch die Überlappung der Gene *mvaK*, *mvaD* und *mvaK2* (siehe Abb. 11), liegt die Vermutung nahe, dass deren Translationsinitiation aufgrund der nicht vorhandenen RBS (siehe auch Tab. 2 in 4.5) mangelhaft ist. Außerdem verfügen die beiden Kinasegene ein GTG anstatt eines von *M. extorquens* weit häufiger verwendeten ATG als Startcodon (77,4 zu 16,8 %, analysiert mit der Software Indidon (Villegas und Kropinski 2008)), was ebenfalls mit einer schlechten Translation verbunden sein kann.

Insgesamt ist es wahrscheinlich, dass durch eine Optimierung des Flusses durch den MVA noch eine deutliche Steigerung der Terpenproduktion mit *M. extorquens* möglich ist. So führte die Expression desselben MVAs aus *M. xanthus* in *Pseudomonas putida* zu einer 7-fachen Steigerung der Produktion des Monoterpens Geraniumsäure (Mi 2014), in *M. extorquens* jedoch nur zu einer dreifachen Steigerung der Humulensynthese. Die Ursache dafür ist vermutlich, neben der unterschiedlichen Verfügbarkeit der Prenyldiphosphate in diesen Organismen, auch die Expression der MVA-Gene. Zu beachten ist allerdings, dass die Produktion unterschiedlicher Terpene, noch dazu aus anderen Vorstufen, nicht vollständig vergleichbar ist.

5.3.2 Besonderheiten des Stoffwechsels von *M. extorquens* für die Terpenbiosynthese

Nur im Falle einer erfolgreichen Entlimitierung des Flusses durch den MVA zur Synthese der Isoprenoidvorstufe IPP könnte die erwähnte Präsenz des MVA-Intermediates Acetoacetyl-CoA im Primärstoffwechsel von *M. extorquens* als Vorteil voll zur Geltung kommen. Denn nur dann würde sich der im Vergleich zu *E. coli* oder *S. cerevisiae* vermutlich höhere Fluss zu Acetoacetyl-CoA positiv auf die Produktion von Terpenen auswirken. Mögliche Schritte für eine Flussoptimierung durch den MVA wurden im vorherigen Abschnitt 5.3.1 diskutiert.

Eine Besonderheit des Stoffwechsels von *M. extorquens* hinsichtlich der Terpenbiosynthese ist dessen natürliche Biosynthese von C30-Carotinoiden

(Sandmann, unveröffentlicht) und Hopanoiden (Bradley 2010). Die Synthese beider Substanzklassen basiert auf der Umsetzung von FPP (siehe Abb. 12), das auch die Vorstufe für die Sesquiterpensynthese ist. Dadurch steht die Hopanoid- und Carotinoidbiosynthese in direkter Konkurrenz zu einer Terpenproduktion in *M. extorquens*. Das bestätigt die erhöhte Produktion von α-Humulen der „weißen" Mutante CM502, deren Defekt in der Carotinoidbiosynthese mit hoher Wahrscheinlichkeit aus der Deletion der Diapolycopin-Oxidase *crtNb* resultiert (siehe Manuskript 4.5). Interessanterweise führte die Verwendung eines Δ*crtN*-(Diapophytoen-Desaturase) Stammes (Horst 2012) nicht zu einer erhöhten α-Humulenproduktion, im Gegensatz zu Stamm CM502 im Vergleich zum *M. extorquens* Wildtyp (Daten nicht in 4.5 gezeigt). Stamm Δ*crtN* zeigte im Vergleich zu CM502 ein starkes Wachstumsdefizit mit dem Humulenproduktionsplasmid pFS62b, was sich vermutlich nachteilig auf die wachstumsgekoppelte Terpensynthese auswirkt. Wohlmöglich verringert die Präsenz der Antioxidantien Diapolycopin und Diaponeurosporen in Stamm CM502, die in Stamm Δ*crtN* nicht gebildet werden, oxidativen Stress beim Wachstum auf Methanol und führt dadurch zu höheren Wachstumsraten.

Die Auswirkung einer kompletten Deletion der Carotinoidbiosynthese in *M. extorquens*, d.h. die Verhinderung der Diapophytoensynthese (siehe Abb. 12), konnte bisher nicht untersucht werden, da der genaue Stoffwechsel bzw. die spezifische Enzymreaktion bisher nicht aufgeklärt ist.

Wie in Abb. 12 dargestellt, könnte Diapophytoen direkt aus FPP durch eine Diapophytoensynthase synthetisiert werden, wie es u.a. für *Staphylococcus aureus* bekannt ist (Raisig und Sandmann 2001). Möglich ist auch eine Überschneidung mit der Hopanoidbiosynthese, welche über Squalen und Didehydrosqualen (analoge Bezeichnung zu Diapophytoen) erfolgt und u.a. in *Methylomonas* sp. 16 vermutet wird (Tao 2005). Die Tatsache, dass die Deletion eines *crtM*-Homologs keine phänotypischen Auswirkung zeigte, die Erstellung einer lebensfähigen *hpnC*-Mutante jedoch nicht möglich war (Horst 2012), deutet aber darauf hin, dass auch in *M. extorquens* AM1 die Diapophytoensynthese und die damit verbundene Carotinoidbiosynthese von der Vorstufe Squalen ausgeht. Dafür spricht auch der

„weiße“ Phänotyp einer *M. extorquens* PA1-Mutante mit deletierter Squalen-Zyklase (Nayak und Marx 2014b), wobei in dieser Studie die Carotinoidzusammensetzung der Mutante nicht näher untersucht wurde.

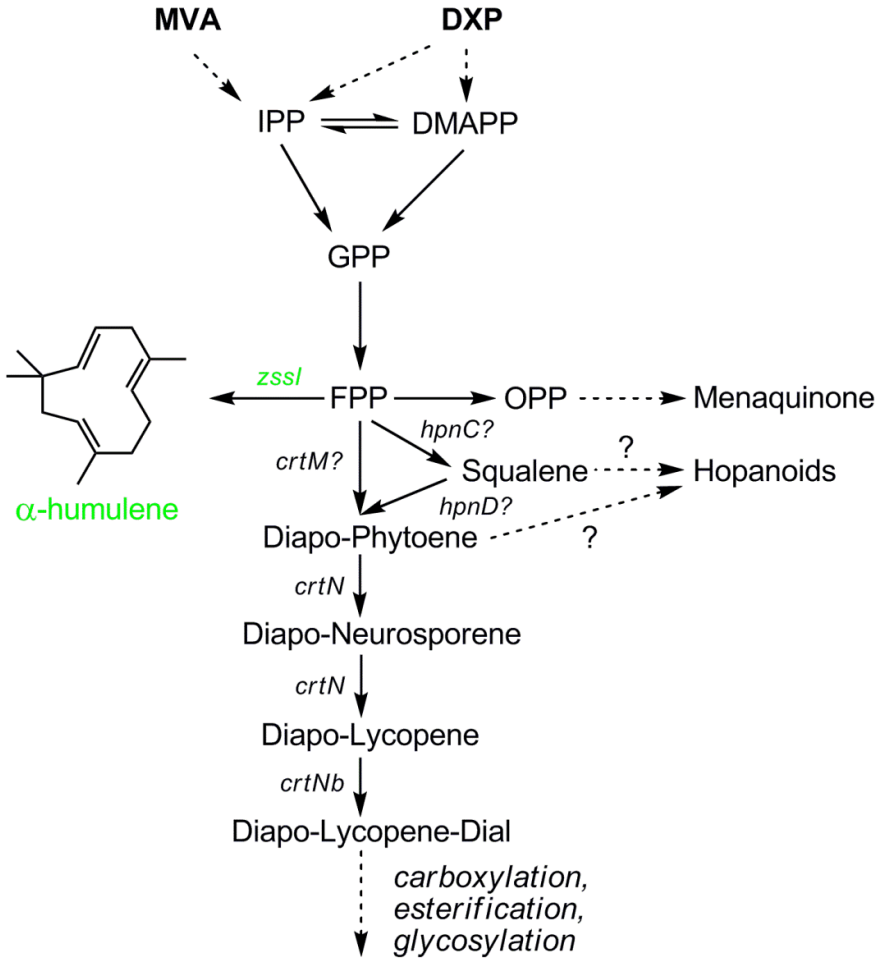


Abbildung 12: Vermutete Biosynthese von C30-Carotinoiden und Hopanoiden, sowie Menaquinon und α-Humulen aus FPP in *M. extorquens* mit Plasmid pFS62b. *crtM*: Diapophytoen-Synthase, *crtN*: Diapophytoen-Reduktase, *crtNb*: Diapolycopin-Oxidase, *hpnC*: Squalen-Zyklase, *hpnD*: Squalen-Reduktase. Modifizierte Abbildung 1 aus Manuskript 4.5.

Theoretisch wäre durch die komplette Entfernung der Carotinoid- und/ oder Hopanoidbiosynthese in *M. extorquens* eine weitere Steigerung der Terpenproduktion möglich. Die schweren Wachstumsdefekte der Δ*crtN*-Mutante von *M. extorquens* AM1 und der Δ*hpnC*-Mutante von Stamm PA1 lassen jedoch an einer Verwendbarkeit dieser Stämme in biotechnologischen Prozessen Zweifel aufkommen.

Die anscheinend ubiquitäre Verbreitung von Hopanoiden in methylotrophen Bakterien lässt vermuten, dass diese für das Wachstum auf Methanol unverzichtbar sind.

Ursächlich dafür könnte die Einkapselung von Formaldehyd bzw. dessen Auswirkung auf die Zellmembran sein (Bradley, unveröffentlicht und persönlicher Kommentar von S. Veuilleumiér). Auch Carotinoide scheinen für das Wachstum von *M. extorquens* auf Methanol notwendig zu sein. Die an der Metabolisierung von Methanol zahlreich beteiligten Oxidationsreaktionen (siehe Abb. 4) führen zwangsweise auch zur Bildung von reaktiven Sauerstoffradikalen, für deren Abfangen die Carotinoide unverzichtbar sein könnten.

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7. Anhang

7.1 Manuskripte

7.1.1 Thioesterases for ethylmalonyl-CoA pathway derived dicarboxylic acid production in Methylobacterium extorquens AM1

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(3) Erstellung der Daten-sammlung und Abbildungen	Abbildungen 1,3 Abbildung 2 Tabellen 1-4	Sonntag F. Sonntag F., Buchhaupt M. Sonntag F.
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Thioesterases for ethylmalonyl–CoA pathway derived dicarboxylic acid production in *Methylobacterium extorquens* AM1

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Abstract The ethylmalonyl–coenzyme A pathway (EMCP) is a recently discovered pathway present in diverse α-proteobacteria such as the well studied methylotroph *Methylobacterium extorquens* AM1. Its glyoxylate regeneration function is obligatory during growth on C1 carbon sources like methanol. The EMCP contains special CoA esters, of which dicarboxylic acid derivatives are of high interest as building blocks for chemical industry. The possible production of dicarboxylic acids out of the alternative, non-food competing C-source methanol could lead to sustainable and economic processes. In this work we present a testing of functional thioesterases being active towards the EMCP CoA esters including in vitro enzymatic assays and in vivo acid production. Five thioesterases including TesB from *Escherichia coli* and *M. extorquens*, YciA from *E. coli*, Bch from *Bacillus subtilis* and Acot4 from *Mus musculus* showed activity towards EMCP CoA esters in vitro at which YciA was most active. Expressing *yciA* in *M. extorquens* AM1 led to release of 70 mg/l mesaconic and 60 mg/l methylsuccinic acid into culture supernatant during exponential growth phase. Our data demonstrates the biotechnological applicability of the thioesterase YciA and the possibility of EMCP dicarboxylic acid production from methanol using *M. extorquens* AM1.

Keywords Methylobacterium extorquens · Thioesterases · Ethylmalonyl–CoA pathway · YciA · Dicarboxylic acid · Coenzyme A

Introduction

Dicarboxylic acids are important building blocks for chemical and pharma industry and can also be used as chelators or food additives (Lee et al. 2002; Werpy and Petersen 2004). Many production processes are still based on chemical synthesis mainly due to economical reasons (Kircher 2006). Nevertheless, substantial progress has been made in the last years for bio-based production of dicarboxylic acids focused on succinic acid (Sauer et al. 2008; Thakker et al. 2012) and adipic acid (Polen et al. 2013). The allocation of new and uncommon dicarboxylic acids by bio-based production processes would provide a variety of sustainable monomers for chemical industry (e.g., novel bioplastics).

The recently elucidated ethylmalonyl–CoA pathway (EMCP) (Erb et al. 2007; Erb et al. 2009; Peyraud et al. 2009) contains several enantiomeric, branched and satured or unsatured carboxylated C4– and C5-acyl-CoA esters (see Fig. 1). Their corresponding dicarboxylic acids ethylmalonic acid, (2*S*)-methylsuccinic acid, mesaconic acid, (2*R*,3*S*)-methylmalic acid and methylmalonic acid are especially interesting as novel synthons being commercially unavailable in bulk quantities to date (Alber 2011). Ethylmalonic acid as well as (2*S*)-methylsuccinic acid are particular interesting because of the unique existence of their corresponding CoA esters in the EMCP. Ethylmalonic acid can be used as co-crystallization additive (Aitipamula et al. 2010) and it has been patented, as well as methylsuccinic acid, as monomer components for various polymers in application for coatings and solvents for cosmetics (Hu and Bailey 1999; Loos et al. 2012; Muller and Richard 2012). Especially the application of 2-methylsuccinate as monomer for cosmetic solvents (Muller and Richard 2012) turns it to an attractive target for biotechnological production of a compound labeled as natural. Mesaconic acid, which also occurs in the mesaconate pathway for L-glutamate fermentation (Kato and Asano 1997), is used as fire retardant (Di Giulio and Bauer 1982).

The EMCP is present in some bacteria such as the methylotrophic model organism *Methylobacterium extorquens* AM1 (Peyraud et al. 2009) offering the possibility to synthesize the novel acids from methanol as a sustainably

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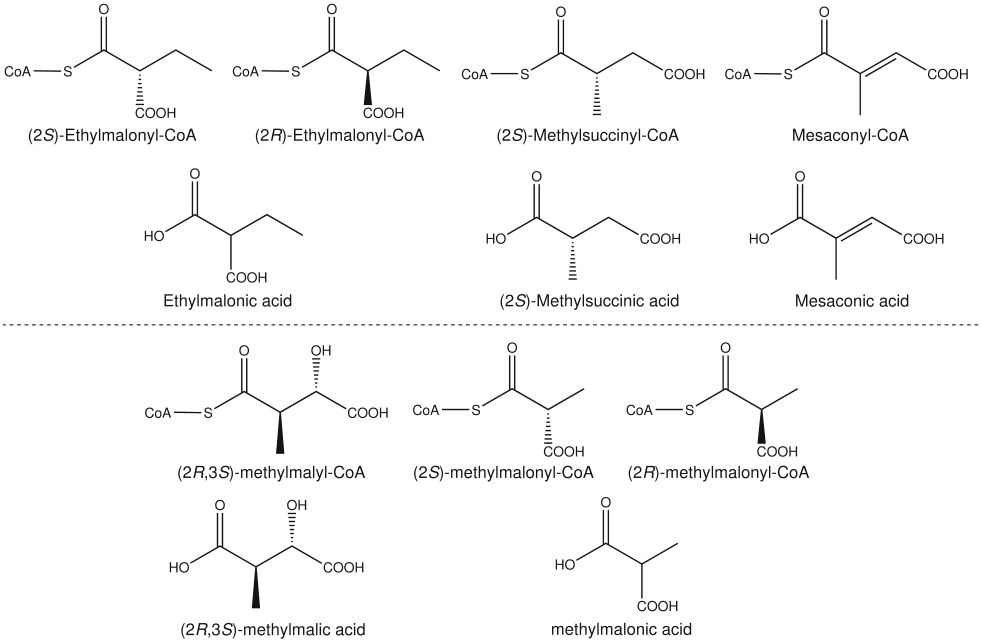

Fig. 1 Selected dicarboxyl-CoA esters of the ethylmalonyl–CoA pathway. Their corresponding dicarboxylic esters after cleavage of the coenzyme A are displayed underneath

producible, non-food competitive substrate (Schrader et al. 2009). The fact of low activity of most EMCP enzymes during growth on C3 or C4 carbon sources (Skovran et al. 2010; Šmejkalová et al. 2010) emphasizes the use of methanol as a substrate.

The presence of the dicarboxylic acids as CoA esters (see Fig. 1) requires a hydrolyzation of their thiol groups, which can be performed by CoA-transferases or thioesterases. The latter do not need an additional donor/acceptor molecule which lowers the chance of adding metabolic imbalances. However, to our knowledge thioesterases being specifically active for EMCP CoA esters have not been described to date. The number of characterized thioesterases is steadily increasing and their classification was excellently reviewed recently (Cantu et al. 2011; Cantu et al. 2010). Unfortunately, most members of the families TE1–TE13, which are acyl-CoA hydrolases, have a rather broad substrate spectrum or are only active on C10– or longer acyl-CoAs (Cantu et al. 2010). Furthermore, the HotDog-fold acyl-CoA thioesterases of mentioned families lack conserved catalytic residues and binding pockets (Zhuang et al. 2008) making an in silico prediction of enzyme activity for the EMCP CoA esters unfeasible.

In this article, we present the identification of thioesterase candidates probably active on EMCP-CoA esters by a comprehensive literature research followed by in vitro enzyme activity analysis of the selected enzymes. *M. extorquens* AM1 expressing suitable candidates were tested for the release of EMCP-related dicarboxylic acids. For the first time, in vivo production of dicarboxylic acids derived from the EMCP with a methylotrophic bacterium growing on methanol is described.

Materials and methods

Chemicals, bacterial strains and growth conditions

M. extorquens AM1 (Peel and Quayle 1961) was grown at 30 °C in minimal medium with increased cobalt concentration of 12.5 μM as described before (Kiefer et al. 2009) containing 123 mM methanol or 31 mM succinate. Utilization of TCA and EMCP-related dicarboxylic acids as sole carbon source was tested in equivalent medium containing 2 mM of a single acid instead of methanol. *Escherichia coli* strains DH5α (Gibco-BRL, Rockville, MD, USA) and BL21(DE3) (Novagen, Madison, WI, USA) were grown in lysogeny broth (LB) medium (Bertani 1951) at 37 °C. Kanamycin was used at concentrations of 30 μg/ml for *E. coli* and 50 μg/ml for *M. extorquens*. Cumate (4-isopropylbenzoic acid) was used as inducer at a final concentration of 5 μg/ml diluted from 1 mg/ml 50:50 methanol–water stock solution.

The dicarboxylic acids ethylmalonic acid, methylmalonic acid, racemic (*rac*-) 2-methylsuccinic acid and mesaconic acid as well as palmitoyl-, decanoyl-, *rac*-methylmalonyl-CoA and succinyl-CoA and cumate were purchased from Sigma-Aldrich (Steinheim, Germany) in their highest available degree of purity. *Rac*-beta-methylmalic acid was synthesized by Otava (Kiev, Ukraine). Coenzyme A was purchased by Chemos (Regenstauf, Germany).

Genetic manipulations and plasmid construction

Polymerase chain reactions (PCR) were performed using High-Fidelity PCR Master and amplified DNA was purified by PCR purification Kit (both from Roche, Basel, Switzerland). Restriction enzymes and T4 ligase were purchased from New England Biolabs (Beverly, USA). Plasmid DNA was purified from *E. coli* by Roti Prep Plasmid MINI Kit (Carl-Roth, Karlsruhe, Germany). Primers were purchased from Sigma-Aldrich. Confirmation of nucleotide sequences was performed by SRD (Bad Homburg, Germany).

Genomic DNA from *E. coli* DH5α and *M. extorquens* AM1 (DSM1338) was purified using GenElute Bacterial Genomic DNA Kit from Sigma-Aldrich. Genomic DNA of *Azoarcus evansii* (DSM6898) and *Bacillus cereus* (DSM31) was purchased from DSMZ (Braunschweig, Germany). *Mus musculus* acyl-CoA thioesterase 4 (acot4) cDNA (Unigene ID Mm.482217) was ordered from Source Bioscience (Nottingham, UK).

All standard cloning techniques were carried out as described before (Sambrook and Russell 2001). Transformation of plasmids into *M. extorquens* was performed as described by Toyama et al. (1998).

Genes encoding the acyl-CoA thioesterase B from *E. coli* DH5α (*tesB*; NP_414986) and *M. extorquens* AM1 (YP002963160), acyl-CoA thioesterase 4 from *Mus musculus* (*acot4*; NP599008), *paaI* from *Azoarcus evansii* (AAG28967), acyl-CoA thioester hydrolase from *E. coli* (*yciA*; YP_002270185) and 3-hydroxyisobutyryl-CoA hydrolase from *Bacillus cereus* (*bch*; NP_832055) were amplified from their genomic DNA using the corresponding primers listed in Table 1. Resulting PCR products were digested with *Nde*I+*Eco*RI and cloned into pET28a(+) for expression in *E. coli* or digested with *Kpn*I+*Eco*RI and cloned into pCM160 for expression in *M. extorquens* (Table 1).

The ribosome binding site of pCM160-thioesterase constructs was optimized using the RBS calculator (Salis 2011). The optimized sequence was introduced by ligation of previously annealed primers RBS_pCM160-fw and RBS-pCM160-rev (Table 1) into *Sph*I+*Kpn*I restricted pCM160-thioesterase vectors.

For inducible expression of thioesterase B from *E. coli* in *M. extorquens*, *tesB* gene from pCM160-*tesBec* was cut out with *Sph*I+*Eco*RI and ligated into equally digested pLC290 (Chubiz et al. 2013).

Protein purification and quantification

TesB from *E. coli* (TesBec) and *M. extorquens* (TesBext), Acot4, PaaI, YciA and Bch were produced heterologously by transformation of each pET28a(+)-thioesterase construct (Table 1) into *E. coli* BL21. Cells were grown in 100 ml LB-medium with 30 μg/ml kanamycin to an OD_{600} of 0.5–0.7. IPTG induction was performed for 16 h at the following concentrations and temperatures: 0.5 mM at 16 °C for TesBec and Bch, 0.4 mM at 16 °C for TesBext and YciA, 0.6 mM at 22 °C for PaaI and 0.1 mM at 22 °C for Acot4. Cells were harvested by centrifugation, washed twice with 50 mM phosphate buffer pH 7.5 and disrupted by sonification (0.5 s pulse, 1 s break for 12 min by an amplitude of 20 %). Cell lysate was centrifuged for 20 min at 15,000×*g* and 4 °C. The supernatant was applied to a Ni-sepharose matrix in a Poly-Prep column (Bio-Rad) that had been equilibrated with 10 bed volumes of buffer A containing 50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole at a pH of 8. The column was washed twice with 4 bed volumes of buffer A containing 20 mM imidazole. Finally, thioesterase was eluted with buffer A containing 250 mM imidazole.

Imidazole was removed and protein concentrated by application of Amicon spin columns (Millipore) at appropriate MWCOs. Enzyme was stored at 4 °C overnight.

Protein was analyzed via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for purity with the help of PageRuler Plus Prestrained Protein Ladder (Fermentas). Enzyme concentration was measured with Pierce BCA Protein Assay Kit (Thermo Scientific).

Synthesis of CoA esters

CoA esters of the EMCP were synthesized from their corresponding acid (see chemicals) via its anhydride as described by Stadtman (1957). The resulting CoA esters have the following conformation: ethylmalonyl-CoA as enantiomeric mixture of (2*R*)- and (2*S*)-ethylmalonyl-CoA (Erb et al. 2007), methylsuccinyl-CoA as a mixture of (2*R*)-(2*S*)-(3*R*)- and (3*S*)-methylsuccinyl-CoA (Erb et al. 2009) and mesaconyl-CoA as mixture of structural isomers mesaconyl-C1-CoA and mesaconyl-C4-CoA. Due to lack of references, methylmalyl-CoA was assumed to be a diastereo-/enantiomeric mixture of (2*R*/3*S*)(2*S*/3*R*)(2*R*/3*R*)(2*S*/3*S*)-beta-methylmalyl-CoA and (2*R*/3*S*)(2*S*/3*R*)(2*R*/3*R*)(2*S*/3*S*)-gamma-methylmalyl-CoA. Product formation of the CoA esters was confirmed by HPLC analysis (Erb et al. 2009). Full conversion was checked by undetectable DTNB absorption indicating fully converted free coenzyme A.

DTNB assay for CoA-ester cleavage

DTNB assay for thioesterase activity measurements was based on a modified protocol of previously described assays from Zheng et al. (2004), Cho and Cronan (1993) and Zhuang et al. (2008). Reaction mixture contained final concentrations of 10 mM HEPES pH 7.5 (25 °C), 150 mM KCL, 10 mM

Table 1 Primers, plasmids and strains used in this work. Underlined sequences (all 5′–3′) indicate restriction enzyme recognition sites

Name	Description	Reference
Primers		
tesBext_pET_fw	ACTCATATGATGCCCGACCCCGTCGATGC	this study
tesBcoli_pET_fw	ACTCATATGATGAGTCAGGCGCTAAAAAA	this study
ACOT4_pET_fw	ACTCATATGATGGCAGCGACACTGAGCG	this study
PaaI_pet_fw	ACTCATATGATGACTGAGGCGGGCTATCG	this study
YciA_pET_fw	ACTCATATGATGTCTACAACACATAACGTC	this study
bch_pET28_fw	ACTCATATGATGACTGAACAAGTTTTATTTTC	this study
tesBext_rev	ACGTGAATTCTCAGCTGCGCCGGGAGCGG	this study
tesBcoli_rev	ACGTGAATTCTTAATTGTGATTACGCATCAC	this study
ACOT4_rev	ACGTGAATTCCTACAGTCTACAGGAGGCTC	this study
PaaI_rev	ACGTGAATTCTCACAACTCGACGACCGCC	this study
YCiA_rev	ACGTGAATTCTTACTCAACAGGTAAGGCG	this study
bch_rev	ACGTGAATTCTTATGCATTAAGTAAGTTAAAG	this study
tesBext_pCM_fw	ACTGGTACCATGCCCGACCCCGTCGATGC	this study
tesBcoli_pCM_fw	ACTGGTACCATGAGTCAGGCGCTAAAAAA	this study
ACOT4_pCM_fw	ACTGGTACCATGGCAGCGACACTGAGCG	this study
PaaI_pCM_fw	ACTGGTACCATGACTGAGGCGGGCTATCG	this study
YciA_pCM_fw	ACTGGTACCATGTCTACAACACATAACGTC	this study
bch_pCM160_fw	ACTGGTACCATGACTGAACAAGTTTTATTTTC	this study
RBS_pCM160-fw	CGCGACGGTCTCGTAAAAAGGAAGGAGGTATTAAGGTAC	this study
RBS_pCM160-rev	CTTAATACCTCCTTCCTTTTTACGAGACCGTCGCGCATG	this study
Plasmids		
pET28a(+)	Inducible expression vector for *E. coli*; pT7, Kan^R, pBR322ori, His-Tag	Novagen
pCM160	constitutive expression vector for *Methylobacterium extorquens*; Kan^R, pmxaF, oriT, pBR322ori	Marx and Lidstrom 2001
pLC290	Cumate inducible expression vector for *M. extorquens*; ; Kan^R, oriT, pBR322ori, ttrnB	Chubiz 2013
pET28-*tesBext*	pET28 containing thioesterase B gene from *M. extorquens*	this study
pET28-*tesBec*	pET28 containing thioesterase B gene from *E. coli*	this study
pET28-*yciA*	pET28 containing acyl-CoA thioesterase *yciA* gene from *E. coli*	this study
pET28-*acot4*	pET28 containing succinyl-CoA thioesterase gene from *Mus musculus*	this study
pET28-*paaI*	pET28 containing paaI gene from *Azoarcus evansii*	this study
pET28-*bch*	pET28 containing 3-hydroxyisobutyryl-CoA hydrolase gene from *Bacillus cereus*	this study
pCM160-*tesBext*	pCM160 containing thioesterase B gene from *M. extorquens*	this study
pCM160-*tesBec*	pCM160 containing thioesterase B gene from *E. coli*	this study
pCM160-*yciA*	pCM160 containing acyl-CoA thioesterase gene from *E. coli*	this study
pCM160-*acot4*	pCM160 containing succinyl-CoA thioesterase gene from *M. musculus*	this study
pCM160-*bch*	pET28 containing 3-hydroxyisobutyryl-CoA hydrolase gene from *B. cereus*	this study
pCM160-RBS-*yciA*	pCM160-yciA containing an optimized RBS	this study
pLC290-*tesBec*	pLC290 containing thioesterase B from *E. coli*	this study
Strains		
E. coli DH5alpha	F^-, Φ80dlacZΔM15, Δ(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(rK^- mK^+), phoA, supE44, λ^-, thi-1	ATCC
E. coli BL21(DE3)	F^- ompT gal dcm lon $hsdS_B(r_B^-$ $m_B^-)$ λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])	Novagen
M. extorquens AM1	Facultative methylotrophic, obligate aerobic, gram-negative, pink pigmented α-proteobacterium, Cm^R	Peel and Quayle 1961 DSM1338

KH_2PO_4, 0.1 mM DTNB, 10–200 μM CoA ester and 1.5 μM protein. The reaction was monitored for 5 min at 25 °C and 412 nm in 96-well microtiter plates using a microplate reader (TECAN, Switzerland).

Absorption slope was an average of three measurements. Specific enzyme activity was calculated by the division of the volumetric enzyme activity by the protein concentration determined as described above.

Analysis and quantification of dicarboxylic acids

First, 1 ml of culture broth was centrifuged for 3 min at 16,000×*g* for sedimentation of cells. Then, supernatant was passed through a 0.22-μm filter and analyzed on HPLC.

Samples were analyzed on SLC10-A system equipped with a SPD20A UV–Vis detector and a SPD10Avp PDA detector (all from Shimadzu) together with an Aminex HPX-87H column (length: 300 mm, diameter: 7.8 mm; Bio-Rad). Separation was performed isocratically for 30 min with a mobile phase consisting of 2 mM H_2SO_4 in MilliQ water. Column temperature was maintained at 30 °C. Elution was monitored continuously at 205 nm with the UV–Vis detector for quantification and at a range of 190–600 nm with PDA-detector for absorption spectra scanning. Ethylmalonic acid, methylmalonic acid, *rac*-2-methylsuccinic acid, *rac*-b-methylmalic acid and mesaconic acid were quantified with the help of calibration curves prepared by the use of their corresponding standards.

Results

Identification of thioesterase candidates acting on EMCP intermediates

Acyl-CoA thioesterases (families TE1–TE13) (Cantu et al. 2010) are most promising for investigation of their activity towards dicarboxyl-CoAs of the EMCP. Most described members of this group cleave off CoA of a broad range of acyl-CoAs with increasing activity by increasing C length. Only a few specific short-chain acyl-CoA thioesterases have been investigated but so far not for their activity towards the EMCP CoA esters. Two species of acyl-CoA thioesterases were chosen for in vitro analysis: "broad range" thioesterase cleaving CoA off acyl-chains from four to 16 or more carbon atoms and specific short-chain thioesterases preferring substrates with a related structure to the EMCP-CoA esters, i.e., C4–C5 (di)carboxyl-CoA esters.

Thioesterase II/B (TesB) of *E. coli* is one of the longest known and investigated broad-range thioesterase originally described in 1970 by Barnes and co-workers. It cleaves CoA off acyl moieties of 4–18 carbon atoms with highest activity for palmityl-CoA (Barnes et al. 1970; Naggert et al. 1991). In addition, it has already been used for biotechnological production of several other acids such as hydroxyvalerates, polyketides, 3-hydroxybutyric acid or 3-hydroxydecanoic acid (Chung et al. 2009; Liu et al. 2008; Martin and Prather 2009; Tseng and Prather 2012) and is therefore a promising candidate for cleaving CoA off the EMCP-CoA esters. A TesB homolog also exists in *M. extorquens* (Vuilleumier et al. 2009), which makes it the second candidate for EMCP-CoA activity investigation.

YciA is another broad range thioesterase which is active on acyl-CoAs with C2 (acetyl-CoA) to C18 moieties (oleyl-CoA) including substrates of the EMCP such as methylmalonyl-CoA and structural related compounds like isobutyryl-CoA or b-methylcrotonyl-CoA (Zhuang et al. 2008). Therefore, YciA of *E. coli* was chosen as another candidate.

The PaaI of *Azoarcus evansii* (Song et al. 2006) is mainly active towards aromatic CoA esters but also on short-chain acyl-CoAs such as methylmalonyl-CoA or isobutyryl-CoA and is therefore an additional candidate.

Succinyl-CoA thioesterase Acot4 from *Mus musculus* and 3-hydroxyisobutyryl-CoA hydrolase Bch from *Bacillus cereus* were chosen as candidates of the specific short-chain acyl-CoA thioesterase group. Both enzymes are active on substrates which show similarity to the EMCP-CoA esters. Acot4 (Westin et al. 2005) shows activity for succinyl- (C4) and glutaryl-CoA (C5) but not on acyl-CoAs with a shorter or longer carbon chain. Bch has been successfully used by Lee and co-workers (2008) for cleavage of the C4-carboxyl CoA-ester 3-hydroxyisobutyryl-CoA in *E. coli.*

The six selected thioesterases provide a starting point for in vitro enzyme analysis to identify possible EMCP CoA-ester hydrolyzation abilities.

Protein purification

The genes coding for TesB from *E. coli* and *M. extorquens*, YciA, Acot4, PaaI and Bch were cloned into pET28a(+) vector and resulting constructs transformed in *E. coli* BL21(DE3). Enzymes were heterologously expressed by IPTG induction and purified by the use of their N-terminal HIS-Tag. As shown in Fig. 2, expected bands of ~32 kDa for thioesterase B of *E. coli* and *M. extorquens*, ~15 kDa for YciA, ~50 kDa for Acot4, ~14 kDa for PaaI and ~36 kDa for Bch are visible for every enzyme after the final purification step. Strong bands at the expected sizes in all samples and only minor impurities in the TesBext, YciA and Acot4 sample allow enzymatic assay for substrate specificity and activity of the thioesterases in vitro.

Testing selected thioesterases for their activity towards EMCP-CoA esters by in vitro DTNB assay

Low hydrolyzation activities for short-chain acyl-CoAs (C6 or lower), mostly tested by CoA-cleavage of substrates having unbranched carbonyl chains like succinyl-CoA or hexanoyl-CoA, has been shown in the literature for all selected thioesterases except TesBext. The carbon chains of all

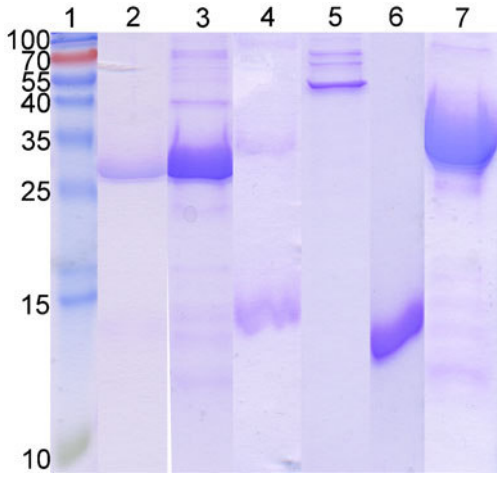

Fig. 2 Sections of several SDS-PAGE gels showing protein bands of thioesterases after final HIS-Tag purification and Amicon concentration. *Lane 1*: marker; *lane 2*: thioesterase B from *E. coli* (TesBec); *lane 3*: thioesterase B homologue from *M. extorquens* (TesBext); *lane 4*: bacterial acyl-CoA thioesterase from *E. coli* (YciA); *lane 5*: succinyl-CoA hydrolase from *M. musculus* (Acot4); *lane 6*: PaaI from *A. evansii*; *lane 7*: 3-hydroxyisobutyryl-CoA hydrolase from *B. cereus* (Bch)

EMCP CoA esters are branched with an additional unsaturated bond for mesaconyl-CoA or hydroxyl group for (2*R*,3*S*)-methylmalyl-CoA (see Fig. 1). This might have strong influence on acceptance by the thioesterases. Due to the only recent elucidation of the EMCP and the current unpurchasability of most of its CoA esters (exception: *rac*-methylmalonyl-CoA), none of them has been tested as a substrate for thioesterases yet. Therefore, the enzyme activities of the six purified thioesterases were measured for *rac*-methylmalonyl-, *rac*-ethylmalonyl-, *rac*-methylsuccinyl-, *rac*-methylmalyl-, mesaconyl- and succinyl-CoA as a control (HPLC data of synthesized CoA esters not shown).

Table 2 summarizes the determined specific activity and K_m values of all purified thioesterases for the different EMCP CoA esters. Although PaaI is active for *rac*-methylmalonyl-, *rac*-ethylmalonyl- and mesaconyl-CoA, its high K_m values make it unattractive for in vivo application. The acyl-CoA thioesterase YciA of *E. coli* shows the highest specific activities for the EMCP CoA esters (especially for mesaconyl-CoA). Thioesterase B of *M. extorquens* seems to have similar substrate preferences of C10 or longer acyl-CoAs as its *E. coli* homologue. In contrast, TesBext has distinct higher K_m values for the EMCP-CoA esters than TesBec at comparable activities. Although (3*S*)-isobutyryl-CoA hydrolase of *B. cereus* (Bch) shows activity for all EMCP CoA esters, the corresponding K_m values are very high with the exception for *rac*-ethylmalonyl-CoA.

We identified thioesterase YciA as an effective enzyme for EMCP CoA-ester hydrolyzation with v_{max} values up to 12.3 U/mg protein in vitro, making it a promising candidate for release of EMCP derived acids in vivo. Thioesterase B, Acot4 and Bch might also be suitable for the release of some EMCP related acids, especially methyl- and ethylmalonate.

Release of dicarboxylic acids by *M. extorquens* AM1 expressing thioesterases

Enzyme properties determined in vitro may vary from its in vivo characteristics due to protein folding, different reaction conditions, inhibitors and so on. To verify whether the identified thioesterase candidates are suitable for cleaving the EMCP CoA esters in vivo, their genes were cloned into pCM160 vector and transformed in *M. extorquens* cells, thereafter plated on methanol agar.

Colonies were obtained for *M. extorquens* transformed with pCM160-*tesBext*, -*yciA*, -*bch* and -*acot4* after 4 days but not for pCM160-*tesBec*. The strong constitutive, methanol inducible pmxaF promoter of pCM160 probably cause high TesBec production which seems to be lethal for *M. extorquens* growing on methanol. Therefore, pCM160-*tesBec* was transformed again and cells plated on minimal medium containing succinate as sole carbon source. On these plates colonies appeared after 4 days. Additionally, *tesBec* was cloned into pLC290 vector containing a cumate inducible promotor. Transformants containing the respective expression plasmid were able to grow on methanol medium.

Production of EMCP derived dicarboxylic acids and growth of *tesBext*-, *ycia*-, *acot4*- and *bch*-expressing *M. extorquens* was investigated by cultivation of cells harboring the pCM160-thioesterase constructs and the empty vector control in shake flasks, measuring OD_{600} and analyzing supernatants for dicarboxylic acids after certain time points. Growth characteristics of strains harboring pCM160-*acot4*, -*bch* and -*yciA* were similar to the empty vector control with μ_{max} values of about 0.12–0.14 per hour. AM1_pCM160-*tesBext* showed a reduced maximal growth rate of 0.09 per hour.

Profile of EMCP derived dicarboxylic acids in culture supernatant is not altered by overexpression of *bch* and *tesBext* compared to empty vector control considering the detection limit of 0.05 mg/l for mesaconate and 2 mg/l for the remaining acids. Mesaconate is released in small amounts by cells containing pCM160-*acot4* accumulating up to 1.5 mg/l after 30 h of cultivation, whereas other EMCP derived dicarboxylic acids were not detectable. In contrast, the supernatant of AM1_pCM160-*yciA* showed concentrations of up to 10 mg/l mesaconate and 2-methylsuccinate compared to AM1 harboring the empty vector where no 2-methylsuccinate and maximal amounts of 0.8 mg/l mesaconate were detectable (data not shown). To further raise acid release, the ribosome binding site of the pCM160_*yciA* construct was optimized for *M. extorquens* and the *yciA* gene.

Table 2 Specific activities (U/mg) and K_m values (μM) of different thioesterases for succinyl-CoA, decanoyl-CoA, palmitoyl-CoA and various CoA-ester intermediates of the ethylmalonyl-CoA pathway in their following conformation: methyl- and ethylmalonyl-CoA as (2R/2S)-enantiomeric mixtures; methylsuccinyl-CoA as mixture of (2*R*)-(2*S*)-(3*R*)- and (3*S*)-methylsuccinyl-CoA; mesaconyl-CoA as mixture of structural isomers mesaconyl-C1– and mesaconyl-C4-CoA and methylmalyl-CoA as diastereo-/enatiomeric mixture of (2*R*/3*S*)(2*S*/3*R*)(2*R*/3*R*)(2*S*/3*S*)-beta-methylmalyl-CoA and (2*R*/3*S*)(2*S*/3*R*)(2*R*/3*R*)(2*S*/3*S*)-gamma-methylmalyl-CoA

Substrate	TesBec	TesBext	Acot4	PaaI	YciA	Bch
Specific activity [U/mg]						
Succinyl-CoA	n.d.	3.5 (±0.13)	1.8 (±0.28) [3.98[b]]	n.d.	31.2 (±0.3)	n.d.
Methylmalonyl-CoA	8.8 (±1.23)	2.7 (±0.33)	0.5 (±0.08)	5.4 (±1.5) [2.9[c]]	10.6 (±3.5)	3.5 (±0.09)
Ethylmalonyl-CoA	3.6 (±0.54)	2.3 (±0.51)	0.2 (±0.02)	3.6 (±2.4)	6.2 (±0.1)	1 (±0.42)
Methylsuccinyl-CoA	0.5 (±0.24)	1.4 (±0.04)	0.3 (±0.04)	n.d.	7.2 (±0.48)	2.6 (±0.56)
Mesaconyl-CoA	2.2 (±0.43)	0.4 (±0.07)	0.2 (±0.01)	2.1 (±0.39)	12.3(±0.46)	0.6 (±0.2)
Methylmalyl-CoA	2.2 (±0.49)	1.9 (±0.29)	0.7 (±0.04)	n.d.	3.5 (±1.3)	1.3 (±0.03)
Decanoyl-CoA	n.m.	94.3 (±13)				
Palmitoyl-CoA	181 (±32) [93.8[a]]	112 (±2.4)				
K_m [μM]						
Succinyl-CoA	n.d.	118 (±4)	18 (±3) [13.3[b]]	n.d.	17 (±0.2)	n.d.
Methylmalonyl-CoA	98 (±13)	54 (±6)	23 (±4)	771 (±189) [1100[c]]	145 (±43)	199 (±5)
Ethylmalonyl-CoA	99 (±15)	112 (±22)	26 (±3)	1269(±724)	43 (±1)	43 (±12)
Methylsuccinyl-CoA	71 (±26)	411 (±12)	45 (±5)	n.d.	50 (±3)	4580 (±864)
Mesaconyl-CoA	24 (±5)	115 (±20)	8 (±1)	254 (±46)	168 (±6)	n.m.
Methylmalyl-CoA	51 (±12)	75 (±10)	35 (±2)	n.d.	13 (±4)	3566 (±82)

Values were determined at pH 7.5 and 25 °C using the DTNB spectrophotometric assay (see Materials and methods). All measurements were performed in triplicates. Corresponding standard deviations are given in parentheses. If available, values described in literature are shown in *square brackets* behind the determined values

TesBec thioesterase B from *E. coli*, *TesBext* thioesterase B homologue from *M. extorquens*, *YciA* bacterial acyl-CoA thioesterase from *E. coli*, *Acot4* succinyl-CoA hydrolase from *M. musculus*, *PaaI* phenylacetate thioesterase from *A. evansii*, *Bch* 3-hydroxyisobutyryl-CoA hydrolase from *B. cereus*, *n.d.* not detectable (detection limits: ≤0.1 U/mg; K_m ≥5,000 μM), *n.m.* not measured

[a] Naggert (1991)

[b] Westin (2005)

[c] Song (2006)

Growth characteristics of cells containing pCM160-RBS-*yciA* and their mesaconate and 2-methylsuccinate supernatant accumulation are shown in Fig. 3.

The optimized ribosome binding site of the new pCM160_RBS-*yciA* construct further increased the amount of mesaconate and 2-methylsuccinate released into the culture supernatant of *M. extorquens* as shown in Fig. 3a. The identity of mesaconate and 2-methylsuccinate peak was confirmed by retention time and identical absorption spectra shown in Fig. 3b and c. As 2-methylsuccinyl-CoA is present in its (2*S*) conformation in the EMCP, a release of (2*S*)-methylsuccinate can be expected although it was not directly confirmed. Up to 70 mg/l mesaconate and 58 mg/l 2-methylsuccinate accumulated after 30 h. That point of time correlates to the maximum optical density of the AM1_pCM160-RBS-*yciA* culture. During the stationary growth phase (30–48 h), the concentration of both acids in supernatant is reduced by more than 50 % from 70 to 38 mg/l mesaconate and 58 to 30 mg/l 2-methylsuccinate.

As already mentioned, colonies for AM1_pCM160-*tesBec* could not be obtained on minimal medium containing methanol. Therefore two approaches were followed to determine a possible EMCP derived acid production by TesBec. In a first approach, the preculture of AM1_pCM160-*tesBec* was cultivated in succinate minimal medium, washed with sterile water and further used to inoculate a main culture growing on methanol. The growth profile of the main culture showed a significantly prolonged lag phase of 24 h compared to 8–10 h of the other thioesterase expressing strains or the empty vector control. However, maximum growth rate during exponential growth phase was not altered compared to the empty vector control as well as the reached maximum optical density of 3–3.5 and the dicarboxylic acid profile in culture supernatant (data not shown). To ensure that the prolonged lag phase and succinate precultivation does not lead to, e.g., plasmid alteration, pLC290-*tesBec* construct harboring a cumate inducible promotor was used as a second approach. AM1_pLC290-*tesBec* grew in methanol minimal medium and was induced

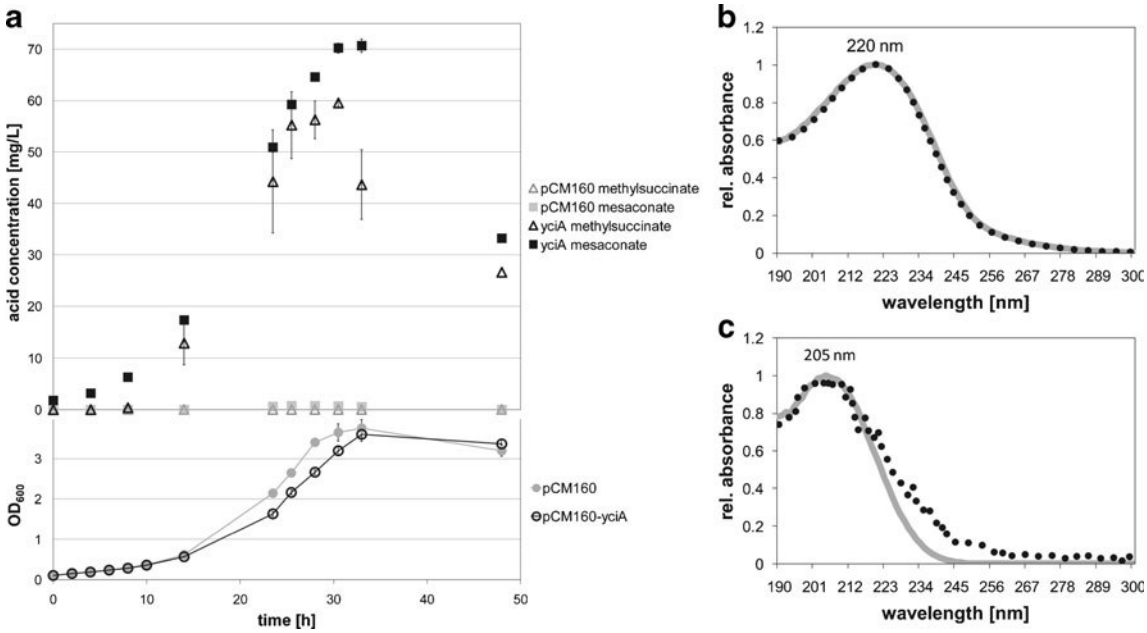

Fig. 3 Time-dependent release of mesaconate and 2-methylsuccinate into culture supernatant and growth characteristics of *M. extorquens* AM1 carrying pCM160-RBS-*yciA* compared to the empty vector control (**a**). All measurements were performed from three independent cultures. **b** Absorption spectra of mesaconate standard (*grey line*) and mesaconate peak of pCM160-RBS-*yciA* (*black dotted line*). **c** Absorption spectra of 2-methylsuccinate standard (*grey line*) and 2-methylsuccinate peak of pCM160-RBS-*yciA* (*black dotted line*). Maximum absorbance of each substance is shown *above the curves*

with cumate after reaching middle exponential growth phase. Compared to the empty vector control and the non-induced culture, induction of *tesBec* did not lead to reduced growth or altered acid accumulations.

We identified thioesterase YciA as an appropriate enzyme for cleavage of the EMCP CoA-esters 2-methylsuccinyl-CoA and mesaconyl-CoA leading to release of 2-methylsuccinic- and mesaconic acid into culture supernatant of *M. extorquens* AM1. During stationary growth phase a significant reduction of the previously released acids was observed.

Utilization of (EMCP)-dicarboxylic acids by AM1

Dicarboxylic acids like mesaconate and 2-methylsuccinate are very stable when dissolved in water which strongly suggests an uptake of the acids during stationary growth phase. This consequently leads to the question whether *M. extorquens* is able to utilize the EMCP related dicarboxylic acids as energy and carbon source. *M. extorquens* AM1 is able to use several dicarboxylic acids as sole carbon source which was already shown by Peel and Quayle (1961) in the paper of AM1's first characterization. Therefore, ethylmalonate, 2-methylsuccinate, mesaconate and methylmalonate were tested as sole carbon source for growth. The TCA-cycle intermediate α-ketoglutarate was used as a comparable C source as the chosen EMCP derived dicarboxylic acids, except methylmalonate, also consist of 5C atoms.

As shown in Table 3, *M. extorquens* AM1 is able to use every tested EMCP related dicarboxylic acid as sole carbon source at which growth on methylmalonate (C4) was faster than on the C5-dicarboxylic acids ethylmalonate, 2-methylsuccinate or mesaconate. Growth on the C5 acid α-ketoglutarate as a part of the TCA was also observable.

The utilization of the EMCP derived dicarboxylic acids as sole carbon source and the observed uptake of mesaconate and 2-methylsuccinate by AM1_pCM160-*yciA* implicates a functional transport system for these acids in *M. extorquens* AM1. Protein BLAST analysis was performed to identify possible dicarboxylate carriers responsible for transport of the EMCP

Table 3 Ability of *Methylobacterium extorquens* AM1 to grow on various dicarboxylic acids of the TCA and EMCP (derived from CoA esters) cycle

Dicarboxylic acid	Pathway	Growth	Reference
Succinate	TCA	++	Peel and Quayle 1961
Fumarate	TCA	++	Peel and Quayle 1961
Malate	TCA	++	Peel and Quayle 1961
α-Ketoglutarate	TCA	++	This study
Ethylmalonate	EMCP	+	This study
2-Methylsuccinate	EMCP	+	This study
Mesaconate	EMCP	(+)	This study
Methylmalonate	EMCP	++	This study

++ growth similar to succinate, + growth slower than on succinate, (+) minimal growth, − no growth

related acids in *M. extorquens* AM1. Results are summarized in Table 4. Several homologues of the DctA dicarboxylic acid transport system in the genome of AM1 were identified.

Discussion

Applications of acyl-CoA thioesterases in biotechnology are steadily increasing as several publications for the production of hydroxylated fatty acids (Zheng et al. 2004) and carboxylic acids (Chung et al. 2009; Liu et al. 2007; Martin et al. 2013; Martin and Prather 2009) by microorganisms illustrate. Many production systems were established with the help of thioesterase B/II (TesB), which is beside thioesterase A/I (TesA) one of the most thoroughly investigated thioesterases including a 3D X-ray structure (Li et al. 2000). Its broad substrate spectrum (Barnes et al. 1970; Naggert et al. 1991) turns TesB to an universally applicable enzyme which, however, might also be often used due to lacking alternatives especially for short-chain acyl-CoAs. Our results show that TesB does not necessarily have to be the thioesterase of choice for cleaving all types of acyl-CoAs. Bacterial thioesterase YciA showed higher CoA-hydrolyzation activities towards five tested EMCP CoA esters than TesB from *E. coli* (see Table 2). This might be due to the branched and unsaturated carbon chains of the CoA esters as most of the substrates investigated for TesB (Barnes et al. 1970; Martin and Prather 2009; Naggert et al. 1991; Zheng et al. 2004) consist of linear hydroxylated carbon chains such as hydroxyvalerate, hydroxydecanoate or hydroxybutyrate. The unobservable EMCP derived dicarboxylic acid release of AM1_pCM160-*tesBec* underline the low activities of TesB from *E. coli* measured in vitro.

Annotated TesB of *M. extorquens* AM1 (TesBext (Vuilleumier et al. 2009)) shares 45 % identity and 60 % positives to its *E. coli* homologue. TesBext prefers longer acyl-CoAs as substrates as well as TesBec does, revealed by in vitro enzymatic analysis (Table 2). Enzyme properties for EMCP CoA esters are in the similar orders of magnitude as well. In contrast, constitutive expression of *tesBext* does not have a lethal effect as *tesBec*, which is probably a result of a disrupted metabolism as the broad range thioesterase TesB might act on various CoA esters pools of *M. extorquens* AM1. The preferred codon usage of *tesBext* in contrast to *tesBec* makes a malfunction of expression highly improbable although TesBext protein occurrence in AM1_pCM160-*tesBext* was not directly assured.

The selected specific short-chain acyl-CoA hydrolases Acot4 and Bch showed low activities towards all EMCP CoA esters and very high K_m values in case of Bch (see Table 2). Although small amounts of mesaconate were detectable in AM1_pCM160-*acot4* supernatant, Acot4 was not further applied due to the higher yield of the same product by AM1_pCM160-*yciA*. Although the measured K_m values of 3-hydroxyisobutyryl-CoA hydrolase (Bch) might suggest it as a candidate for specific *rac*-ethylmalonyl- or *rac*-methylmalonyl-CoA cleavage, the supernatant dicarboxylic acid profile of AM1_pCM160-*bch* was not altered. The only described application of Bch which is also the first publication of this enzyme so far does not provide any further information on other substrates than 3-hydroxyisobutyryl-CoA (Lee et al. 2008). Homologues from *Rattus norvegicus* or *Homo sapiens* show only very limited activities towards structural related compounds like methylmalonyl-CoA or 3-hydroxybutyryl-CoA or are even inhibited by these CoA esters (Shimomura et al. 1994; Shimomura et al. 2000), which might also be the case for Bch.

There might be additional promising candidates for EMCP CoA-ester hydrolyzation, but most characterized acyl-CoA thioesterases which could be an alternative show too low or no activity on acyl-CoA esters with six or less C atoms, e.g., TesA (Bonner and Bloch 1972; Cho and Cronan 1993), EntH

Table 4 Proteins of *M. extorquens* AM1 showing highest homology to different dicarboxylic acid transporters identified by protein BLAST analysis

	Homologues in *M. extorquens* AM1		
Transporter	Protein ID and gene locus taq	Identitiy	Similarity
DctA[a] (succinate importer)	YP_002964290.1 MexAM1_META1p3271	63 %	80 %
	YP_002964292.1 MexAM1_META1p2326	55 %	73 %
	YP_002963386.1 MexAM1_META1p4339	48 %	69 %
DctB[a] (transport sensor protein)	YP_002962215.1 MexAM1_META1p1044	32 %	49 %
DctD[a] (transcriptional regulator of dctA)	YP_002964292.1 MexAM1_META1p4339	39 %	53 %
KgpT[b] (α-ketoglutarate permease)	YP_002966015.1 MexAM1_META1p5134	61 %	75 %

[a] Homology to *Rhodobacter capsulatus* SB1003

[b] Homology to *Escherichia coli* K12 proteins

(Chen et al. 2009), YbgC (Zhuang et al. 2002) or thioesterase III (Nie et al. 2008). Other candidates being active on short-chain acyl-CoAs like Acot12 seem to be difficult to express in bacteria (Suematsu et al. 2002).

The release of 2-methylsuccinate and mesaconate into culture supernatant of *M. extorquens* AM1 expressing *yciA* confirms that the bacterial thioesterase is also active in vivo. The cleavage of mesaconyl-CoA is consistent with the measured in vitro activity towards this substrate which was the highest among the EMCP CoA esters (Table 2). Note that an isomeric mixture of mesaconyl-C1– and mesaconyl-C4-CoA was used for in vitro enzyme assays whereas only mesaconyl-C1-CoA is present in vivo. Though not all values measured in vitro fit exactly to the in vivo observations as the in vitro activities towards succinyl- and *rac*-methylmalonyl-CoA are higher than those for *rac*-methylsuccinyl-CoA, but a release of succinate or methylmalonate was not detectable. This might be due to various reasons: the intracellular environment can be very different to the in vitro assay conditions such as pH, salt concentrations and additional inhibitor or activator substances shifting the enzyme activities and substrate preferences. Moreover, the pool sizes of the EMCP CoA esters in *M. extorquens* AM1 growing on methanol are rather low — between 16 and 95 μM (Kiefer 2009) — additionally weighting any shifted K_m values and activities resulting from in vivo conditions.

The drop of mesaconate and 2-methylsuccinate concentrations after the end of the exponential growth phase in culture supernatant of *M. extorquens* expressing *yciA* (Fig. 3) demonstrates the uptake of these acids. *M. extorquens* AM1 is able to use several C4– dicarboxylic acids of the TCA and methylmalonate of the EMCP as sole carbon source as well as α-ketoglutarate and ethylmalonate, 2-methylsuccinate and mesaconate which consist of 5C atoms (Table 3). Cellular acid uptake may be triggered upon transition from exponential to stationary growth phase. Another possibility might be a permanent acid uptake which is lower than the release during exponential growth phase but higher during stationary growth phase where the metabolic flux towards the CoA-ester substrates ceases. As *M. extorquens* AM1 is able to co-consume methanol and succinate (Peyraud et al. 2012) it might also co-consume other acids with methanol. Because of the CoA ester forms of all EMCP intermediates, the acids may be condensed to a CoA molecule by CoA-ligases or -transferases before they are further metabolized.

Several dicarboxylic acid uptake systems exist in bacteria such as the DctA family mainly occurring in aerobic bacteria, the CitT family, SdcS described for *Staphylococcus aureus*, the DcuAB family present in anaerobic bacteria or the tripartite ATP-independent periplasmic (TRAP) transport carriers (Janausch et al. 2002). An α-ketoglutarate permease was identified in *E. coli* by Seol and Shatkin (1991). *M. extorquens* AM1 very likely harbors the DctA dicarboxylic acid transporter including its regulators DctB and DctD (see Table 4), which are located downstream of annotated *dctA* gene MexAM1_META1p3271 in the AM1 genome suggesting YP_002964290.1 as most likely DctA homologue. An α-ketoglutarate permease KgpT also seems to be existent in AM1. Proteins with high homology to CitT, CitA, DcuC/AB/SR, DctQM or SdcS were not found.

All of these transporters might be able to take up the EMCP derived acids, and it can be speculated that the succinate importer DctA might be responsible for the uptake of the C4 dicarboxylic acid methylmalonate whereas KgpT transports the other EMCP related dicarboxylic acids ethylmalonate, methylsuccinate and mesaconate which consist of 5C atoms such as α-ketoglutarate. Investigations of Van Dien and co-workers (2003) show that an *M. extorquens* AM1 deletion mutant for *dctA* is not able to grow on succinate or pyruvate anymore. Therefore application of this mutant — alone or in combination with a *kgpT* knockout — might be interesting to test towards their EMCP acid uptake.

In summary, we identified bacterial thioesterase YciA which has not been used to date for biotechnological applications as the first thioesterase shown to cleave EMCP CoA esters (2*S*)-methylsuccinyl-CoA and mesaconyl-C1-CoA. Consequently, we also report the production of the corresponding acids 2-methylsuccinate and mesaconate by expression of *yciA* in EMCP harboring *M. extorquens* AM1 for the first time. Increasing CoA-ester concentrations de novo by metabolic engineering will lead to further production increase of mesaconate or 2-methylsuccinate and might also reveal other thioesterases as being able to release other CoA-ester related acids.

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7.1.2 High-level production of ethylmalonyl-CoA pathway derived dicarboxylic acids by *Methylobacterium extorquens* under cobalt-deficient conditions and by polyhydroxybutyrate negative strains

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(3) Erstellung der Daten-sammlung und Abbildungen	Abbildungen 1-3, 5, 6 Abbildung 4 Tabellen 1-2	Sonntag F., Buchhaupt M. Sonntag F., Müller J., Kiefer P. Sonntag F.
(4) Analyse/Interpretation der Daten	komplette Analyse der Daten und Einordnung in den Gesamtkontext	Sonntag F., Buchhaupt M., Kiefer P., Müller J.
(5) übergeordnete Einleitung/ Ergebnisse/Diskussion	60% 25% je 5%	Sonntag F. Buchhaupt M. Müller J., Kiefer P., Vorholt J.A.

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High-level production of ethylmalonyl-CoA pathway-derived dicarboxylic acids by *Methylobacterium extorquens* under cobalt-deficient conditions and by polyhydroxybutyrate negative strains

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Abstract Bio-based production of dicarboxylic acids is an emerging research field with remarkable progress during the last decades. The recently established synthesis of the ethylmalonyl-CoA pathway (EMCP)-derived dicarboxylic acids, mesaconic acid and (2*S*)-methylsuccinic acid, from the alternative carbon source methanol (Sonntag et al., Appl Microbiol Biotechnol 98:4533–4544, 2014) gave a proof of concept for the sustainable production of hitherto biotechnologically inaccessible monomers. In this study, substantial optimizations of the process by different approaches are presented. Abolishment of mesaconic and (2*S*)-methylsuccinic acid reuptake from culture supernatant and a productivity increase were achieved by 30-fold decreased sodium ion availability in culture medium. Undesired flux from EMCP into polyhydroxybutyrate (PHB) cycle was hindered by the knockout of polyhydroxyalkanoate synthase *phaC* which was concomitant with 5-fold increased product concentrations. However, frequently occurring suppressors of strain Δ*phaC* lost their beneficial properties probably due to redirected channeling of acetyl-CoA. Pool sizes of the product precursors were increased by exploiting the presence of two cobalt-dependent mutases in the EMCP: Fine-tuned growth-limiting cobalt concentrations led to 16-fold accumulation of mesaconyl- and (2*S*)-methylsuccinyl-CoA which in turn resulted in 6-fold increased concentrations of mesaconic and (2*S*)-methylsuccinic acids, with a combined titer of 0.65 g/l, representing a yield of 0.17 g/g methanol. This work represents an important step toward an industrially relevant production of ethylmalonyl-CoA pathway-derived dicarboxylic acids and the generation of a stable PHB synthesis negative *Methylobacterium extorquens* strain.

Keywords Dicarboxylic acids · Ethylmalonyl-CoA pathway · *Methylobacterium extorquens* · Methanol · PHB

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Introduction

Dicarboxylic acids consisting of four or five carbon atoms are important platform chemicals for the synthesis of polyamides, diols and plastics with an annual market volume of more than 15 billion US$ (Jang et al. 2012; Lee et al. 2011). The increasing oil price and societal need for sustainable production processes turned the focus from petrochemical to bio-based production with tremendous productivity advances during the last years (Cao et al. 2011; Yu et al. 2011). Besides compounds with established biotech production possibilities such as succinic, fumaric, adipic, or itaconic acid, specialty dicarboxylic acids for new applications and respective production processes are increasingly gaining importance (Alonso et al. 2014).

Recently, we demonstrated a proof of concept for the de novo synthesis of hitherto biotechnologically inaccessible dicarboxylic acids, mesaconic acid and (2*S*)-methylsuccinic acid, by the methylotrophic bacterium *Methylobacterium extorquens* (Sonntag et al. 2014). Both acids are attractive synthons and additionally have useful applications as fire

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retardants or coatings and solvent ingredients for cosmetics (Di Giulio and Bauer 1982; Loos et al. 2012; Muller and Richard 2012). Moreover, the production of both dicarboxylic acids out of the alternative raw material methanol provides several advantages over most biotechnological acid production routes using the classical carbon source glucose. Methanol can be produced either from petrochemicals or from biological waste materials such as wood and even pilot plants for the production from CO_2 have started recently, making it ubiquitously available and noncompeting with food production (Ochsner et al. 2014; Schrader et al. 2009). In addition, methanol is subjected to only minor price fluctuations and is comparably cheap with probably further decreasing prices due to planned construction of new large-scale operation plants in the future (Bertau et al. 2014). Taken together, methanol is a promising substrate for the production of bulk chemicals such as dicarboxylic acids.

Despite the successful production of 70 mg/l mesaconic acid and 60 mg/l (2*S*)-methylsuccinic acid, the yield and productivity have to be increased significantly to allow a commercially attractive process such as it is achieved for succinic or itaconic acid (Alonso et al. 2014).

Mesaconic and (2*S*)-methylsuccinic acids are synthesized by the hydrolytic release of coenzyme A from mesaconyl- and (2*S*)-methylsuccinyl-CoA by heterologous thioesterase YciA (see Fig. 1) which is constitutively produced via plasmid pCM160-RBS-*yciA* in *M. extorquens* growing in methanol minimal medium. Mesaconyl- and (2*S*)-methylsuccinyl-CoA are intermediates of the ethylmalonyl-CoA pathway (EMCP), an essential pathway for methylotrophic growth of *M. extorquens* that fulfills the task of glyoxylate regeneration and includes two CO_2 fixation steps (Erb et al. 2007; Peyraud et al. 2009). The EMCP comprises 12 enzymatic steps catalyzed by 11 enzymes which require different cofactors including NADPH, cobalt ion-containing vitamin B_{12}, ATP and FAD (Alber 2011). Note that the pathway is not specific for methylotrophy and also occurs in nonmethylotrophic organisms where it is used as linear pathway for assimilation of C2 carbon sources (Chistoserdova et al. 2009).

Furthermore, the EMCP overlaps with several central metabolic pathways, such as the tricarboxylic acid cycle (TCA) and serine cycle as well as the polyhydroxybutyrate (PHB) cycle. PHB is an important energy and carbon storage compound and main redox sink for methanol-grown *M. extorquens* cells and can add up to 40 % of the cell dry mass (Korotkova and Lidstrom 2001). Hence, avoiding PHB synthesis would not only avoid carbon flux, in the context of this study, into an undesired by-product but also likely increase metabolic flux through the EMCP to the product precursors of mesaconic acid and (2*S*)-methylsuccinic acid.

In this study, we present different approaches to improve the recently established de novo synthesis of the uncommon EMCP-derived dicarboxylic acids mesaconic acid and (2*S*)-methylsuccinic acid. Besides the avoidance of carbon loss caused by PHB synthesis, our strategies comprised the prevention of product reuptake and the increase of product formation rate by modulation of precursor pools.

By demonstrating a production yield of 0.17 g EMCP-derived acids/g methanol, this work is an important step toward the establishment of an economically viable biotechnological synthesis of mesaconic acid and (2*S*)-methylsuccinic acid from methanol.

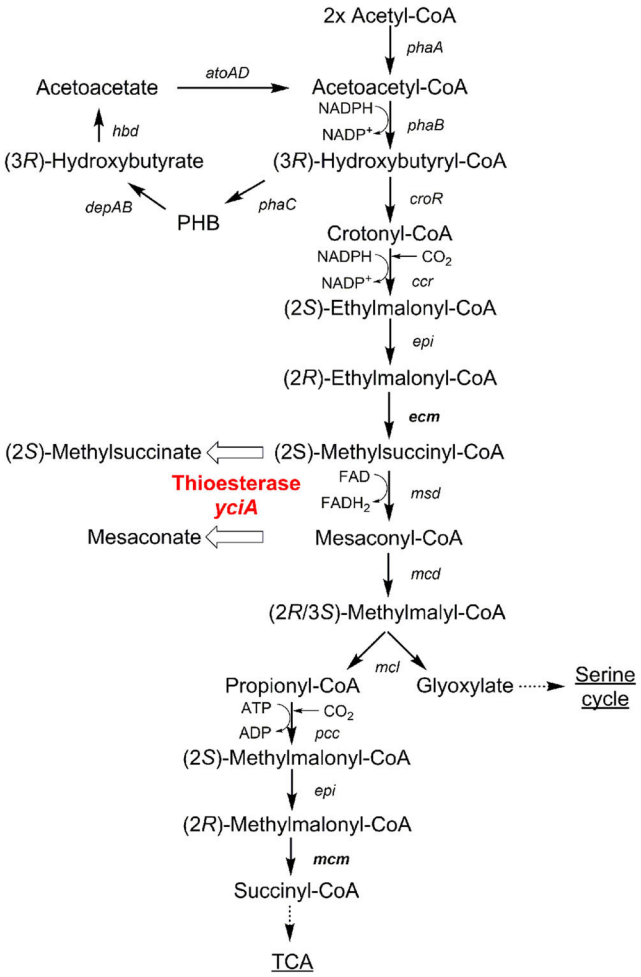

Fig. 1 Ethylmalonyl-CoA pathway (EMCP) and polyhydroxybutyrate (PHB) cycle in *M. extorquens*. Expression of thioesterase *yciA* (marked in *red*) leads to hydrolytic release of coenzyme A from (2*S*)-methylsuccinyl-CoA and mesaconyl-CoA and to the release of the corresponding acids (2*S*)-methylsuccinate and mesaconate into supernatant as indicated by *broad arrows* (Sonntag et al. 2014). Genes encoding ethylmalonyl-CoA mutase (*ecm*) and methylmalonyl-CoA mutase (*mcm*) using cobalt ion-containing vitamin B_{12} as cofactor are marked in *bold letters*. Further genes: *phaA*: β-ketothiolase; *phaB*: acetoacetyl-CoA dehydrogenase; *phaC*: PHB synthase; *croR*: crotonase; *ccr*: crotonyl-CoA carboxylase/reductase; *epi*: m/ethylmalonyl-CoA epimerase; *msd*: methylsuccinyl-CoA dehydrogenase; *mcd*: mesaconyl-CoA dehydratase; *mcl*: malyl-CoA/beta-methylmalyl-CoA lyase; *pcc*: propionyl-CoA carboxylase

Material and methods

Chemicals, media, and bacterial strains

M. extorquens AM1 (Peel and Quayle 1961) and deletion strains were grown at 30 °C in minimal medium as described before (Kiefer et al. 2009) containing 123 mM methanol. Standard cobalt concentration was 12 μM. Media containing cobalt concentrations of 0.6, 0.4, 0.2 or 0 μM were prepared by using trace element solutions with the respective cobalt concentrations. Standard sodium concentration was 486 μM from 6.9 mM $NaH_2PO_4 \cdot 2H_2O$ used as buffer substance. Methanol minimal medium containing reduced amount of sodium was prepared by replacement of the 6.9 mM $NaH_2PO_4 \cdot 2H_2O$ by 7.66 mM KH_2PO_4 (equal molar amount) as phosphate buffer substance besides K_2HPO_4 resulting in a final Na^+ concentration of 0.25 μM from $Na_2MoO_4 \cdot H_2O$ and Na_2EDTA contained in the trace element solution. Succinate minimal medium was prepared as methanol minimal medium containing 30.7 mM succinate instead of methanol as sole carbon source. Minimal media with malate, fumarate, ethylmalonate, *rac*-2-methylsuccinate, mesaconate, or methylmalonate were prepared according to succinate medium with 2 mM of the respective acid.

Escherichia coli strain DH5α (Gibco-BRL, Rockville, USA) was grown in lysogeny broth (LB) medium (Bertani 1951) at 37 °C. The following antibiotic concentrations were used: kanamycin at 30 μg/ml for *E. coli*, 50 μg/ml for *M. extorquens* and tetracycline-hydrochloride at 10 μg/ml for *E. coli* and *M. extorquens*.

The dicarboxylic acids (3*R*)-hydroxybutyric acid, ethylmalonic acid, methylmalonic acid, *rac*-2-methylsuccinic acid, and mesaconic acid were purchased from Sigma-Aldrich (Steinheim, Germany) in their highest available degree of purity. *Rac*-beta-methylmalic acid was synthesized by Otava (Kiev, Ukraine).

Genetic manipulations and plasmid constructions

Primers were purchased from Sigma-Aldrich (Steinheim, Germany). All standard cloning techniques were carried out as described before (Sambrook 2001). Transformation of plasmids into *M. extorquens* was performed as described by Toyama et al. (1998).

Generation of deletion mutants Δ*phaC*, Δ*phaR*, and Δ*dctA*

Knockout of polyhydroxybutyrate synthase (*phaC*; MexA1_META1p3304) was carried out by using pCM184 plasmid system (Marx 2002); 500 bp upstream and downstream genomic flanking regions of *phaC* were amplified with primer pairs phaC_up-fw/phaC_up-down and phaC_down-fw/phaC_down-rev, respectively. The upstream flanking region fragment was digested with restriction enzymes *Aat*II and *Nco*I and ligated into equally digested pCM184 resulting in pCM184_Δ*phaC*-up. Subsequently, *Sna*B1 and *Sac*I digested downstream flanking region fragment was ligated into pCM184_Δ*phaC*-up cut with the same restriction enzymes finally leading to pCM184Δ*phaC*. pCM184Δp*haC* was transformed into electrocompetent *M. extorquens* AM1 cells. Double recombination mutant selection and removal of the kanamycin resistance marker with the help of plasmid pCM157 was performed as described by Marx (2002) on solid methanol medium containing the appropriate antibiotic. Colony PCR for verification of the correct integration site was performed with primers phaC-up_fw/Kan-check_rev and phaC-up_rev/Kan-check_fw.

M. extorquens AM1 strain Δ*phaR* (MexAM1_META1p3699) was constructed as described for strain Δ*phaC* with the use of plasmids pCM184 and pCM157 according to the procedure described by Korotkova et al. (2002). Genomic flanking sites of *phaR* for integration into pCM184 were amplified with primers phaR-down_fw, phaR-down_rev, phaR-up_fw and phaR-up_rev.

Strain Δ*dctA::kanR* (MexAM1_META1p3271) was constructed in the same manner as Δ*phaC* and Δ*phaR* using primers dctA-down_fw, dctA-down_rev, dctA-up_fw and dctA-up_rev for amplification of the genomic flanking sites of the *dctA* gene which were digested with *Aat*II/*Nde*I (upstream) and *Sna*B1/*Sac*I (downstream) for ligation into pCM184. All oligonucleotides used are listed in Table 1.

PHB analysis

PHB was analyzed and quantified as described before (Braunegg et al. 1978). Gas chromatographic analysis was performed on a Shimadzu GC17A system equipped with an FID and a DB-WAXextr column (*l* 30 m, *d* 0.25 mm; Agilent, USA).

Cultivations for dicarboxylic acid production

For all cultivations, precultures were grown for 48 h in test tubes containing 5 ml methanol minimal medium with 12 μM Co^{2+} and kanamycin. Dicarboxylic acid production under non-limiting cobalt conditions (12 μM) was carried out by directly inoculating main cultures (50 ml methanol minimal medium with 12 μM Co^{2+} and kanamycin in 300 ml unbaffled shake flasks) to an OD_{600} of 0.1 from precultures. For production under limiting cobalt conditions (0, 0.2, 0.4, or 0.6 μM), preculture cells were washed with ddH_2O (3 min, 4000*g*, 20 °C) prior to inoculation of the main cultures containing methanol minimal medium with the respective lower amounts of cobalt.

Effects of lowered amounts of sodium ions were investigated by directly inoculating main cultures containing methanol minimal medium without NaH_2PO_4 but KH_2PO_4 as buffer substance with precultures grown at standard sodium

Table 1 Primers, plasmids and strains used in this work

Name	Description	Reference
Primers		
phaC-up_fw	acta*gacgtc*ACGATCACTCCACCGGGTTG	This study
phaC-up_rev	acta*ccatgg*GCGACACGTCCTCCCAAAGGT	This study
phaC-down_fw	acta*tacgta*AGGGCATGAAGGTGTGAGGG	This study
phaC-down_rev	acta*gagctc*TTCGAGCGCCCGCAGCAGC	This study
phaR-up_fw	aaa*agatct*GCATGCGCCGGATTCTAC	This study
phaR-up_rev	aaa*ccatgg*AGAATTCGAGGAAGCTCG	This study
phaR-down_fw	aaa*gggccc*CGTCAGCGGCCCGGCCGA	This study
phaR-down_rev	aaa*gagctc*CGTGCGTTCGAGGCGTGC	This study
dctA-up_fw	actagacgtcAGCGGAAGCGAACTCTGCG	This study
dctA-up_rev	acta*catatg*GGGCGTTTCTCCCTGTCGGA	This study
dctA-down_fw	acta*tacgta*TCCGGTCAGGAGGGCGCAC	This study
dctA-down_rev	acta*gagctc*AGGGCTTCGGGCGTATCGAG	This study
Kan-check_fw	AGTTTCATTTGATGCTCGATGAG	This study
Kan-check_rev	AGACGTTTCCCGTTGAATATG	This study
Plasmids		
pCM160	Constitutive expression vector for *Methylobacterium extorquens*; Kan^R, pmxaF, oriT, pBR322ori	Marx and Lidstrom (2001)
pCM184	Allelic exchange vector for gene deletion in *Methylobacterium extorquens*; Kan^R, Tc^R, Amp^R oriT, pBR322ori	Marx (2002)
pCM157	Cre recombinase expression plasmid; Tc^R, oriT, pBR322ori	Marx (2002)
pCM160-RBS-*yciA*	pCM160 containing thioesterase *yciA* including an optimized RBS	Sonntag et al. (2014)
pCM184-Δ*phaC*	pCM184 containing 500 bp flanking sites of *phaC*	This study
pCM184-Δ*phaR*	pCM184 containing 500 bp flanking sites of *phaR*	This study
pCM184-Δ*dctA*	pCM184 containing 500 bp flanking sites of *dctA*	This study
Strains		
E. coli DH5α	F^-, Φ80dlacZΔM15, Δ(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(rK^- mK^+), phoA, supE44, λ^-, thi-1	ATCC
M. extorquens AM1	Facultative methylotrophic, obligate aerobic, gram-negative, pink pigmented α-proteobacterium, Cm^R	Peel and Quayle (1961)
DSM1338		
M. extorquens Δ*phaC*	*M. extorquens* AM1 with chromosomal deletion of PHB synthase *phaC* (MexA1_META1p3304)	This study
M. extorquens Δ*phaR*	*M. extorquens* AM1 with chromosomal deletion of PHB synthesis regulator gene *phaR* (MexA1_META1p3699)	This study
M. extorquens Δ*dctA*	*M. extorquens* AM1 with chromosomal deletion of dicarboxylic acid transporter *dctA* (MexA1_META1p3271)	This study

Restriction endonuclease recognition sites are marked in italics. Binding domains of primers are indicated by capital letters

concentration (486 μM) with a dilution of 1/30. This resulted in a final sodium concentration in the main cultures of 486 μM/30=16 μM.

Mesaconic and (2*S*)-methylsuccinic acid analysis

Mesaconic acid and (2*S*)-methylsuccinic acid were analyzed and quantified as described before (Sonntag et al. 2014). Yield per substrate ($Y_{P/S}$) calculations were based on the assumption that the initial amount of methanol (3.94 g/l=123 mM) in a batch culture was consumed completely. OD_{600} values at the time point of the highest product concentration were converted to biomass concentrations by using OD–biomass correlation of 0.33 $g_{cdw}l^{-1}$ $OD_{600}{}^{-1}$ (Peyraud et al. 2012) to calculate yields per biomass ($Y_{P/X}$).

CoA ester pool determination

Coenzyme A esters were extracted from culture samples as described previously (Peyraud et al. 2009) except that formic acid concentration in quenching solution was 4-fold increased (100 mM instead of 25 mM).

Quantification of CoA esters was achieved by applying the isotope dilution mass spectrometry approach as described by

Wu et al. (2005). To this end, a uniformly ^{13}C-labeled cell extract of *M. extorquens* AM1 was prepared (Peyraud et al. 2009) and absolute concentrations of uniformly ^{13}C-labeled coenzyme A esters were determined as described by Bennett et al. (2008). All samples were spiked with amounts of labeled cell extract corresponding to biomass amounts of sample. Labeled cell extract was always added directly after mixing of the quenching solution and sample.

LC-MS analysis of coenzyme A esters was performed on a Rheos 2200 HPLC system (Flux Instruments, Basel, Switzerland), equipped with an electrospray ionization probe, that was coupled to an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) as described previously (Peyraud et al. 2009) with slight modifications (Schneider et al. 2012).

Results

Prevention of dicarboxylic acid reuptake

The release of the dicarboxylic acids mesaconic acid and (2*S*)-methylsuccinic acid into culture supernatant of *M. extorquens* requires a transport mechanism over the cytoplasmic membrane. As discussed in our previous study (Sonntag et al. 2014), *M. extorquens* possesses two dicarboxylic acid transporters, DctA and KgpT, of which one or both are likely involved in (2*S*)-methylsuccinic acid and mesaconic acid transport. Gene knockouts or functional inhibition of one or both transporters might lower the observed concentration decrease of the acids during stationary growth phase which is likely caused by a reuptake by the cells.

To determine the effect of the DctA transporter on (2*S*)-methylsuccinic and mesaconic acid production kinetics, a complete knockout of an annotated *dctA* gene with the highest similarity to the gene encoding protein A33597 given by Van Dien and coworkers was created by using the pCM184 system. Removal of the gene MexAM1_META1p3271 was verified by colony PCR, but the Δ*dctA::kanR* strain was still able to grow on succinate, malate, α-ketoglutarate, fumarate, mesaconate, *rac*-2-methylsuccinate, ethylmalonate and methylmalonate such as AM1 wild type (data not shown). This is in contrast to an earlier study that described the inability of an AM1 strain containing a transposon insertion in *dctA* (A33597) to grow on succinate or pyruvate (Van Dien et al. 2003a).

As the transport function of some DctA transporters has been shown to be sodium ion dependent (Janausch et al. 2002), we examined the effects of a reduced sodium concentration in the methanol minimal medium on the production and uptake of mesaconic acid and (2*S*)-methylsuccinic acid.

A 30-fold decreased sodium ion concentration (486 to 16 μM) in methanol minimal medium led to a 2-fold increased mesaconic and (2*S*)-methylsuccinic acid production combined with a reduced growth rate (0.107 h^{-1} (±0.001) to 0.121 h^{-1} (±0.002)) of AM1_pCM160-RBS-*yciA* as shown in Fig. 2. In addition, acid concentration remained almost constant after the end of the exponential growth phase when medium with a sodium ion concentration of 16 μM was used, while it decreased by more than half in medium with the standard sodium concentration of 486 μM.

Deletion of PHB synthesis as competing pathway for EMCP-derived acid production

The EMCP in *M. extorquens* overlaps with several other central metabolic pathways such as the serine, the TCA and the PHB cycle (Ochsner et al. 2014; Peyraud et al. 2011). In the latter, (3*R*)-hydroxybutyryl-CoA molecules are condensed by the polyhydroxyalkanoate synthase PhaC to PHB (see Fig. 1). During production of mesaconic acid and (2*S*)-methylsuccinic acid, PHB synthesis is a competing reaction for (3*R*)-hydroxybutyryl-CoA which is converted in the EMCP to the product precursor molecules (2*S*)-methylsuccinyl- and mesaconyl-CoA. Hence, reduction of PHB synthesis would probably lead to higher metabolic flux toward the substrates

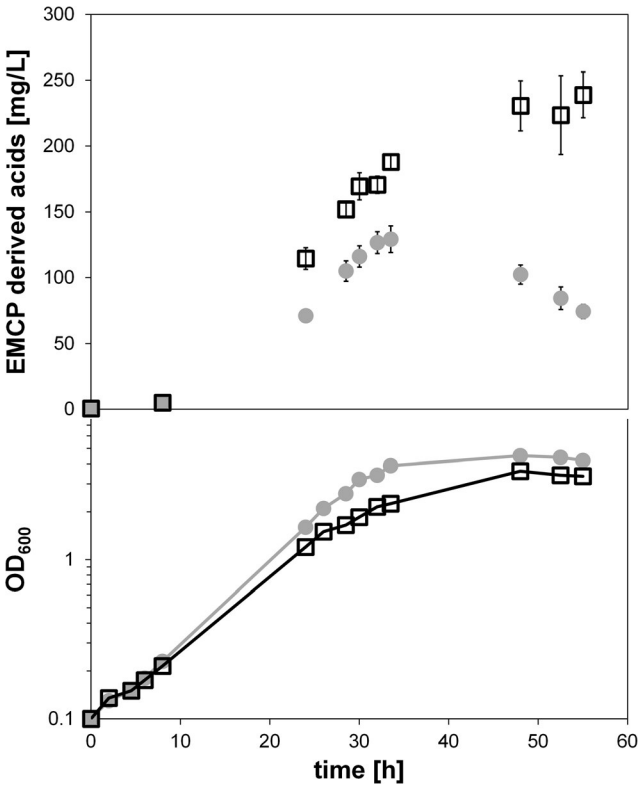

Fig. 2 Growth kinetics and time-dependent EMCP-derived acid concentration (combined concentration of mesaconic acid and (2*S*)-methylsuccinic acid) in supernatant of AM1_pCM160-RBS-*yciA* growing in methanol minimal medium containing 16 μM sodium (*empty black squares*) or 486 μM sodium (*filled gray circles*). *n*=3; *error bars* represent standard deviations

of thioesterase YciA likely resulting in an increased production of mesaconic acid and (2*S*)-methylsuccinic acid.

A knockout of the *phaC* gene encoding polyhydroxyalkanoate (PHA) synthase was already performed by Korotkova and Lidstrom (2001) who described *M. extorquens* Δ*phaC* as unable to produce PHB concomitant with a reduced growth rate. The mutant was furthermore characterized as highly unstable due to the frequent occurrence of suppressor cells which regained wild-type-like growth but were still unable to produce PHB and carried the introduced antibiotic marker cassette. As the described characteristics of the Δ*phaC* suppressor strains (wild-type-like growth but no PHB synthesis) seemed to be beneficial for the production of EMCP-derived acids, *M. extorquens* PHA synthase deletion strain was constructed and tested. Production of PHB was not detectable in Δ*phaC* cells grown in methanol minimal medium in contrast to *M. extorquens* AM1 wild type (data not shown). Consequently, electrocompetent Δ*phaC* cells were transformed with plasmids pCM160 and pCM160-RBS-*yciA* leading to strains Δ*phaC*_pCM160 and Δ*phaC*_pCM160-RBS-*yciA*, respectively.

EMCP-derived acid production and growth was compared to the respective AM1 wild-type strains containing the plasmids. The maximum growth rate of the control strain Δ*phaC*_pCM160 was 0.123 h^{-1} (±0.002) which was similar to AM1_pCM160 (0.131 h^{-1} (±0.005)) and AM1_pCM160-RBS-*yciA* (0.111 h^{-1} (±0.005)). As expected, Δ*phaC* without thioesterase *yciA* produced only minor amounts (<3 mg/l) of mesaconic acid and undetectable amounts of (2*S*)-methylsuccinic acid which is similar to observations for AM1_pCM160 (Sonntag et al. 2014).

Δ*phaC*_pCM160-RBS-*yciA* showed reduced growth and synthesized up to 160 mg/l mesaconic acid and 120 mg/l (2*S*)-methylsuccinic acid, i.e. a combined titer of EMCP-derived acids of 280 mg/l (see Fig. 3a) which is a 2.5-fold increase compared to the product concentrations in AM1_pCM160-RBS-*yciA* cultures. However, as illustrated in Fig. 3a, strain Δ*phaC*_pCM160-RBS-*yciA* started to grow faster during cultivation and recovered wild-type-like growth rate after more than 30 h of very slow growth. This drastic increase in growth rate was accompanied by barely increasing concentrations of EMCP-derived acids in culture supernatant. Reinoculation of the Δ*phaC*_pCM160-RBS-*yciA* strain from the same methanol agar plate after storage for 2 weeks at 4 °C led to instant wild-type-like growth and acid titers which did not exceed a maximum concentration of 30 mg/l (see Fig. 3a) which is 10-fold less than that of Δ*phaC* in the first experiment and even 4-fold less than AM1_pCM160-RBS-*yciA*. Our Δ*phaC* suppressor strain did not produce PHB and the *phaC* gene was not detectable by colony PCR (data not shown) thereby exhibiting similar properties as the Δ*phaC* suppressor characterized by Korotkova and Lidstrom (2001) and showing that the suppressor is not a revertant.

To check whether the suppressor characteristics are the result of a mutation or of an adaptation, the Δ*phaC*_pCM160-RBS-*yciA* suppressor strain was plated three times consecutively on solid succinate minimal medium or once on solid methanol minimal medium (both media contained kanamycin for plasmid retention). In case of a metabolic adaptation, the suppressor effect might disappear or be reduced after adaptation to succinate as sole carbon source, whereas it should be maintained in case of a mutation. As expected, Δ*phaC*_pCM160-RBS-*yciA* replated once on methanol minimal medium maintained growth and EMCP-derived acid production characteristics of the suppressor (see Fig. 3b). In

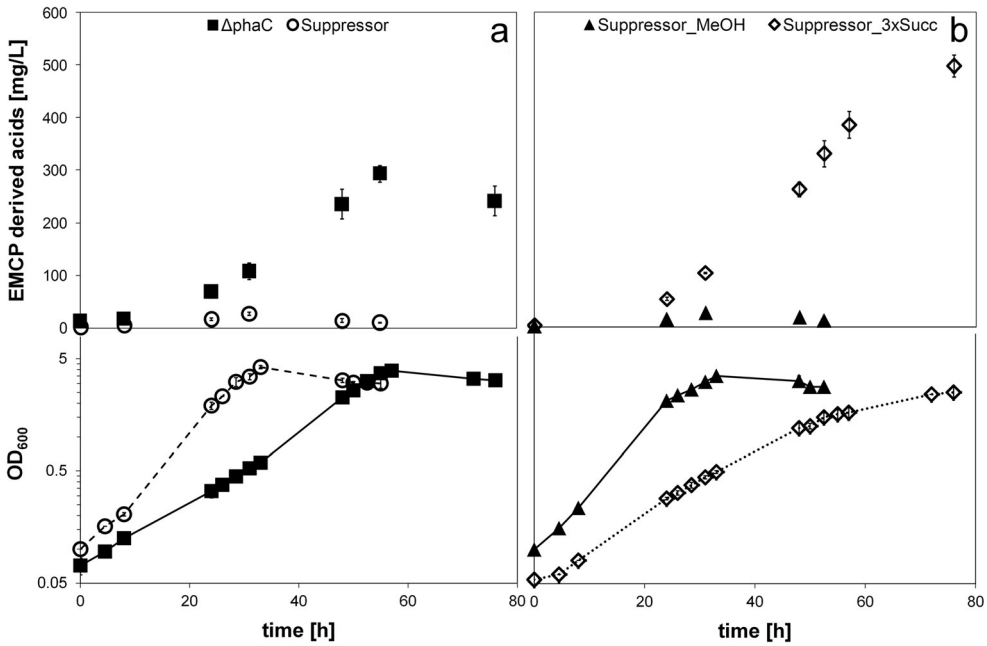

Fig. 3 Growth and EMCP-derived acid production (combined concentration of mesaconic acid and (2*S*)-methylsuccinic acid) comparison of strain Δ*phaC*_pCM160-RBS-*yciA* and Δ*phaC*_pCM160-RBS-*yciA* suppressor strain (**a**) and Δ*phaC*_pCM160-RBS-*yciA* suppressor strain replated once on methanol agar or replated three times on succinate agar (**b**). *n*=3; *error bars* represent standard deviations

contrast, after replating on succinate minimal medium, the Δ*phaC*_pCM160-RBS-*yciA* suppressor grew again like the original deletion strain including increased EMCP-derived acid production. During the cultivation time of 76 h, succinate-replated Δ*phaC*_pCM160-RBS-*yciA* accumulated a combined titer of 500 mg/l mesaconic acid and (2S)-methylsuccinic acid. However, cultivation of the recovered Δ*phaC* strain after storage on solid methanol minimal medium for 2 weeks at 4 °C again led to the emergence of the suppressor strain with the characteristics shown in Fig. 3.

Despite the obvious beneficial effect of PHB synthesis deletion on the production of mesaconic acid and (2*S*)-methylsuccinic acid, its instability renders the Δ*phaC* strain not applicable for a robust EMCP-derived dicarboxylic acid production process and prompted our search for alternative mutants hindered in PHB synthesis.

PHB synthesis is a complex process whose regulation strongly differs between different organisms. The overlap with an essential primary pathway, the EMCP, as it exists in *M. extorquens*, is a special case because PHB synthesis regulation is connected to the primary metabolic flux. One regulator for PHB synthesis in AM1 was described by Korotkova et al. (2002): PhaR is a regulator for phasin proteins binding to PHB granules and thereby promoting PHB synthesis. A *phaR* knockout strain grows slower on methanol and produces nearly no PHB compared to AM1 wild type. Despite the slower growth, the strongly reduced PHB accumulation and the observed stability of Δ*phaR* might also improve EMCP-derived dicarboxylic acid production due to less bypath flux.

A knockout strain Δ*phaR* was constructed according to Korotkova et al. (2002) and transformed with plasmids pCM160 and pCM160-RBS-*yciA*. As observed for Δ*phaC*, also Δ*phaR* did not produce PHB in detectable amounts (data not shown). Growth and production of mesaconic acid and (2*S*)-methylsuccinic acid in methanol minimal medium were compared to AM1_pCM160-RBS-*yciA*.

Growth rate of Δ*phaR*_pCM160-RBS-*yciA* was reduced by about 25 % compared to AM1_pCM160-RBS-*yciA* which is consistent to the results of Korotkova et al. (2002), but the overall production of EMCP-derived acids was not changed but delayed according to the shifted exponential growth phase (data not shown). In contrast to Δ*phaC*, the growth and production characteristics of Δ*phaR* remained constant over 110 h of cultivation. Obviously, the Δ*phaR* strain does not provide an increased flux toward the EMCP product precursors although PHB was not detectable.

Accumulation of EMCP intermediates by limitation of cobalamin-dependent mutase activities

It is described in the literature that *M. extorquens* growing with methanol as carbon source and insufficient amounts of cobalt accumulates EMCP intermediates due to inadequate cofactor availability for the ethyl- and methylmalonyl-CoA mutase (Kiefer et al. 2009). Here, we aimed to identify a cobalt concentration that leads to an increase of the (2*S*)-methylsuccinyl- and mesaconyl-CoA pool sizes as direct product precursors while still maintaining methylotrophic growth and thereby a metabolic flux through the EMCP. Therefore, pool size measurements of the EMCP CoA esters were carried out for *M. extorquens* growing in methanol minimal medium containing 0.4 μM Co^{2+}, which is 30-fold lower than the standard, non-growth-limiting concentration. Under these conditions, AM1 still grows with a maximum growth rate of 0.07 h^{-1} compared to 0.16 h^{-1} under non-limited growth conditions (12 μM Co^{2+}) in shake flask cultivations which probably fit the mentioned prerequisite of a still existing flux through the EMCP.

Figure 4 illustrates the different EMCP intermediate pool sizes of *M. extorquens* cells in exponential growth phase with methanol as carbon source under growth-limiting (0.4 μM) and non-growth-limiting (12 μM) cobalt concentrations. The methylmalonyl-CoA pool showed the highest accumulation (32x), but also the ethylmalonyl-, (2*S*)-methylsuccinyl-, mesaconyl- and methylmalyl-CoA pools increased 3- to 15-fold under cobalt-limited growth conditions, whereas the 3-hydroxybutyryl-CoA pool was not affected.

The significantly higher pool sizes of (2*S*)-methylsuccinyl-CoA and mesaconyl-CoA of *M. extorquens* growing on methanol with only 0.4 μM instead of 12 μM Co^{2+} make this altered medium a promising approach for improved production of mesaconic acid and (2*S*)-methylsuccinic acid by AM1 expressing thioesterase *yciA* via plasmid pCM160-RBS-*yciA*.

Mesaconic and (2*S*)-methylsuccinic acid production under cobalt-limited growth conditions

Although concentrations of (2*S*)-methylsuccinyl-CoA and mesaconyl-CoA were considerably increased during growth of AM1 in medium containing only 0.4 μM Co^{2+}, this concentration does not necessarily have to be the optimum for de novo synthesis of mesaconic acid and (2*S*)-methylsuccinic acid. Balance between accumulation of the CoA ester precursors and growth, i.e. flux through the EMCP, has to be maintained for optimum production rates of AM1 expressing thioesterase *yciA*. Therefore, it is likely that cobalt concentrations below a certain threshold would be rather worse for productivity as well as concentrations which do not cause sufficiently high accumulations. Consequently, EMCP-derived acid production and growth of AM1_pCM160-RBS-*yciA* during cultivation in methanol minimal medium containing cobalt concentrations of 0, 0.2, 0.4, 0.6 or 12 μM were investigated.

Growth rates of *M. extorquens* harboring pCM160-RBS-*yciA* increased from 0.05 h^{-1} at 0 μM Co^{2+} to 0.07 h^{-1} at 0.6 μM Co^{2+} and 0.11 h^{-1} at the standard concentration of 12 μM, as illustrated in Fig. 5b. Concentrations of mesaconic acid and (2*S*)-methylsuccinic acid measured in culture

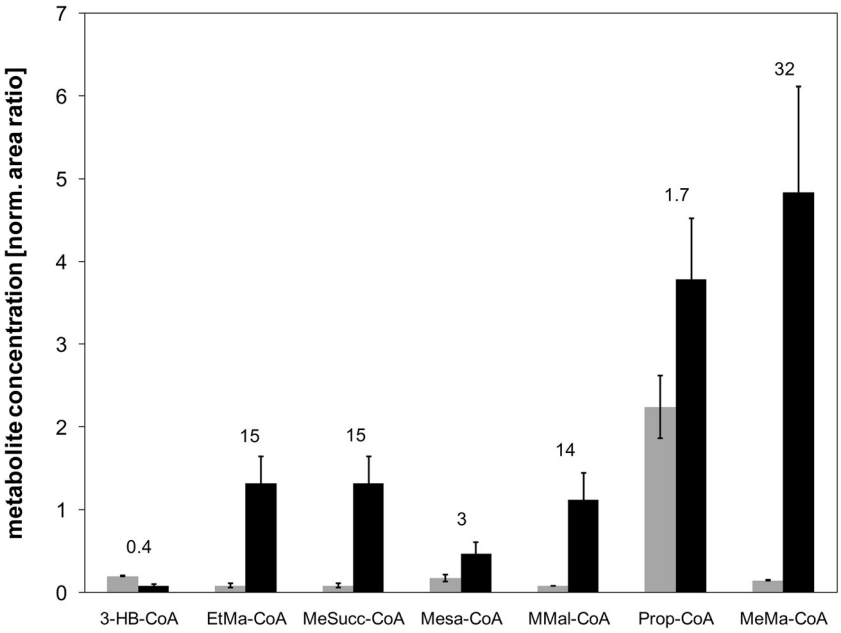

Fig. 4 Concentrations of EMCP intermediates of *M. extorquens* AM1 growing in methanol minimal medium under non-limiting (12 μM; *gray*) and limiting (0.4 μM; *black*) cobalt concentrations. Metabolite concentrations are given as normalized area ratios for (3*R*)-hydroxybutyryl-CoA (*3-HB-CoA*), ethylmalonyl-CoA (*EtMa-CoA*), (2*S*)-methylsuccinyl-CoA (*MeSucc-CoA*), mesaconyl-CoA (*Mesa-CoA*), beta-methylmalyl-CoA (*MMal-CoA*), propionyl-CoA (*Prop-CoA*), and methylmalonyl-CoA (*MeMa-CoA*). Accumulation factors are displayed above the corresponding bars. $n=2$; *error bars* represent standard deviations

supernatants decreased with increasing growth rates. Cultures growing with 0.2 μM cobalt showed a 6-fold increased acid production compared to cultures growing with 12 μM Co^{2+} (see Fig. 5a). It should be noted that growth with 0 μM cobalt is probably occurring due to minor amounts of cobalt left after preculture washing and residual vitamin B_{12} and mutase

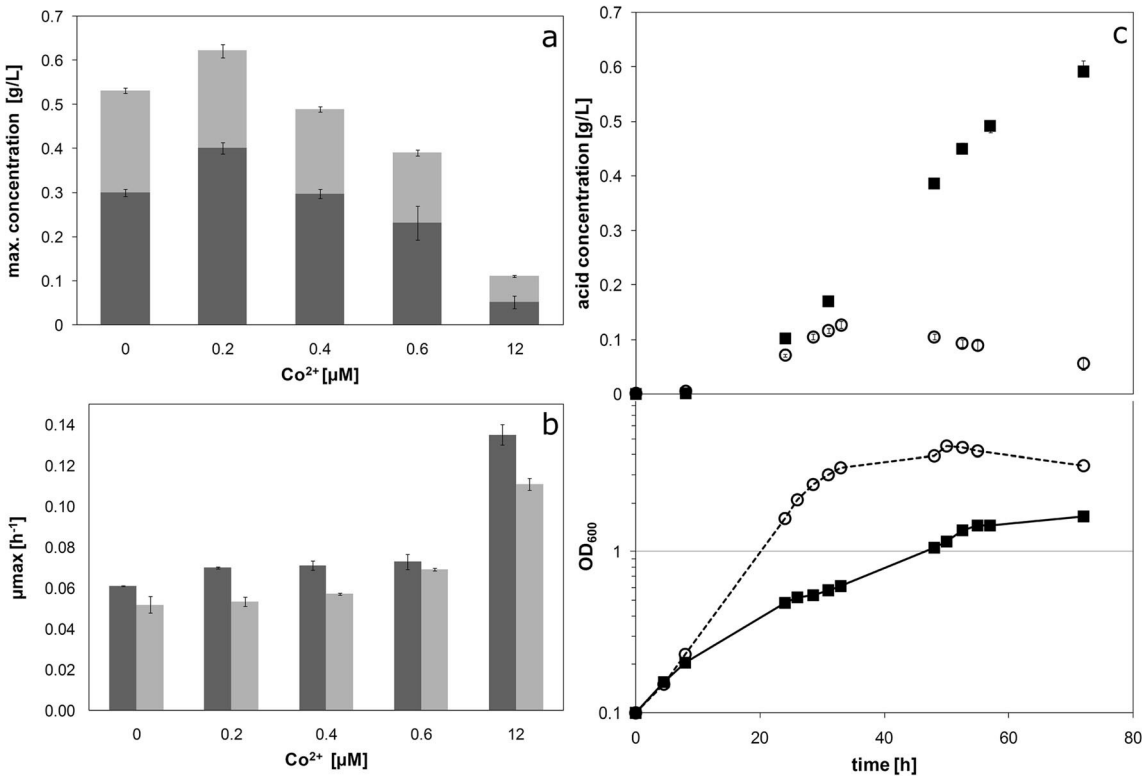

Fig. 5 Comparison of the production of EMCP-derived dicarboxylic acids (2*S*)-methylsuccinic acid and mesaconic acid by *M. extorquens* AM1 expressing thioesterase *yciA* under cobalt-limited and non-limited growth conditions. **a** Total EMCP-derived acid concentrations in culture supernatant with respective shares of mesaconic acid (*dark gray*) and (2*S*)-methylsuccinic acid (*light gray*) at different cobalt concentrations. **b** Growth rate comparison of *M. extorquens* AM1 harboring pCM160 (*black bars*) or pCM160-RBS-*yciA* (*empty bars*) at different cobalt concentrations. **c** Comparison of total EMCP-derived acid concentrations in supernatant and growth of AM1_pCM160-RBS-*yciA* in medium with 12 μM (*empty circles*) or 0.2 μM cobalt (*filled squares*). $n=3$; *error bars* represent standard deviations

enzymes built during preculture cultivation. However, production of mesaconic acid and (2*S*)-methylsuccinic acid with 0 μM was lower than with 0.2 μM cobalt.

In contrast to the experiments with AM1_pCM160-RBS-*yciA* growing under non-limiting cobalt concentrations (12 μM Co^{2+}), a decrease of the acid concentration after the end of the exponential growth phase was not observed for limiting cobalt concentrations (see Fig. 5c). Also, after further incubation of the culture grown with 0.2 μM Co^{2+} for 2 days, acid concentration remained almost constant (data not shown).

The cultivation of *M. extorquens* AM1 expressing thioesterase *yciA* in methanol minimal medium containing reduced cobalt concentration of 0.2 μM increased the final combined titer of mesaconic acid and (2*S*)-methylsuccinic acid from 0.1 to 0.65 g/l compared to cultivations in standard medium containing 12 μM cobalt. Besides, the observed effect of decreasing acid concentrations during stationary growth phase was not detectable.

Combination of EMCP-derived acid production under cobalt-limited growth conditions with reduced sodium ion concentration and PHB synthesis negative strains

As described above, the observed decrease of mesaconic acid and (2*S*)-methylsuccinic acid during stationary growth phase of AM1_pCM160-RBS-*yciA* grown in medium containing 12 μM cobalt was not detectable in the medium with 0.2 μM cobalt, making efforts toward the reduction of the acid uptake unnecessary. Nevertheless, we analyzed the possible effects of a reduced sodium concentration on EMCP-derived acid production in medium with growth-limiting cobalt concentration.

In summary, neither growth rate nor production of mesaconic acid or (2*S*)-methylsuccinic acid of AM1_pCM160-RBS-*yciA* was changed in cobalt-limited medium when the sodium ion concentration was reduced from 486 to 16 μM (data not shown).

Strain Δ*phaC* expressing thioesterase *yciA* showed increased EMCP-derived dicarboxylic acid production under non-growth-limiting cobalt concentrations, but acid production by frequently occurring suppressors was even below that of AM1_pCM160-RBS-*yciA*. Hence, EMCP-derived acid production of Δ*phaC*_pCM160-RBS-*yciA* growing in cobalt-limited medium might be further increased and was therefore investigated.

Growth of Δ*phaC*_pCM160-RBS-*yciA* was extremely slow in medium containing 0.4 μM Co^{2+} (see Fig. 6) with a maximum growth rate of 0.033 h^{-1} (±0.004) which was about two thirds of the growth rate of control strain Δ*phaC*_pCM160 (0.053 h^{-1} (±0.002)) and half of the growth rate of AM1_pCM160-RBS-*yciA*. The minor growth of Δ*phaC*_pCM160-RBS-*yciA* was concomitant with a production of 82 mg/l mesaconic acid and 46 mg/l (2*S*)-methylsuccinic acid

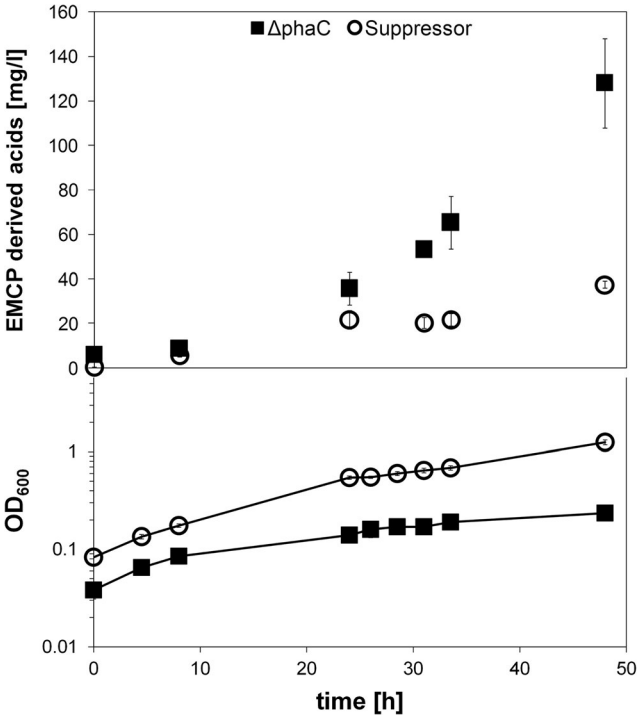

Fig. 6 Growth and EMCP-derived acid production (combined concentration of mesaconic acid and (2*S*)-methylsuccinic acid) of Δ*phaC*_pCM160-RBS-*yciA* and a Δ*phaC*_pCM160-RBS-*yciA* suppressor strain growing in medium containing 0.4 μM cobalt. n=2; *error bars* represent standard deviations

after 48 h which was even less than the product concentrations synthesized by AM1_pCM160-RBS-*yciA* at cobalt concentrations of 0.4 or 0.2 μM (see Fig. 5). Furthermore, growth-limiting cobalt concentrations did not prevent the occurrence of Δ*phaC* suppressors. Reinoculation of Δ*phaC*_pCM160-RBS-*yciA* after storage on solid methanol minimal medium for 2 weeks at 4 °C in methanol medium containing 0.4 μM Co^{2+} resulted in faster growth and strongly reduced production of mesaconic acid and (2*S*)-methylsuccinic acid (see Fig. 6) which is comparable to the observations for non-limiting cobalt concentrations.

EMCP-derived acid production and growth of Δ*phaR*_pCM160-RBS-*yciA* grown in cobalt-limited medium (0.2 μM Co^{2+}) was not changed compared to AM1_pCM160-RBS-*yciA* (data not shown). This result confirms that flux through the EMCP to mesaconyl- and (2*S*)-methylsuccinyl-CoA is not enhanced in strain Δ*phaR*.

In summary, the combination of growth-limiting cobalt concentrations with reduced sodium ion concentration or PHB synthesis negative strains did not further improve EMCP-derived acid production achieved with AM1_pCM160-RBS-*yciA* grown in methanol minimal medium with growth-limiting cobalt concentrations (see Table 2). Table 2 also illustrates that AM1_pCM160-RBS-*yciA* grown in medium containing 0.2 μM Co^{2+} led to the highest product yield per substrate of 0.17 g/g, whereas the highest yield per biomass (1.63 g/g) was

Table 2 Overview of maximum concentrations of mesaconate and (2*S*)-methylsuccinate produced by *M. extorquens* AM1, Δ*phaC*, or Δ*phaR* expressing thioesterase *yciA* cultivated in methanol minimal medium containing 0.2 or 12 μM cobalt in combination with 468 (+) or 46 (−) μM sodium

Strain:		AM1				Δ*phaC*[a]				Δ*phaR*			
Co^{2+} [μM]:		0.2		12		0.2[b]		12		0.2		12	
Medium containing 468 (+) or 46 (−) μM sodium:		+	−	+	−	+	−	+	−	+	−	+	−
Mesaconate	c_{max} [g/l]	0.389	n.s.c.	0.051	0.109	0.082	ND	0.311	ND	0.443	ND	0.046	ND
(2*S*)-Methylsuccinate	c_{max} [g/l]	0.266	n.s.c.	0.059	0.142	0.046	ND	0.186	ND	0.222	ND	0.037	ND
Total EMCP acids	c_{max} [g/l]	0.655	n.s.c.	0.11	0.251	0.128	ND	0.497	ND	0.665	ND	0.084	ND
	$Y_{P/S}$ (g/g)	0.17	n.s.c.	0.03	0.06	0.03	ND	0.13	ND	0.17	ND	0.02	ND
	$Y_{P/X}$ (g/g)	1.12	n.s.c.	0.08	0.21	1.63	ND	0.6	ND	1.17	ND	0.07	ND

ND not determined, *n.s.c.* not significantly changed compared to 12 μM Co^{2+}, $Y_{P/S}$ product yield per substrate, $Y_{P/X}$ product yield per dry biomass

[a] Values given for nonsuppressor Δ*phaC*

[b] Note that growth of nonsuppressor Δ*phaC* in methanol minimal medium is almost not detectable

achieved by slow-growing Δ*phaC*_pCM160-RBS-*yciA* under growth-limiting cobalt concentrations.

Discussion

In this study, we focused on the optimization of EMCP-derived dicarboxylic acid production with *M. extorquens* from methanol by investigating the impact of dicarboxylic acid transport, abolishment of PHB synthesis as unwanted side reaction and improved precursor provision.

Understanding the function and regulation of organic acid transporters is an important issue for the microbial synthesis of dicarboxylic acids and has been investigated in detail over the last two decades for classic production hosts such as *E. coli* or *Corynebacterium glutamicum* (Janausch et al. 2002; Teramoto et al. 2008). In bacteria, dicarboxylic acid uptake under aerobic conditions is mainly carried out by transporter DctA, and the corresponding gene including some regulator genes is present in the genome of strictly aerobic *M. extorquens* AM1 (Sonntag et al. 2014). Therefore, it was obvious to investigate whether a deletion of *dctA* has an impact on the transport and thereby on the production of EMCP-derived dicarboxylic acids.

Knockout of the *dctA* homologue had no effect on the utilization of eight tested EMCP-derived and TCA dicarboxylic acids as sole carbon source, although Van Dien et al. (2003a) reported that a transposon mutant carrying a deletion inside a *dctA* transporter gene was unable to use succinate as sole carbon source. Unfortunately, the reported accession number of this gene is not directly assignable to one of the three *dctA* homologues identified in the genome of *M. extorquens* AM1 (Sonntag et al. 2014). Therefore, the homologue with the highest similarity on amino acid level, MexAM1_META1p3271, was deleted. The different phenotypes of our mutant and the mutant of Van Dien and coworkers might result from the knockout of different transporter genes or the possibility that the transposon also inactivated the gene of the DcuS sensor kinase which is required for a functional acid transport by DctA (Witan et al. 2012) and is located directly downstream of MexAM1_META1p3271 (Vuilleumier et al. 2009). For rational engineering of the dicarboxylic acid reuptake and export systems in the future, characterization of the putative factors involved would be necessary.

However, a clear reduction of product reuptake and an increased maximal product concentration could be achieved by the use of a medium with reduced sodium concentration. The ratio behind this approach was the finding of sodium dependency for some DctA-type dicarboxylic acid transporters (Janausch et al. 2002) which are the only known dicarboxylic acid transporters encoded in *M. extorquens* genome (Sonntag et al. 2014), although most of the DctA transporters in other Gram-negative bacteria were shown to be proton coupled (Groeneveld et al. 2010; Youn et al. 2009). More detailed investigations will be necessary to clarify if reduction of the sodium concentration really has an impact on the dicarboxylic acid import or if further metabolization of mesaconic acid and (2*S*)-methylsuccinic acid is affected by this change in medium composition.

Metabolic flux redirection into desired product compounds is a widely applied strategy for optimization of biotechnological production processes. Thereby, deletion of genes encoding enzymes converting the desired product or its precursor(s) often results in significantly increased product titers (Cao et al. 2011; Litsanov et al. 2012; Peters-Wendisch et al. 2005). The polyhydroxyalkanoate synthase PhaC of *M. extorquens* uses the EMCP intermediate (3*R*)-hydroxybutyryl-CoA as substrate for the synthesis of the undesired by-product PHB, thereby reducing the flux through the EMCP toward the dicarboxylic acid products (see Fig. 1). In addition, PHB can make up to 40 % of the dry

biomass of *M. extorquens* grown on methanol as sole carbon source (Korotkova and Lidstrom 2001), representing a drastic loss of carbon resulting in an unfavorable product yield.

The constructed Δ*phaC*_pCM160-RBS-*yciA* strain, which was evidently deficient in PHB synthesis, showed a 2-fold improved production of mesaconic acid and (2*S*)-methylsuccinic acid, confirming the assumption that more metabolic flux is directed toward the EMCP in strain Δ*phaC*. However, as already reported by Korotkova and Lidstrom (2001), Δ*phaC* produced suppressors at high frequency that recovered wild-type-like growth. In addition, the Δ*phaC*_-pCM160-RBS-*yciA* suppressor strain showed a strongly decreased maximal product concentration which was 10-fold lower than that of the nonsuppressor strain and even 4-fold lower than that of the wild-type strain.

The growth defect caused by the PHB synthesis knockout in *M. extorquens* is a phenomenon that has not been reported for other PHB-producing organisms harboring an EMCP such as *Rhodobacter capsulatus* or *Rhizobium meliloti* (Kranz et al. 1997a, b; Povolo et al. 1994; Schlegel et al. 1970). One exception is *Rhizobium etli* whose Δ*phaC* strain has a growth defect concomitant with 3- to 17-fold increased NADH levels and secreted TCA cycle-derived acids (Cevallos et al. 1996). Regarding the effects of *R. etli* Δ*phaC*, Korotkova and Lidstrom speculated about an NADH accumulation in *M. extorquens* Δ*phaC* caused by the absence of PHB which is an important redox sink in methanol-grown cells (Korotkova and Lidstrom 2001). *M. extorquens* citrate synthase is inhibited by NADH (Anthony 1982) resulting in a block of the TCA cycle which in turn could explain the growth defect of the Δ*phaC* strain. The strongly decreased EMCP-derived acid production of the Δ*phaC* suppressor is in turn a hint for a redirected acetyl-CoA flux. An increased flux of acetyl-CoA into the TCA cycle concomitant with a reduced flux into the EMCP would explain the recovery of the wild type-like growth as well as the reduced acid release due to less available EMCP CoA esters as substrate for thioesterase YciA.

A promising candidate for a regulator protein responsible for the control of acetyl-CoA flux toward the TCA cycle and EMCP is the regulator protein PhaR which plays an important role in acetyl-CoA flux direction in AM1's close relative *Methlyobacterium rhodesianum* (Mothes et al. 1996, 1998). *M. extorquens* Δ*phaR* is not able to synthesize PHB just as Δ*phaC* but does not produce any second site suppressors (Korotkova et al. 2002). Hence, Δ*phaR* was tested for improved EMCP-derived acid production. However, the observed mesaconic and (2*S*)-methylsuccinic acid concentrations did not distinctly differ between Δ*phaR* and AM1 wild type expressing thioesterase *yciA*.

PhaR has been studied extensively in *E. coli* and *Pseudomonas denitrificans* where it can bind simultaneously to the PHB granule-associated protein PhaP and upstream of the *phaP* gene, thereby regulating PHB granule formation in a complex manner (Yamada et al. 2013, 2007a, b). Assuming a similar PHB cycle regulation in *M. extorquens*, the formation of PHB granules in AM1Δ*phaR* would be inhibited but the undesired synthesis of PHB from (3*R*)-hydroxybutyryl-CoA catalyzed by PhaC is probably still existent. Furthermore, it has been shown that about 70 % more acetyl-CoA is directed into the TCA cycle in AM1Δ*phaR* (Van Dien et al. 2003b) which is likely accompanied with a reduced EMCP flux. However, the final reaction sequence of the EMCP was not identified in 2003 so that this obvious assumption was not verified. In consequence, Δ*phaR* does not seem to represent a suitable alternative to Δ*phaC* to increase EMCP-derived acid production via shutdown of PHB synthesis.

As the product precursor molecules mesaconyl- and (2*S*)-methylsuccinyl-CoA are intermediates in the essential EMCP, deletion of the gene encoding the respective downstream enzyme is not applicable for an increase of product precursor pools as it would either require expensive feeding of intermediates or the establishment of a metabolic shunt. Therefore, downregulation of the corresponding enzymatic steps, i.e. (2*S*)-methylsuccinyl-CoA dehydrogenase and mesaconyl-CoA dehydratase (see Fig. **1**), is a more straightforward approach, and such a strategy has been applied for other essential biosyntheses routes (Asadollahi et al. 2008).

The EMCP contains two mutases that are dependent on cobalt in the form of vitamin B_{12} (Alber 2011; Erb et al. 2008; Peyraud et al. 2009). Cobalt limitation caused by stress-induced uptake deficiency leads to significantly increased ethyl- and methylmalonyl-CoA pools (Kiefer et al. 2009) which are the substrates of the two cobalt-dependent mutases. Under the conditions used in our experiments, cobalt limitations led to a strong accumulation of nearly all EMCP CoA esters. Methylmalonyl-CoA, for whose synthesis both mutase steps are required, was most affected. The pool of ethylmalonyl-CoA and that of all following CoA esters accumulated 3- to 15-fold, whereas the pool sizes of all CoA esters in the EMCP occurring before the ethylmalonyl-CoA mutase step showed no significant changes. Apparently, methylmalonyl-CoA mutase is more affected by cobalt limitation which creates the major EMCP block, but ethylmalonyl-CoA mutase still provides enough activity which is sufficient for accumulation of subsequent EMCP intermediates. These data show that cobalt limitation cannot only be used to increase the ethyl- or methylmalonyl-CoA pools but also most of the other EMCP CoA ester pools. This effect was also used in another study to provide sufficient amounts of EMCP-derived intermediates for the de novo biosynthesis of functionalized PHAs (Orita et al. 2014).

The increased precursor pools of mesaconyl- and (2*S*)-methylsuccinyl-CoA under growth-limiting cobalt conditions led to significantly increased production of mesaconic acid and (2*S*)-methylsuccinic acid when thioesterase *yciA* was expressed in *M. extorquens* (see Fig. **5**). This is likely

attributed to the increased substrate availability of the thioesterase as well as substrate concentrations which are higher or at the level of YciA's K_m values (Sonntag et al. 2014). In addition, growth under cobalt limitation implies another benefit for mesaconic and (2*S*)-methylsuccinic acid production: Decrease of the concentrations in supernatant during stationary growth phase was not or hardly observable which would simplify a future process implementation.

The cultivation of Δ*phaC* under growth-limiting cobalt concentrations did neither increase EMCP-derived acid product concentrations nor prevented the occurrence of the suppressors of strain Δ*phaC* concomitant with the already discussed disadvantages. The low concentration of EMCP-derived acids produced by the nonsuppressor strain is an effect of its very slow growth, but the yield per biomass of this strain was clearly above the value of AM1_pCM160-RBS-*yciA* (see Table 2). Hence, with a stable PHB^- strain in hand, decoupling biomass and product formation could allow combination of abolished PHB synthesis and increased EMCP intermediate availability due to cobalt limitation.

In summary, we increased the production of the EMCP-derived dicarboxylic acids, mesaconic acid and (2*S*)-methylsuccinic acid, 6-fold to a combined total titer of 0.65 g/l, representing a yield of 0.17 g/g methanol, by growing *M. extorquens* that expresses thioesterase *yciA* under cobalt-limited conditions (see Table 2). Reuptake of the released acids during stationary growth phase does not take place to a relevant extent under growth-limiting cobalt concentrations in contrast to non-growth-limiting cobalt concentrations (12 μM Co^{2+}). Furthermore, EMCP-derived acid production is strongly increased in PHB synthase knockout strain Δ*phaC* but decreased even below concentrations released by the wild type in the frequently occurring suppressors of Δ*phaC* in which the acetyl-CoA flux is probably redirected toward the TCA cycle. The lack of the PHB synthesis regulator protein PhaR prevents PHB accumulation but does not improve production of mesaconic or (2*S*)-methylsuccinic acid in thioesterase *yciA*-expressing cells.

This work is not only an important step toward achieving industrially relevant EMCP-derived acid productivities and yields but also illustrates the complex regulation and metabolic interplay of EMCP-connected PHB synthesis in *M. extorquens*.

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7.1.3 *Methylobacterium extorquens*: methylotrophy and biotechnological applications

Type: Review

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Anlage 1

Erklärung über Anteile der Autoren/Autorinnen an den einzelnen Kapiteln der Promotionsarbeit

Titel der Publikation/ des Manuskripts: Methylobacterium extorquens: methylotrophy and biotechnological applications

	Was hat der/die Promovierende bzw. was haben die Co-Autoren/Autorinnen beigetragen[#]	Name des/der jeweiligen Autors/Autoren/Autorin*
(1) Entwicklung und Planung	je 30%	Ochsner A.M., Sonntag F.
	25%	Vorholt J.A.
	10%	Schrader J.
	5%	Buchhaupt M.
(2) Durchführung der einzelnen Untersuchungen/ Experimente	nicht durchgeführt (Review)	
(3) Erstellung der Daten-sammlung und Abbildungen	Abbildung 1	Ochsner A.M., Vorholt J.A.
	Abbildung 2	Sonntag F., Schrader J.
	Tabellen 1-3	Ochsner A.M., Vorholt J.A.
	Tabelle 4, S1	Sonntag F.
(4) Analyse/Interpretation der Daten	nicht durchgeführt (Review)	
(5) übergeordnete Einleitung/ Ergebnisse/Diskussion	je 35%	Ochsner A.M., Sonntag F.
	20%	Vorholt J.A.
	8%	Schrader J.
	2%	Buchhaupt M.

[#]Bei 2, 3 und 4 bitte kurze inhaltliche Angaben der jeweiligen Anteile, bei 1 und 5 reichen prozentuale Angaben

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MINI-REVIEW

Methylobacterium extorquens: methylotrophy and biotechnological applications

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Abstract Methylotrophy is the ability to use reduced one-carbon compounds, such as methanol, as a single source of carbon and energy. Methanol is, due to its availability and potential for production from renewable resources, a valuable feedstock for biotechnology. Nature offers a variety of methylotrophic microorganisms that differ in their metabolism and represent resources for engineering of value-added products from methanol. The most extensively studied methylotroph is the Alphaproteobacterium *Methylobacterium extorquens*. Over the past five decades, the metabolism of *M. extorquens* has been investigated physiologically, biochemically, and more recently, using complementary omics technologies such as transcriptomics, proteomics, metabolomics, and fluxomics. These approaches, together with a genome-scale metabolic model, facilitate system-wide studies and the development of rational strategies for the successful generation of desired products from methanol. This review summarizes the knowledge of methylotrophy in *M. extorquens*, as well as the available tools and biotechnological applications.

Keywords *Methylobacterium extorquens* · Methanol · Methylotrophy · Industrial biotechnology

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Introduction

Methylobacterium is a widespread genus that can be readily isolated from various habitats including wastewater (Kohler-Staub et al. 1986), soil (Doronina and Trotsenko 1996), and the phyllosphere where they are present in large numbers (Delmotte et al. 2009; Vorholt 2012). Its ability to use plant-derived methanol as an energy substrate provides an advantage during plant colonization under competitive conditions (Abanda-Nkpwatt et al. 2006; Fall and Benson 1996; Sy et al. 2005). One of the most intensively studied species of the genus is *Methylobacterium extorquens* and in particular strain AM1 which has been investigated as a model organism for methylotrophy for more than 50 years (Anthony 2011). Detailed information on the methylotrophic metabolism has been uncovered from core enzyme functions to pathways up to a genome-scale metabolic model. In addition, omics approaches have helped to describe methylotrophy on a systems level. The latter approaches initially became possible through the determination of the genome of *M. extorquens* AM1, for which a fully annotated genome sequence is available, as well as a genome-scale metabolic model. Furthermore, a versatile set of genetic tools has been developed and applied for gene deletions and overexpression, as well as random mutagenesis and chromosomal gene integration. Finally, bioprocess engineering led to the development of high cell density fermentation protocols for several *M. extorquens* strains (see below).

The accumulated knowledge about the biology and technical use of *M. extorquens* together with the availability of genetic tools has led to a constant increase in interest in the use of this methylotroph as a microbial host for the production of value-added compounds. Accretive industrialization and growing world population encourage the discussion about alternative raw materials to become more independent of steadily

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increasing oil prices but also to avoid direct competition with resources used for food production (Schrader et al. 2009).

Methanol is already an important carbon feedstock for the chemical industry with a worldwide production of approximately 53 million tons in 2011 and an expected annual growth rate in the range of 10–20 % (Bertau et al. 2014), and a methanol-based bioeconomy has been proposed (Olah 2013). In addition to its worldwide availability, methanol production is flexible. The majority of today's methanol is produced from fossil raw materials, such as crude oil, coal, and natural gas, but in the future an increasing share is expected to be produced from renewables or sustainable sources, such as biogas, glycerol, wood, or solid municipal waste (Schrader et al. 2009). For example, a biomass-to-methanol (BtM) plant (that produces methanol starting from forest residues) with a capacity of 100,000 t/a is currently being projected in Sweden (Bertau et al. 2014). Such endeavors illustrate that methanol is producible in various ways, and consequently, industrial biotechnology based on this compound is less dependent on market price fluctuations of raw materials. However, compared to sugars, the market price of which is approximately 0.2$/kg (Peralta-Yahya et al. 2012) corresponding to 0.5$ per kilogram substrate carbon, methanol is still more expensive with a price of approximately 0.45$/kg (Bertau et al. 2014), corresponding to approximately 1.2$ per kilogram substrate carbon. On the other hand, sugar is an agricultural commodity, which is subject to strict price regulations and import limitations in Europe. This impairs free-market conditions which apply for any intermediate in the chemical industry, including methanol. As the price for sugar derived from arable land—the common fermentation substrate in industrial biotechnology—is expected to steadily increase due to its linkage to the food sector, and sugar from lignocellulosics still suffers from severe technical challenges, "bio-methanol" will become an attractive alternative for the establishment of sustainable bioprocesses in the future (Schrader et al. 2009).

Within this review, we summarize the current knowledge of methylotrophy in *Methylobacterium* and its use for methanol conversion to value-added products. Additional applied aspects of *Methylobacterium* strains, such as plant growth-promoting microbials in agriculture (Fedorov et al. 2011; Joe et al. 2013; Tani et al. 2012), are beyond the scope of this review. For a recent review on a thermophilic methylotroph, *Bacillus methanolicus*, the reader is referred to Müller et al. (2014).

Model strains of *M. extorquens*, growth substrates and genomes

The first model methylotrophic bacterium and the most extensively studied to date is *M. extorquens* AM1. It was initially called *Pseudomonas* AM1 and was isolated in Oxford as an airborne contaminant in methylamine medium (Peel and Quayle 1961), hence the name AM1 for airborne methylamine. It grows on the reduced C1 substrates methanol and methylamine but also as a facultative methylotroph on multicarbon substrates such as succinate (C4) and acetate (C2) (Large et al. 1961; Schneider et al. 2012a) (for a more detailed list, see Table S1). The genome sequence of *M. extorquens* AM1 revealed a 6.88-Mb genome consisting of a chromosome, a megaplasmid, and three smaller plasmids (Vuilleumier et al. 2009). An additional number of *Methylobacterium* strains have been sequenced (Marx et al. 2012; Vuilleumier et al. 2009) (Table 1) including *M. extorquens* PA1 that was recently selected as a competitive colonizer of the phyllosphere of *Arabidopsis thaliana* (Knief et al. 2010) (PA1 for phyllosphere of *Arabidopsis*). Further *M. extorquens* strains that have been characterized and sequenced include strains that are able to grow on chloromethane (strain CM4) and dichloromethane (strain DM4). All of the strains harbor a genome of 5.4–5.9 Mb and one or two plasmids (except strain PA1 which has none); as mentioned above, strain AM1 also contains a megaplasmid. While the function of the plasmids of *M. extorquens* AM1, DM4, and BJ001 is unknown, the 380-kb plasmid of strain CM4 was shown to carry genes for chloromethane dehalogenation (Roselli et al. 2013).

This review will mainly focus on the model strain *M. extorquens* AM1 and occasionally refer to strain PA1, which in our opinion represents a valuable alternative to *M. extorquens* AM1 for further research on methylotrophy. This is due to the simpler genome structure, the applicability of genetic screens (Metzger et al. 2013), and the overall conserved methylotrophy features compared to the AM1 strain (Nayak and Marx 2014b). In addition, strain PA1 lacks the long history of domestication, which led to divergence from the initially isolated strain (Carroll et al. 2014).

Tools developed and applied for *M. extorquens*

Genetic tools have been used since the early research of *M. extorquens* AM1 to identify essential genes for methylotrophy. Historically, chemical mutagenesis was applied (Heptinstall and Quayle 1970) in combination with complementation analysis to classify mutants into different complementation groups (Nunn and Lidstrom 1986). Subsequently, site-directed knockouts (Chistoserdova and Lidstrom 1992) and transposon mutagenesis (Springer et al. 1995) were established. To date, a broad selection of genetic tools for expression, knockout, and transposon mutagenesis, has been described (Table 2), and the genetic toolbox for *M. extorquens* and other Alphaproteobacteria is constantly

Table 1 List of genome sequenced *M. extorquens* strains

Strain	Isolation	C1 substrates	Genome
AM1	Airborne contaminant in methylamine (Peel and Quayle 1961)	Methanol, methylamine	Chromosome: 5.511 Mb Megaplasmid: 1.26 Mb Plasmids: 44 kb, 38 kb, 25 kb (Vuilleumier et al. 2009)
DM4[a]	Soil from a treatment plant for halogenated hydrocarbon waste (Kohler-Staub et al. 1986)	Methanol, dichloromethane (Kohler-Staub et al. 1986)	Chromosome: 5.94 Mb Plasmids: 140 kb, 39 kb (Vuilleumier et al. 2009)
PA1	*Arabidopsis thaliana* phyllosphere (Knief et al. 2010)	Methanol, methylamine (Knief et al. 2010)	Chromosome: 5.471 Mb (Marx et al. 2012)
CM4[b]	Soil of petrochemical factory (Doronina and Trotsenko 1996)	Methanol, methylamine, chloromethane (Doronina and Trotsenko 1996)	Chromosome: 5.778 Mb Plasmids: 380 kb, 23 kb (Marx et al. 2012)
BJ001[c]	*Populus deltoides* × *nigra* DN34 endophyte (Van Aken et al. 2004)	Methanol, methane[d] methylamine (Van Aken et al. 2004)	Chromosome: 5.8 Mb plasmids: 25 kb, 23 kb (Marx et al. 2012)

[a] Previously *Methylobacterium dichloromethanicum* DM4

[b] Previously *Methylobacterium chloromethanicum* CM4

[c] Previously *Methylobacterium populi* BJ001

[d] Was disputed (Dedysh et al. 2004); genome sequencing later revealed the lack of characteristic genes for methane monooxygenase (*pmoABC* and *mmoXYBZDC*)

expanding. Moreover, a recent study provided a modified medium and a genetically engineered strain, which lacks cellulose synthase, optimized for reproducible high-throughput growth experiments (Delaney et al. 2013).

Table 2 List of available genetic tools for *M. extorquens* AM1

Genetic tools	Description	Reference
Expression systems		
Plasmid-borne		
Constitutive		
P_{mxaF}/P_{lac}	pCM80 (TetR), pCM160 (KanR) IncP *oriV*, *oriT* (RK2-derived)	Marx and Lidstrom (2001)
Methylobricks (P_{mxaF}), P_{fumC}, P_{coxB}, P_{tuf}	Derived from promoter-less pCM80 (TetR) (also KanR available)	Schada von Borzyskowski et al. (2014)
Inducible		
Cumate-inducible P_{mxaF}	Regulator cymR chromosomally integrated	Choi et al. (2006)
Cumate-inducible P_{mxaF}	pHC115, cymR encoded on plasmid, comparatively high background expression	Chou and Marx (2012)
Cumate/anhydrotetracycline-inducible P_R	pLC290, pLC291 (both KanR)	Chubiz et al. (2013)
Cumate-inducible P_{syn2}	pQ2148, tight promoter	Kaczmarczyk et al. (2013)
Insertional		
pCM168/pCM172	Chromosomal insertion of desired gene into *katA* gene *loxP*-flanked resistance marker	Marx and Lidstrom (2004)
Transposons used for genetic screens		
pCM639	Mini Tn-5 derivative IsphoA/hah-Tc	Marx et al. (2003c)
pAlmar3	Mariner transposon (used in strain PA1)	Metzger et al. (2013)
Site-directed knockout		
pAYC61		Chistoserdov et al. (1994)
pCM184	Cre-lox-based system allows unmarked deletions	Marx and Lidstrom (2002)
pCM433	SacB-based bhr vector	Marx (2008)

The fact that *M. extorquens* is a facultative methylotroph represents a useful trait to study methylotrophy. Knockout mutants in essential genes for methylotrophy can be generated on an alternative carbon source and then selected on methanol, thus facilitating the discrimination between true essential genes for methylotrophy and potential technical obstacles to generate a particular gene knockout.

Much of the knowledge generated for *M. extorquens* AM1 is based on classical biochemical studies resulting from enzyme discovery to gene discovery or vice versa. More recently, a number of complementary omics approaches have been applied including transcriptomics, proteomics, and metabolomics to characterize methylotrophy more systematically (Table 3). In these studies, cell analytes detected under methylotrophic conditions were compared to those detected under non-methylotrophic growth conditions, usually succinate, which results in similar growth rates (Peyraud et al. 2012). These studies gave valuable insights into methylotrophic metabolism and represent useful tools for further studies. Comparative proteomics and a microarray study revealed proteins/transcripts that were differentially expressed under methylotrophic conditions compared to non-methylotrophic conditions (Bosch et al. 2008; Laukel et al. 2004; Okubo et al. 2007). These experiments demonstrated the induction of proteins/transcripts known to be involved in methylotrophy, as well as many with unknown functions.

Different methods to study the metabolome have been applied to *M. extorquens*. These methods include nucleotide determination (including ATP and NAD) (Guo and Lidstrom 2006), metabolite profiling (Guo and Lidstrom 2008), and quantitative metabolome analyses (Kiefer et al. 2011; Kiefer et al. 2008). In addition, a method to quantify CoA-esters has been established and was employed to demonstrate the ethylmalonyl-CoA pathway (EMCP) in *M. extorquens* AM1 (Peyraud et al. 2009).

Recently, a genome-scale model of the methylotrophic metabolism of *M. extorquens* was established by integrating all available information including omics, biochemical, and mutation studies (Peyraud et al. 2011). The functionality of the metabolic network was analyzed in silico including elementary flux mode analysis, and the model was tested for network topology and features. In addition, methylotrophy was examined experimentally using ^{13}C-fluxomics via NMR and mass spectrometry (Peyraud et al. 2011). The study provided valuable information about the different pathways utilized during methylotrophic growth and demonstrated the presence of a degree of connectivity of the core metabolism as a mosaic of common and specific pathways. It was also proposed that methylotrophy is under constant selection in nature, which is in line with experimental evolution experiments, where prolonged selection on succinate resulted in a loss of the ability to grow on C1 compounds in a part of the population (Lee et al. 2009).

Methylotrophy in *M. extorquens*

Methylotrophy can be divided into different parts, which altogether allow metabolization of the reduced C1 carbon source for energetic and biosynthetic purposes: (1) oxidation of primary C1 substrate to formaldehyde, (2) oxidation of formaldehyde to CO_2, and (3) assimilation into biomass (Chistoserdova 2011). Different combinations of enzymes and pathways exist, which represent "solutions" found in nature. In the following section, the crucial steps of methylotrophy in *M. extorquens* AM1 are described after a short historical perspective. A graphical overview of methylotrophy in *M. extorquens* AM1 is shown in Fig. 1 and a list of the involved genes including information on their essentiality and regulation is given in Table S2.

M. extorquens AM1 as a model organism for enzyme and pathway discovery

As mentioned above, *M. extorquens* AM1 has been an important model organism for methylotrophy in the past half century. In fact, many enzymes and pathways relevant for C1 metabolism in more general terms have been discovered in this bacterium and are now known to be widespread among methylotrophs (Chistoserdova 2011).

The serine cycle for C1 assimilation was elucidated in *M. extorquens* AM1 already more than five decades ago through the seminal work of Quayle and colleagues (Large et al. 1961, 1962a, b; Large and Quayle 1963). The continuous operation of this pathway requires the regeneration of glyoxylate. Interestingly, *M. extorquens* lacks isocitrate lyase (Dunstan and Anthony 1973), the key enzyme of the known glyoxylate cycle (Kornberg and Krebs 1957), and uses an alternative route for glyoxylate regeneration. The exact sequence of reactions of this pathway, termed the EMCP, was only solved a few years ago (Erb et al. 2007; Peyraud et al. 2009). For a historic overview of the elucidation of the serine cycle and the EMCP, we refer to the recent review by Anthony (2011).

Another important discovery was that the catabolic formaldehyde oxidation in *M. extorquens* AM1 depends on tetrahydromethanopterin (H_4MPT), which was previously believed to be unique to methanogenic and sulfate-reducing Archaea (Chistoserdova et al. 1998). In *M. extorquens*, the H_4MPT-dependent pathway functions in the reverse direction compared to the known mode of operation in hydrogenotrophic methanogens and catalyzes the oxidation of formaldehyde to formate. The pathways described above were subsequently discovered in other microorganisms capable of growth on C1 compounds, including aerobic proteobacterial methanotrophs (Chistoserdova et al. 2005b; Vorholt et al. 1999) as well as an anaerobic bacterial methanotroph (Ettwig et al. 2010).

Table 3 Overview of omics studies investigating methylotrophy in *M. extorquens* AM1

Omics study	Reference	Description
Genome	Vuilleumier et al. (2009)	
Genome scale model	Peyraud et al. (2011)	
Proteomics	Laukel et al. (2004), Bosch et al. (2008)	Comparison of methanol and succinate: gel-based approach (2004) and multidimensional protein identifications technology (MudPIT) (2008)
Transcriptomics	Okubo et al. (2007)	Comparison of methanol and succinate microarray
Metabolomics		
Metabolite profiling	Guo and Lidstrom (2008)	Metabolite profiles during growth on methanol and succinate
Quantitative metabolome analysis	Kiefer et al. (2011); Kiefer et al. (2008)	Concentration of central metabolites on methanol and succinate
CoA-esters determination	Peyraud et al. (2009)	Quantification and dynamic labeling of CoA-esters during methylotrophic growth
^{13}C-Fluxomics	Peyraud et al. (2011)	Investigation of metabolic fluxes during methylotrophic growth
Metabolite discovery	(Yang et al. 2013)	Determination of metabolites labeled during growth on ^{13}C-labeled methanol

Methanol oxidation

In *M. extorquens*, methanol oxidation is catalyzed by a periplasmatic pyrroloquinoline quinone (PQQ)-dependent methanol dehydrogenase (MDH) (Anthony 1986). MDH has a hetero-tetrameric $\alpha_2\beta_2$ structure where each α-subunit contains one Ca^{2+} ion and one PQQ as a prosthetic group (reviewed in Anthony and Williams 2003). The large (66 kDa) α-subunit is encoded by *mxaF* and the small (9 kDa) β-subunit by *mxaI*. The electrons taken up by the MDH-bound PQQ during the oxidation of methanol are transferred to the unique cytochrome c_L (*mxaG*), then further via the type I cytochrome c_H to cytochrome *c* oxidase and eventually to oxygen (Anthony 1992). These genes are located in a gene cluster together with genes thought to have auxiliary functions (Morris et al. 1995; Richardson and Anthony 1992). The genes for the biosynthesis of the cofactor PQQ (*pqqABC/DE*, *pqqFG*) are located in two clusters elsewhere in

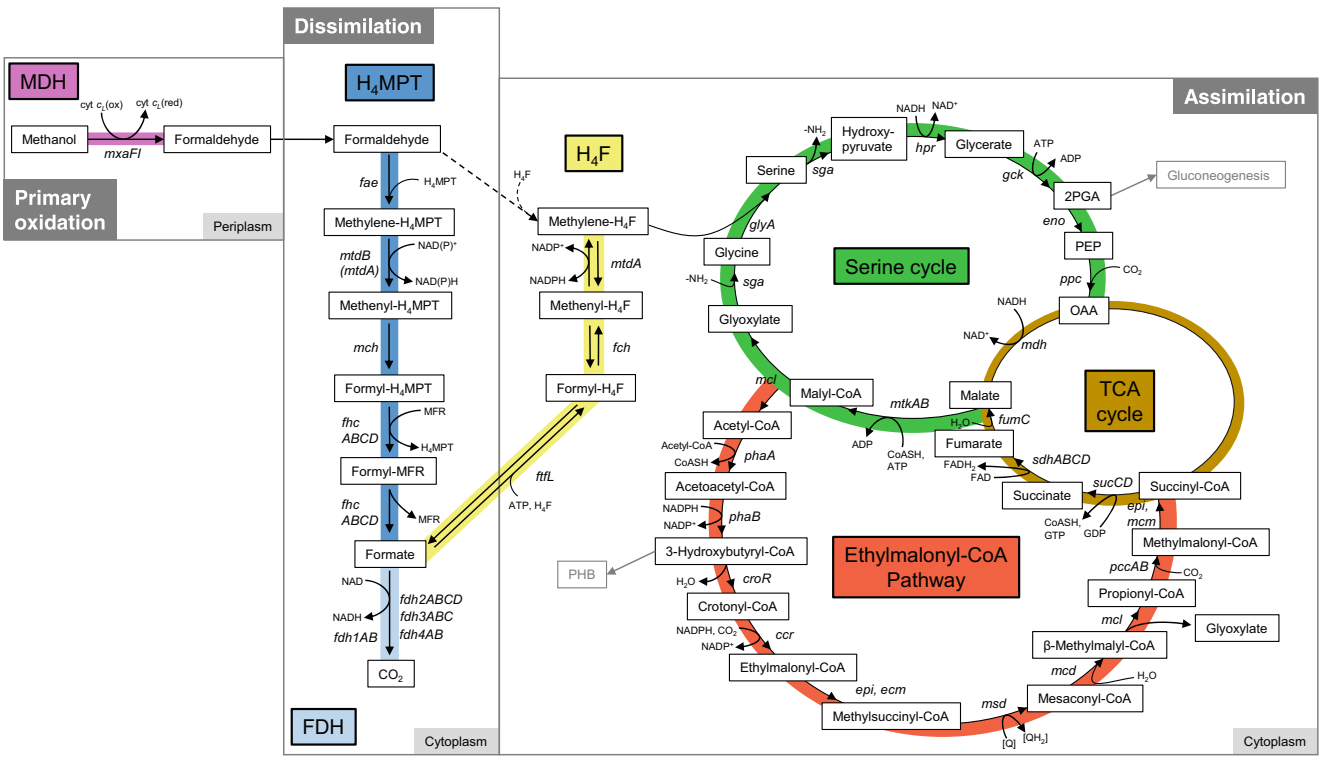

Fig. 1 Overview of methanol metabolism in *M. extorquens* AM1. The coloring marks the different pathways. Genes encoding for the corresponding enzymes are shown in *italics*. For a detailed list of the involved genes, refer to Table S2

the genome (Chistoserdova et al. 2003; Morris et al. 1994; Toyama et al. 1997).

The genome sequence of *M. extorquens* AM1 revealed the presence of several gene-encoding paralogs of the large subunit of methanol dehydrogenase, such as *xoxF1* (Chistoserdova and Lidstrom 1997), which has a sequence identity of approximately 50 %. The enzyme has methanol and formaldehyde oxidation activity (Schmidt et al. 2010) and is able to replace MxaF as a La^{3+}-dependent methanol dehydrogenases under distinct conditions (Nakagawa et al. 2012). In addition to its catalytic role, *xoxF1* is, together with its paralog *xoxF2*, postulated to be involved in regulation (Skovran et al. 2011). Interestingly, *xoxF* is highly expressed in the phyllosphere (Delmotte et al. 2009) and plays a role in plant colonization (Schmidt et al. 2010).

Formaldehyde dissimilation

Formaldehyde produced by methanol dehydrogenase in *M. extorquens* AM1 is further oxidized by a H_4MPT-dependent pathway (Chistoserdova et al. 1998) which, in addition to its catabolic role, can also participate in formaldehyde detoxification (Marx et al. 2003a) (Fig. 1). *M. extorquens* AM1 produces a dephosphorylated form of H_4MPT lacking the terminal α-hydroxyglutaryl phosphate unit (dH_4MPT) (Chistoserdova et al. 1998). Several genes involved in the biosynthesis of dH_4MPT in methylotrophs have been identified (Chistoserdova et al. 2005a) and the gene products of two of them, *mptG* (Rasche et al. 2004) and *dmrA* (Caccamo et al. 2004; Marx et al. 2003c)), have been characterized.

The first step in formaldehyde conversion consists of the condensation of formaldehyde with dH_4MPT to methylene-dH_4MPT, a step which occurs spontaneously (Escalante-Semerena et al. 1984). Nevertheless, an enzyme accelerating the condensation of formaldehyde with dH_4MPT, termed formaldehyde-activating enzyme (Fae), could be identified in *M. extorquens* AM1 and is essential for methylotrophy (Acharya et al. 2005; Vorholt et al. 2000). Fae is produced in high amounts under both methylotrophic and non-methylotrophic conditions, whereby the presence of the enzyme on methanol is slightly higher (Bosch et al. 2008). Mutants of the *fae* gene are sensitive to formaldehyde, suggesting that the spontaneous reaction is not efficient enough to detoxify formaldehyde (Vorholt et al. 2000).

The formed methylene-dH_4MPT is oxidized to methenyl-dH_4MPT by methylene-H_4MPT dehydrogenases MtdA and MtdB, which in contrast to their F_{420}-dependent archaeal counterparts (Thauer 1998) represent unique enzymes because they use pyridine nucleotides (NAD and NADP) (Vorholt et al. 1998). The NADP-specific MtdA catalyzes the reaction for methylene-tetrahydrofolate (H_4F) as well as methylene-H_4MPT (Ermler et al. 2002; Hagemeier et al. 2000; Vorholt et al. 1998), while the NAD(P)-dependent MtdB is specific for methylene-dH_4MPT (Hagemeier et al. 2000). They both are essential under methylotrophic growth conditions and play distinct roles in controlling the flux of C1 units (Martinez-Gomez et al. 2013; Marx and Lidstrom 2004).

Methenyl-dH_4MPT is converted to N^5-formyl-dH_4MPT by methenyl-dH_4MPT cyclohydrolase (Mch) (Pomper et al. 1999). The formyl group is then transferred to methanofuran (MFR) by formyltransferase (Ftr). The Ftr-homolog of AM1 (FhcD) forms a complex with three polypeptides that possess sequence identity to formyl-MFR dehydrogenase (FhcABC) (Pomper and Vorholt 2001). Contrary to initial expectations deduced from the analogous enzymatic steps in methanogenic archaea (Vorholt and Thauer 2002), this complex did not catalyze the oxidation of formyl-MFR but instead catalyzed the hydrolysis to formate. The complex was therefore renamed formyltransferase/hydrolase complex (Fhc) (Pomper et al. 2002).

The final oxidation step from formate to CO_2 is catalyzed by several formate dehydrogenases (Fdh), of which only one has been biochemically characterized thus far (Laukel et al. 2003). Subsequently, three more formate dehydrogenases were discovered (Chistoserdova et al. 2007; Chistoserdova et al. 2004). A triple mutant was still able to grow on methanol, but accumulated formate, whereas a quadruple mutant was unable to grow on methanol, which indicates that formate oxidation is essential for growth on methanol (Chistoserdova et al. 2007) and points towards an important role of formate as an intermediate with tightly regulated fluxes and/or yet undiscovered regulatory processes.

Assimilatory pathways

The serine cycle for the assimilation of C1 substrates was first shown in *M. extorquens* AM1 (Large et al. 1961, 1962a, b; Large and Quayle 1963). The C1 unit enters the serine cycle via condensation of methylene-H_4F (see below) with glycine to serine catalyzed by serine hydroxymethyltransferase (GlyA). GlyA is also present in most non-methylotrophic organisms where it provides methylene-H_4F for biosynthesis (Maden 2000). The produced serine is transformed to glyoxylate (to regenerate glycine) and acetyl-CoA. The pathway occurs via intermediates partly shared with gluconeogenesis and the tricarboxylic acid (TCA) cycle and involves a CO_2 fixation step (Anthony 1982) (Fig. 1). None of the genes of the serine cycle are specific for methylotrophic metabolism, but a characteristic feature for methylotrophs is the clustering of the involved genes on the genome (Chistoserdova 2011). In *M. extorquens* AM1, most serine cycle genes are present in one cluster and are transcribed in two operons (Chistoserdova et al. 2003).

The C1-donor methylene-H_4F for the conversion of glycine to serine can principally be produced in two ways: via a direct and indirect route. The direct pathway involves the spontaneous condensation of formaldehyde with H_4F to methylene-H_4F, whereas the indirect route involves the oxidation

of formaldehyde to formate via the H_4MPT-dependent pathway followed by ATP-dependent condensation with H_4F catalyzed by formate tetrahydrofolate ligase (FtfL) (Marx et al. 2003b). The produced formyl-H_4F is converted to methenyl-H_4F (catalyzed by Fch), and subsequently to methylene-H_4F catalyzed by the bifunctional NADP(P)-dependent MtdA enzyme (Vorholt et al. 1998). Experimental data on the operation of the H_4F-dependent pathway in situ is controversial. An earlier study suggested a direct condensation of formaldehyde with H_4F and an indirect route only during a substrate switch (Marx et al. 2005); however, a more recent paper provides evidence for the condensation of formate with H_4F followed by reduction being more important than the direct condensation of formaldehyde with H_4F (Crowther et al. 2008). Future studies using dynamic ^{13}C labeling from methanol will be required to directly demonstrate the carbon flow via cofactor-bound C1 intermediates.

Because metabolic intermediates are withdrawn from the serine cycle, glyoxylate must be regenerated to keep the pathway running. *M. extorquens* AM1 does not use the classical pathway involving isocitrate lyase but instead uses a series of CoA intermediates (Korotkova et al. 2002a; Korotkova et al. 2005). Based on the discovery of the carboxylation activity of crotonyl-CoA reductase (Ccr) (renamed crotonyl-CoA carboxylase/reductase), the EMCP was proposed and shown to operate in *M. extorquens* (Alber et al. 2006; Erb et al. 2007; Meister et al. 2005; Peyraud et al. 2009). The first step of the pathway is the condensation of two acetyl-CoA units, which are then transformed into methylmalyl-CoA over a series of CoA thioester derivatives involving a CO_2 fixation step. Methylmalyl-CoA is cleaved to glyoxylate and propionyl-CoA, which can be converted to C4 compounds, such as succinate, via another carboxylation reaction. This pathway shares many enzymes with other pathways, but unlike the serine cycle, it also possesses unique reactions. Two of these are ethylmalonyl-CoA mutase (Ecm) (Erb et al. 2008) and the above-mentioned crotonyl-CoA reductase/carboxylase (Ccr) (Erb et al. 2007). Unlike other methylotrophy genes, the genes of the EMCP are not localized in large operons but are loosely clustered or not co-localized at all (Hu and Lidstrom 2012).

Alternative C1 carbon sources

Different *M. extorquens* strains grow on a number of additional C1 compounds, including methylamine, chloromethane, and dichloromethane (Chistoserdova and Lidstrom 2013). For all these different substrates, specific metabolic pathways can be identified (Chistoserdova 2011). Methylamine is oxidized to formaldehyde by a tryptophan tryptophylquinone (TTQ)-dependent methylamine dehydrogenase (encoded by *mau* cluster in strain AM1) with amicyanin as the electron acceptor (Chistoserdov et al. 1994). Strains PA1 and DM4, which both lack the *mau* cluster, were recently shown to use the *N*-methylglutamate pathway for growth on methylamine (Gruffaz et al. 2014; Nayak and Marx 2014b). Chloromethane is dehalogenated by the transfer of the methyl group to H_4F (catalyzed by CmuA and CmuB in the strain CM4) (Studer et al. 2001), which is then further oxidized via the H_4F-dependent pathway using specific enzymes (Studer et al. 2002). Dichloromethane is converted to *S*-chloromethyl glutathione and inorganic chloride by dichloromethane dehydrogenase (DcmA in the strain DM4) (Leisinger et al. 1994). The instable *S*-chloromethyl glutathione decomposes spontaneously to formaldehyde, inorganic chloride, and glutathione (Muller et al. 2011).

Facultative methylotrophy and co-consumption of substrates

As mentioned above, *M. extorquens* is a facultative methylotroph. The alternative non-C1 growth substrates, which have been investigated in detail, are succinate (C4), acetate (C2), and oxalate (C2). Succinate and acetate enter the metabolism on the level of the TCA, and in both cases gluconeogenesis is employed to produce sugars. In the case of growth on acetate, glyoxylate needs to be regenerated via the EMCP (Schneider et al. 2012a). Oxalate is converted to oxalyl-CoA, which is decarboxylated to formyl-CoA and further metabolized via a variant of the serine cycle where glyoxylate is provided by the reduction of oxalyl-CoA (Schneider et al. 2012b). A recent study investigated the activities of all enzymes involved in methanol assimilation under C1, C2, and C4 substrate conditions (Smejkalova et al. 2010).

It can be expected that *M. extorquens* needs to switch carbon sources regularly in its natural environment depending on carbon availability. The adaptation involved in such a switch from multi-carbon (succinate) to single carbon (methanol) was investigated using a systems-level approach (Skovran et al. 2010). This study revealed that immediately after the substrate switch a significant amount of methanol is oxidized to formate and further to CO_2. It was proposed that *M. extorquens* uses a downstream priming approach; i.e. the assimilation cycles (serine cycle and EMCP) are induced before the pathways delivering the corresponding precursors (H_4F-dependent pathway). The study also identified potential control points including the conversion of methenyl- to methylene-H_4F (catalyzed by MtdA) and the conversion of mesaconyl-CoA to β-methylmalyl-CoA (catalyzed by Mcd). The expression of the latter is initially decreased but later increased again, suggesting that in this way the buildup of the toxic intermediate glyoxylate, which is produced in the subsequent step, is blocked. Interestingly, the expression of genes common for both growth substrates is changed greatly

during the switch, but the metabolite pools are relatively constant, suggesting the presence of metabolic set points (Skovran et al. 2010).

In addition to alternative carbon utilization and substrate switches, mixed substrate availability has also been examined. Interestingly, succinate and methanol are co-consumed (Peyraud et al. 2012) if fed simultaneously to a batch culture. This is achieved by partitioning of methanol and succinate to specific metabolic pathways. Methanol is primarily used for energy generation via linear oxidation and dedicated biosynthetic reactions involving C1 precursors such as purine biosynthesis (Peyraud et al. 2012). Succinate is mainly used to provide precursors for biomass production with the concomitant production of reducing equivalents that can be used for ATP generation. The repression of methanol assimilation occurred at the level of the serine cycle, and no operation of the EMCP was observed. It was suggested that co-consumption is a strategy to optimize substrate utilization under environmental conditions (Peyraud et al. 2012).

Taken together, these findings suggest that the metabolism of the facultative methylotroph is optimized to exploit the availability of methanol at any time. This is additionally supported by the fact that MxaF is the most abundant protein not only on C1 but also on multi-carbon substrates (Bosch et al. 2008).

Regulation of methylotrophic pathways

Several transcriptional regulators for methylotrophy have been identified thus far. Each of the gene clusters involved in methanol oxidation (*mxa*, *mxb*, *mxc*) are transcribed in one unit, and the promoter regions for the *mxa* and *mxb* cluster have been identified (showing similarity to the *Escherichia coli* σ^{70} promoter consensus) (Zhang and Lidstrom 2003). In addition, a multiple A-tract regulatory sequence was identified upstream of the promoter regions of five methanol oxidation genes and is essential for normal expression of these genes (Zhang et al. 2005). Five essential genes encoded together with methanol oxidation genes are involved in the regulation of gene expression. The *mxbDM* genes are involved in transcriptional regulation (*mxbM* encodes for a response regulator, *mxbD* for a sensor kinase) of different methanol oxidation genes including *mxaF*, *pqqE*, and *mxaW* (Springer et al. 1997). Another regulator-sensor pair involved in *mxaF* transcription is encoded by *mxcQE* (Springer et al. 1995). One of these couples (*mxcQE*) positively regulates the expression of the other (*mxbDM*) (Springer et al. 1997). The knockout mutants of *mxcQE* and especially *mxbDM* do not express cytochrome c_L but an alternative cytochrome termed c_{553} (Springer et al. 1995), suggesting that c_{553} is negatively regulated by *mxbDM*. Another gene, *mxaB*, shows homology to response regulators (Springer et al. 1998) and is required for the expression of *mxaF* (Morris and Lidstrom 1992). Two additional genes involved in the regulation of methanol oxidation are the *mxaF*-homologs *xoxF1* and *xoxF2*, which appear to fulfill a dual role: a catalytic role (see methanol oxidation chapter) and a regulatory role (Skovran et al. 2011). The double mutant of *xoxF12* is not able to grow on methanol and shows decreased expression of *mxbDM* and *mxcQE*, suggesting that it functions via these regulators (Skovran et al. 2011).

A LysR-type transcriptional regulator, QscR, positively controls the expression of the two serine cycle operons: *qsc1* (*sga*, *hpr*, *mtdA*, *fch*) and *qsc2* (*mtkAB*, *ppc*, *mclA*) and the separate gene *glyA*. The expression of genes that are also involved in central metabolism (*mdh* and *eno*) was not regulated by QscR (Kalyuzhnaya and Lidstrom 2003, 2005). The expression of *qscR* is independent of the growth substrate, but the DNA-binding activity of QscR is increased by formyl-H_4F and decreased by $NADP^+$ and acetyl-CoA (Kalyuzhnaya and Lidstrom 2005). Surprisingly, a recent proteomics study revealed that QscR is strongly down-regulated on methanol (Bosch et al. 2008). Further investigation will be required to understand this finding.

Unlike other methylotrophy genes, the genes of the EMCP are not transcribed in operons (Hu and Lidstrom 2012) and are expressed in multiple patterns during a substrate switch from succinate to methanol and not as a unit (Skovran et al. 2010). Very little is known about their regulation. Recently, a TetR family regulator for the expression of *ccr*, encoding for a key enzyme of the EMCP, was discovered and termed CcrR. Interestingly, this regulator did not affect other genes of the EMCP (Hu and Lidstrom 2012).

M. extorquens in biotechnology

The profound knowledge of the metabolism, proteome, genome, and transcriptome of *M. extorquens*, in addition to the availability of genetic tools and well-established bioprocess regimes, makes this bacterium an attractive candidate for the bioproduction of chemicals from non-sugar substrates (see Fig. 2). Such value-added products might be general substances from precursors common to all organisms such as amino acids, or products based on specific intermediates of the metabolism of *M. extorquens*, including PHB or EMCP intermediates, for the biosynthesis of novel dicarboxylic acids, polyketides, or butanol.

A further benefit of *M. extorquens* as production host is the standard use of minimal media, which significantly facilitates product recovery during downstream processing compared to conventional bioprocesses, where complex carbon sources, such as molasses or corn steep liquor, are often employed. The toxicity of methanol is another advantage, as most microbes are not able to grow and therefore to contaminate

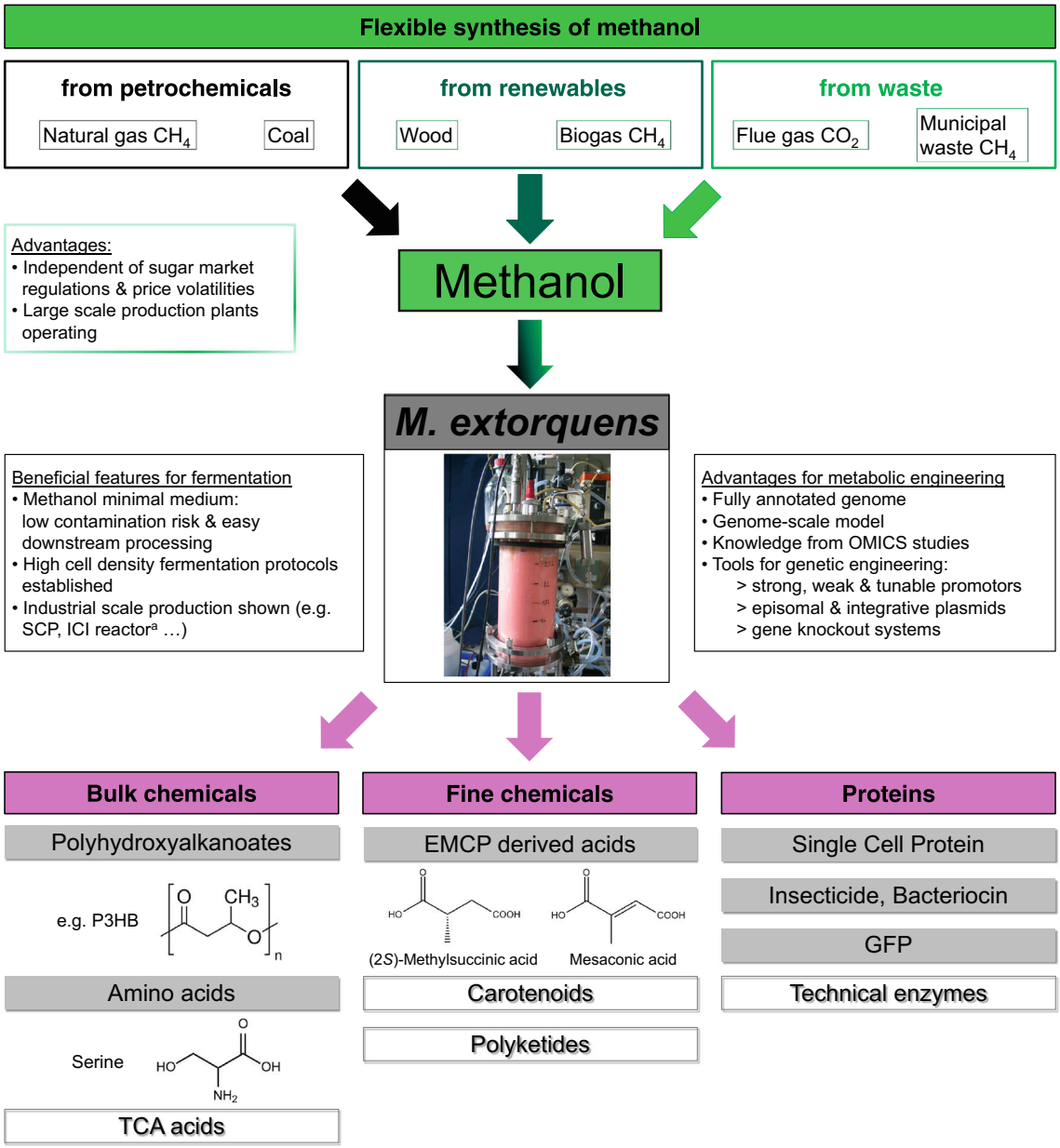

Fig. 2 Use of *M. extorquens* as microbial host for methanol-based product syntheses. Established products are shaded in *gray*, potential products in *white*. Please refer to Table 4 for references of the distinct processes. [a] single cell protein production by Imperial Chemical Industries in 1,500 m^3 air-lift reactor performed in the 1970s and 1980s (Senior and Windass 1980)

methanol minimal media-based fermentation processes. Although *M. extorquens* is able to utilize a wide range of different carbon sources consisting of one-, two-, three-, four-, or five-carbon atoms including alcohols, amines, mono-, and dicarboxylic acids (see Table S1), all the reported biotechnological processes established with *M. extorquens* thus far are based on the substrate methanol. A recent exception is the synthesis of 1-butanol using ethylamine (see Table 4). In addition to methanol, (bio-)ethanol might also become an attractive alternative as a non-sugar-based (and renewable) substrate for the biotechnological production of chemicals by *M. extorquens*. However, no ethanol-based process has been reported to date. In this respect, it should be noted that the maximum growth rates on methanol, which varied from 0.169 to 0.19 h^{-1} (Bélanger et al. 2004; Peyraud et al. 2011), are considerably higher than the 0.069 h^{-1} reported for growth on ethanol (Dunstan et al. 1972). The slow growth of *M. extorquens* AM1 on ethanol is a drawback for competitive industrial applications. In this respect, strain PA1 might represent an attractive alternative candidate with higher growth rates on methanol, ethanol, and multi-carbon substrates (Nayak and Marx 2014a).

Until now, *M. extorquens* has been used for the biotechnological production of various chemicals from methanol, such as polyhydroxyalkanoates (precisely polyhydroxybutyrates),

Table 4 Biotechnological production processes for various compounds established with *M. extorquens* AM1 as a host strain

Substance class	Product	Max. concentration and yield	Description	Reference
Amino acids	L-serine	54.9 g/l (8.3 % from methanol, 39.3 % from glycine	Fermentation of frozen-thawed resting cells at pH 8.2; methanol and glycine as substrates	Sirirote et al. (1986)
		11.3 g/l (4.5 % from methanol, 95.1 % from glycine)	Ca-alginate immobilized resting cells with methanol and glycine as substrates	Sirirote et al. (1988)
PHAs	PHB (600 kDa)	n.s.	Shake flask cultivations	Anderson et al. (1992); Taidi et al. (1994)
	PHB (900–1,800 kDa)	52.9 g/l (0.09–0.12 g/g MeOH; up to 45 % of cdw)	Methanol limited (<0.1 g/l) fed-batch fermentation	Bourque et al. (1995)
	PHB (size n.d.)	149 g/l (0.2 g/g MeOH; 64 % of cdw)	Fed-batch fermentation with defined carbon/nitrogen ratio feed	Suzuki et al. (1986)
		3.3 g/l (33 % of cdw)	Fed-batch fermentation at 1.7 g/l methanol	Bourque et al. (1992)
	PHB-co-3HV	~1 g/l; ratio 0.8–0.2	Addition of 0.5 % (*w*/*v*) valerate to fed-batch fermentation	Bourque et al. (1992)
		0.33 g/l (33 % of cdw); ratio 0.62–0.38	Shake flask cultivation with 0.5 % methanol and 0.05 % *n*-amyl alcohol (both *v*/*v*)	Ueda et al. (1992)
	PHB-co-3HV and PHB-co-3HV-co-3HHx	total 43 % of cdw; ratio of 0.91 to 0.06–0.003	Genomic substitution of native *phaC* by *Aeromonas caviae phaC* + deletion of propionyl-CoA carboxylase *pcc*; growth under cobalt limitation in shake flasks	Orita et al. (2014)
	Functionalized PHB	n.d.; up to 6 % of C5:0, 6:5, 6:0, 8:7, or 8:0 monomers	Overexpression of native or heterologous *phaC*; C5-C11 saturated and unsaturated carboxylic acids as co-substrates	Hofer et al. (2010)
Dicarboxylic acids	Mesaconate	70 mg/l (0.0175 g/g MeOH)	Constitutive overexpression of acyl-CoA thioesterase *yciA*; shake flask cultivation	Sonntag et al. (2014)
	2-Methlysuccinate	60 mg/l (0.015 g/g MeOH)		
Alcohols	1-Butanol	15.2 mg/l	Ethylamine as sole carbon source; co-expression of *adhE*2 from *Clostridium acetobutylicum*, crotonyl-CoA reductase *ter* from *Treponema denticola* and endogenous *croR*	Hu and Lidstrom (2014)
Proteins	Green fluorescent protein (GFP)	0.85–1 mg/g cdw	Construct: pRK310-p*lacZ*-GFP; shake flask cultivation	Figueira et al. (2000)
		1.4 g/l (80 mg/g cdw; 16 % of total protein)	Construct: $pCM110_2$-p*mxaF*-GFP; fed-batch fermentation	Bélanger et al. (2004)
	Haloalkane dehalogenase DhlA	10 % of total protein	pCM80-*dhlA* in ΔphaC strain; shake-flask cultivation	Fitzgerald and Lidstrom (2003)
	Enterocin P (EntP)	150 μg/l	pCM80-*entP*; shake flask cultivation	Gutierrez et al. (2005)
	Insectidical protein Cry1Aa	12.6 mg/l (9 mg/g cdw; 4 % of total protein)	pCM80-*cry1Aa*; shake flask cultivation	Choi et al. (2008)

n.s. not specified, *n.d.* not determined, *PHA* polyhydroxyalkanoates, *PHB* poly-(*R*)-3-hydroxybutyrate, *3HV* (*R*)-3-hydroxyvalerate, *3HHx* (*R*)-3-hydroxyhexanoate, *cdw* cell dry weight

amino acids, dicarboxylic acids, as well as proteins and alcohols from ethylamine (see Table 4). Here, we provide an overview regarding all the reported bioprocesses using *M. extorquens* followed by a short assessment of the future potential of *M. extorquens* as a microbial production host.

One group of bulk chemicals are polymers, such as polyhydroxyalkanoates (PHAs), which have a wide range of applications in packaging, medicine, or as textile and household materials (Keshavarz and Roy 2010). *M. extorquens* is a natural producer of poly-3-hydroxybutyrate (P3HB), whose production from methanol has been investigated for more than 25 years. Suzuki and colleagues reached a remarkable concentration of 149 g/l P3HB (64 % of cell dry weight (cdw)) at a yield of 0.2 g/g methanol in 1986. Similar yields and total amounts were later achieved by Mokhtari-Hosseini et al. (2009). Both groups used accurately adjusted methanol and nitrogen-limited fed-batch fermentations of *M. extorquens*. The total amounts, yield per hour or yield per cdw of produced PHB by *M. extorquens*, are the highest reported compared to other one-carbon- (carbon dioxide, methane, or methanol) based production processes (Khosravi-Darani et al. 2013). Even in comparison to classic production hosts, such as *Cupriavidis necator* (former *Ralstonia eutropha*) or recombinant *E. coli* converting non C1-carbon sources up to 180 g/l PHB, the production outcomes described for *M. extorquens* were in the same range, although space time yields were lower (Chen 2009). Pure P3HB has, however, only limited fields of application due to its stiffness and brittleness caused by its high crystallinity (Verlinden et al. 2007). Investigations of copolymerization with other hydroxyalkanoate co-monomer(s), which overcome the poor mechanical properties of pure P3HB, were therefore carried out and successfully established by the addition of valerate or n-amyl alcohols to the medium (Bourque et al. 1992; Ueda et al. 1992). The production of an even wider range of functionalized PHBs was established using a combination of different native or heterologously expressed PHB synthases and saturated or unsaturated carboxylic acids as co-substrates (Hofer et al. 2010). However, one must be aware that the addition of precursors increases the production costs, which is especially unfavorable for bulk chemicals such as PHBs. A recent study described the de novo production of the copolymer poly[(3-hydroxybutyrate-co-3-hydroxyvalerate] and the terpolymer poly[(3-hydroxybutyrate-co-3-hydroxyvalerate-co-3-hydroxyhexanoate)] by a metabolically engineered *M. extorquens* strain growing under cobalt-limited conditions (Orita et al. 2014). Cobalt limitation provides limited flux through the EMCP (Chou et al. 2009; Kiefer et al. 2009), resulting in higher pools of propionyl- and butyryl-CoA, which act as precursors for PHB functionalization. Although the PHA content of the cells is still lower than shown for pure P3HB (see Table 4) production processes, newly available tools, e.g., plasmids harboring inducible promoters, (see Table 2) for metabolic engineering might enable higher yields for the production of functionalized PHAs from methanol in the future.

Amino acids are also considered as chemical bulk products with an overall worldwide market volume of more than 3 million tons per year (Sanchez and Demain 2008). Most of the commercially available amino acids are produced by microbial fermentations, such as serine, which is mainly used in pharmaceutical and cosmetic industry and as a building block for the chemical industry (Ikeda 2003). In contrast to other amino acids such as lysine or glutamate, serine is a central metabolic intermediate and precursor for many other metabolites in the classical amino acid producer *Corynebacterium glutamicum*. This has been a drawback for large-scale and high-yield de novo production of serine; thus, despite intense metabolic engineering efforts, the produced serine concentrations of 36.5 g/l (Stolz et al. 2007) are still comparatively low. Methylotrophs harboring the serine cycle, such as *M. extorquens*, offer the possibility to produce serine directly out of cheap methanol and glycine because glycine serves as acceptor molecule for methylene H_4F leading to serine (see above). Sirriote and coworkers used immobilized freeze-thawed resting cells of *M. extorquens* in a controlled fermentation process to produce serine (Sirirote et al. 1988; Sirirote et al. 1986). Freeze-thawing causes porous cell membranes and enables serine export over the cellular membrane. The fermentation of freeze-thawed cells was carried out at methanol concentrations high enough to inhibit growth of *M. extorquens* but below the lethal dose. Thus, biomass formation was prevented and remarkable serine product concentrations of 54.9 g/l were achieved. Today's processes are based on *Methylobacterium* sp. MN43 (Hagishita et al. 1996), which has been reported to produce up to 65 g/l serine. Further increases might be possible by the application of presently available genetic tools, although the challenges of registering food additives produced by genetically modified organisms must be considered.

The heterologous production of (recombinant) proteins is a multi-billion dollar market ranging from pharmaceutical proteins to industrial enzymes (Goodman 2009; Group F 2009). Only approximately 50 % of biopharmaceutical proteins are produced in bacteria (mostly *E. coli*) or yeast (mostly *Saccharomyces cerevisiae*) (Ferrer-Miralles et al. 2009), whereas industrial enzymes are produced by fungi (e.g., *Aspergillus* spp.), yeast (e.g., *S. cerevisiae*, *Pichia pastoris*), or bacteria (e.g., *Bacillus* spp., *E. coli*) (Adrio and Demain 2010). Using *M. extorquens* for the production of enzymes or recombinant proteins from the carbon source methanol would be an interesting alternative to conventional sugar-based production processes as the use of substrates competing with food production could be avoided and product recovery facilitated by the use of minimal medium and the relatively "clean" supernatant of *M. extorquens* cultures.

To date, the heterologous production of four different proteins by *M. extorquens* (GFP, insecticidal protein Cry1Aa, a haloalkane dehalogenase, and Enterocin P) has been reported (see Table 4). These studies provide a proof-of-concept for the applicability of *M. extorquens* as a production host for different heterologous proteins. However, protein yields and final concentrations vary highly from microgram per liter scale for Enterocin P to 1.4 g/l (16 % of total protein) reported for GFP by Bélanger et al. (2004). Commercial production processes would require considerably higher yields because yields up to 50 % of cdw and product concentrations of more than 5 g/l protein were achieved for *E. coli* (Panda 2003) and methylotrophic yeast *P. pastoris* (Macauley-Patrick et al. 2005), respectively. It must also be considered that the use of *M. extorquens* as a bacterial host has several disadvantages compared to already established methylotrophic yeast *P. pastoris*, such as a lacking posttranscriptional modification machinery. In particular, more research concerning the protein secretion machinery of *M. extorquens* would be needed to adjust its capabilities to the purpose of high-efficiency protein production. Nevertheless, *M. extorquens* might be very suitable for the production of industrial enzymes due to its relatively "clean" supernatant.

In addition to the possibility of producing long-known bulk chemicals from the alternative carbon source methanol, the C1 metabolism of *M. extorquens* itself provides potential precursors for the production of special chemical compounds. One rather unusual pathway is the EMCP (see above), which comprises of several branched, saturated or unsaturated, and in some cases, chiral C4- and C5-acyl-CoA esters (Erb et al. 2007; Peyraud et al. 2009). Their corresponding dicarboxylic acids, e.g., ethylmalonic acid, (2*S*)-methylsuccinic acid, mesaconic acid, or (2*R*/3*S*)-methylmalic acid, represent new and interesting building blocks for the chemical industry (Alber 2011). Recently, a bacterial thioesterase YciA was identified that is capable of cleaving CoA from mesaconyl- and (2S)-methylsuccinyl-CoA, resulting in de novo production of mesaconic and 2-methylsuccinic acid from methanol (Sonntag et al. 2014). Although still in the milligram per liter range, EMCP-derived dicarboxylic acid production offers the possibility of the sustainable production of novel monomers. The research to increase process efficiency is currently ongoing in our laboratories.

Beside the dicarboxylic acid derivatives, the EMCP intermediates can also be used as precursors for the biosynthesis of next generation fuels. Hu and Lidstrom (2014) used a combined overexpression of a crotonoyl-CoA reductase and alcohol dehydrogenase for the production of 1-butanol out of crotonyl-CoA. Interestingly, these approach required ethylamine as carbon source instead of methanol to synthesize detectable butanol concentrations. Further optimization might not only increase the product concentrations which are in low miligram per liter range but also enable the use of methanol as carbon source.

Taken together, *M. extorquens* is an efficient bacterial host for the production of various chemical compounds starting from methanol as shown by the synthesis of (functionalized) PHB, amino acid serine, proteins, and uncommon dicarboxylic acids. Final product concentrations and yields are often already competitive, and the increasing interest in the use of alternative carbon sources will most likely focus efforts on establishing application processes with the methylotroph *M. extorquens*.

Future potential of *M. extorquens* as production host

Proof of concepts for the production of PHAs, amino acids, proteins, and dicarboxylic acids from methanol by *M. extorquens* have been delivered to date. In these laboratory processes, product concentrations and yields for PHB and serine synthesis have already reached high values. Nevertheless, new available genetic tools and metabolic insights might provide possibilities to maximize the efficiency of these processes.

The natural PHB synthesis pathway in *M. extorquens* will likely play a key role for the further optimization of de novo production processes, as PHB is in most cases an unwanted by-product. This is particularly true for the production of EMCP-derived dicarboxylic acids due to an overlap of the PHB cycle and EMCP (see Fig. 1). Knockout of the PHB synthase gene *phaC* was already described by Korotkova and Lidstrom (2001), but this mutant suffered from a strong growth defect and was highly instable because it produced second-site suppressor mutants at a high frequency. A deeper insight into PHB synthesis regulation showed that regulator protein PhaR might act as a central flux regulator for acetyl-CoA adjusting its distribution either to the EMCP and therefore also to PHB or to the TCA cycle (Korotkova et al. 2002b). The knockout strain Δ*phaR* is as a stable mutant unable to produce PHB, but metabolomics revealed increased flux through the TCA (Van Dien et al. 2003), which is likely disadvantageous for increasing EMCP-related product formation. A deeper understanding of the mechanism of PhaR may not only provide a way to establish a stable PHB-synthase knockout strain without drastic growth defect, but may also be useful for the controlled increase of TCA fluxes. The latter may open opportunities for TCA cycle-derived dicarboxylic acid or amino acid production from methanol by *M. extorquens*.

In addition to mesaconic acid and (2*S*)-methylsuccinic acid, other EMCP-derived dicarboxylic acids, such as ethylmalonic acid, methylmalonic acid, or the chiral (2*R*/3*S*)-methylmalic acid (Alber 2011), are interesting chemical building blocks unavailable in bulk quantities to date. Their production would require another thioesterase that ideally has

specific activity with one of the related CoA-esters. As in vitro screens are hindered by the fact that many (EMCP) CoA-esters are expensive or commercially unavailable, in vivo screening of potential thioesterases might be an option. McMahon and Prather (2014) recently published a screen for thioesterases showing increased activity in comparison to broad-range thioesterase TesB. They expressed different thioesterases in an engineered *E. coli* strain harboring a heterologous pathway with several short-chain acyl-CoA intermediates followed by supernatant analysis for an altered spectrum of released acids or alcohols as the cleave-off products of the acyl-CoAs. This method is most likely also applicable for screening thioesterases active on CoA-ester intermediates of the EMCP in *M. extorquens*. However, lethal effects resulting from unspecific cleavage of essential CoA-esters and insufficient CoA-ester pools for measurable acid concentrations must be taken into consideration when applying this approach.

Along with the function as precursors for the production of novel dicarboxylic acids, CoA-esters of the EMCP, especially ethyl- and methylmalonyl-CoA, could act as monomers for heterologous polyketide synthesis, as they are the most important building blocks for polyketide synthases (Wilson and Moore 2012). Sufficient supply of heterologous polyketide synthesis in *E. coli* or other hosts often suffers from the low amount of available precursors (e.g., methylmalonyl-CoA) or their complete inexistence (e.g., ethylmalonyl-CoA in *E. coli*). Therefore, the expensive addition of necessary substrates or the high effort of establishing additional pathways for polyketide precursor synthesis is required (Gao et al. 2010; Pfeifer and Khosla 2001). This could be avoided by using *M. extorquens* as a host for polyketide biosynthesis, as most of the known precursors such as acetyl-, methylmalonyl-, ethylmalonyl-, malonyl-, and propionyl- are part of the primary metabolism with high metabolic fluxes and likely comparatively high pool sizes. However, the functional expression of a heterologous polyketide synthases in *M. extorquens* has not been proven to date.

M. extorquens is a pink-pigmented methylotroph (Peel and Quayle 1961), and the color is a result of natural carotenoid production, which are likely C30 carotenoids instead of common C40 carotenoids (Sandmann, unpublished). As a native carotenoid producer, *M. extorquens* might be suitable for the production of other commercially interesting carotenoids such as β-carotene. However, exact information on the natural carotenoid biosynthesis, which most likely overlaps with hopane biosynthesis (Bradley et al. 2010), would be necessary for efficient pathway manipulation.

In summary, there is tremendous potential for *M. extorquens* to be applied as a microbial cell factory for the synthesis of various compounds from methanol, in addition to the already established processes (see above). In addition to other bulk chemicals such as TCA- or EMCP-derived dicarboxylic acid, *M. extorquens* metabolism also offers an opportunity for the production of fine chemicals such as polyketides, carotenoids, or terpenoids.

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7.1.4 Methanol in Biotechnology

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(2) Durchführung der einzelnen Untersuchungen/ Experimente	nicht durchgeführt (Buchkapitel)	
(3) Erstellung der Daten-sammlung und Abbildungen	Abbildungen 1, 3, 4 Abbildung 2 Tabellen 1, 3 Tabellen 2, 4	Veith T., Holtmann D. Hippmann S., Bertau M. Hippmann S., Bertau M. Holtmann D., Veith T., Schrader J., Sonntag F.
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6.4.3 Methanol in Biotechnology

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Introduction

Methylotrophs play a key role in the global cycling of C_1 compounds and offer biotechnological opportunities for the production of commodity chemicals from methanol.[1] The major proportion of the annual plant-released methanol does not enter the atmosphere. The methanol is converted by methanol-oxidizing prokaryotes. These methylotrophic bacteria belong to different classes including Proteobacteria, Verrucomicrobia, Firmicutes, and Actinobacteria. The wide variety of bacteria and also yeasts are able to grow in inexpensive synthetic media with methanol as the sole or major source of carbon and energy. This is due to the presence of a few unique enzymes which enable these organisms to generate metabolic energy and synthesize cell constituents from this one-carbon substrate. As a feedstock for industrial fermentations, methanol is also attractive because of its low cost, ease of handling and abundant availability.[2] Furthermore, methanol is often used as carbon source in biological wastewater treatment plants, as fuel in biofuel cells or can be produced by biological components.

6.4.3.1 Metabolism and physiology

Metabolism of methylotrophic bacteria

Methylotrophy can be defined as the ability of (micro-) organisms "to grow at the expense of reduced carbon compounds containing one or more carbon atoms but containing no carbon-carbon bonds".[3] Thus, beside methane and methanol also methanesulfonate and other methylated sulphur species, methylated amines, the halogenated hydrocarbons chloromethane, bromomethane, and dichloromethane can serve as sole carbon and energy source, either exclusively or in addition to methanol. Methylotrophy research dates back to 1906 with the discovery of N.L. Soehngen and his first description of a bacterium growing on methane or methanol, *Bacillus methanicus,* which was later renamed as *Methylomonas methanica*. Nowadays, numerous bacterial methylotrophs are known and several genomes are published including the smallest known genome of a (non-parasitic) free living cell to date: the marine methylotroph *Methylophilales bacterium* HTCC2181.[4] Phylogenetically bacterial

methylotrophs belong to the alpha, beta and gamma subclasses of Proteobacteria or the group of Gram-positive bacteria, mainly.[5]

In a dissimilatory process methylotrophic bacteria oxidize the reduced one carbon (C_1) source stepwise to CO_2. Thereby, ATP is generated due to the involvement of electron-transport-coupled phosphorylation. In this process formaldehyde represents the key intermediate. It is the first intermediate after the oxidation of methanol and beside its function in the dissimilatory pathway leading to CO_2 it also serves as input for C_1 assimilation pathways in methylotrophs (see below). The oxidation of methane to methanol is performed by a subgroup of methylotrophic bacteria, the methanotrophs. Two versions of the respective enzyme, methane monooxygenase (MMO) have been described: a soluble (s), cytoplasmic MMO, and a particulate (p), membrane-bound MMO. Both enzymes are regulated due to the availability of copper, e.g. sMMO a non-heme iron-containing enzyme is expressed under low copper conditions, the pMMO with a di-copper centre in the active site is expressed under high copper conditions.[6-8] This biological methane oxidation is remarkable as the chemical methanol synthesis is a three stage catalyist-requiring process whereas the MMO reaction is carried out directly with dioxygen.[9] Most attention has been focused on the iron-containing sMMO, especially from the species *Methylosinus trichosporium* and *Methylococcus capsulatus.* The ability of MMO to activate methane at room temperature and ambient pressure makes it an attractive target for research toward a potential enzymatic large-scale production of methanol.[10]

After the uptake of methanol or its generation from methane in case of methanotrophs, methanol is further converted by methanol dehydrogenase to formaldehyde. So far, periplasmic (PQQ)-dependent methanol dehydrogenases have been described for Gram-negative bacteria as well as NAD(P)-dependent enzymes in Gram-positive bacteria.[11, 12] The following conversion of toxic formaldehyde to CO_2 by methylotrophs can be achieved by several, linear or cyclic, pathways of which some can occur in parallel within one organism.[13] Exemplarily, co-factor dependent linear pathways such as the tetrahydromethanopterin (H_4MPT) pathway, discovered in the Alphaproteobacterium *Methylobacterium extorquens*, and glutathione (*Paracoccus denitrificans*) and mycothiol (Gram-positive methylotrophs) pathways should be mentioned as well as the cyclic ribulose monophosphate (RuMP) pathway of Gram-negative Proteobacteria e.g. *Methylobacillus flagellates*.[3, 14-16] The latter being nearly identical to the assimilatory RuMP pathway mentioned afterwards (see fig. 1).[3]

The assimilatory incorporation of C_1 compounds of bacterial methylotrophs can be roughly divided into two pathways both requiring the aforementioned formaldehyde as precursor (reviewed in e.g. [3]). Assimilation via the RuMP pathway utilizes all carbon needed from formaldehyde by catalysing the reaction from ribulose-5-phosphate to hexulose-6-phosphate by hexulose phosphate synthase (see fig 1). In contrast the serine cycle for carbon assimilation of Alphaproteobacteria (e.g. *M. extorquens*) incorporates CO_2 additionally by carboxylation reactions beside formaldehyde assimilation via serine. This remarkable combination of pathways leads to accumulation of about 50 % biomass carbon from CO_2 and thus to a refixation of CO_2 produced during the dissimilatory process described above.[17, 18] The fixation of CO_2 occurring within central pathways can be calculated from the difference

between CO_2-utilizing and CO_2-releasing fluxes.[19] 20 % of the formed CO_2 was recovered. This correlates with 16 % of the consumed methanol.

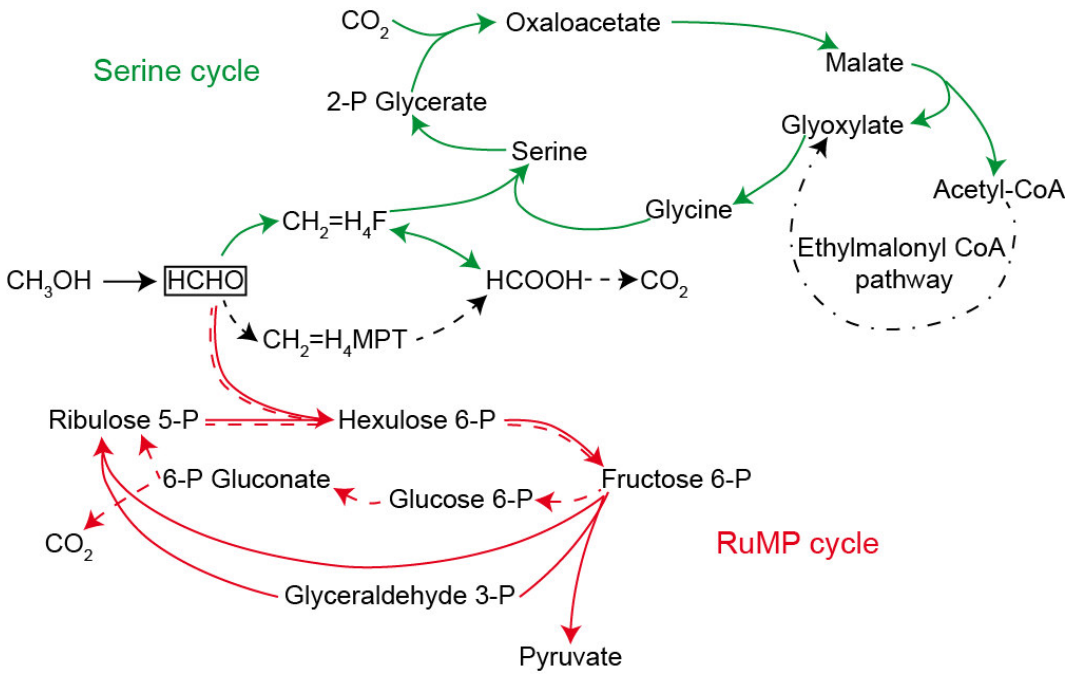

Figure 1: Generalized scheme of methanol assimilation via the serine (green) and RuMP (red) cycles of methylotrophic bacteria. Note that formaldehyde plays a central role as branch point for all shown pathways (box). Dissimilatory processes are indicated by dashed arrows. The multi-step conversion of acetyl-CoA to glyoxylate via the ethylmalonyl-CoA pathway is indicated by a dashed-pointed arrow.

Although the general understanding of metabolism of methylotrophic bacteria is not as advanced as for model organisms like *E. coli* or baker`s yeast, research on methylotrophy is advancing, especially considering *M. extorquens* the probably best understood organism within methylotrophic bacteria. With the genome published [20] and a variety of genetic tools at hand ubiquitous efforts have been made to study its (one carbon and multi-carbon) metabolism including proteome and transcriptome analyses as well as metabolite profiling (reviewed e.g. in [2]). Recently, the activities of all (postulated) enzymes required for methanol assimilation and their regulation in comparison to acetate and succinate grown cells as well as metabolic adaptation processes occurring during the shift to C1 carbon metabolism were published.[21, 22]

Physiology and Metabolism of methylotrophic yeasts

Contrary to prokaryotic cells, eukaryotic cells are substantially larger and possess membrane-surrounded compartments in which the reaction conditions for metabolic processes are ideally adjusted: short diffusion ways, the enrichment of the intermediates in sufficient concentrations, necessary enzymes and pH value. Thus different reactions can take place at the same time in the cell without interacting.[23] The only known eukaryotes which can use methanol as carbon and energy source for growth belong to the yeasts, i.e. single cell fungi.

Yeasts are widely spread in nature and occur particularly in the ground and on plants. They have been in use by man for thousands of years in order to manufacture alcoholic beverages and bread. It is not until the 19th century that they are cultivated in larger quantities. Yeast cells are 5 to 10 µm large and they mostly belong to the group of the ascomycota. Their cell shape is predominantly round oval to cylindrical and reproduction takes place either asexually via budding or sexually via formation of ascospores.[24]

Yeasts using methanol for growth are called facultative methylotrophs and were first mentioned in 1969 by Ogata.[25] Facultative means that they are not able to metabolize methane. But they can use higher oxidized C1-substrates, like methanol, or substrates with C-C-bonds, like glucose as carbon and energy source.[26] Some important representatives of this group are shown in table 1.

Table 1: Name of some methylotrophic yeast (year of renaming)[27]

Common Name	Scientific Name	Other Synonyms
Pichia angusta	*Ogataea angusta* (2010)	*Pichia angusta* (1984) *Hansenula polymorpha* (1959) *Hansenula angusta* (1961) *Ogataea polymorpha* (1994)
Pichia pastoris	*Komagataella pastoris* (1995)	*Pichia pastoris* (1956) *Saccharomyces pastoris* (1952) *Zygowillia pastoris* (1954) *Zymopichia pastoris* (1961)
Pichia guilliermondii	*Meyerozyma guilliermondii* (2010)	*Pichia guilliermondii* (1966) *Yamadazyma guilliermondii* (1989) *Candida carpophila* (2005)
Candida boidinii	*Candida boidinii* (1953)	*Candida methanolica* (1972) *Candida methylica* (1974) *Candida queretana* (1978) *Hansenula alcolica* (1975) *Kloeckera boidinii* (1975)

In nature they can be found in spoiled fruits and vegetable products as well as in exudates and the bark of trees.[28] The existence of these eukaryotes in those habitats can be attributed to the fact that methanol becomes available by the degradation of the methoxy-moieties of lignin. Equally, most of the tested methylotrophic yeasts are able to grow on a medium with pectin, a polymer which is rich in methoxy-groups and which is ubiquitous in fruit.[29] Unlike bacteria

yeasts are not equipped with methanol dehydrogenase to form formaldehyde. Instead they possess specialized cell compartments, peroxisomes, in which methanol oxidation occurs. Peroxisomes are round shapes, approx. 0.5 µm large organelles surrounded by a membrane which are located in the cytoplasm of eukaryotes. These vesicles proliferate if the cells are exposed to nutrients which requires metabolism where peroxisomal functions and enzymes are involved. Therefore, in the presence of methanol they account for up to 80% of the total cell volume.[30] An overview of methanol metabolic pathways in methylotrophic yeasts is summarized in figure 2.[31]

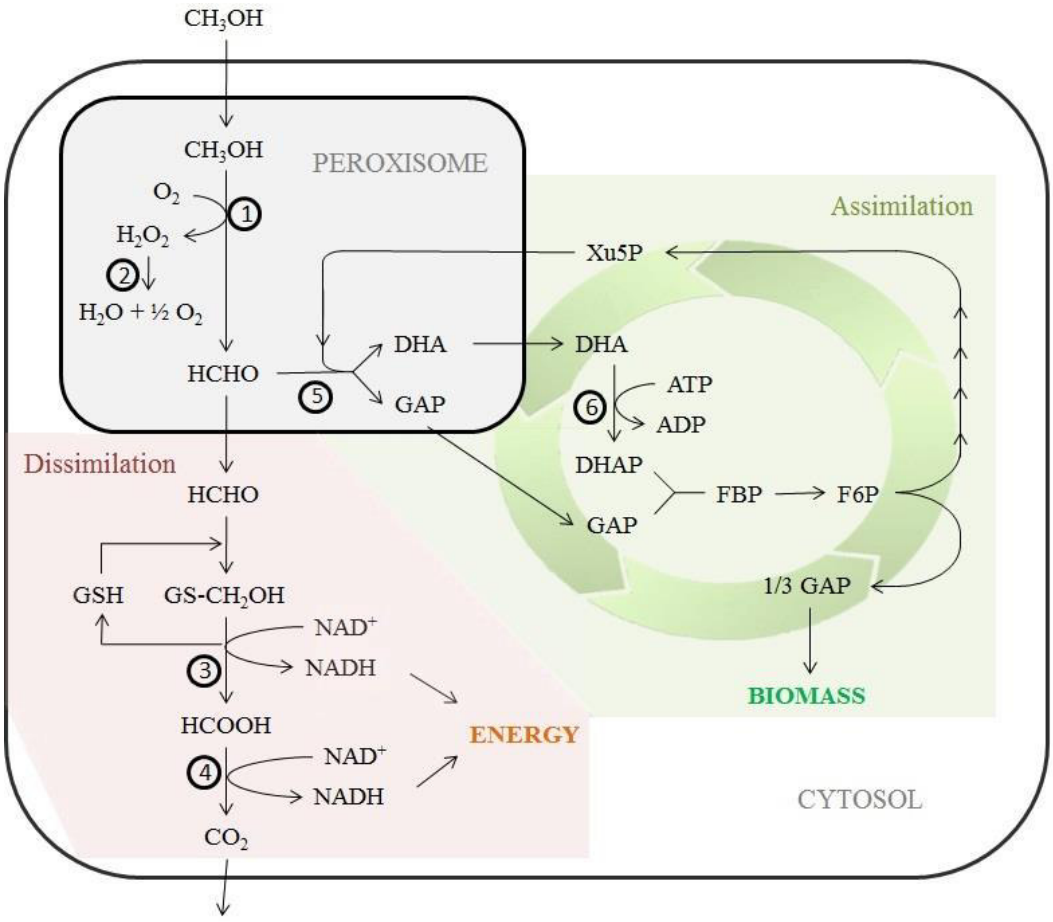

Figure 2: Methanol metabolism pathway in methylotrophic yeasts[31] 1 – alcohol oxidase, 2 – catalase, 3 – formate dehydrogenase, 4 – formaldehyde dehydrogenase, 5 – dihydroxyacetone synthase, 6 – dihydroxyacetone kinase, GSH – glutathione, Xu5P – xylulose-5-phosphate, DHA – dihydroxyacetone, DHAP – dihydroxyacetone phosphate, GAP – glyceraldehyde-3-phosphate, FBP – fructose-1,6-bisphosphate, F6P – fructose-6-phosphate

Methanol metabolism is initiated by an alcohol oxidase mediated oxidation to formaldehyde, the key intermediate of methylotrophs, which takes place under oxygen consumption. This reaction goes along with hydrogen peroxide formation upon which H_2O_2 degradation by catalase sets in. Therefore, compartmentalization of this reaction is an elementary strategy to prevent cell damage caused by formaldehyde and hydrogen peroxide.[32] Subsequently formaldehyde can be successively dissimilated to CO_2 and reduced nucleotides (energy). In detail, this process encompasses formaldehyde oxidation through the action of NAD^+ and

glutathione dependent formaldehyde dehydrogenase to form *S*-formyl-glutathione. This latter compound can now either be oxidized to CO_2 by formate dehydrogenase (NAD^+ dependent) or it can be hydrolysed by formylglutathione-hydrolase to formate which is then oxidized to CO_2 by formate dehydrogenase. The assimilation of formaldehyde is accomplished through the xylulose monophosphate cycle. In this pathway the C_1-entity formaldehyde reacts with C_5-building block xylulose-5-phosphate (Xu5P) to form two C_3-compounds: glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone (DHA) which reaction is catalysed by dihydroxyacetone synthase. Dihydroxyacetone kinase, the next enzyme involved, phosphorylates DHA to dihydroxyacetone phosphate (DHAP) which in a next step reacts with glyceraldehyde-3-phosphate to form fructose-1,6-bisphosphate (FBP), a C_6-compound. Dephosphorylation leads to fructose-6-phosphate which is further converted to GAP and Xu5P, thus rendering the pathway ready for the next cycle (figure 2, figure 3). To produce one molecule of the C_3-body GAP which is then funnelled into central metabolism to form biomass the Xu5P-cycle has to be run three times.

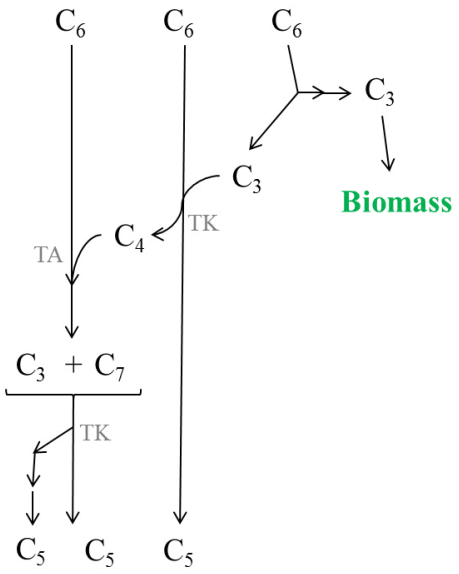

Figure 3: Rearrangement reactions to convert three molecules fructose-6-phosphat (C_6) to three xylulose-5-phosphate (C_5) to keep Xu5P-cycle running.[33] C_3 – glyceraldehyde-3-phosphate, C_4 – erythrose-4-phosphate, C_7 – sedoheptulose-7-phosphate, TK – transketolase, TA – transaldolase

Most yeasts, including the methylotrophic ones, possess GRAS status („generally recognized as safe") for which reason they nowadays are used for a huge number of fermentations, as food additives, as a source of vitamins, and also as "bio-factories" for the production of various antibiotics, steroid hormones, homologous and heterologous proteins as well as models for studying genetic regulation in eukaryotic cells.[34]

6.4.3.2 Growth and product formation

Methylotrophic bacteria

The key issues in the use of methylotrophic bacteria are the productivity, the carbon conversion efficiency and the achievable product concentration. Various authors developed stoichiometric equations for biomass formation on methanol via the RuMP pathway and the average values are shown in the following equation:[35-37]

$$1.63\ CH_3OH + 1.39\ O_2 + 0.23\ NH_3 + \text{nutrients} \rightarrow$$

$$CH_{1.69}O_{0.38}N_{0.24}\ (\text{biomass}) + 0.63\ CO_2 + 2.76\ H_2O \qquad \Delta H = -\ 449.7\ kJ/mol_{MeOH}$$

The carbon balance shows that approximately 38 % of the methanol is dissimilated and 62 % is used for the formation of cellular mass. An amount of 0.85 g oxygen is required for the oxidation of 1 g of methanol and 0.52 g of carbon dioxide is produced. In table 2 published data for biomass productivities and concentrations as well as yields are summarized. The table shows that high biomass productivities over 25 g biomass per litre and hour can be achieved. Surprisingly, the highest reported biomass concentration was 250 g/L.[38] This value has not been reproduced in the last 15 years. Optimum growth rates for biomass accumulation can be obtained by maintaining low and stable methanol concentrations. High methanol concentrations and sudden concentration shifts cause toxic effects on the bacteria due to the accumulation of formaldehyde.[39, 40]

Table 2: Examples of growth parameters of microorganisms on methanol

Microorganisms	Pathway	Biomass productivity in g_{CDM}/L/h	Cell dry mass in g/L	Cell yield in g_{CDM}/g_{MeOH}	Comment	Literature
Methylomonas methanolica	RuMP	25 (potential)	50	0.5	Industrial SCP process	[35]
Methylomonas sp.	RuMP	28.4	114	0.49	Continuous process, μ = 0.25/h	[41]
Methylobacterium organophilum	serine	3.6	250	0.34	PHA process	[38]
Methylobacterium sp.	serine	2	172		PHA process	[42]
Methylobacterium	serine	1.2	56.1	0.28	GFP	[43]

extorquens					production, recombinant	
Mixed culture		4.55	10.6	0.44	Continuous process	[44]

Typical products of methylotrophic prokaryotes are polyhydroxyalkanoates (PHA) and amino acids. On the basis of known metabolic pathways, Leak compared predicted carbon conversion efficiencies for diverse products in different microorganisms either growing on methanol or glucose.[45] For extracellular polysaccharides, polyhydroxyalkanoates and glutamate the predicted conversion efficiencies are similar. This indicates that for high yielding organisms methanol based bioprocesses could be economically competitive. The higher oxygen demand of methylotrophs could be considered as a disadvantage. In the case of glutamate, the oxygen-based yield $Y_{P/O2}$ is predicted to be 2- to 3-fold lower for the growth on methanol than for the growth on glucose. A comparison of the stoichiometric conversion of methanol to glutamate via either the RuMP or the serine pathway indicates predicted yields of 0.76 g/g and 0.92 g/g[45], respectively, whereas the theoretical yield for glutamate production of organisms growing on glucose is 0.82 g/g. Comparison of lysine production by *Bacillus methanolicus* that starts from methanol with lysine production in *Corynebacterium glutamicum*, which employs glucose shows that the yields were comparable.[46] The theoretical yields for both conversions of methanol to lysine with *B. methanolicus* were $Y_{P/S}$ = 0.82 $g_{Lysin\text{-}HCl}/g_{methanol}$ and 0.71 $g_{Lysin\text{-}HCl}/g_{methanol}$ respectively. The highest productivity of PHA was described by Kim et al..[38] The biomass and PHA formation rate reached 3.57 and 1.86 g/L/h, respectively. The highest product yield was 0.2 g PHA per g methanol. A recombinant *M. extorquens* strain produces PHA derivates. By co-feeding methanol and 5-hexenoic acid, functionalized PHA containing C–C double bonds were produced.[47, 48] A genetically modified strain of *Methylobacterium rhodesianum* was used for the production of the chiral compound (R)-3-hydroxybutyrate.[49] The product is formed during intra-cellular degradation of a PHA. In fed batch cultivation 2.8 g/L of (R)-3-hydroxybutyrate were produced. Wild-type strains of *B. methanolicus* can secrete more than 58 g/L of L-glutamate in fed-batch cultures[50] and mutants of *B. methanolicus* secreting 69 g/L of L-glutamate.[51] A further mutant of *B. methanolicus* produce L-Lysine up to 65 g/L.[51] Further products from the metabolism of methylotrophic microorganisms are polysaccharides[52, 53], indole-3-acetic acid[54], trans-zeatin[55, 56] as well as different proteins such as GFP[43, 57, 58], enterocin P[59], acylamide amidohydrolase[60], haloalkane dehalogenase[61] and an esterase[57, 62, 63]. Belanger et al.[43] has carried out fermentation to produce the green fluorescent protein as a model protein to study the recombinant protein production. The maximum specific GFP production was 80 mg/g representing approximately 16% of the total cell protein.

Methylotrophic yeasts

After discovering methylotrophic yeasts' ability to metabolize inexpensive substrates like methanol, attempts were undertaken to use them for production of single cell protein (SCP)

and metabolites. Yeasts possess the advantages of a fast cell growth, the possibility of simple genetic engineering and of the simple process preparation due to their size.[64]

Among the various yeast cultures studied, *Pichia pastoris* showed particular high cell yield from methanol, high protein content and stable fermentation characteristics. Without optimisation *P. pastoris* was slowly growing (doubling time 5.5…6.0hrs).[65] The reason is that yeasts consume more metabolic energy for synthesis of one C_3-molecule compared to methylotrophic bacteria using the ribulose monophosphate cycle (RuMP cycle) for formaldehyde fixation. A stoichiometric equation with the average values for biomass formation on methanol via the Xu5P-cycle for the methylotrophic yeast *Kloeckera* sp. 2201 is shown in the following equation:[66]

$$2.06\ CH_3OH + 2.02\ O_2 + 0.15\ NH_3 + \text{nutrients} \rightarrow$$

$$CH_{1.75}O_{0.5}N_{0.15}\ (\text{biomass}) + 1.06\ CO_2 + 3.47\ H_2O \qquad \Delta H = -268.4\ kJ/mol_{MeOH}$$

The ratio of methanol and the formed biomass shows that approximately 49 % of the methanol is used to produce cellular mass und 51 % is dissimilated to gain energy. This is reflected in lower growth yields of yeasts (yeasts: 0.37…0.45g/g methanol; bacteria: 0.55g/g methanol).[67] Additionally yeasts have higher oxygen consumption when using methanol as carbon source. E.g. for the oxidation of 1 g of methanol to 0.71 g of carbon dioxide by *Kloeckera* sp. 2201 an amount of 0.98 g of oxygen is required. This means this yeast needs 15 % more oxygen than bacteria (0.85 g O_2/g methanol). Therefore, in order to use these microorganisms in biotechnological applications with methanol as sole carbon and energy source a specific reactor design is needed (see 6.4.3.3).

The singular enzymes of methanol metabolism of methylotrophic yeasts offer special applications which are discussed in the following. Formate dehydrogenase (FDH; EC 1.2.1.2) which can be obtained from the methylotrophic yeast *Candida boidinii* catalyses the last step of the methanol oxidation in methylotrophs and is located in the cytosol (figure 4). It consists of two identical subunits of approx. 36kDa. FDH is moderately thermostable (30…60°C), is insensitive against oxygen and is active along a broad pH range (pH 5…10).[68] An important application of this enzyme is, in combination with formate, the in situ regeneration of NADPH or NADH in catalytic systems, for the gaseous, volatile product CO_2 allows for easily shifting the reaction equilibrium to the right (figure 4).[69, 70]

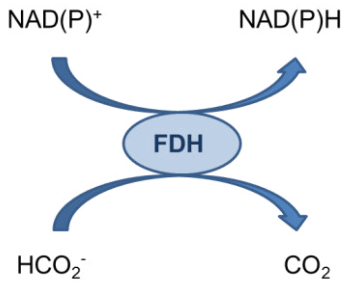

Figure 4: In situ regeneration of NAD(P)H by formate dehydrogenase (FDH)

Because the concept proved successful and fields of applications are so broad, FDH is commercially available and used on an industrial scale in a large set of applications, e.g. production of *tert*-L-leucine.[69-71] Also, it was discovered that FDH cleaves selectively formic acid esters to the respective alcohol and carbon dioxide. The fact, that FDH differentiates between formic acid esters and non-formic acid esters, contrary to hydrolases, renders this enzyme particularly suitable for protective group chemistry. It was shown that even rather disubstituted esters like 1-acetoxy-4-formoxy butane are a substrate for FDH which catalysed the cleavage of the formate group while the acetate group remained unaffected.[72]

Another enzyme belonging to the metabolism of methylotrophic yeasts is methanol oxidase or alcohol oxidase (AOX, EC 1.1.3.13), catalysing oxidation of methanol in the peroxisomes to formaldehyde and hydrogen peroxide using oxygen as electron acceptor (figure 2). AOX consist of eight identical subunits (74kDa[34]), and each one is non-covalently bound to one FAD as prosthetic group. The AOX has a large temperature and pH range, e.g. that of *P. pastoris*, $18 \leq T \leq 45$°C and $6.5 \leq pH \leq 8.3$ under which conditions the enzyme displays at least 50% of its original activity.[73] AOX oxidizes most of the primary short chain alcohols to the corresponding aldehydes for which reason it is used in combination with oxygen and hydrogen peroxide sensors for the determination of lower alcohols.[34] Additionally it could be used as a potential catalyst for organic synthesis. It was found that AOX is able to oxidize 2-chloroethanol, 2-cyanoethanol and 2-methoxyethanol to their aldehydes which are important intermediates in heterocycle synthesis.[74] Further AOX applications are the production of formaldehyde and hydrogen peroxide. Both was examined in laboratory scale obtaining 0.95M formaldehyde by a mutant of *Candida boidinii* [75] and 10mM H_2O_2 by chemical treated *Pichia pastoris* cells. The latter could be used for *in situ* bleaching, oxidizing toxic organic compounds and disinfection.[76]

Heterologous gene expression in methylotrophic yeasts

In methylotrophic yeasts enzymes of the methanol metabolism can be produced in high quantities only by growing on methanol. This circumstance renders them very interesting targets for genetic engineering. Responsible for the high expression of these proteins is a promoter which tightly regulates the AOX gene.[77]

Methylotrophic yeasts, in particular *Pichia pastoris* and *Pichia angusta*, are used preferentially as expression systems for the production of heterologous proteins because of the easy handling, the inexpensive substrate and the strong methanol-induced promoter which is missing in the model organism baker yeast (*Saccharomyces cerevisiae*). In addition, high protein yield, the possibility for high cell density approaches and the option for posttranslational modification of proteins render these methylotrophic yeasts attractive in industrial biotechnology. Thus far, over 500 foreign proteins, including eukaryotic proteins, were successfully expressed which were accessible in *Escherichia coli* only as inactive inclusion bodies. Foreign proteins can be secreted into the medium, if behind the AOX

promoter a secretion-signal sequence is cloned. In table 3 some heterologous proteins are listed which are produced by methylotrophic yeasts.[78,79]

Table 3: Selection of heterologous proteins expressed in *Pichia pastoris* (Pp), *Pichia angusta* (Pa), *Candida boidinii* (Cb) and *Pichia methanolica* (Pm) successfully; Mode: C – cytosolic, S – secreted; Yield: g (protein)/l (medium)

Protein	Mode	Host	Yield	Reference
Bacteria				
Pertussis pertactin protein (*Bordetella*)	C	Pp	3g/l	[80]
Subtilisin inhibitor (*Streptomyces*)	S	Pp	0,5g/l	[81]
Tetanus toxin C fragment (*Clostridium*)	C	Pp	12g/l	[82]
Fungi				
1,2-Mannosyltransferase (*Saccharomyces*)	S	Pp	0,4g/l	[83]
Adenylate kinase (*Saccharomyces*)	C	Cb	2g/l	[84]
Alt a 1 allergen (*Alternaria*)	S	Pp		[85]
Catalase T (*Saccharomyces*)	C	Pa		[86]
Dipeptidyl-peptidase V (*Aspergillus*)	S	Pp		[87]
Glucoamylase (*Schwanniomyces*)	S	Pa	1,4g/l	[88]
Glucose oxidase (*Aspergillus*)	S	Pa	0,9g/l	[89]
Invertase (*Saccharomyces*)	S	Pa	1g/l	[90]
Phytase (*Aspergillus*)	S	Pa	13,5g/l	[91]
Plants				
Cyn d 1 allergen (Bermuda grass)	S	Pp		[92]
Glycolate oxidase (spinach)	C	Pp; Pa		[86, 93]
Malate dehydrogenase (water melon)	C	Pa		[94]
Phytochrome (oat)	C	Pa		[95]
Phytochromes A and B (potato)	C	Pp		[96]
Seed storage protein	C	Pa		[97]

Animal				
Aprotinin (bovine)	S	Pa	0,35g/l	[98]
Bm86 antigen (tick)	C	Pp		[99]
Green fluorescent protein (jellyfish)	C	Pp		[100]
Hirudin	S	Pp; Pa	1,5g/l	[101, 102]
Human				
µ-Opioid receptor	S	Pp		[103]
Hemoglobin	C	Pa		[105, 106]
Hepatitis B surface antigen	C	Pp; Pa	0,4g/l	[106]
Human endostatin	S	Pp	0,02g/l	[107]
Human glutamate decarboxylase	C	Pm	0,5g/l	[108]
Human Lewis fucosyltransferase (Fuc-TIII)	S	Pp	0,03g/l	[109]

6.4.3.3 Specific bioprocess characteristics

Compared to other microbiological cultivation techniques for the production of bulk chemicals, two important differences have to be considered. First of all, inexpensive, defined mineral media can be used as there is no need to supply the carbon source in a complex matrix, which is usually done to save cost in sugar based fermentations. In addition, ammonia can be added as an inexpensive nitrogen source and costly vitamins or other organic molecules are not required. This also reduces downstream processing costs of products that are secreted to the medium, significantly. Furthermore, process instabilities caused by compositional variations in complex raw materials can be avoided. The second important difference to sugar based processes is the toxicity of the carbon source.[39, 42, 110] On the one hand, this tremendously reduces the risk of contaminations. On the other hand, toxicity has to be considered during the development of appropriate feeding strategies to maximize growth and product formation rates. Methanol has a higher reduction state than sugars, thus methanol fermentations are characterized by higher oxygen demands. As heat evolution increases with oxygen consumption, also the cooling requirements rise for fermentations using methanol instead of sugar.

Bourque et al.[111] developed high cell density processes with *M. extorquens* for the production of PHA. Control algorithms were used to maintain both the methanol and the

dissolved oxygen concentration at the desired set-point value. Suzuki et al.[112] also described a process for PHA production. Concentration of methanol was maintained automatically at 0.5 ± 0.2 g/L. Dissolved oxygen in the culture broth was controlled in the range of 2 - 3 mg/L. Kim et al.[38] carried out fed batch cultures to avoid the inhibitory effect of methanol for cell growth in a 2.5 L fermenter equipped with standard control units and instrumentations. Schendel et al.[113] carried out fed-batch cultivation of *Bacillus methanolicus* at 50°C to produce the amino acid L-lysine. The higher fermentation temperature reduces the costs for cooling the reaction system. The production of single cell protein with methanol as substrate reached the industrial-scale. The ICI process[37, 114-116] used a pressure airlift reactor with inner loop. The working volume was 1500 m³ capable of producing up to 50000 tonnes per annum. Under continuous fermentation conditions runs in excess of 100 days without contaminations have been reported. To optimize the oxygen transfer the reactor was driven with an overpressure of 3 bar in the head of the reactor.[117] To avoid high potentially toxic, local concentrations of methanol (>0.2 g/L), the carbon substrate was sparged via 3000 outlets in the reactor.[118] The ICI process used the methylotrophic bacterium *Methylophilus methylotrophus.* The maximum specific growth rate was approximately 0.55/h, the continuous process was carried out at dilution rates of 0.16-0.19/h.[45] The biomass concentration during fermentations reached 30 g/L and the maximum cell yield was up to 0.5 g/g. The organism *Methylomonas clara* was used by the company Hoechst/Uhde. They used 20 m³ reactors to reach an annual production capacity up to 1000 tons. The substrate concentration was controlled to a value of 0.005%. The maximum growth rate was 0.5/h und dilution rates of 0.3-0.5/h were applied. Under these conditions biomass production rates of 5 g/L/h and a methanol based biomass yield of 50 % were achieved.

In order to be able to run an industrial process economically, high biomass yields are required bringing about the necessity to optimize medium component concentrations. It was observed that the methanol concentration should be less than 6% (v/v), to avoid toxic effects.[119] *P. pastoris* is able to grow at high cell density with a continuous mineral nutrient supply. Yeasts prefer usually temperatures of 25…35°C and have a high oxygen demand when growing on methanol.[120] Consequently the result of scale-up experiments showed that heat removal and oxygen transfer are the limiting factors on the large scale. In order to minimize these limitations, reactors were modified or developed further, eventually leading to the Phillips/Provesta continuous high cell density, direct-dry process of Phillips Petroleum Company.[65] Cell densities of 125…150g/l dry cell weight were achieved at a pH of 3.5 and a temperature between 30...40°C.[121] The reactor design allowed an effective heat exchange and oxygen transfer up to 1 mol O_2 l^{-1} h^{-1}. The main benefit of this particular process was the replacement of cell concentration steps by a direct drying of the culture broth through heat treatment. 100% recovery of yeast soluble products was achieved, on combination with lower operating costs and easier processing.[65] Although, SCP from methylotrophic yeasts are rich in essential amino acids and have a high protein content, all SCP production systems based on methylotrophic yeast were not produced any further, because it was feared that the cells contain toxic residuals.[122] Additionally the methanol price is not low enough that the fermentations can be run economically compared to other commercial processes.[123] Yet, the advantages of the Phillips process led to the fermenter design being used for other yeast and bacteria, e.g. *Torula* yeast grown on sucrose to produce yeast products for human and animal

food sector.[124] Today SCP won by non-methylotrophic yeast is used in form of yeast extract in small quantities as flavour-enhancing component in food products or as meat substitutes.[123]

6.4.3.4 Further application of methanol in biotechnological processes

Methanol as carbon source in biological wastewater treatment

The denitrification is the biological reduction of nitrate to nitrogen gas by facultative heterotrophic bacteria. Denitrification occurs when oxygen levels are depleted and nitrate becomes the primary oxygen source. The process is performed under anoxic conditions, when the dissolved oxygen concentration is less than 0.5 mg/L. For the denitrification process the bacteria need a readily degradable carbon source such as methanol. The denitrification process can be described by the following equation:

$$6\ NO_3^{2-} + 5\ CH_3OH \rightarrow 3\ N_2 + 5\ CO_2 + 7\ H_2O + 6\ OH^-$$

Methanol as carbon source in denitrification has different advantages; it contains no solids and no additional nutrients, has a neutral pH, is inexpensive and contains 100% readily degradable substrate.[125] The denitrification with methanol as carbon source is an established technique in municipal waste water treatment plants.[126-129] Nevertheless, there are still some parameters to be optimized. A precise feeding of methanol is necessary. Low dosage rates can lead to excess of NO_3^{2-} in the effluent. If methanol is overfed, it may result in elevated effluent biochemical oxygen demand concentrations. The addition of methanol can also improve biological phosphorus removal by creating anaerobic conditions and increasing the availability of organic carbon in wastewater for polyphosphate accumulating organisms.[127] Unlike acetate, long-term application of methanol has no negative impact on the settling properties of the sludge.

Methanol biofuel cell

Biofuel cells are fuel cells that employ biocatalysts to convert chemical energy into electrical energy. The main types of biofuel cells are defined by the type of biocatalyst. Microbial biofuel cells employ living cells to catalyse the oxidation of the fuel, whereas enzymatic biofuel cells use enzymes for this purpose.[130] There is growing interest in enzyme-based biofuel cells as a source of renewable and sustainable power.[131] They are attractive for special applications, such as implantable devices, sensors, drug delivery, microchips, and portable power supplies. Several drawbacks, such as short lifetime and low power density, limited enzyme-based biofuel cells from being used for practical applications. The oxidation of methanol follows the methylotrophic pathway sequentially to formaldehyde, formate and carbon dioxide.[132] Each oxidation step releases two electrons, yielding six electrons per molecule of methanol. The cofactor nicotinamide adenine dinucleotide (NAD^+) is reduced in the enzyme catalysed reactions and can be used as mediator to transport the electrons to an

electrode. The formal redox potential of NAD^+/NADH is -0.56 V vs. standard calomel electrode.[133] However, the oxidation of NADH by electrodes has poor kinetics and requires large overpotentials. Mediators can be used to reduce the overpotentials and improve the electron transfer rates (table 4). The first methanol biofuel cell used a diaphorase/benzyl viologen system as NADH oxidation catalyst.[134] Electropolymerized mediators such as methylene green, toluidine blue and neutral red can be used as stable catalysts with enhanced activity toward NADH oxidation.[133, 135-138] Often the stability of the mediator restricts the performance of the biofuel cell.[139] By addition of aluminium dioxide into electrode paste the mediator tetramethyl phenylenediamine can be stabilized.

Table 4: Characterization of methanol biofuel cells

Catalyst	Mediator	Fuel cell characteristic	literature
Alcohol dehydrogenase, aldehyde dehydrogenase and formate dehydrogenase	Benzyl viologen	open-circuit voltage of the cell was 0.8 V, power output 0.67 mW cm^{-2} at 0.49 V	[134]
methanol dehydrogenase from *Methylobacterium extorquens*	tetra methyl phenylene diamine	open circuit voltage 1.4 V, power density 0.25 mW cm^{-2} and current density 0.38 mA cm^{-2} at 0.67 V	[139]
alcohol dehydrogenase, aldehyde dehydrogenase and formate dehydrogenase	poly(methylene green)	power density of 0.26 mW cm^{-2} and current density of 0.85 mA/cm^{-2}	[137]

Miscellaneous

Enzymatic reduction of CO_2 and formaldehyde to methanol

The NADH-mediated reduction of CO_2 for production of methanol can be described as a multistep reaction process. The reaction consists of three reversible enzymatic steps: reduction of CO_2 to formate catalysed by formate dehydrogenase (FateDH), reduction of formate to formaldehyde by formaldehyde dehydrogenase (FaldDH), and reduction of formaldehyde to methanol by alcohol dehydrogenase (ADH).[140, 141] Reduced nicotinamide adenine dinucleotide (NADH) acts as an electron donor for each dehydrogenase-catalysed

reduction. Thermodynamic studies have shown that the enzymatic conversion of carbon dioxide is highly sensitive to the pH value and ionic strength of the reaction solution.[142] It is possible to shift the metabolic reaction equilibrium constants by a factor of several orders of magnitude to favour the synthesis of methanol. Electrolysis of carbon dioxide-saturated buffer solution in the presence of the enzymes formate dehydrogenase and methanol dehydrogenase together with methylviologen or pyrroloquinoline quinone as an electron relay yielded formaldehyde and methanol as the reduction products.[143, 144] The combined photochemical and enzymatic synthesis of methanol from formaldehyde is possible by using alcohol dehydrogenase (ADH) from *Saccharomyces cerevisiae* and photoreduction of NAD^+ by zinc tetraphenylporphyrin tetrasulfonate (ZnTPPS) in the presence of methylviologen, diaphorase and triethanolamine.[145] In a similar approach the synthesis of methanol from HCO_3^- using formate dehydrogenase, aldehyde dehydrogenase and alcohol dehydrogenase was shown.[146]

Biotechnological conversion of methane to methanol by P450s

Besides methane monooxygenases enzymes of the cytochrome P-450 family, with a less complicated structure than MMO, have been found to catalyse the oxidation of methane to methanol.[147] Cytochrome P450 enzymes are heme-dependent monooxygenases that catalyse the oxidation of non-activated C-H bonds. The addition of chemically inert perfluoro carboxylic acids to P450 BM3 causes an activation of the enzyme for short-chain aliphatic substrates as a result of the conversion of the Fe/heme from a low-spin to a high-spin state, and the reduction of the binding-pocket size. Together these effects allow otherwise inert substrates such as propane and even methane to be oxidized.[147]

6.4.3.5 Conclusion and outlook

The disadvantages associated with the conversion of food substrates into biofuels and bulk chemical have stimulated the search for alternative raw materials. Methanol is an already important carbon feedstock in the chemical industries. As a feedstock for industrial fermentations methanol is also attractive because of its similar costs compared with other raw materials and its abundant availability. Recent advances in understanding the physiology and biochemistry of methylotrophs made it possible to evaluate their potential in biotechnological processes. Compared with chemical syntheses starting from methanol, biotechnological processes are particular promising in cases where high selectivities are needed or complex products are desired, e.g. branched C_3 to C_6 metabolites. Furthermore, many metabolites of methylotrophic bacteria are not found in the metabolism of established microorganisms, such as *E. coli*. The special metabolites can be suitable building blocks for chemicals, for example for novel fuels and polymers.[2, 47, 49, 148]

6.4.3.6 References

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7.1.5 Engineering *Methylobacterium extorquens* for de novo synthesis of the sesquiterpenoid α-humulene from methanol

Type: Research article

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Status: accepted

Anlage 1

Erklärung über Anteile der Autoren/Autorinnen an den einzelnen Kapiteln der Promotionsarbeit

Titel der Publikation/ des Manuskripts: Engineering Methylobacterium extorquens for de novo synthesis of the sesquiterpenoid -humulene from methanol

	Was hat der/die Promovierende bzw. was haben die Co-Autoren/Autorinnen beigetragen[#]	Name des/der jeweiligen Autors/Autoren/Autorin*
(1) Entwicklung und Planung	60%	Sonntag F.
	je 15%	Buchhaupt M., Schrader J.
	je 4%	Lubuta P., Kroner C.
	2%	Peyraud, R.
(2) Durchführung der einzelnen Untersuchungen/ Experimente	Klonierung der Plasmide zur Expression der Terpensynthase, FPPS und des MVA	Sonntag F., Lubuta P.
	Analytiketablierung und Toxizitätstest von Alpha-Humulen	Sonntag F.
	Produktivitätsbestimmung im Schüttelkolbenmaßstab vom Wildtyp und "weißen" Mutanten	Sonntag F., Kroner C.
	Charakterisierung der in der Carotinoidbiosynthese inhibierten Stämme ("weiße" Mutanten)	Horst A.
	Alpha-Humulen-Synthese in Fed-Batch-Fermentationen	Kroner C., Sonntag F.
	In silico Modellierung und Berechnung der maximalen theoretischen Ausbeute an Alpha-Humulen aus Methanol	Peyraud, R.
(3) Erstellung der Daten-sammlung und Abbildungen	Abbildungen 1-5, S1	Sonntag F.
	Abbildung 6	Horst A., Sonntag F.
	Abbildung 7	Sonntag F., Kroner C.
	Tabellen 1, 2, S1	Sonntag F.
	Tabellen S2, S3	Peyraud R.
(4) Analyse/Interpretation der Daten	komplette Analyse der Daten und Einordnung in den Gesamtkontext	Sonntag F., Buchhaupt M., Schrader J., Peyraud R., Kroner C.
(5) übergeordnete Einleitung/ Ergebnisse/Diskussion	75%	Sonntag F.
	10%	Buchhaupt M.
	je 5%	Kroner C., Peyraud R., Schrader J.

[#]Bei 2, 3 und 4 bitte kurze inhaltliche Angaben der jeweiligen Anteile, bei 1 und 5 reichen prozentuale Angaben

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Datum/Ort

Langen, 29.03.15

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Title:

Engineering *Methylobacterium extorquens* for *de novo* synthesis of the sesquiterpenoid α-humulene from methanol

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Highlights

- first *de novo* terpenoid production in an engineered methylotrophic bacterium
- introduction of the mevalonate pathway from *Myxococcus xanthus*
- flux balancing by ribosome binding site optimizations
- up to 75 mg/L α-humulene in shake flask cultivations
- up to 1.65 g/L α-humulene in methanol limited fed-batch fermentation

Abstract

Over the last 10 to 15 years, metabolic engineering of microbes has become a versatile tool for high-level *de novo* synthesis of terpenoids, with the sesquiterpenoids armopha-1,4-diene, farnesene and artemisinic acid as prime examples. However, almost all cell factory approaches towards terpenoids to date have been based on sugar as the raw material, which is mainly used as a food resource and subject to high price volatilities. In this study we present *de novo* synthesis of the sesquiterpenoid α-humulene from the abundantly available non-food carbon source methanol by metabolically engineered *Methylobacterium extorquens* AM1. Expression of α-humulene synthase from *Zingiber zerumbet* in combination with farnesyl pyrophosphate (FPP) synthase from *Saccharomyces cerevisiae* led to concentrations of up to 18 mg/L α-humulene. Introduction of a prokaryotic mevalonate pathway from *Myxococcus xanthus* in combination with ribosome binding site optimization of α-humulene and FPP synthases increased product concentration 3-fold. This value was additionally raised by 30% using a carotenoid synthesis deficient mutant strain. Final product concentrations of up to 1.65 g/L were obtained in methanol limited fed-batch cultivations, which is the highest titer of *de novo* synthesized α-humulene reported to date. This study demonstrates the potential of *Methylobacterium extorquens* as a future platform strain for the production of high-value terpenoids from the alternative carbon source methanol.

Keywords:

Alpha-humulene, diapocarotenoids, methanol, *Methylobacterium extorquens*, mevalonate pathway, terpenoids

1. Introduction

Sesquiterpenoids form the largest subgroup of terpenoids with more than 7000 known compounds known to date (Breitmaier, 2006; Fraga, 2013). They exert a wide range of functions as regulators, structural components or metabolites in all kingdoms of life (Ajikumar et al., 2008). Plant terpenoids, in particular, are of increasing interest as biological resources for the industrialized society: many compounds have important applications, e.g. artemisinin and taxol as drugs (White, 2008), (-)-menthol as flavor and fragrance compound (Hanson and Hanson, 1996) and farnesene as biofuel precursor (Renninger and McPhee, 2008).

In nature, sesquiterpenoids, which harbor a C_{15} skeleton, are synthesized by terpene synthases from FPP which itself is formed by an FPP synthase through conjugation of three C_5 units, i.e. two molecules isopentenyl pyrophosphate (IPP) and one molecule dimethylallyl pyrophosphate (DMAPP) (Stevens, 1992). IPP and DMAPP are generated either by the mevalonate (MVA) pathway, which is ubiquitous to eukaryotes including the cytoplasm and ER of plants and which can also be found in archaea and some Gram-positive bacteria, or by the methylerythritol 4-phosphate (MEP) pathway, found in eukaryotic organelles such as plastids and mitochondria and in most bacteria (Kuzuyama, 2002). Enhanced terpenoid production in common bacterial hosts such as *Escherichia coli* can therefore be accomplished either by introduction of a heterologous MVA pathway (a concept first introduced by Martin et al. 2003) or by upregulation of the native MEP pathway (Ajikumar et al., 2010). However, thus far the MVA pathway has been the superior route for high level terpenoid production in bacteria as it circumvents the regulatory control of the native MEP pathway (Zhao et al., 2013). The most prominent example for high-level sesquiterpenoid production by heterologous overexpression of the MVA pathway in

E. coli is dedicated to the production of the artemisinin precursor amorphadiene. Product concentrations of up to 300 $mg*L^{-1}*OD^{-1}$ were achieved by flux optimization including promoter, ribosome binding site (RBS) and codon improvements (Anthony et al., 2009; Martin et al., 2003; Nowroozi et al., 2014; Pitera et al., 2007).

In the last years, the monocyclic sesquiterpenoid α-humulene (also called α-caryophyllene) has received increased attention due to its anti-inflammatory and potential anti-cancerogenic properties (Fernandes et al., 2007; Passos et al., 2007). In addition, it is a precursor in the biosynthetic route to zerumbone which itself shows striking anti-inflammatory and anti-cancerogenic effects (Kitayama, 2011; Prasannan et al., 2012). α-Humulene is a natural constituent of *Humulus lupulus* (Katsiotis et al., 1989) and the shampoo ginger plant *Zingiber zerumbet* (Yu et al., 2008). However, terpenoid extraction from plants would be accompanied by several disadvantages such as weather, location and harvest dependency as well as high amounts of waste production (Peralta-Yahya and Keasling, 2010). Chemical synthesis of α-humulene requires numerous steps as well as expensive and environmental hazardous catalysts (Hu and Corey, 2002) making biotechnological production an attractive alternative for a sustainable large-scale production of α-humulene.

After the identification of the α-humulene synthase gene ZSSI from *Zingiber zerumbet* in 2008 by Yu and coworkers (Yu et al., 2008), the gene was introduced in *E. coli* harboring a heterologous MVA pathway for the production of α-humulene (Harada et al., 2009). A product concentration of 1 g/L was achieved in TB medium with additional feeding of lithium acetoacetate and MVA to provide sufficient supply of MVA and its precursor molecule acetoacetyl-CoA.

120 The huge majority of microbial sesquiterpenoid productions described so far rely on
121 sugar-based or complex media fermentations (Chandran et al., 2011). However,
122 despite its pivotal role in the cellular metabolism of most microbes used in industrial
123 biotechnology, the carbon source sugar has also a severe disadvantage as long as it
124 cannot be economically derived from lignocellulosics: the growing world population
125 needs sugar for food and nutrition. Sugar is therefore likely to further increase in
126 price, and, especially in Europe, free market conditions are not given due to trade
127 regulations and high import duties. Therefore, the use of non-food competing carbon
128 sources is highly attractive to industrial biotechnology.
129 One such alternative carbon source is methanol, which is available from
130 petrochemical resources in high quantities and at low cost, and may also be derived
131 from biomass, such as wood or glycerol. In addition, methanol brings along a strongly
132 reduced contamination risk and is typically provided in minimal media which facilitate
133 downstream processing (Bertau et al., 2014; Schrader et al., 2009). Hence, methanol
134 consuming bacteria, which belong to the methylotrophs, represent promising
135 candidates for new microbial cell factories. One of the best studied methylotroph is
136 the model organism *Methylobacterium extorquens* AM1, for which a fully sequenced
137 genome, genetic tools and detailed metabolome, proteome and fluxome data are
138 available (Ochsner et al., 2014). Methanol metabolism of *M. extorquens* was also the
139 basis for recent efforts to transfer methylotrophy to *E. coli* by metabolic engineering
140 (Müller et al., 2015). However, with the availability of the omics techniques and the
141 steadily increasing amount of data, research with *M. extorquens* is now entering a
142 new stage, where rational strain development by metabolic engineering opens new
143 avenues towards the production of desired chemicals with natural methylotrophs.
144 Only few examples have been described so far, such as the production of
145 polyhydroxyalkanoate copolymers (Orita et al., 2014) and dicarboxylic acids (Sonntag

et al., 2014; Sonntag et al., 2015) from methanol or 1-butanol from ethylamine (Hu and Lidstrom, 2014). From a physiological viewpoint, *M. extorquens* AM1 seems to be particularly suitable for the production of (sesqui)terpenoids as it already synthesizes terpenoids naturally, more precisely carotenoids, via the MEP pathway (Peel and Quayle, 1961; Vuilleumier et al., 2009). The naturally produced carotenoids are responsible for the pink pigmentation of *M. extorquens*. In addition, it does not harbor the MVA pathway. This opens the possibility to introduce and overexpress a heterologous MVA pathway without expecting interferences by endogenous regulation. Furthermore, the first enzymatic reaction step of the MVA pathway converting two molecules of acetyl-CoA to acetoacetyl-CoA is already present in the primary metabolism of *M. extorquens*, namely the ethylmalonyl-CoA pathway (EMCP) (Peyraud et al., 2009) (Fig. 1). This feature could be an advantage for engineering the terpenoid biosynthesis, because the heterologous expression of an acetoacetyl-CoA synthase has often turned out to be difficult to establish in other microbial hosts (e.g. *atoB* in *E. coli*; (Harada et al., 2009; Martin et al., 2003)). Finally, docking the heterologous MVA pathway directly to the EMCP, which is essential for *M. extorquens* during growth on methanol (Ochsner et al., 2014), may allow for a reasonable flux diversion towards the desired terpenoid biosynthesis.

In this study, we report the first biotechnological production of a terpenoid in the g/L scale from the C-source methanol by a metabolically engineered methylotrophic bacterium. For this purpose, we chose the model methylotroph *M. extorquens* AM1. After functional establishment of α-humulene synthase in *M. extorquens*, a complete prokaryotic MVA pathway from the bacterium *Myxoccous xanthus* was used to increase terpenoid precursor supply which required fine-tuning by adjustment of the translation initiation rate of ribosome binding sites of several pathway genes. Production of α-humulene was further improved by the use of a carotenoid synthesis

deficient strain preventing unwanted flux from FPP into carotenoid biosynthesis. Finally, α-humulene concentrations were pushed beyond the 1 g/L line by methanol limited fed-batch fermentation.

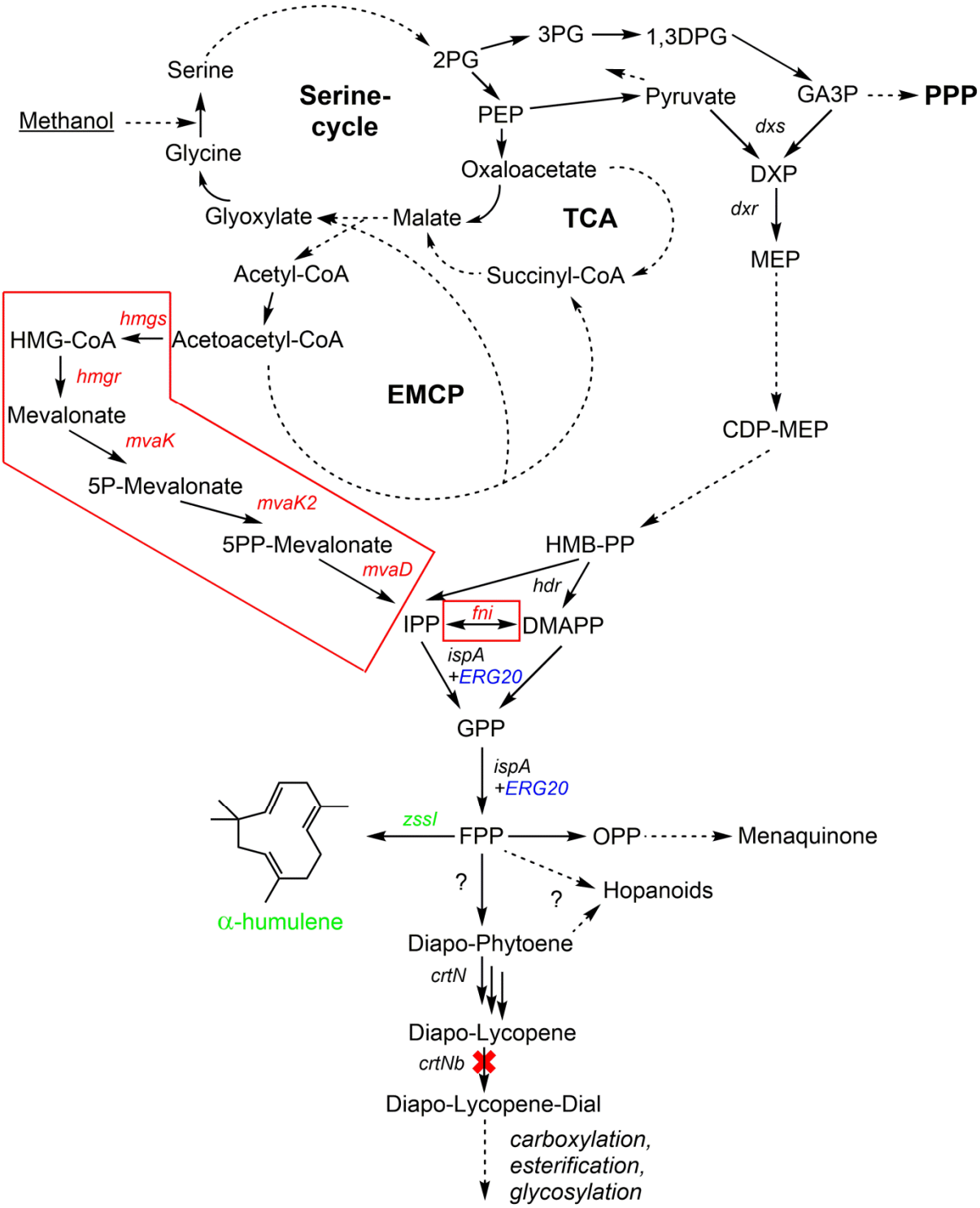


Fig. 1. Schematic overview of the central metabolism of *Methylobacterium extorquens* AM1 including serine cycle, ethylmalonyl-CoA pathway (EMCP), tricarboxylic acid cycle (TCA), pentose-phosphate-pathway (PPP), endogenous terpenoid synthesis via methylerythritol 4-phosphate pathway (MEP) and heterologously integrated mevalonate (MVA) pathway (red boxed), α-humulene synthase *zss*I (green) and additional FPP synthase *ERG20* (blue). Also, IPP isomerase (*fni*), not present in *M. extorquens* naturally, is shown. Hopanoid biosynthesis might be coupled to carotenoid biosynthesis although details have not been elucidated (Bradley et al., 2010). Carotenoids in *Methylobacterium* species have been shown to have a C_{30} skeleton (Sandmann, University of Frankfurt am Main, unpublished), therefore a putative C_{30} carotenoid pathway is depicted although its occurrence in *M. extorquens* remains to be finally proven. Red cross indicates deletion of carotenoid synthesis gene *crtNb* (carotinoid oxidase), putatively responsible for the white phenotype of *M. extorquens* CM502 (Van Dien et al., 2003).

Heterologously integrated MVA pathway from *Myxococcus xanthus* includes hydroxymethylglutaryl-CoA synthase (*hmgs*), hydroxymethylglutaryl-CoA reductase (*hmgr*), mevalonate kinase (*mvaK*), phosphomevalonate kinase (*mvaK2*), pyrophosphomevalonate decarboxylase (*mvaD*) and isopentenyl pyrophosphate isomerase (*fni*).

Further genes: *dxs*: 1-deoxy-D-xylulose-5-phosphate synthase, *dxr*: 1-deoxy-D-xylulose-5-phosphate reductase , *hrd*: HMB-PP reductase, *ispA*: FPP synthase; molecule abbreviations: 2PG: 2-phosphoglycerate, 3PG: 3-phosphoglycerate, 1,3-DPG: 1,3-diphosphoglycerate, GA3P: glyceraldehyde 3-phosphate, PEP: phosphoenolpyruvate, 3-HB: 3-hydroxybutyrate, HMG-CoA: hydroxymethylglutaryl-CoA, DXP: 1-deoxy-D-xylulose-5-phosphate, MEP: 2-C-methyl-D-erythritol-4-phosphate, CDP-MEP: 4-diphosphocytidyl-2-C-methyl-D-erythritol-2-phosphate, HMB-PP: (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate, IPP: isopentenyl pyrophosphate, GPP: geranyl pyrophosphate, FPP: farnesyl pyrophosphate, OPP: octaprenyl pyrophosphate.

201 ## 2. Material and methods

202

203 ### 2.1. Chemicals, media, plasmids and strains

204

205 *Methylobacterium extorquens* strain AM1 (Peel and Quayle, 1961) and "white
206 mutant" strain of AM1, CM502 (Van Dien et al., 2003), were grown at 30°C in minimal
207 medium with a cobalt concentration of 12.5 µM as described before (Kiefer et al.,
208 2009) containing 123 mM methanol or 30 mM succinate. Solid medium was prepared
209 by the addition of 1.5% agar-agar (m/v). Fermentation medium contained final
210 concentrations of 30 mM PIPES, 1.45 mM NaH_2PO_4, 1.88 mM K_2HPO_4, 1.5 mM
211 $MgCl_2$, 11.36 mM $(NH_4)_2SO_4$, 20 µM $CaCl_2$, 45.6 µM sodium citrate
212 ($Na_3C_6H_5O_7$*$2H_2O$), 8.7 µM $ZnSO_4$*$7H_2O$, 15.2 µM $MnCl_2$*$4H_2O$, 36 µM
213 $FeSO_4$*$7H_2O$, 1 µM $(NH_4)_6Mo_7O_{24}$*$4H_2O$, 0.3 µM $CuSO_4$*$5H_2O$ and 12.6 µM
214 $CoCl_2$*$6H_2O$. *Escherichia coli* strain DH5α (Gibco-BRL, Rockville, USA) was grown in
215 lysogeny broth (LB) medium (Bertani, 1951) at 37 °C. Tetracycline-hydrochloride was
216 used at concentrations of 10 µg/ml (10 mg/ml stock solution in ddH_2O) for *E. coli* and
217 *M. extorquens*. Cumate (4-isopropylbenzoic acid) was used as inducer at a final
218 concentration of 100 µM diluted from a 100 mM stock solution dissolved in ethanol
219 (shake flask cultivations) or methanol (bioreactor cultivations).
220 Cumate, tetracycline-hydrochloride, α-humulene, zerumbone and (*RS*)-mevalonic
221 acid lithium salt were purchased from Sigma-Aldrich (Steinheim, Germany) in their
222 highest available degree of purity. Dodecane (>99% purity) was obtained from VWR
223 (Darmstadt, Germany). Plasmids and strains used in this work are summarized in
224 table 1.

225 **Table 1**

226 Plasmids and strains used in this work. TIR: translation initiation rate according to Salis Lab RBS
227 calculator (Salis et al., 2009); op: operon, MVA: mevalonate pathway.

228

Name	Description	reference
plasmids		
pUC18	expression vector for *Escherichia coli*; Amp^R, lacZ promoter, pBR322ori	Norrander et al. (1983)
pACCRT-MN	expression vector for *Escherichia coli*; Amp^R; contains diapophytoene synthase (*crtM*) and diapophytoene desaturase (*crtN*) from *Staphylococcus aureus* under control of lacZ promotor	Sandmann, unpublished
pCM80	constitutive expression vector for *Methylobacterium extorquens*; Tet^R, pmxaF, oriT, pBR322ori	Marx and Lidstrom (2001)
pHC115	expression vector for *Methylobacterium extorquens* harboring cumate inducible pmxaF promoter variant; Kan^R , oriT, pBR322ori	Chou and Marx (2012)
pQ2148	expression vector for *Methylobacterium extorquens* harboring cumate inducible promoter 2148; Tet^R , oriT, pBR322ori	Kaczmarczyk et al. (2013)
pQ2148F	pQ2148 with adapted multiple cloning site	this work
pUC18-MVA-op	pUC18 harboring *Myxococcus xanthus* mevalonate pathway operon (*hmgr*, *mvaK, mvaK2*, *mvaD*, *fni*)	Mi et al. (2014)
pCM80-HMGS	pCM80 harboring hydroxymethylglutaryl synthase *hmgs*	this work
pCM80-MVA	pCM80 harboring complete mevalonate pathway	this work
pCM80-MVA-ERG20	pCM80 harboring complete mevalonate pathway and FPP synthase ERG20	this work
pFS33	pCM80 harboring α-humulene synthase *zssI*	this work
pFS34	pCM80 harboring α-humulene synthase *zssI* and FPP synthase ERG20	this work
pFS44	pFS34-*hmgs*-MVAop (pCM80-*zss*I-ERG20-*hmgs*-MVAop)	this work
pFS45	pHC115 harboring α-humulene synthase *zssI*	this work
pFS46	pHC115 harboring α-humulene synthase *zssI* and FPP synthase ERG20	this work
pFS47	pFS46-*hmgs*-MVAop (pHC115-*zss*I-ERG20-*hmgs*-MVAop)	this work
pFS49	pQ2148F harboring α-humulene synthase *zssI*	this work
pFS50	pQ2148F harboring α-humulene synthase *zssI* and FPP synthase ERG20	this work
pFS52	pFS50- *hmgs*-MVAop (pQ2148F-*zss*I-ERG20-*hmgs*-MVAop)	this work
pFS57	pQ2148F harboring α-humulene synthase *zssI* with optimized RBS (TIR of 225000)	this work
pFS58	pFS57-ERG20 (pQ2148F-*zss*I^{225k}-ERG20)	this work
pFS59	pFS58- *hmgs*-MVAop (pQ2148F-*zss*I^{225k}-ERG20-*hmgs*-MVA)	this work
pFS60a	pFS57-$ERG20^{35k}$ (RBS with TIR of 35000)	this work
pFS60b	pFS57-$ERG20^{20k}$ (RBS with TIR of 22000)	this work
pFS61a	pFS60a-*hmgs*-MVAop (pQ2148F-*zss*I^{225k}-$ERG20^{35k}$-*hmgs*-MVA)	this work
pFS61b	pFS60b-*hmgs*-MVAop (pQ2148F-*zss*I^{225k}-$ERG20^{20k}$-*hmgs*-MVA)	this work
pFS62a	pFS61a with RBS optimized IPP isomerase *fni* (TIR of 65000)	this work
pFS62b	pFS61b with RBS optimized IPP isomerase *fni* (TIR of 65000)	this work
strains		
E. coli DH5α	F^-, Φ80dlacZΔM15, Δ(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(rK^- mK^+), phoA, supE44, λ^-, thi-1	ATCC

M. extorquens AM1	Facultative methylotrophic, obligate aerobic, gram-negative, pink pigmented α-proteobacterium, Cm^R	Peel and Quayle (1961) DSM1338
M. extorquens CM502	Carotenoid biosynthesis deficient strain (assumed as knockout of phytoene desaturase by Van Dien et al.)	Van Dien et al. (2003)
Saccharomyces cerevisiae CEN.PK2-1c	MATa; ura3-52; trp1-289; leu2-3,112; his3Δ 1; $MAL2\text{-}8^C$; SUC2	Entian and Koetter (1998)

2.2. Materials and tools for genetic modifications

Primers were purchased from Sigma-Aldrich (Steinheim, Germany). All standard cloning techniques were carried out as described before (Sambrook, 2001). Transformation of plasmids into *M. extorquens* AM1 or CM502 was performed as described by Toyama et al. (1998).

Ribosome binding sites were designed with the help of the ribosome binding site calculator (Salis, 2011). Codon adaptation index (CAI) (Sharp and Li, 1987) was determined by the CAI-calculator (Puigbo et al., 2008).

2.3. Cloning of mevalonate pathway genes from *Myxococcus xanthus*

Genomic DNA from *Myxococcus xanthus* DSM16525 was purchased from DSMZ (Braunschweig, Germany). *Eco*RI-restriction site contained in gene *hmgs* encoding HMG-CoA synthase was removed by overlap extension PCR inserting a silent mutation (gaattc→gagttc). Therefore first part of the gene was amplified using primers HMGS-fw and HMGS-over-rev while HMGS-over-fw and HMGS-rev were used for the second part. Resulting PCR-products were used as “mega”-primers together with HMGS-fw and HMGS-rev for final amplification of *hmgs* lacking *Eco*RI-restriction site.

Mevalonate pathway operon of *M. xanthus* including genes *hmgr*, *mva*K1, *mva*K2, *mva*D and *fni* coding for HMG-CoA reductase, mevalonate kinase,

phosphomevalonate kinase, pyrophosphomevalonate reductase and isopentenyl pyrophosphate isomerase, respectively, was present on plasmid pUC18-mva-op (Mi et al., 2014).

2.4. Cloning of plasmids containing α-humulene synthase

The multiple cloning site of plasmid pQ2148 (Kaczmarczyk et al., 2013) was altered for increased flexibility in cloning. Therefore primers pQF-MCS-fw and pQF-MCS-rev were annealed by heating 100µl annealing buffer (10 mM TRIS pH7.5, 50mM NaCl, 1mM EDTA) containing 10 µM of each primer for 15 minutes followed by slow cooling to room temperature for 3 hours. Annealed primers were ligated into pQ2148 digested with *Spe*I and *Xho*I leading to plasmid pQ2148F.

α-Humulene synthase *zss*I originating from *Zingiber zerumbet* (Yu et al., 2008) was ordered codon optimized for *M. extorquens* AM1 from Life technologies (Thermo Fisher scientific; sequence see supplemental material Info S1). It was amplified for insertion into pCM80 (Marx and Lidstrom, 2001) and pHC115 (Chou and Marx, 2012) using primers ZSSI-fw and ZSSI-rev. RBS-optimized variant (translation initiation rate (TIR) of 221625) for pQ2148F was amplified using ZSSI-RBS-fw and ZSSI-rev. FPP synthase *ERG20* from *Saccharomyces cerevisiae* CEN.PK2-1c (Entian and Koetter, 1998) was amplified from genomic DNA using primers ERG20-fw and ERG20-rev. RBS optimized variants were amplified with ERG20-RBS35k-fw or ERG20-RBS20k-fw in combination with ERG20-rev-2 resulting in two ERG20 PCR products with RBS having an TIR of 35000 or 20000.

A schematic overview of the cloning procedure, which will be described in the following paragraphs of this subchapter, is given in figure 2. For detailed description of the plasmids, please refer to table 1.

The *zss*I PCR product amplified with ZSSI-fw and ZSSI-rev was digested with *Sph*I and *Xba*I and inserted into equally digested plasmid pCM80 leading to plasmid pFS33 (pCM80-*zss*I). *Cla*I and *Sna*B1 digested PCR product of *ERG20* was subsequently cloned into same restriction sites of pFS33 resulting in pFS34 (pCM80-*zss*I-ERG20). The *hmgs* gene lacking *Eco*RI-restriction site (see above) was inserted behind *ERG20* using restriction sites *Xba*I and *Bam*H1, resulting in plasmid pFS34-*hmgs*. Finally, *M. xanthus* mevalonate operon was excised from pUC18-mva-op by digestion with *Bam*H1 and *Eco*RI and re-inserted into equally digested pFS34-*hmgs* leading to pFS44 (pCM80-*zss*I-ERG20-*hmgs*-MVAop).

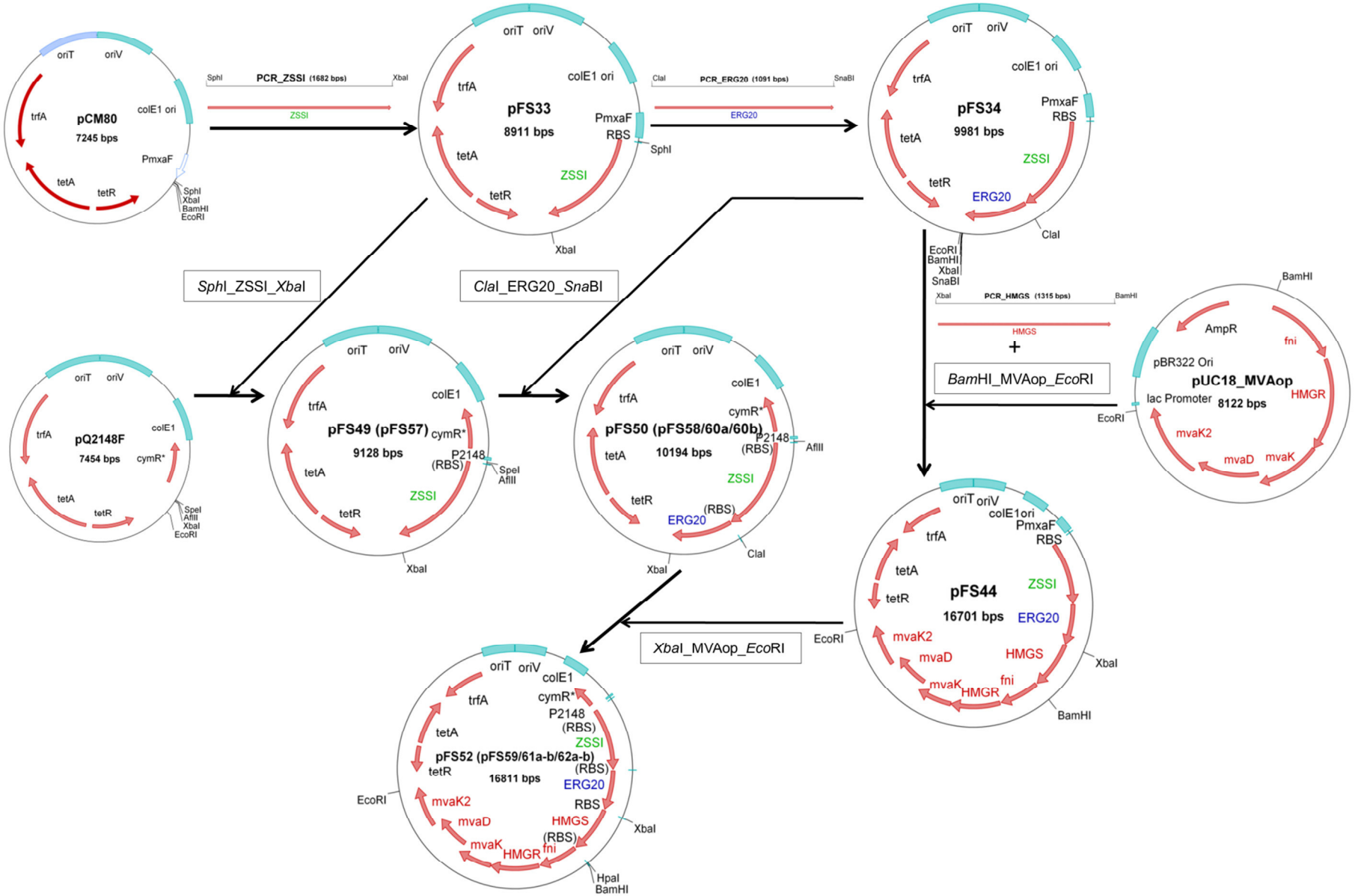
Plasmids pFS45 (pHC115-*zss*I), pFS46 (pHC115-*zss*I-ERG20) and pFS47 (pHC115-*zss*I-ERG20-*hmgs*-MVAop) were constructed by excising *zss*I from pFS33, *zss*I-*ERG20* from pFS34 and *zss*I-ERG20-*hmgs*-MVAop from pFS44 with *Afl*II and *Eco*RI followed by their insertion into equally digested pHC115.

Plasmids pFS49 (pQ2148F-*zss*I) and pFS50 (pQ2148F-*zss*I-ERG20) were constructed by excising *zss*I and *zss*I-ERG20 with *Afl*II and *Xba*I from pFS33 and pFS34, respectively, followed by their insertion into equally digested pQ2148F. *Hmgs* and mevalonate pathway operon (MVAop) were excised from pFS44 by the use of *Xba*1 and *Eco*RI and subsequently inserted into same restriction sites of pFS50 resulting in pFS52 (pQ2148F-*zss*I-ERG20-*hmgs*-MVAop).

PCR product of α-humulene synthase *zss*I containing the optimized RBS was digested by *Spe*I and *Xba*I and ligated into equally digested pQ2148F leading to pFS57 (pQ2148F-*zss*I^{225k}). PCR product of *ERG20* containing non-optimized RBS was cloned behind *zss*I of pFS57 using restriction sites *Cla*I and *Xba*I resulting in pFS58 (pQ2148F-zssI^{RBSopt}-ERG20). *Hmgs*-MVAop was inserted into pFS58 as described for pFS52 which led to pFS59 (pQ2148F-*zss*I^{225k}-ERG20-*hmgs*-MVAop).

PCR products containing RBS optimized variants for *ERG20* (TIR = 35000 and

20000) were digested by *Cla*I and *Xba*I and inserted into pFS57 cut by the same restriction enzymes leading to pFS60a (pQ2148F-zssI^{225k}-ERG20^{35k}) and pFS60b (pQ2148F-*zss*I^{225k}-ERG20^{20k}), respectively. Insertion of *hmgs*-MVAop into pFS60a and pFS60b resulted in plasmids pFS61a (*zss*I^{225k}-ERG20^{35k}-*hmgs*-MVAop) and pFS61b (*zss*I^{225k}-ERG20^{35k}-*hmgs*-MVAop). RBS of IPP isomerase *fni* was optimized for pFS61a and pFS61b by insertion of initially annealed primers fni-RBSopt-fw and fni-RBSopt-rev (annealing method see above) into restriction sites *Hpa*I and *Bam*H1. Resulting plasmids had a TIR of 65000 for *fni* RBS and were named pFS62a and pFS62b (Table 1).


pCM80
7245 bps
pFS33
8911 bps
pFS34
9981 bps
pQ2148F
7454 bps
pFS49 (pFS57)
9128 bps
pFS50 (pFS58/60a/60b)
10194 bps
pFS44
16701 bps
pFS52 (pFS59/61a-b/62a-b)
16811 bps
pUC18_MVAop
8122 bps
PCR_ZSSI (1682 bps)
PCR_ERG20 (1091 bps)
PCR_HMGS (1315 bps)
SphI_ZSSI_XbaI
ClaI_ERG20_SnaBI
XbaI_MVAop_EcoRI
BamHI_MVAop_EcoRI
oriT
oriV
colE1 ori
trfA
tetA
tetR
PmxaF
RBS
ZSSI
ERG20
HMGS
mvaK2
mvaD
mvaK
HMGR
fni
AmpR
pBR322 Ori
lac Promoter
cymR*
P2148
(RBS)
colE1
SphI
XbaI
BamHI
EcoRI
SpeI
AflII
ClaI
SnaBI
HpaI

313 **Fig. 2.** Cloning scheme comprising the genes *zss*I (α-humulene synthase), ERG20 (FFP synthase) as well as *Myxococcus xanthu*s mevalonate pathway genes
314 *hmgs* (3-hydroxymethylglutaryl-CoA synthase), *hmgr (*3-hydroxymethylglutaryl-CoA reductase), *mvaK* (mevalonate kinase), *mvaK*2 (phosphomevalonate kinase),
315 *mvaD* (pyrophosphomevalonate reductase) and *fni* (IPP isomerase) for constitutive (via pFS33, 34 and 44) or inducible expression (via pFS49, 50, 52, 58, 59,
316 60a/b, 61a/b and 62a/b) in *Methylobacterium extorquens*.

317

2.5 α-Humulene production in aqueous-organic two-phase shake flask cultivations

Methylobacterium extorquens AM1 or CM502 harboring α-humulene production plasmids were cultivated in minimal medium containing tetracycline-hydrochloride and methanol as carbon source (see 2.1). Precultures were inoculated from agar plates into reaction tubes containing 5 ml medium and shaken for 48-72 hours at 30°C and 180 rpm. Main cultures with 12 ml medium in 100 ml baffled shake flasks and were inoculated from one preculture to an OD_{600} of 0.1. After cultivation for 16 hours at 30°C and 120 rpm, main cultures reached the early exponential growth phase (OD_{600} 0.3-0.6). Consequently, cumate was added for induction as well as 3 ml dodecane as organic phase. After 48 hours of induction, total culture volume of 15 ml was decanted into a 15 ml falcon tube followed by centrifugation for 10 minutes at 3220 g. One ml of upper dodecane layer was taken for α-humulene analysis. Remaining liquid phase was discarded completely. Cell pellet was resuspended in 1 ml dH_2O for intracellular α-humulene analysis.

2.6 Dodecane and α-humulene tolerance of *M. extorquens* AM1

AM1 precultures were cultivated in reaction tubes containing 5 ml methanol minimal medium (MM) for 48 hours. Tolerance of AM1 towards 20 % (v/v) dodecane was investigated by growth comparison (OD_{600}) of cultures containing 15 ml MM and cultures containing 12 ml MM and 3 ml dodecane. One culture with and without dodecane was inoculated from one preculture.

α-Humulene tolerance was analyzed in two ways: tolerance towards α-humulene directly added to the aqueous phase and α-humulene solved in organic dodecane layer. For the former experiment, pure α-humulene was diluted 5, 10 and 1000 fold in

342 ethanol resulting in concentrations of 177.2, 88.8 and 8.8 g/L, respectively. Addition
343 of 84.7 µl from 5 fold, 84.5, 42.2 and 16.9 µl from 10 fold as wells as 85.2, 42.6, 17
344 and 8.5 µl from 100 fold dilution to 100ml baffled shake flasks containing 15 ml MM
345 led to final concentrations of 1000, 500, 250, 100, 50, 25, 10 and 5 mg/L,
346 respectively. Equal amounts of ethanol were used as negative controls. The flasks
347 containing the different concentrations of α-humulene and its corresponding negative
348 controls (equal ethanol amount) were inoculated from a preculture without α-
349 humulene to an OD_{600} of 0.1. OD_{600} was monitored for 30 hours at which MM
350 containing equivalent amounts of ethanol or ethanol and α-humulene, respectively,
351 were used as blanks.
352 For the later experiment, pure α-humulene was diluted 100 fold in dodecane resulting
353 in a concentration of 8.8 g/L. This stock was used to prepare five 6ml-solutions in
354 dodecane containing α-humulene in concentrations of 1000, 500, 100, 50 and 10
355 mg/L. Two cultures containing 12 ml MM and 3 ml of dodecane with α-humulene
356 were inoculated to an OD_{600} of 0.1 for each concentration from a preculture of AM1
357 grown without dodecane. Two cultures containing dodecane without α-humulene
358 acted as negative controls. OD_{600} was monitored for 30 hours.

359 2.7 α-Humulene analysis

360 One ml dodecane sample was dried with $NaSO_4$. As internal standard, 25 µl of 1 mM
361 zerumbone dissolved in dodecane were added to 225 µl dodecane sample.
362 Intracellular α-humulene was extracted as follows: resuspended cell pellet was added
363 to 4 ml GC-vial in combination with approximately 300 mg 0.2 mm glass beads. Cells
364 were vortexed vigorously 3x 30 s with interim ice cooling. Lysed cells were extracted
365 three times with 1 ml hexane followed by volume reduction to 1 ml using a nitrogen

366 stream. As internal standard, 25 µl of 1 mM zerumbone dissolved in hexane were
367 added to 225 µl sample.
368 α-Humulene was analyzed and quantified via GC-MS (GC17A with Q5050 mass
369 spectrometer, Shimadzu, Kyoto, Japan) equipped with an Equity 5 column (Supelco,
370 30 m x 0.25 mm x 0.25 µM). Measurements were conducted in duplicates as follows:
371 carrier gas: helium; split injections (8:1) at 250°C; column flow: 2.2 ml/min; interface
372 temperature: 250°C; program: 80°C hold for 3 min, 16°C/min to 240°C, hold for 2
373 min. Retention time was 9.3 min for α-humulene and 11.5 min for zerumbone. α-
374 Humulene in samples was identified by comparison of three major fragment ions of
375 its mass spectra to purchased α-humulene standard (rel. intensity in parentheses):
376 93 (15.5), 41 (11.4), 80 (6.7). For quantification, a calibration curve with
377 concentrations of 4500, 2250, 900, 675, 450, 225, 90, 67.5, 22.5, 9 and 4.5 µM α-
378 humulene in each case with 100 µM zerumbone as internal standard, was used.
379

380 2.8 Carotenoid extraction and analysis

381 For carotenoid extraction, cells of *M. extorquens* AM1 or *E. coli* were pelleted via
382 centrifugation, washed with ddH_2O and lyophilized in the dark.
383 For unsaponified extracts, 2 ml of methanol was added to 50 mg crushed freeze-
384 dried cells followed by incubation at 65°C for 30 minutes. After centrifugation (10 min,
385 4000g, 4°C), supernatant was dried with nitrogen and resuspended in 0.5 ml of a
386 petrolether(40-60°C):diethylether:acetone:methanol (40:10:15:5) mixture.
387 Precipitated protein was separated by centrifugation (5 min, 16,000g, 4°C) and
388 supernatant was resuspended in 100 µl tetrahydrofurane (THF) for HPLC analysis
389 after its drying with nitrogen. For saponified extracts, supernatant after protein
390 removal (see above) was incubated with 10% KOH solution (dissolved in methanol)

391 for 2 hours at room temperature. Upper organic phase was subsequently dried with
392 nitrogen and resuspended in 100 µl THF for HPLC analysis.
393 HPLC analysis was performed with a Shimadzu SCL10 system (SPD10A UV/VIS-
394 detector, SPD-M10A diode array detector, SIL10A autosampler, CTO-10AC column
395 oven; all Shimadzu, Kyoto, Japan). Carotenoids were separated on a reverse phase
396 C18 column (250 mm x 4.5 mm x 5µ; Alltech, Deerfield, USA) using a gradient
397 program of acetonitrile:methanol:2-propanol (85:10:5) as solvent A and 100% 2-
398 propanol as solvent B. At a flow rate of 1ml/min at 32°C the elution program was the
399 following: 100% solvent A, 0% solvent B 0-31 minutes, 0% solvent A, 100% solvent B
400 31-36 minutes, 100% solvent A and 0% solvent B 36-45 minutes. Wavelength range
401 of 190-600 nm was monitored by diode array detector. Retention time of lycopene
402 was 25.38 minutes verified by corresponding standard. Diapolycopene was identified
403 by carotenoid extract comparison to *E. coli* heterologously expressing diapophytoene
404 synthase and diapophytoene desaturase from *Staphylococcus aureus* via pACCRT-
405 MN (Table 1) which was a kind gift of Prof. Gerhard Sandmann (Biosynthesis Group,
406 Molecular Bioscience, Goethe University Frankfurt).
407

408 ## 2.9 Fermentation

409 Fed-batch cultures were carried out in a 2.4 L KLF 2000 fermenter (Bioengineering
410 AG, Wald, Switzerland) equipped with a pH and pO_2 electrode from Mettler-Toledo
411 (Greifensee, Switzerland), two six-disc-turbine impellers and one downward adjusted
412 disk impeller. Filter-sterilized air or oxygen was supplied at a flow rate of 50 L/h. All
413 experiments were conducted at 30°C and at a pH of 6.75 which was controlled by
414 automated addition of NH_4OH (30% v/v). Dissolved oxygen (DO) concentration was
415 controlled (DO > 30%) by adjusting the agitation rate, starting at 700 rpm. Oxygen

and carbon dioxide in exhaust gas was measured using BINOS 1001 gas analyzer (Rosemount Analytical, Hanau, Germany). Methanol concentration was online monitored and adjusted half-hourly by ProcessTRACE 1.21 MT system (Trace Analytics, Braunschweig, Germany) equipped with a dialysis probe. Methanol concentration was measured every 15 min and methanol feed was set as follows: at a concentration below 1 g/L, 0.79 g (1 ml) and below 0.5 g/L, 1.42 g (1.8 ml) methanol was added by Watson-Marlow 505Du peristaltic pump (Cornwall, England). Anti foam B Emulsion (Sigma-Aldrich) was added manually to reduce foaming in addition to a six-disc-turbine impeller installed right above the liquid phase acting as a mechanical foam breaker.

After *in situ* sterilization of 900 ml fermentation medium (see 2.1.), the fermenter was inoculated to an OD_{600} of 0.5-1 using a preculture culture grown for 72h in shake flasks. After reaching an OD_{600} of 5-10, 100 µM cumate from a freshly prepared stock solution in methanol as wells as 15% dodecane was added. Methanol feeding rate was doubled after induction. Induced culture was cultivated for 120 hours. Samples were taken manually from which cell dry mass and OD_{600} was determined from aqueous phase and α-humulene was measured in organic dodecane phase (see 2.7).

2.10 *In silico* reconstruction of the strain carrying the mevalonate pathway

In silico reconstruction of the synthetic strains was performed by adding the heterologous reactions catalyzed by the mevalonate pathway genes plus sesquiterpene synthase as well as an exchange reaction for each compound of interest (geranyl pyrophosphate, isopentenyl pyrophosphate, all-trans-farnesyl pyrophosphate, and α-humulene) into the genome-scale metabolic model of

M. extorquens AM1 (Peyraud et al., 2011). Identifiers of the synthetic reactions were tagged by a prefix R_S_ in order to differentiate them from the endogenous reactions. The synthetic model, iRP911MEV, was converted in system biology markup langage (sbml) prior simulation and is available in Supplemental material Info S2.

2.11 Theoretical yield calculation by flux balance analysis using genome-scale model

Theoretical yields were calculated by flux balance analysis using the reconstructed model of the synthetic strain, iRP911MEV (see 2.10). Calculation were performed with the software Optflux v3.0.1 (Rocha et al., 2010) using the exchange fluxes of the compounds of interested as the objective functions. The constraints applied for simulation are i) the setting of the exchange fluxes corresponding to the available substrates in minimal medium plus methanol as sole source of carbon and energy, ii) the removing of the stoichiometrically balanced cycles converting freely NADH and NADPH in order to obtain an accurate redox balance through the network, and iii) the non-growth associated maintenance (NGAM) flux was not taken into account, i.e. no constraint was set on this flux. The list of constraints is available in supplemental material table S2. The parsimonious flux balance analysis method was used to obtain a solution minimising the sum of fluxes (Parsimonious enzyme usage), and a knock-out in the genes of the mevalonate pathways was used to calculate the yield via the endogenous methylerythritol phosphate pathway. Details of the simulation results are available in the supplemental material table S3.

3 Results and discussion

3.1. Constitutive expression of α-humulene synthase in *M. extorquens* AM1 resulted in production of 2.3 mg/L α-humulene

Methylobacterium extorquens AM1 produces endogenously FPP which is converted to menaquinone, hopanoids and carotenoids (Fig.1) and should therefore be able to synthesize α-humulene upon expression of a heterologous α-humulene synthase. Plasmid pCM80 harboring the strong pmxaF promotor from the methanol dehydrogenase operon of *Methylobacterium* (Marx and Lidstrom, 2001) has been applied several times for heterologous production of proteins in *M. extorquens* AM1 (Figueira et al., 2000; Fitzgerald and Lidstrom, 2003; Hofer et al., 2010) and was therefore chosen as vector for the expression of α-humulene synthase *zss*I from *Zingiber zerumbet*. A codon optimized *zss*I variant (Table 1 and Supplemental Info S1) was introduced into pCM80 leading to pFS33. To further increase α-humulene production, FPP synthase from *Saccharomyces cerevisiae* was cloned behind *zss*I into pFS33 leading to pFS34 (pCM80-*zss*I-ERG20).

Cultivations were carried out as aqueous-organic two-phase cultures (see 2.6.) wherein dodecane acts as organic phase. The strong hydrophobicity of α-humulene leads to its complete accumulation in the dodecane phase as intracellular concentrations were not measureable (data not shown), which has two advantages: production of α-humulene can be directly quantified by measuring its concentration in the dodecane phase and evaporation of α-humulene is reduced due to the high boiling point of dodecane. A concentration of 20% (v/v) dodecane was used which

had previously been shown to be not growth inhibiting for *M. extorquens* AM1 (data not shown).

M. extorquens harboring pFS33 was able to produce α-humulene as evidenced by GC-MS analysis (see 2.7). A peak having similar retention time and mass spectrum as the α-humulene standard was identified (Fig. 3) whereas α-humulene production was not detectable for the AM1 control strain harboring the empty vector. A concentration of 2.3 mg/L α-humulene was produced by AM1_pFS33 and AM1_pFS34 indicating that FPP-synthase *ERG20* present on pFS34 does not increase α-humulene concentration. This might be due to inactive protein or, more probably, insufficient supply of IPP/DMAPP, the metabolic substrates of FPP synthases. Further increase in α-humulene production may therefore require an elevated IPP synthesis which can be achieved either by upregulation of native MEP pathway or by expression of a heterologous MVA pathway.

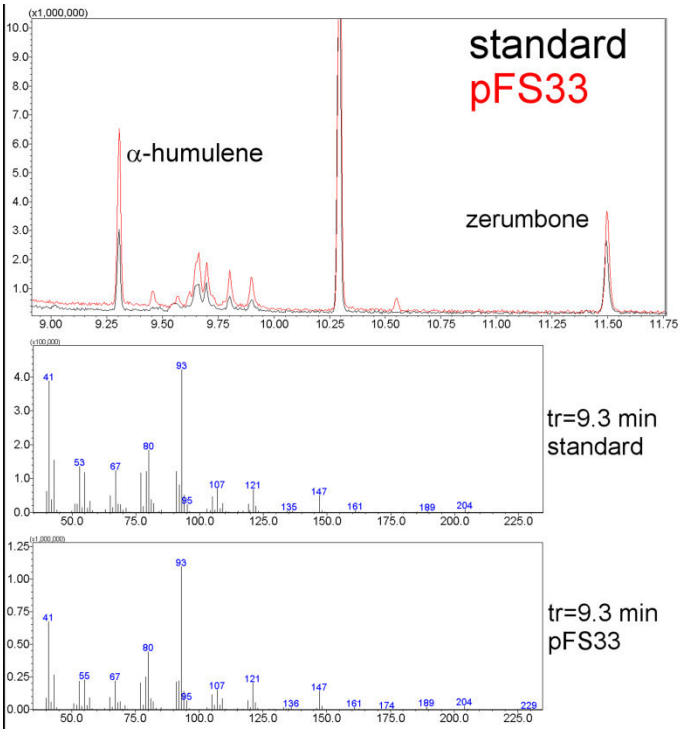


Fig. 3. GC-MS chromatogram comparison of α-humulene standard (black line) and sample of *M. extorquens* harboring pFS33 (pCM80-*zss*I, red line). Internal standard zerumbone elutes after 11.5

503 minutes. α-Humulene in pFS33 sample was identified by comparison of mass spectra displayed
504 underneath the chromatogram.

3.2. Constitutive expression of the mevalonate pathway genes from *Myxococcus xanthus* in *M. extorquens* AM1 did not lead to viable transformants

Using a heterologous MVA pathway to increase IPP supply has the advantage of circumventing native regulation of the MEP pathway. In addition, the initial MVA precursor, acetoacetyl-CoA, is already part of the primary metabolism of *M. extorquens* (see chapter 1 and Fig.1). We therefore aimed at channeling part of the flux through this intermediate to terpenoid biosynthesis via overexpression of the genes of the MVA pathway to further increase α-humulene production.

Previous heterologous MVA pathway integrations into *E. coli* mostly used sequential assembly of eukaryotic genes from *S. cerevisiae* and/or partially prokaryotic genes from *Staphylococcus aureus* or other Gram-positive cocci (Martin et al., 2003; Tsuruta et al., 2009; Yoon et al., 2009; Yoon et al., 2006). Beside the cloning effort of this strategy, the low GC-content of *S. cerevisisae*, *S. aureus*, *Streptococcus pyogenes* or *Enterococcus faecalis* (<40%) in contrast to *M. extorquens* AM1 (68.5%, (Vuilleumier et al., 2009)) would very likely lead to an inacceptable codon bias. Therefore, we focused on cloning a complete prokaryotic MVA pathway originating from *Myxococcus xanthus*. The similar GC content of 68.9% (Muller et al., 2013) results in excellent Codon Adaptation Indices (CAI) between 0.742 and 0.853 for the MVA pathway genes.

Transformation of plasmid pFS44 containing *zss*I, *ERG20* and *M. xanthus* MVA pathway genes *hmgs*, *fni*, *hmgr*, *mvaK*, *mvaK2* and *mvaD*, into electrocompetent *M. extorquens* AM1 did not lead to transformant colonies on solid minimal medium, neither containing methanol nor succinate as sole carbon source. Although stability problems of the large plasmid could not be excluded at this stage of the project, we hypothesized that the lethal effect observed was rather based on the constitutive

530 expression of the MVA pathway genes due to different possible factors: α-humulene
531 is produced in toxic concentrations, toxic accumulation of MVA pathway
532 intermediates due to imbalanced flux or a general metabolic burden due to high
533 protein expression (Glick, 1995). To exclude toxicity of α-humulene, growth inhibiting
534 effects on *M. extorquens* were investigated.
535

536 ### 3.3. Extracellular α-humulene has no growth inhibiting effect on *M. extorquens*
537 AM1 up to concentrations of 1g/l

538

539 Terpenoids often have toxic effects on bacteria although highest inhibiting effects are
540 mostly reported for monoterpenoids (Sarria et al., 2014; Trombetta et al., 2005).
541 Sesquiterpenoids, especially those which are highly hydrophobic such as farnesene,
542 are often described as less toxic (Renninger et al., 2010) although inhibiting effects
543 may vary depending on the compound and microbial host used.
544 Potential toxicity of α-humulene on *M. extorquens* was examined by monitoring
545 growth in the presence of different α-humulene concentrations. As cultivations for α-
546 humulene production were carried out in aqueous-organic two-phase systems with
547 dodecane as organic phase, liquid α-humulene was diluted into dodecane in various
548 concentrations for the toxicity tests. The dodecane layer containing α-humulene was
549 added to the *M. extorquens* culture as a second phase. In a second approach, α-
550 humulene was directly added into the aqueous phase to verify toxic effects in the
551 absence of an organic layer which would be the case for α-humulene producing
552 colonies harboring pFS33, pFS34 or pFS44 after transformation.

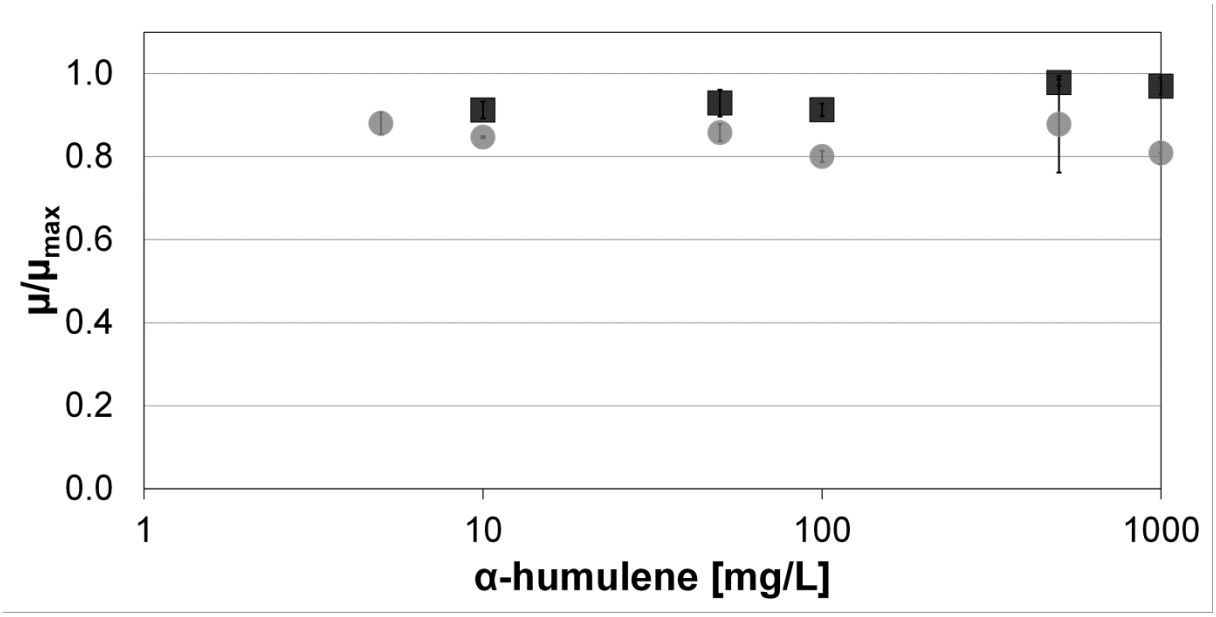


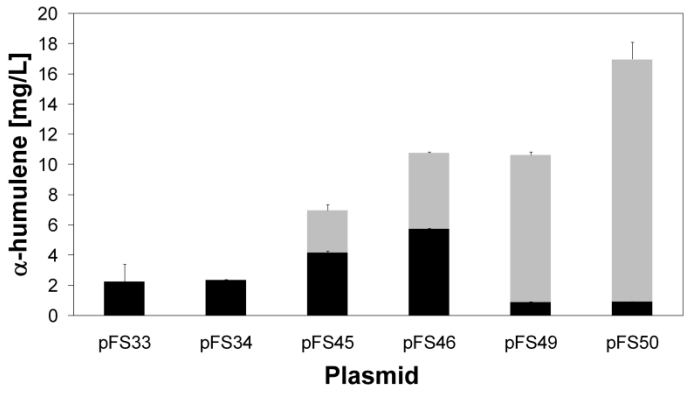
Fig. 4. Tolerance of *M. extorquens* AM1 towards α-humulene directly dissolved in aqueous phase or dissolved in dodecane as second organic phase. Maximum specific growth rates in corresponding medium without α-humulene (μ_{max}) are compared to maximum specific growth rates (μ) obtained at different α-humulene concentrations. n=3; error bars represent standard deviations. Grey circles indicate "dissolved in aqueous phase", black squares indicate "dissolved in dodecane phase".

Figure 4 clearly demonstrates that α-humulene has no significant growth inhibiting effect on *M. extorquens* even at concentrations of 1 g/L, independent of whether it is delivered via the dodecane phaseor directly in the aqueous phase.

The results indicate that α-humulene toxicity is unlikely to be the reason for the lethal effect of pFS44 on *M. extorquens*.

3.4. Inducible expression of α-humulene and FPP synthases in *M. extorquens* AM1 leads to increased α-humulene synthesis but transformants harboring an additional *M. xanthus* mevalonate pathway still grow poorly

569 Identification of the reason for MVA pathway toxicity on *M. extorquens* is difficult
570 without positive transformants harboring the MVA pathway. To avoid immediate
571 transcription of MVA pathway genes, a plasmid system containing an inducible
572 promoter is required. Therefore, α-humulene synthase *zss*I alone, in combination
573 with FPP synthase *ERG20* and in combination with *ERG20* and the genes of the
574 MVA pathway were cloned into plasmid pHC115 harboring a cumate inducible
575 promoter (Chou and Marx, 2012), resulting in plasmids pFS45, pFS46 and pFS47,
576 respectively (Fig. 2). After transformation, colonies were obtained for pFS45 and
577 pFS46 but not for pFS47 containing the MVA pathway. A potential reason for these
578 phenotypes may be derived from data for pFS45 and pFS46 shown in figure 5: more
579 than 50% α-humulene was produced without induction leading to the conclusion that
580 repression of gene expression from pHC115 is not tight and remaining expression of
581 MVA pathway genes is still toxic.



582

583 **Fig. 5.** α-Humulene production of *M. extorquens* AM1 harboring plasmids pFS33 (pCM80-*zss*I), pFS34
584 (pCM80-*zss*I-ERG20), pFS45 (pHC115-*zss*I), pFS46 (pHC115-*zss*I-ERG20), pFS49 (pQ2148F-*zss*I)
585 and pFS50 (pQ2148F-*zss*I-ERG20). Black bar parts indicate production without induction whereas
586 grey bar parts indicate production with induction. Please note that pCM80 harbors a constitutive
587 promoter. Concentrations were compared after 48 hours of cultivation (pFS33, pFS34) or induction
588 (pFS45, pFS46, pFS49, pFS50), respectively. n=3; error bars represent standard deviations.

589

To overcome this problem, a more tightly regulated inducible promoter system recently published by Kaczmarczyk et al. (2013) and coworkers was used. Plasmid pQ2148 contains cumate inducible 2148 promotor which was shown to be suitable for tightly regulated high level protein expression in *M. extorquens*. *Zss*I alone and again in combination with *ERG20* as well as the MVA pathway genes were introduced into pQ2148F resulting in pFS49 (*zss*I), pFS50 (*zss*I-ERG20) and pFS52 (*zss*I-ERG20-MVA). Colonies were obtained after transformation into *M. extorquens* for pFS49, pFS50 and also pFS52, though colonies for pFS52 were very small even after 8 days of growth at 30°C in contrast to colonies containing pFS49 or pFS50 which were obtained after 5 days. α-Humulene concentrations reached 11 mg/L in AM1_pFS49 and 17 mg/L in AM1_pFS50 cultures (Fig. 5) which is a 6-fold or 1.6 fold increase for the *zss*I-ERG20 construct compared to pFS34 (pCM80-*zss*I-ERG20) or pFS46 (pHC115-*zss*I-ERG20), respectively. In contrast to pHC115 constructs, background production, i.e. without induction, was only 5%. However, the low background expression when using the pQ2148 system still seems to be highly inhibiting as shown by the very slow growth of AM1_pFS52 (pQ2148F-*zss*I-ERG20-MVA).

3.5. Ribosome binding site optimization of α-humulene and FPP synthases as well as IPP isomerase leads to viable transformants of *M. extorquens* AM1 able to synthesize up to 58 mg/l α-humulene in shake flask cultivations

Imbalances in the flux of a heterologously expressed MVA pathway can cause accumulation of intermediates which were shown to have inhibitory effects on *E. coli*:

614 especially the accumulation of 3-hydroxymethylglutaryl-CoA (HMG-CoA) and FPP
615 results in strong growth defects (Dahl et al., 2013; Pitera et al., 2007).
616 Direct determination of a potential HMG-CoA or FPP accumulation was not possible
617 for AM1_pFS52 (pQ2148F-*zss*I-ERG20-MVA) due to its very slow growth. An indirect
618 test was performed by transformation of pCM80 (constitutive promoter) harboring 3-
619 hydroxymethylglutaryl-CoA synthase (*hmgs*) and pCM80 harboring the complete
620 MVA pathway with and without *ERG20* but no α-humulene synthase. pCM80-*hmgs*
621 should result in the accumulation of HMG-CoA as *M. extorquens* does not harbor any
622 native HMG-CoA converting enzymes and pCM80-MVA-ERG20 should result in the
623 accumulation of prenyl phosphates IPP, DMAPP and FPP. Colonies were obtained
624 for AM1_pCM80-*hmgs* after 4 days but neither for pCM80-MVA nor for pCM80-MVA-
625 ERG20 after 8 days. These results give a strong hint that accumulation of prenyl
626 phosphates is most likely the reason for toxicity of the heterologous MVA pathway in
627 *M. extorquens* even at low expression levels.
628
629 Flux balancing of pathways might be achieved by adjusting various parameters such
630 as promoter strength, inducer concentration, plasmid copy number or translation
631 initiation rates (TIR) of ribosome binding sites (RBS). RBS optimization has the
632 advantage of low cloning effort and maintaining the existing plasmid system.
633 Improvement of expression of MVA pathway genes on translational level has already
634 been realized by RBS optimization leading to increased terpenoid production in
635 *E. coli* (Nowroozi et al., 2014). Hence, to prevent prenyl phosphate accumulation in
636 *M. extorquens*, TIR of α-humulene synthase RBS was increased 146 fold in plasmids
637 pFS57 (*zss*I^{225k}), pFS58 (*zss*I^{225k}-ERG20) and pFS59 (*zss*I^{225k}-ERG20-MVA) (see

638 table 2) to obtain higher levels of ZSSI and thus more conversion of toxic FPP to α-
639 humulene.

Table 2

Translation initiation rates (TIR) of the native and optimized ribosome binding sites (RBS) of the heterologous mevalonate pathway genes from *Myxococcus xanthus,* FPP synthase *ERG20* and α-humulene synthase *zss*I present on different plasmids and growth behavior of the respective strains. TIRs of RBS for mevalonate kinase (*mvaK*), phosphomevalonate kinase (*mvaK2*) and pyrophosphomevalonate decarboxylase (*mvaD*) are given in parentheses because these genes overlap in the MVA pathway operon so that RBS are not ascertainable in the same way as for the remaining genes. Growth: colony appearance of *M. extorquens* on methanol agar after transformation: ++++: as empty vector (3-4 days), +++: 4-5 days, ++: 5-6 days, +: 6-7 days, -: no colonies visible within 8 days

wt: only native FPP synthase of *M. extorquens* AM1 with unknown RBS; Intermediates: AAc-CoA: acetoacetyl-CoA, HMG-CoA: hydroxymethylglutaryl-CoA, Mev: mevalonate, P-Mev: phosphomevalonate, PP-mev: pyrophosphomevalonate, IPP: isopentenyl pyrophosphate, DMAPP: dimethylallyl pyrophosphate, FPP: farnesyl pyrpophosphate; genes: *hmgs*: hydroxymethylglutaryl-CoA synthase, *hmgr*: hydroxymethylglutaryl-CoA reductase, *fni*: isopentenyl pyrophosphate isomerase

Intermediate:	AAc-CoA	→	HMG-CoA	→	Mev	→	P-Mev	→	PP-Mev	→	IPP / DMAPP	→	FPP	→	α-humulene

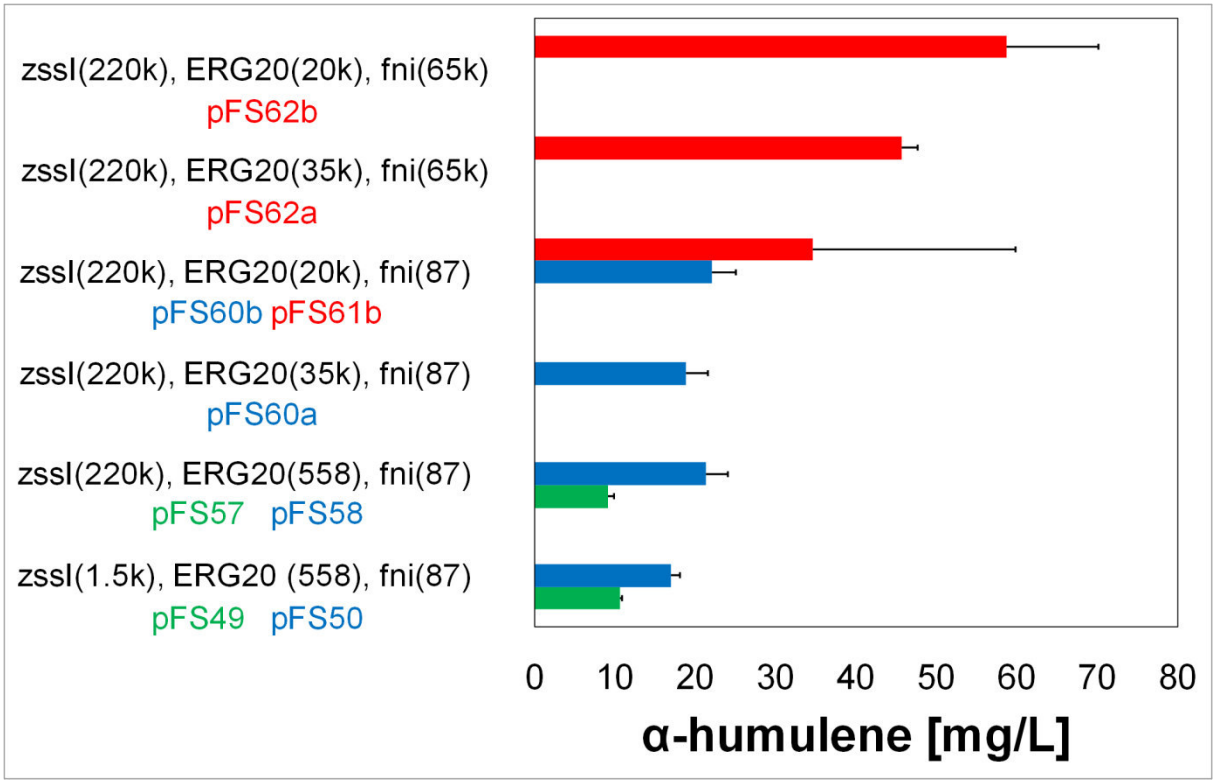
Gene:	*hmgs*	*hmgr*	*mvaK*	*mvaK2*	*mvaD*	*fni*	*ERG20*	*zss*l	
Plasmid	**Translation initiation rate (au)**								**Growth**
pFS49	-	-	-	-	-	-	wt	1514	+++
pFS50	-	-	-	-	-	-	558	1514	++
pFS52	1995	701	(0.87)	(0.43)	(317)	87.3	558	1514	-§
pFS57	-	-	-	-	-	-	wt	221,625	++
pFS58	-	-	-	-	-	-	558	221,625	+++
pFS59	1995	701	(0.87)*	(0.43)	(317)	87.3	558	221,625	-§
pFS60a	-	-	-	-	-	-	36,800	221,625	++
pFS60b	-	-	-	-	-	-	22,000	221,625	+++
pFS61a	6345	701	(0.87)	(0.43)	(317)	87.3	36,800	221,625	-
pFS61b	6345	701	(0.87)	(0.43)	(317)	87.3	22,000	221,625	+
pFS62a	6345	701	(0.87)	(0.43)	(317)	65,000	36,800	221,625	+
pFS62b	6345	701	(0.87)	(0.43)	(317)	65,000	22,000	221,625	++

§ different colony sizes whereas bigger colonies usually showed low or undetectable α-humulene production

As shown in figure 6, RBS optimization of *zss*I did not result in higher α-humulene productions without additional precursor supply from the MVA pathway (pFS57 and pFS58). Transformants with pFS59 (*zss*I^{225k}-ERG20-MVA) grew very slowly, comparable to pFS52 containing strains without *zss*I RBS optimization (see table 2). Interestingly, some bigger colonies appeared additionally which showed a significantly reduced production level or did not lead to detectable product concentrations at all when propagated in liquid culture (data not shown).

Growth inhibition caused by pFS59 might be due to toxic accumulation of other prenyl phosphates than FPP, i.e. probably of IPP, as TIR of *ERG20* in pFS59 is rather low. Therefore, TIR of *ERG20* RBS was increased to a 1:6 (pFS61a) and 1:4 (pFS61b) ratio to the TIR of *zss*I RBS (Table 2) to provide adequate FPP supply for α-humulene synthase on the one hand but prevent toxic FPP accumulation on the other hand. RBS optimization of *ERG20* in combination with *zss*I RBS optimization did not lead to increased α-humulene production without the MVA pathway (pFS60a and pFS60b, Fig. 6). The combination of RBS optimized α-humulene synthase, FPP synthase and the MVA pathway finally resulted in non-lethal plasmid pFS61b (TIR of *ERG20* is 22,000). Interestingly, in contrast to AM1_pFS61b, AM1_pFS61a (TIR of *ERG20* is 36,800) was not able to grow which is probably due to a still toxic FPP pool. Concentrations of up to 60 mg/L α-humulene were produced by some AM1 clones harboring pFS61b. However, production rates varied dramatically between different transformants as shown by the high standard deviation in figure 6 (average production was 35 mg/L). Colonies harboring pFS61b seem to be under high selection pressure to decrease terpene production probably by plasmid alteration. Therefore, the varying production rates of AM1_pFS61b did not allow the development of a stable process.

682 IPP and DMAPP are produced in a ratio of 5:1 (Zhao et al., 2013) by the HMB-PP
683 reductase of the MEP pathway occurring naturally in *M. extorquens* (Fig.1). In
684 contrast, only IPP is produced via the heterologous MVA pathway which makes an
685 IPP isomerase necessary to provide sufficient supply of DMAPP for FPP synthesis.
686 Table 2 illustrates that TIR of IPP isomerase *fni* RBS is very low compared to TIR of
687 *ERG20* or *zss*I RBS which probably results in insufficient DMAPP supply for FPP
688 synthesis. Therefore TIR of *fni* RBS was increased to 65,000 in plasmids pFS62a
689 and 62b (see table 2). AM1_pFS62b and AM1_pFS62a showed an improved growth
690 in contrast to AM1_pFS61b or AM1_pFS60b suggesting an IPP – DMAPP imbalance
691 without *fni* RBS optimization. An α-humulene concentration of 58 mg/L was produced
692 by AM1_pFS62b with a significantly reduced variance between different
693 transformants compared to AM1_pFS61b (Fig. 6). Optical density was around 3 after
694 48 hours of induction which corresponds to a cell dry weight of 1 g/L.



695

696 **Fig. 6.** α-Humulene production of *M. extorquens* harboring plasmids containing ribosome binding site
697 (RBS) optimized α-humulene synthase (*zss*I), FPP synthase (*ERG20*) and IPP isomerase (*fni*) in
698 different combinations. Translation initiation rates for the genes are listed in parentheses on the y-axis
699 (Salis 2011). Concentrations are average product concentrations of 3 transformants, each one
700 cultivated in two separate cultures (see 2.6.). Green: plasmids only containing *zss*I, Blue: plasmids
701 containing *zss*I and *ERG20*, Red: plasmids containing *zss*I, *ERG20* and the complete mevalonate
702 pathway. Error bars represent standard deviations.

703 Thus, stable heterologous expression of the MVA pathway in *M. extorquens* was
704 finally achieved by adaption of the RBS of α-humulene synthase, FPP synthase and
705 IPP isomerase. The concentration of 58 mg/L α-humulene obtained with
706 *M. extorquens* harboring pFS62b (*zss*I^{220k}-ERG20^{20k}-*fni*^{65k}-MVA) corresponds to a
707 threefold increase compared to overexpression of α-humulene synthase and FPP
708 synthase without mevalonate pathway.

709 Addition of 5 mM mevalonic acid preliminary to induction of α-humulene synthesis
710 genes did not significantly change α-humulene production of AM1_pFS62b (data not

711 shown). The manifold acid utilization capacity of *M. extorquens* (Sonntag et al., 2014)
712 makes a limitation in mevalonic acid uptake rather unlikely. Hence, the conversion of
713 acetoacetyl-CoA to mevalonate is most probably not limiting α-humulene production
714 in AM1_pFS62b and does, therefore, not represent a valuable target for further
715 optimization. Instead, the conversion of mevalonate to α-humulene seems to be a
716 bottleneck to be addressed by further strain engineering. Here, the lower mevalonate
717 pathway reactions from mevalonate to IPP catalyzed by MvaK, MvaK2 and MvaD are
718 promising targets as their TIR values are comparatively low (see table 2).
719

720 3.6. α-Humulene production in carotenoid biosynthesis deficient
721 *M. extorquens* strain CM502 is increased by 30% compared to strain AM1
722

723 Several pathways compete with α-humulene synthase for its precursor FPP in
724 *M. extorquens* including quinone, hopanoid and carotenoid biosynthesis (Fig. 1).
725 Menaquinones or ubiquinones are very likely indispensable for cell growth and
726 maintenance of obligate aerobic *M. extorquens* due to their role in cell respiration.
727 Hopanoids have been shown to be dispensable for growth of other bacteria under
728 non-stressful growth conditions (Welander et al., 2009). However, biosynthesis of
729 hopanoids and their precise role for *M. extorquens* have not been elucidated in detail
730 making a shutdown of this pathway rather unfeasible. In contrast, carotenoids are not
731 essential as already shown by the isolation of a colorless mutant of *M. extorquens*
732 AM1 by Van Dien et al. (2003). The use of a carotenoid synthesis deficient mutant
733 could therefore provide a higher FPP supply for α-humulene synthesis.
734 The colorless *M. extorquens* AM1 mutant strain CM502 isolated by van Dien and
735 coworkers was predicted as Δ*crtN*, i.e. a phytoene desaturase mutant. Although not

finally proven, it is very likely that *M. extorquens* AM1 does not harbor GGPP-derived C_{40} carotenoids but FPP-derived C_{30} diapocarotenoids (G. Sandmann, Goethe University Frankfurt, personal communication). This hypothesis is substantiated by the fact, that C_{30} diapocarotenoids have been identified in other species of the same genus, such as *Methylobacterium rhodinum*, previously named *Pseudomonas rhodos* (Kleinig et al. 1979) and in a series of six *Methylobacterium* species including *M. populi* and *M. radiotolerans* (Osawa et al. 2015). In addition, *M. extorquens* seems to add aldehyde groups to diapolycopene and further oxidizes the diapolycopene-dial to diapolycopene-dioate which, in turn, can be further esterified and/or glycosylated (Fig. 1) as shown for *Staphylococcus aureus* or *M. extorquens'* close relatives *Methylobacterium rhodinum* and *Methylomonas* sp. strain 16a (Pelz et al., 2005; Tao et al., 2005). In their screening of *Methylobacterium* species, Osawa et al. (2015) also identified new C_{30} diapocarotenoid derivatives, all having the core structure "ß-D-glucosyl 4,4'-diapolycopene-4,4'-dioic acid" in common. Carotenoid extraction and analysis (see 2.8.) of strain CM502 revealed that it produces diapolycopene but not lycopene which have identical UV-spectra but different retention times (Fig.7A). Saponified extract of CM502 showed less peaks than that of AM1 wildtype although an assignment of peak identities was not possible (data not shown). Therefore it can be assumed that strain CM502 is not a (diapo)phytoene desaturase mutant but more likely a diapolycopene oxidase mutant (*crtNb*) as it still contains diapolycopene but presumably no esterified/glycosylated derivatives.

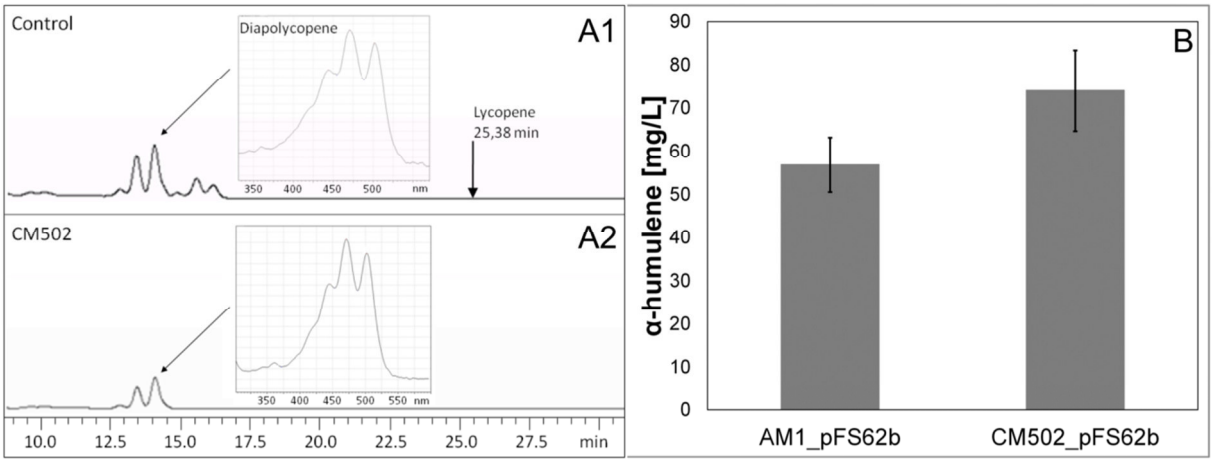


Fig. 7. A: Chromatograms (n= 502 nm) of unsaponified carotenoid extract of *E. coli* expressing diapophytoene synthase and diapophytoene desaturase from *S. aureus* via pACCRT-MN (A1) and *M. extorquens* carotenoid biosynthesis deficient strain CM502 (A2). Theoretical retention time of lycopene is indicated by the arrow. B: α-Humulene production of *M. extorquens* AM1 and CM502, both harboring plasmid pFS62b (pQ2148F-*zss*I^{225k}-ERG20^{22k}-*fni*^{65k}-MVA), in shake flasks after 48 hours induction (n=3; error bars represent standard deviations).

α-Humulene production of CM502 containing plasmid pFS62b was significantly increased by about 30% compared to *M. extorquens* AM1 wildtype harboring pFS62b (Fig.7B). Although non-oxidized (apo)carotenoids are still produced by strain CM502_pFS62b, the overall flux into FPP consuming carotenoid biosynthesis is obviously lowered which can explain the enhanced α-humulene production observed. To the authors' knowledge, such a downregulation of a competing pathway to enhance terpenoid production - as known from engineering *S. cerevisiae,* e.g. by lowering native sterol biosynthesis (Asadollahi et al., 2008) - has not been reported for terpenoid producing bacteria before. A knockout or downregulation of hopanoid biosynthesis may therefore be another promising strategy to further increase terpenoid production by *M. extorquens*. As already mentioned, this would require

776 detailed clarification of hopanoid biosynthesis in *M. extorquens* and its cellular
777 importance which has been only partly investigated so far (Bradley et al., 2010).
778 Our final shake flask production titer of 75 mg/L α-humulene is below the titer of 1000
779 mg/L described by Harada et al. (2009) who used recombinant *E. coli* cells. However,
780 in the latter case the medium was supplemented with expensive lithium acetoacetate
781 or DL-mevalonate synthesized from mevalonolactone (both ~105€/g; Sigma-Aldrich),
782 which should be taken into account. Without precursor supplementation, Harada et
783 al. obtained a maximum titer of 250 mg/L.
784

785 3.7. Cultivation of *M. extorquens* strains harboring pFS62b in a fed-batch bioreactor
786 results in α-humulene titers of up to 1.6 g/l

787 To evaluate the performance of *M. extorquens* for α-humulene production, methanol
788 limited fed-batch fermentations were carried out. High cell density fermentations of
789 *M. extorquens* AM1 have already been described by Bourque et al. (1995) and
790 Bélanger et al. (2004) whose process parameters were adapted to our aqueous-
791 organic two phase cultivation system (see 2.9.).
792 *M. extorquens* AM1 or CM502 carrying plasmid pFS62b were grown to an OD_{600} of 5
793 to 10 before expression of α-humulene synthesis pathway was induced with cumate
794 and a dodecane phase was added. Further cultivation was carried out at constant pH
795 of 6.75, dissolved oxygen of > 30% and a methanol concentration controlled to a
796 value of about 1 g/l. Average OD_{600} values of 80 to 90 were reached per fermentation
797 (Table 3) corresponding to a cell density of about 30 g/L. As shown in figure 8, α-
798 humulene production was clearly growth dependent indicated by no further increase
799 in product concentration during stationary growth phase. A maximum α-humulene
800 concentration of 1.65 g/L related to the aqueous phase volume was produced by

strain CM502_pFS62b representing a 57% increase compared to the highest concentration of 1.02 g/L achieved by strain AM1_pFS62b. The concentration of 1.65 g/L in the fed-batch cultivation corresponds to a 22 fold increase compared to highest concentrations achieved by shake flask cultivation, while the α-humulene/OD_{600} ratio remained equal at ca. 20 $mg*L^{-1}*OD_{600}^{-1}$.

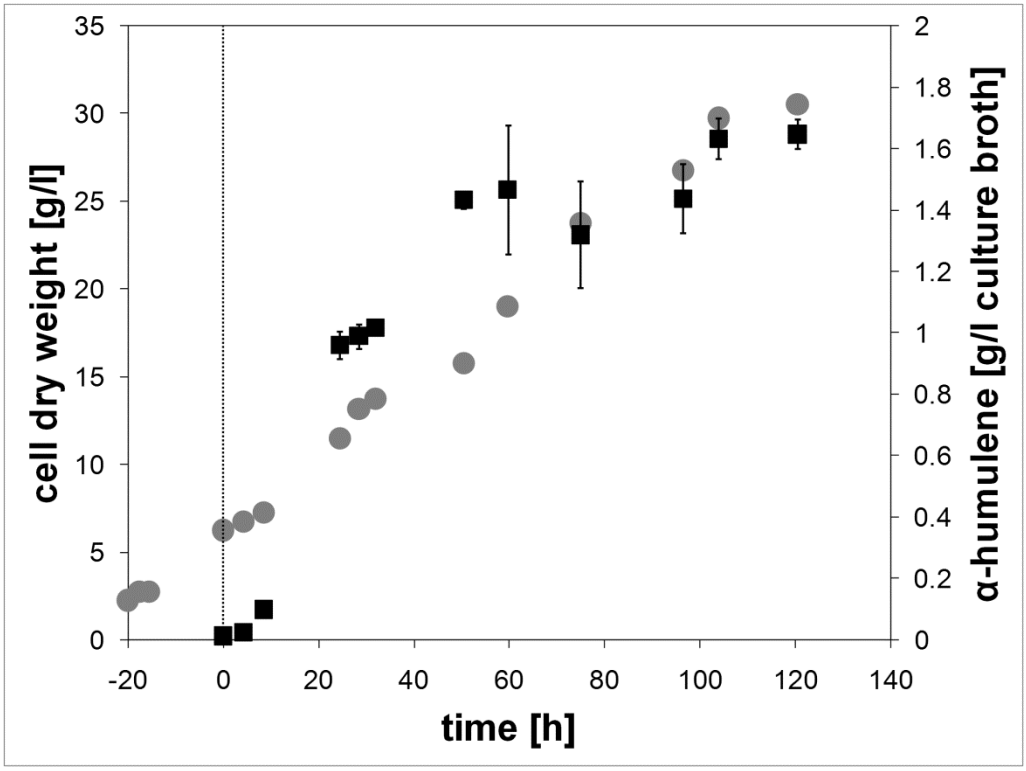


Fig. 8. Time course of cell dry weight and α-humulene concentration of strain CM502 containing pFS62b in fermentation 5 (Table 3). Time point zero represents point of induction with cumate indicated by the dashed vertical black line. Standard deviations (represented by error bars) of α-humulene concentrations are calculated from same sample analyzed three times. Black squares: α-humulene concentration, grey circles: cell dry weight; both values calculated related to the volume of the aqueous phase (= culture broth).

The maximum theoretical yield of α-humulene per methanol is 0.26 g/g according to calculations based on an *in silico* model of *M. extorquens* AM1 harboring all introduced heterologous production modules (see 2.10 and 2.11). Therefore, the

817 maximum yield of 0.031 $g_{\alpha\text{-humulene}}/g_{MeOH}$ obtained in fermentation 5 (see table 3)
818 corresponds to 12% of the maximum theoretical yield. Other (sesqui-)terpenoid
819 production processes based on glucose and the classical hosts *E. coli* or
820 *S. cerevisiae* vary greatly in their yield: from only 0.52% or 1.2% of the theoretical
821 maximum yield for α-santalene (Scalcinati et al., 2012) and α-pinene (Sarria et al.,
822 2014), respectively, up to 36% for bisabolene (Peralta-Yahya et al., 2011) and even
823 more than 50% for farnesene (Renninger et al., 2010). Therefore, the product yield
824 obtained in the present work is already in a competitive range especially in view of
825 the use of an alternative carbon source as substrate.
826 Although the market price of 0.45$/kg methanol (Bertau et al., 2014) is still higher
827 than 0.2$/kg sugar (Peralta-Yahya et al., 2011), several facts should be taken into
828 consideration when comparing the different C sources and bioprocess systems: i)
829 usually, sugar based bioprocesses use glucose instead of raw sugar, i.e. a
830 purification step may be needed resulting in increased costs, ii) sugar prices can be
831 expected to increase over the next decades whereas methanol prices will most likely
832 decrease due to highly expanding production capacities (Bertau et al., 2014), iii)
833 methanol strongly reduces contamination risk in comparison to sugar based
834 fermentations which, in turn, reduces sterilization expenditures and iv) in contrast to
835 technical sugar sources and complex media (e.g. corn steep liquor or molasses)
836 methanol minimal medium does not contain complex chemical compounds which
837 significantly facilitates downstream processing. Furthermore, compared to methane
838 as another potential C_1 source for industrial biotechnology (for a recent review on
839 metabolic engineering of methanotrophs see Kalyuzhnaya et al., 2015), methanol,
840 though being more expensive, has the advantage of being a well soluble liquid
841 instead of being a gaseous substrate. This fact makes methanol far easier to be fed
842 and controlled in a conventional bioreactor and due to its lower degree of reduction,

the substrate methanol is accompanied by a lower oxygen / cooling energy demand than methane. The engineered strain and fermentation process described in the present work may be easily adaptable towards the production of other industrially useful sesquiterpenoids, such as flavor and fragrance compounds or potential biofuels (Peralta-Yahya et al., 2012) and could therefore provide a basis for future bioproduction of terpenoids from the non-food carbon source methanol.

Table 3

Process characteristics of methanol limited fed-batch fermentations performed with strains AM1 and CM502 harboring pFS62b. Cumate induction was carried out after reaching early exponential growth phase (OD_{600} around 10). Given data represent measurements at time point after induction. n.d.: not determined, cdw: cell dry weight, $Y_{P/S}$: substrate-specific product yield, STY: space time yield

strain	fermentation #	time [h] till max. α-humulene concentration	max. α-humulene concentration [g/L]	OD_{600}[a]	cdw[a] [g/L]	$Y_{P/S}$[a,b] [g/g_{MeOH}]	STY[c] [$mgL^{-1} h^{-1}$]
AM1	1	63	0.74	90	30	0.023	11.7
	2	70.5	1.02	148	n.d.	0.024	15
	3	93	0.73	84	27.9	0.015	7.8
CM502	4	80	1.37	79	28.4	0.023	17.1
	5	104	1.65	85	30	0.031	14.6

a: at stationary phase

b: maximum theoretical yield is 0.26 g/g_{MeOH}

c: average STY, calculated from time point of induction (t=0) until process end (end of stationary growth phase)

Conclusion

We engineered the model methylotroph *Methylobacterium extorquens* for *de novo* synthesis of the sesquiterpenoid α-humulene from methanol. Expression of a codon and ribosome binding site optimized α-humulene synthase from *Zingiber zerumbet* together with FPP synthase from *Sacchormyces cerevisiae* as well as a prokaryotic mevalonate pathway from *Myxococcus xanthus* led to α-humulene production levels of 58 mg/L in shake flask cultivations. Reducing undesired flux of the precursor FPP towards carotenoids by the use of carotenoid biosynthesis deficient mutant CM502 (Van Dien et al., 2003) further increased product concentration to 74 mg/L. Methanol limited fed-batch cultivations of the latter strain finally resulted in α-humulene titers of up to 1.65 g/L, which is, to our knowledge, the highest reported value for *de novo* synthesized α-humulene so far.

This study demonstrates for the first time, that sesquiterpenoids can be efficiently produced from the non-food carbon source methanol as an alternative to sugar. The engineered *M. extorquens* strain can serve as a platform for future production of various other terpenoids. Our results also highlight the importance of precisely balancing production and subsequent conversion of prenyl phosphates, the metabolic terpenoid precursor molecules. For *M. extorquens*, accumulation of prenyl phosphates has not only growth reducing effects as described for *E. coli* (Ma et al., 2011; Martin et al., 2003; Pitera et al., 2007) but is completely growth inhibiting. In the future, we will focus on the mevalonate pathway to identify further bottlenecks and, thus, to maximize IPP and DMAPP supply for terpenoid production in *M. extorquens*.

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Supplemental material

Table S1: Primers used in this work

Info S1: Gene sequence of codon optimized α-humulene synthase *zss*I

Info S2: sbml file of the genome scale metabolic model iRP911MEV

Table S2: List of constraints used in flux balance analysis

Table S3: Metabolic model simulation results

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Table S1: Primers used in this work. Underlined and italic sequences (all 5'→3') indicate restriction enzyme recognition sites. Bold letters indicate ribosome binding site (RBS) sequences.

Name	Sequence
primers	
HMGS-fw	AGTCTAGA**GAGGAG**CGCAGGATGAAGAAGCGCGTGGGAAT
HMGS-rev	ATCTG*GATCCG*TTTAAAC*CCTGCAGG*ACCGGT*GTTAAC*TCAGTTCCCTTCGGCGTAC
HMGS-over-fw	GCTGCGCGGCCGAGTTCTACTCCGGCACG
HMGS-over-rev	CGTGCCGGAGTAGAACTCGGCCGCGCAGC
MVA1_fw	ATCTGGATCCT**AGGAGG**AATAATATGGGCGACGACATCACTG
MVA-SacIA-rev	AACACCATGGCGAGCTCTC
MVA-SacIA-fw	GAGAGCTCGCCATGGTGTT
MVA-SacIB-rev	GTGCCCGTTGAGCTCCACCT
MVA-SacIB_fw	AGGTGGAGCTCAACGGGCAC
MVA2_rev	ATCGAATTC*AAGCTT*TCAGCTCAGCGCGCGCACC
pQF_MCS-fw	CTAGTCTGCAGCTTAAGCATGCTCTAGAAGATC
pQF_MCS-rev	TCGAGATCTTCTAGAGCATGCTTAAGCTGCAGA
ZSSI-fw	TA*GCATGC*TTA**AGAAGG**ATCAGTCATAATGGAACGCCAGTCGATGG
ZSSI-RBS-fw	ATACACTAGT**AGCTTAAGGATAAAGAAGGAGGTAAAAC**ATGGAACGCCAGTCGATGG
ZSSI-rev	AG*TCTAGA*TACGTA*ATCGAT*TCAGATGAGGAACGACTCGA
ERG20_fw	ATCGTATCGAT**AGGAG**CGCAGGATGGCTTCAGAAAAAGAAATTAG
ERG20-RBS(35k)-fw	ATCGTATCGAT**GAGAAGAGCAGACTCGATCATAACAGGGGACTAG**ATGGCTTCAGAAAAAGAAATTAG
ERG20-RBS(20k)-fw	ATCGTATCGAT**ACATCAAACCAAAGGACTTTACAGGTAGTAGAA**ATGGCTTCAGAAAAAGAAATTAG
ERG20_rev	atcgtacgtaCTATTTGCTTCTCTTGTAAACT
ERG20_rev-2	ACTATCTAGATAAAGTAGAGGAGGATTAATCTATTTGCTTCTCTTGTAAACT
fni-RBSopt-fw	AACCTAAAATTAACGAGGAAAGAGGGAGGTTACAG
fni-RBSopt-rev	GATCTGTAACCTCCCTCTTTCCTCGTTAATTTTAGGTT

Info S1: DNA-sequence of α-humulene synthase *zss*I codon optimized for *Methylobacterium extorquens* AM1

ATGGAACGCCAGTCGATGGCCCTCGTCGGCGACAAGGAGGAGATCATCCGCAAGTCGTTC
GAGTACCACCCGACCGTCTGGGGCGACTACTTCATCCGCAACTACTCGTGCCTCCCGCTC
GAGAAGGAGTGCATGATCAAGCGCGTCGAAGAGCTGAAGGACCGCGTCCGCAACCTCTTC
GAAGAGACGCACGACGTCCTCCAGATCATGATCCTCGTCGACTCGATCCAGCTCCTCGGC
CTCGACTACCACTTCGAGAAGGAGATCACCGCCGCCCTCCGCCTCATCTACGAGGCCGAC
GTCGAGAACTACGGCCTCTACGAGGTGTCGCTCCGCTTCCGCCTCCTCCGCCAGCACGGC
TACAACCTCTCGCCGGACGTGTTCAACAAGTTCAAGGACGACAAGGGCCGCTTCCTCCCG
ACCCTCAACGGCGACGCCAAGGGCCTCCTCAACCTCTACAACGCCGCCTACCTCGGCACC
CACGAAGAGACGATCCTCGACGAGGCGATCTCGTTCACCAAGTGCCAGCTCGAGTCGCTC
CTCGGCGAGCTGGAACAGCCGCTCGCCATCGAGGTGTCGCTGTTCCTCGAGACGCCGCTC
TATCGCCGCACCCGCCGCCTGCTCGTCCGCAAGTACATCCCGATCTATCAGGAGAAGGTC
ATGCGCAACGACACCATCCTCGAGCTGGCCAAGCTGGACTTCAACCTCCTCCAGTCGCTC
CACCAGGAGGAGGTCAAGAAGATCACCATCTGGTGGAACGACCTCGCCCTCACCAAGTCG
CTCAAGTTCGCCCGCGACCGCGTCGTCGAGTGCTACTACTGGATCGTCGCCGTCTACTTC
GAGCCGCAGTACTCGCGCGCCCGCGTCATCACCTCGAAGGCCATCTCGCTCATGTCGATC
ATGGACGACATCTACGACAACTACTCGACCCTCGAAGAGTCGCGCCTCCTCACCGAGGCC
ATCGAGCGCTGGGAGCCGCAGGCCGTCGACTGCGTCCCCGAGTACCTCAAGGACTTCTAC
CTCAAGCTCCTCAAGACCTACAAGGACTTCGAGGACGAGCTGGAACCGAACGAGAAGTAC
CGCATCCCGTACCTCCAGGAGGAGATCAAGGTCCTCTCGCGCGCCTACTTCCAGGAGGCC
AAGTGGGGCGTCGAGCGCTACGTCCCGGCCCTCGAAGAGCACCTCCTCGTGTCGCTCATC
ACCGCCGGCTACTTCGCCGTCGCCTGCGCCTCGTACGTGGGCCTCGGCGAGGACGCCACC
AAGGAGACGTTCGAGTGGGTCGCCTCGTCGCCGAAGATCCTCAAGTCGTGCTCGATCCAC
TGCCGCCTGATGGACGACATCACCTCGCACCAGCGCGAGCAGGAGCGCGACCACTTCGCC
TCGACCGTGGAGTCGTACATGAAGGAGCACGGCACCTCGGCCAAGGTCGCCTGCGAGAAG
CTCCAGGTCATGGTCGAGCAGAAGTGGAAGGACCTCAACGAGGAGTGCCTCCGCCCGACC
CAGGTCGCCCGCCCGCTCATCGAGATCATCCTCAACCTGTCGCGCGCCATGGAAGACATC
TACAAGCACAAGGACACCTACACCAACTCGAACACCCGCATGAAGGACAACGTGTCGCTG
ATCTTCGTCGAGTCGTTCCTCATCTGA

Table S2: Constraints applied on the FBA performed using the software Optflux v3.0.1 (Rocha et al., 2010) and which correspondes to a minimal medium with methanol as sole source of carbon and energy.

Exchange flux	Lower bound	upper bound
EX_0001	-15	-15
EX_0002	-10000	10000
EX_0003	-10000	10000
EX_0004	-10000	10000
EX_0007	0	10000
EX_0008	-10000	10000
EX_0009	0	10000
EX_0010	0	10000
EX_0011	0	10000
EX_0012	0	10000
EX_0013	0	10000
EX_0014	0	10000
EX_0015	0	10000
EX_0016	0	10000
EX_0017	0	10000
EX_0019	0	10000
EX_0020	-10000	10000
EX_0021	-10000	10000
EX_0022	0	10000
EX_0023	0	10000
EX_0024	0	10000
EX_0025	0	10000
EX_0026	-10000	10000
EX_0027	0	10000
EX_0028	0	10000
EX_0029	0	10000
EX_0030	0	10000
EX_0031	0	10000
EX_0032	0	10000
EX_0033	-10000	10000
EX_0036	-10000	10000
EX_0037	0	10000
EX_0038	-10000	10000
EX_0039	-10000	10000
EX_0040	-10000	10000
EX_0041	-10000	10000
EX_0042	-10000	10000
EX_0043	-10000	10000
EX_0044	-10000	10000
EX_0045	-10000	10000

EX_0046	-10000	10000
EX_0047	0	0
EX_0048	0	10000
EX_0049	0	10000
EX_0050	0	10000
EX_0051	0	10000
EX_0052	0	10000
EX_0053	0	10000
EX_0054	0	10000
EX_0055	0	10000
EX_0056	0	10000
EX_0057	0	10000
EX_0058	0	10000
EX_0059	0	10000
EX_0060	0	10000
EX_0061	0	10000
EX_0062	0	10000
EX_0063	0	10000
EX_0064	0	10000
EX_0065	0	10000
EX_0066	0	10000
EX_0067	0	10000
EX_0068	0	10000
EX_0069	0	10000
EX_0070	0	10000
R_0234	0	0
R_0890	0	0
R_0002	0	0

Rocha, I., Maia, P., Evangelista, P., Vilaca, P., Soares, S., Pinto, J., Nielsen, J., Patil, K., Ferreira, E., Rocha, M., 2010. OptFlux: an open-source software platform for in silico metabolic engineering. BMC Systems Biology. 4**,** 45.

7.2 Weiterführende Forschungsarbeiten

7.2.1 Rational attempts towards replacement of the glyoxylate-regenerating ethylmalonyl-CoA pathway in *Methylobacterium extorquens* by a heterologous glyoxylate cycle

Sonntag F., Schrader J., Buchhaupt M., unveröffentlicht

Zusammenfassung:

In diesem unveröffentlichten Manuskript wird die Wiederherstellung der Methylotrophie von *M. extorquens* AM1-Stämmen mit verschiedenen Deletionen im EMCP durch die Expression eines heterologen Glyoxylat-Zyklusses untersucht. Soweit bekannt, handelt es sich dabei um den ersten Versuch des kompletten Austausches eines primären Stoffwechselszyklusses.

Durch die Deletion einzelner EMCP-Gene und dem damit verbundenem Verlust der entsprechenden enzymatischen Aktivität kommt es zur Akkumulation des jeweiligen CoA-Ester Substrates, was für weitere Produktsynthesen vorteilhaft sein kann. Jedoch ist die native Funktion der Glyoxylatregeneration des EMCP essentiell für das Wachstum auf C1- und C2-Kohlenstoffquellen, so dass EMCP-Deletionsstämme nicht mehr auf Methanol wachsen können. *M. extorquens* hat keine Isocitrat-Lyase bzw. keinen Glyoxylat-Zyklus, der in anderen Serin-Zyklus-Methylotrophen die Funktion der Glyoxylatregeneration erfüllt. Eine ICL aus *E. coli* konnte aktiv in *M. extorquens* exprimiert werden, führte jedoch trotz hoher gemessener enyzmatischer Aktivität nicht zu einer Wachstumswiederherstellung von EMCP-negativen Stämmen auf Methanol. Metabolomische Analysen inklusive dynamischen 13C-labelling und Konzentrationsmessungen von Zentralstoffwechselintermediaten und Reduktionsäquivalenten zeigten, dass mit hoher Wahrscheinlichkeit weder eine Verarmung noch eine starke Akkumulation eines Zentralstoffwechselintermediates die Ursache für die nicht mögliche Methylotrophiewiederherstellung der EMCP-negativen *M. extorquens* Stämme ist. Da weitere Ursachen im Stoffwechsel nicht offensichtlich waren, wurde nach einem methylotrophen MO mit der gleiche Stoffwechselkombination der EMCP-negativen *M. extorquens*-Stämme gesucht, um dadurch mögliche Anhaltspunkte für die Probleme der Wachstumswiederherstellung

zu erhalten. Eine BLAST-basierte Analyse von mehr als 1500 Genomen konnte nur einen nachweislich methylotrophen und methanolverwertenden MO mit der Stoffwechselkombination Serin-Zyklus^{+}, EMCP^{-}, Glyoxylat-Zyklus^{+}, CBB^{-} und RuMP^{-} identifiziert werden, welche der der EMCP-negativen *M. extorquens*-Stämme mit heterologer ICL entspricht. Für dieses Bakterium namens *Granulibacter bethesdensis* CGDNIH1 konnte gezeigt werden, dass eine funktionelle ICL essentiell für das Wachstum auf Methanol als alleiniger Kohlenstoff- und Energiequelle ist, jedoch nicht für das Wachstum auf Glukose.

Titel:

Rational attempts towards replacement of the glyoxylate-regenerating ethylmalonyl-CoA pathway in *Methylobacterium extorquens* by a heterologous glyoxylate cycle

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Keywords:

Methylobacterium extorquens, ethylmalonyl-CoA pathway, glyoxylate cycle, serine cycle, methylotrophy, metabolomics, Granulibacter bethesdensis

Abstract

Methylotrophs using the serine cycle for assimilation of C1 carbon sources require regeneration of glyoxylate which is either achieved by the glyoxylate cycle or the ethylmalonyl-CoA pathway (EMCP). Substitution of the EMCP by a glyoxylate cycle is theoretically possible but has not been accomplished so far for serine cycle methylotrophs such as the model organism *Methylobacterium extorquens*. In this study, a comprehensive approach for methylotrophy restoration in an EMCP negative *M. extorquens* strain by expression of a heterologous glyoxylate shunt is presented. Expression of the respective genes from *Escherichia coli* resulted in high activities of isocitrate lyase and malate synthase but did not restore growth of EMCP negative *M. extorquens* strains on C1 or C2 carbon sources. Metabolomics and determination of NAD(P)H/NAD(P)$^+$ ratios in strains with reduced EMCP flux expressing isocitrate lyase showed that neither depletion nor tremendous accumulation of a central metabolite or nicotinamide cofactor species occurred.

With the prospect of getting clues for reengineering of this central metabolic pathway on a long term we attempted to identify natural serine cycle methylotrophs that use only the glyoxylate shunt. Analysis of more than 1500 bacterial genomes surprisingly revealed only one unambiguously experimentally evidenced methylotroph containing a serine cycle and a glyoxylate shunt without EMCP, Calvin-Benssom Bassham cycle or ribulose-monophosphate pathway, namely *Granulibacter bethesdensis* CGDNIH1. Further analysis of this bacterium might help to identify the subtle differences in the metabolic networks of *G. bethesdensis* and EMCP-negative *M. extorquens* with a heterologous glyoxylate shunt. This study provides extensive data surrounding the complex task of glyoxylate regeneration pathway manipulation and gives further insight on glyoxylate cycle distribution within methylotrophic bacteria.

Introduction

Methylotrophic organisms have the ability to utilize reduced compounds without carbon-carbon bonds as sole carbon sources and can therefore grow on substrates such as methylated amines and sulfur species as well as on (halogenated) methane or methanol (Anthony 1982; Chistoserdova 2009). In general, methylotrophy involves oxidation of the substrate via formaldehyde to CO_2 as dissimilatory pathway and the assimilation of C1 units. The latter can take place at the level of formaldehyde via the ribulose-monophosphate pathway (RuMP), at the level of CO_2 via the Calvin-Benson-Bassham (CBB) cycle or with formaldehyde and methylene-H_4F via the serine cycle (Christoserdova and Lidstrom 2013).

In the serine cycle, methylene-H_4F and glycine are initially condensed to serine which is further converted via several enzymatic steps to malyl-CoA that is in turn cleaved into acetyl-CoA and glyoxylate. Glyoxylate is converted back to glycine whereas acetyl-CoA is used to regenerate glyoxylate which finally completes the serine cycle (Anthony 1982). This glyoxylate regeneration can either proceed via the glyoxylate cycle involving isocitrate lyase (icl) and malate synthase or the ethylmalonyl-CoA pathway (EMCP). In the EMCP two acetyl-CoAs are converted to one molecule glyoxylate and one molecule succinyl-CoA via 12 enzymatic steps including two CO_2 fixation and two NADPH consumption steps (see Fig.1) (Anthony 2011; Erb 2007; Erb 2008; Peyraud 2009). The EMCP contains two mutases, namely the ethylmalonyl-CoA and methylmalonyl-CoA mutase (Ecm and Mcm, respectively, see fig.1) using cobalamine as essential cofactor and therefore requiring cobalt for their activity (Erb 2008; Miyamoto 2002).

In addition to glyoxylate regeneration during methylotrophy, the EMCP is also used for the assimilation of C2 carbon sources such as acetate or ethylamine in methylotrophic and non-methylotrophic organisms (Alber 2011).

Beside the elucidation of its function and regulation, the biotechnological use of the EMCP has gained interest during the last years. The possibility of producing fine or platform chemicals out of C1 carbon sources which do not compete with food production by well-established methylotrophs such as *Methylobacterium extorquens* offers a huge potential for chemical industry (Alber 2011; Schrader 2009). Consequently, several applications using the EMCP CoA-esters as valuable

substrates for biosyntheses have been implemented to date including the production of the EMCP-derived dicarboxylic acids mesaconate and (2*S*)-methylsuccinate (Sonntag 2014; Sonntag 2015), valuable PHA co- and ter-polymers (Orita 2014) and 1-butanol (Hu and Lidstrom 2014). Furthermore, the EMCP has the potential to be used as the basis for many other products including other dicarboxylic acid derivatives, polyketides or platform chemicals such as 2-ethyl-3-hydroxypropionate (Alber 2011; Ochsner 2014).

Despite the advantages of the production of EMCP derivatives, the obligatory function of the pathway for growth on C1 and C2 carbon sources is a clear drawback. Provision of a distinct precursor in sufficient amounts for highly efficient product syntheses cannot be achieved by deletion of the gene encoding the enzyme that converts the EMCP CoA-ester of interest. An alternative is the use of medium containing a strongly reduced amount of cobalt which is a simple and also efficient strategy (Orita 2014; Sonntag 2015). The cobalt limitation reduces the EMCP flux due to insufficient cobalamin supply for ethyl- and methylmalonyl-CoA mutase, resulting in the accumulation of ethylmalonyl-CoA and subsequent EMCP intermediates However, this strategy cannot be used to increase EMCP intermediates preceeding ethylmalonyl-CoA (Sonntag 2015) and is not suited to increase specific precursor pools which would be beneficial for unspecific enzymes such as thioesterases or a possible polyketide production (Ochsner 2014). Hence, finding a way to restore methylotrophy of strains harboring a deletion of an EMCP-enzyme encoding gene is of high interest for biotechnological use of this pathway.

One obvious way for methylotrophy restoration in EMCP-negative strains is the introduction of a heterologous glyoxylate cycle as alternative glyoxylate regeneration system, as *M. extorquens* does not harbor an isocitrate lyase naturally (Anthony 1982), i.e. is a serine+ icl- methylotroph. Serine+ icl+ methylotrophs containing a glyoxylate cycle require a minimum isocitrate lyase activity of 115 nmol * min^{-1} * mg^{-1} for growth on C1 carbon sources (Anthony 1982) assuming the lack of other glyoxylate regeneration pathways. Hence, an EMCP-negative *M. extorquens* would require the expression of an isocitrate lyase with the stated activity *in vivo*. Glyoxylate regeneration by a glyoxylate shunt is also theoretically possible in *M. extorquens* as shown by stoichiometric simulations of the isocitrate lyase reaction in a genome scale model of *M. extorquens* AM1 (Peyraud 2011).

In this study, we present a comprehensive approach to restore methylotrophy in various EMCP knockout strains by the integration of a heterologous glyoxylate cycle. Despite verified activity of the integrated glyoxylate cycle enzymes, most strains with a knockout of an EMCP gene were not able to utilize methanol as carbon source which led to intense investigations on the underlying causes.

This work is an important step towards the establishment of EMCP-negative *M. extorquens* strains and the overall knowledge on glyoxylate regeneration cycles in methylotrophs.

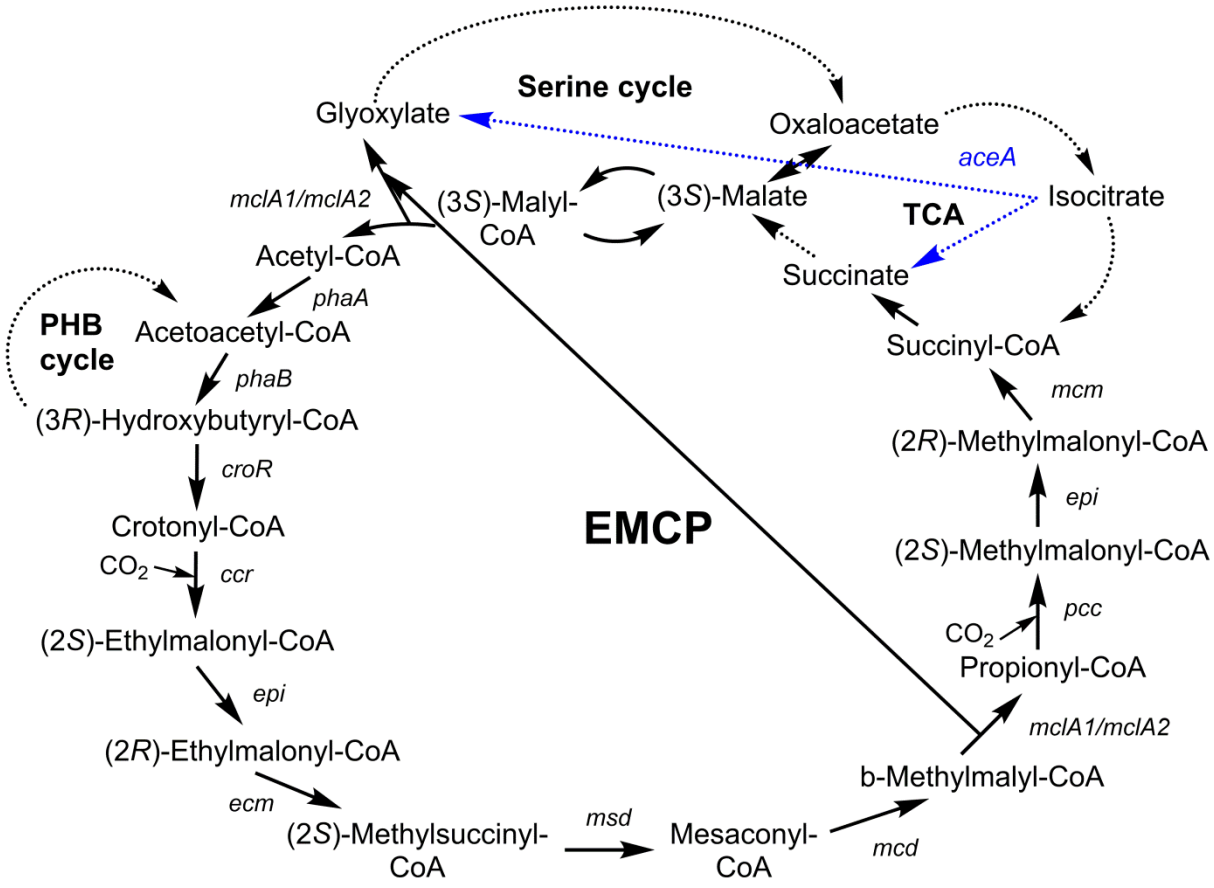

Figure 1: The ethylmalonyl-CoA pathway (EMCP) including overlaps with the polyhydroxybutyrate (PHB) cycle, serine-cycle and tricarbonic acid (TCA) cycle. The reaction of a heterologous isocitrate lyase encoded by *aceA* is shown in blue. Genes: *phaA*: β-ketothiolase; *phaB*: acetoacetyl-CoA reductase; *croR*: crotonase; *ccr*: crotonyl-CoA carboxylase/ reductase; *epi*: ethylmalonyl-/methylmalonyl-CoA epimerase; *ecm*: ethylmalonyl-CoA mutase; *msd*: (2*S*)-methylsuccinyl-CoA reductase; *mcd*: mesaconyl-CoA dehydratase; *mclA*; malyl-CoA/ beta-methylmalyl-CoA lyase; *pcc*: propionyl-CoA carboxylase; *mcm*: methylmalonyl-CoA mutase

Material and Methods

Chemicals, media and growth conditions

Methylobacterium extorquens AM1 (Peel and Quayle 1961) and deletion strains were grown at 30°C in minimal medium as described before (Kiefer 2009) containing 123 mM methanol and 12 µM cobalt unless stated otherwise. Succinate and acetate minimal media were prepared in the same way but contained 30.7 mM succinate or 5 mM acetate instead of methanol as sole carbon source. Glyoxylate was added from a 1000-fold aqueous stock solution to a final concentration of 5 mM to generate MeOH+Glyox-medium.

Escherichia coli strain DH5α (Gibco-BRL, Rockville, USA) was grown in lysogeny broth (LB) medium (Bertani 1951) at 37°C. Following antibiotic concentrations were used: tetracycline-hydrochloride at 10 µg/ml for *E. coli* and *M. extorquens* and kanamycin at 50 µg/ml for *M. extorquens*,

Granulibacter bethesdensis CGDNIH1 was grown in minimal medium 569 (Sievers and Swings 2005) containing 1% (v/v) methanol as sole carbon source or complex medium containing 50 g/l glucose, 12.5 g/l $CaCO_3$ and 5 g/l yeast extract. If stated, 3-nitropropionate purchased from Sigma-Aldrich (Steinheim, Germany) was added after autoclaving the medium to a final concentration of 1 mM from a 2000-fold stock solution solved in pure ethanol.

Construction of plasmids

Primers were purchased from Sigma-Aldrich (Steinheim, Germany). All standard cloning techniques were carried out as described before (Sambrook 2001). Genomic DNA from *E. coli* DH5α was purified using the GenElute Bacterial Genomic DNA Kit from Sigma-Aldrich. Transformation of plasmids into *M. extorquens* was performed by electroporation as described by Toyama (1998).

Gene deletions in *M. extorquens* were carried out by using the pCM184 system in combination with pCM157 for subsequent removal of the integrated kanamycin

marker cassette (Marx 2002). Therefore about 500 bp upstream and downstream flanking sites of ethymalonyl-CoA/methylmalonyl-CoA epimerase (*epi*, MexAM1_META1p0839), ethylmalonyl-CoA mutase (*ecm*, MexAM1_META1p0180), methylsuccinyl-CoA dehydrogenase (*msd*, MexAM1_META1p2223), mesaconyl-CoA dehydratase (*mcd*, MexAM1_META1p4153), malyl-CoA lyase/beta-methylmalyl-CoA lyase (*mclA1*, MexAM1_META1p1733), carbon-carbon lyase (malyl-CoA lyase homolog *mclA2*, MexAM1_META1p4295) and methylmalonyl-CoA mutase (*mcmA*, MexAM1_META1p5251) genes were amplified by PCR using corresponding up- and down primer pairs listed in Table S1. Amplified upstream flanking sites of *epi*, *mclA1*, *mclA2* and *mcmA* were digested with *Eco*RI and *Kpn*I and that of *ecm* and *msd* with *Pvu*II and *Nco*I and subsequently ligated into equally digested pCM184 resulting in pCM184_epi-up, pCM184_ecm-up, pCM184_msd-up, pCM184_mclA1-up, pCM184_mclA2-up and pCM184_mcmA-up, respectively. Ligation of the previously digested downstream flanking sites with *Sac*I and *Sac*II for *epi*, *Sna*B1 and *Age*I for *ecm*, *Sna*B1 and *Sac*I for *msd* and *mclA2*, *Apa*I and *Sac*I for *mclA1* and *mcmA* into the equally digested pCM184 plasmids containing the upstream flanking site of the respective gene finally resulted in plasmids pCM184-Δ*epi*, pCM184-Δ*ecm*, pCM184-Δ*msd*, pCM184-Δ*mclA1*, pCM184-Δ*mclA2* and pCM184-Δ*mcmA*. pCM184-Δ*mcd* was constructed vice versa, i.e. amplified downstream flanking site digested with *Age*I and *Sac*I was ligated first into equally digested pCM184 followed by the insertion of the upstream flanking site into *Pvu*II and *Nco*I restriction sites of pCM184_mcd-down.

For constitutive expression of heterologous isocitrate lyase from *E. coli* in *M. extorquens*, pCM80*icl* which was constructed previously (Kiefer 2009), was used and renamed to pCM80-*aceA* (see Table 1). Malate synthase A or G were amplified from purified genomic DNA of *E. coli* DH5α using primer pairs aceB-fw and aceB-rev or aceB-fw2 and aceB-rev2 for malate synthase A and MSG-fw in combination with MSG-rev for malate synthase G. Plasmids pCM80-*aceB* and pCM80-*aceAB* were created by ligating malate synthase A PCR-product (amplified with aceB-fw and aceB-rev) digested with *Xba*I and *Eco*RI into pCM80 or pCM80-*aceA*, respectively, cut with the same restriction endonucleases. Digesting PCR product of malate synthase A, amplified with primers aceBA-fw and aceBA-rev, with *Hind*III and *Xba*I followed by its ligation into equally digested pCM80-*aceA* resulted in plasmid pCM80-*aceBA*. Plasmid pCM80-*aceA*-G was constructed by ligation of *Xba*I and *EcoR*I

digested PCR product of malate synthase G into pCM80-aceA cut with the same enzymes.

Table 1: Plasmids and strains used in this work.

Name	Description	reference
Plasmids		
pCM80	constitutive expression vector for *Methylobacterium extorquens*; Kan^R, pmxaF, oriT, pBR322ori	Marx 2001
pCM184	Allelic exchange vector for gene deletion in *Methylobacterium extorquens*; Kan^R, Tc^R, Amp^R oriT, pBR322ori	Marx 2002
pCM157	Cre recombinase expression plasmid; Tc^R, oriT, pBR322ori	Marx 2002
pCM80-*aceA*	pCM80 containing isocitrate lyase from *E. coli* (pCM80*icl*)	Kiefer 2009
pCM80-*aceB*	pCM80 containing malate synthase A from *E. coli*	this study
pCM80-*aceBA*	pCM80-aceB containing isocitrate lyase downstream of *aceB*	this study
pCM80-*aceAB*	pCM80-aceA containing malate synthase A downstream of *aceA*	this study
pCM80-*aceA-G*	pCM80-aceA containing malate synthase G from *E. coli* downstream of *aceA*	this study
pCM184-Δ*epi*	pCM184 containing 500bp flanking sites of *epi*	this study
pCM184-Δ*ecm*	pCM184 containing 500bp flanking sites of *ecm*	this study
pCM184-Δ*msd*	pCM184 containing 500bp flanking sites of *msd*	this study
pCM184-Δ*mcd*	pCM184 containing 500bp flanking sites of *mcd*	this study
pCM184-Δ*mclA1*	pCM184 containing 500bp flanking sites of *mclA1*	this study
pCM184-Δ*mclA2*	pCM184 containing 500bp flanking sites of *mclA2*	this study
pCM184-Δ*mcmA*	pCM184 containing 500bp flanking sites of *mcmA*	this study
strains		
E. coli DH5α	F^-, Φ80dlacZΔM15, Δ(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(rK^- mK^+), phoA, supE44, λ^-, thi-1	ATCC
M. extorquens AM1	Facultative methylotrophic, obligate aerobic, gram-negative, pink pigmented α-proteobacterium, Cm^R	Peel & Quayle 1961 DSM1338
M. extorquens Δ*epi*	*M. extorquens* AM1 with chromosomal deletion of ethylmalony-CoA/ methylmalonyl-CoA epimerase gene *epi (*MexAM1_META1p0839; YP_002962037.1)	this study
M. extorquens Δ*ecm*	*M. extorquens* AM1 with chromosomal deletion ethylmalonyl-CoA mutase *ecm (*MexAM1_META1p0180; YP_002961419.1)	this study
M. extorquens Δ*msd*	*M. extorquens* AM1 with chromosomal deletion of methylsuccinyl-CoA dehydrogenase *msd* (MexAM1_META1p2223; YP_002963293.1)	this study
M. extorquens Δ*mcd*	*M. extorquens* AM1 with chromosomal deletion of mesaconyl-CoA dehydratase (MexAM1_META1p4153; YP_002965082.1)	this study
M. extorquens Δ*mclA1*	*M. extorquens* AM1 with chromosomal deletion of malyl-CoA lyase/ beta-methylmalyl-CoA lyase *mclA1* (MexAM1_META1p1733; YP_002962854.1)	this study
M. extorquens Δ*mclA1A2*	*M. extorquens* ΔmclA1 with additional chromosomal deletion of carbon-carbon lyase/ malyl-CoA lyase homolog *mclA2* (MexAM1_META1p4295; YP_002965208)	this study
M. extorquens Δ*mcmA*	*M. extorquens* AM1 with chromosomal deletion of methylmalonyl-CoA mutase mcmA (MexA1_META1p5251; YP_002966130.1)	this study

Construction of EMCP-negative strains

EMCP knockout strains of *M. extorquens* were created according to a procedure described before (Marx 2002). Briefly, 4µl of purified plasmid pCM184-Δ*epi*, pCM184-Δ*ecm*, pCM184-Δ*msd*, pCM184-Δ*mcd*, pCM184-Δ*mclA1* or pCM184-Δ*mcmA* were transformed into electrocompetent *M. extorquens*. Kanamycin-resistant colonies obtained on succinate+kanamycin agar were checked for tetracycline-resistance on succinate agar containing tetracycline for the identification of potential null mutants. Because a functional EMCP is obligatory for growth on C1 carbon sources (Peyraud 2009), growth of kanamycin-resistant colonies was additionally checked on methanol agar for further confirmation of the deletion. Subsequently, two colony PCRs using gene specific primers together with Kan-check-fw and Kan-check-rev (see Table S1; e.g. Kan-epi-fw in combination with Kan-check-rev and Kan-check-fw in combination with Kan-epi-rev for verification of Δ*epi*) were performed for verification of the site specific removal of the corresponding gene in the potential null mutant. Resulting strains were named Δ*epi*::*kanR*, Δ*ecm*::*kanR*, Δ*msd*::*kanR*, Δ*mcd*::*kanR*, Δ*mclA1*::*kanR* or Δ*mcmA*::*kanR*. Chromosomally integrated kanamycin resistance marker flanked by Cre-loxP sites was removed by transformation of pCM157 into electrocompetent knockout strains followed by the steps described by Marx (2002) resulting in strains Δ*epi*, Δ*ecm*, Δ*msd*, Δ*mcd*, Δ*mclA1* and Δ*mcmA* (see table 1). Strain Δ*mclA1A2* was created by transformation of pCM184-Δ*mclA2* into strain Δ*mclA1* followed by the protocol described above.

Enzyme activity measurements of isocitrate lyase and malate synthase

For enzymatic activity measurements, *M. extorquens* AM1 harboring the corresponding plasmid constructs was grown in methanol minimal medium containing tetracycline at 30°C in shake flasks. Main cultures containing 50 ml methanol minimal medium were inoculated from a 48-hour grown pre-culture to an OD_{600} of 0.1 and harvested after overnight-cultivation in mid-exponential growth phase (OD_{600} ~ 2) by centrifugation at 4°C for 10 min and 4500 g. Cells were washed once with ice-cold phosphate buffer (100 mM KH_2PO_4, pH7.5), resuspended in 10 ml of the buffer and lysed by two cycles in a high pressure homogenizer at 137 kPa. Afterwards, cell

debris was separated by centrifugation at 4°C and 14.000 g for 10 min. Supernatant was kept at 4°C for further analysis.
Activities of iscocitrate lyase and malate synthase in *M. extorquens* crude cell extracts were measured spectrophotometrically according to the procedures described by Chell (1978) and Khan (1993), respectively.

Quantification of NAD(P)(H)

For quantification of NAD(P)(H), *M. extorquens* AM1 was grown in methanol minimal medium without or with tetracycline if harboring pCM80 or pCM80-*aceA*. Pre-cultures grown for 48 hours were diluted 1:30 for main culture inoculation in methanol minimal medium without cobalt to inhibit the EMCP. A final concentration of 0.4 µM Co^{2+} in the main culture was estimated assuming no cobalt consumption in the pre-culture. Cultures without EMCP inhibition were grown in standard medium containing non-growth limiting cobalt concentration (12 µM).
NAD(P)(H) was quantified using the EnzyChrom $NADP^+$/NADPH (ECNP-100) and NAD^+/NADH (E2ND) assay kits from BioAssay Systems. Briefly, cells were harvested after overnight cultivation during exponential growth phase (OD_{600} between 0.5 and 2.5) by centrifugation for 5 min at 3220 g and 4°C. After washing of the cells with ice-cold phosphate buffer (100 mM KPP, pH7.5) and subsequent resuspension in 10 ml buffer, the cell suspension was divided into 2 x 5 ml for the separate quantification of oxidized ($NAD(P)^+$) or reduced (NAD(P)H) cofactors. Both samples were centrifuged again, supernatant was discarded and cell pellets were suspended in 500 µl of the NAD(P) or NADP(H) extraction buffers of the kits. After lysing cells via sonification (55% amplitude, 0.5s/ 0.5s for 120 s), the procedure of the kit's manual was further followed for photometric quantification of NAD(P)(H).

Calculation of glutamate concentrations for external addition

Required amounts of external glutamate addition assuming an absent glutamate biosynthesis in *M. extorquens* were estimated as follows: Percentages of glutamate as well as arginine, proline and glutamine which are synthesized out of glutamate, of

the total protein amount of *M. extorquens* AM1 were calculated by summarizing the percentages of codons which are translated into the respective amino acids according to the codon usage database (Nakamura 2000) resulting in a proportion of 14.6%. Cell dry weight (cdw) of *M. extorquens* AM1 consists of 59.1% protein (Peyraud 2011) which results in an amount of 0.086 g glutamate, arginine, proline and glutamine per g cdw. Using an OD_{600} – biomass correlation of 0.33 $g_{cdw} * l^{-1} * OD_{600}^{-1}$ (Peyraud 2012), this results in a concentration of 0.259 g/l (1.53 mM) glutamate that is required for growth of *M.extorquens* to an OD_{600} of 9 which is about twice of the OD_{600} that is reached with the methanol concentration and medium used in this study.

Dynamic labeling experiments

Dynamic labeling experiments including calculation of the ^{13}C-labelling incorporation were performed adapted to the protocol of Schneider (2012b). Therefore, *M. extorquens* AM1 harboring pCM80 or pCM80-*aceA* was cultivated as described in "Quantification of NAD(P)(H)" for simulation of inhibited and non-inhibited EMCP with a natural abundance of ^{13}C-methanol. Exponentially growing culture was spiked with 123 mM of [U-^{13}C]-methanol followed by continuous mixing and incubation of the sample for 0, 5, 10, 30, 60, 120 and 300 s in 50 ml reaction tubes. Subsequent sampling was performed as stated in the next section.

Sampling, quenching and metabolite extraction

Quenching and extraction of CoA-esters was performed as described elsewhere (Peyraud 2012). In brief 1 ml of Culture (OD 1-2.5) was directly injected into 4.5 ml quenching solution and incubated 15 min on ice for metabolite extraction. Core metabolites were sampled by fast filtration as described previously (Kiefer 2008). To this end sample containing biomass equivalent of about 1 mg CDW was filtered and washed with 5 ml Medium. Filters were directly transferred into -20°C cold organic quenching solution acidified with formic acid and incubated for 10 minutes on ice

(Müller 2015). After metabolite extraction, all samples were deep-frozen with liquid nitrogen, freeze dried, and stored at -20°C until analysis.

Metabolomic analysis via HPLC-MS

LC-MS analysis was carried out using a Rheos 2200 HPLC system (Flux Instruments) coupled to an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific) and equipped with an electrospray ionization probe as described previously (Schneider 2012a). For LC-MS analysis of core metabolites a split-free nanoLC Ultra system (Eksigent,Dublin, CA) was hyphenated to the MS instrument instead of the Rheos 2200 HPLC system. Metabolite separation was achieved by nano scale ion-pair reversed phase HPLC as described elsewhere (Kiefer 2011) except that a different C18 column was used C18 column (Dr. Maisch HPLC Markensäule, Reprosil-Gold 120, 100 mm x 0.1 mm, Morvay Analytik GmbH) as stationary phase.

Genome based identification of methylotrophs harboring a serine and glyoxylate cycle without an additional EMCP, CBB cycle or RuMP

To identify candidates for methylotrophic bacteria using the serine cycle and the glyoxylate shunt but do not contain the CBB cycle, the EMCP or the RuMP, we analyzed all bacterial genomes available at the Microbial Nucleotide Blast homepage (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=MicrobialGenomes) for the presence of respective pathway marker genes. If sequences similar to the *M. extorquens* AM1 glyoxylate aminotransferase (Sga) and *M. extorquens* AM1 hydroxypyruvate reductase (Hpr) with e-values lower than 0.8 (Sga) and 10^{-40} (Hpr) could be identified within a tblastn analysis with the standard parameter values, we concluded the presence of the serine cycle. Sequences with similarity to crotonyl-CoA carboxylase/reductase (Ccr) of *M. extorquens* AM1 with a cut-off e-value of 10^{-43} served as marker genes for the EMCP and sequences with similarity to isocitrate lyase (Icl) of *Escherichia coli* with a boundary e-value of 10^{-14}

served as marker genes for the glyoxylate shunt. If sequences similar to the hexulose-6-phosphate synthase (H6ps) and hexulose-6-phosphate isomerase (Hpi) of *Bacillus methanolicus* with e-values lower than 10^{-10} (H6ps) and 10^{-17} (Hpi) could be identified within the tblastn analysis, we concluded the presence of the RuMP cycle. If sequences similar to the large subunit of Rubisco as well as phosphoribulokinase (Prk) of *Paracoccus denitrificans* with e-values lower than 10^{-10} (Rubisco) and 10^{-60} (Prk) could be identified within the tblastn analysis, we concluded the presence of the CBB cycle. The combined presence of sequences with similarity to the large subunit of the methanol dehydrogenase (MoxF) of *M. extorquens* AM1 with a cut-off e-value of 10^{-80} and sequences with similarity to a methanol-dehydrogenase-like protein (XoxF) of *M. extorquens* AM1 with a cut-off e-value of 10^{-60} is a strong indication for the property of methanol oxidation.

Results and Discussion

Methylotrophy of strains harboring EMCP gene deletions is partly restorable by glyoxylate feeding but not by a heterologous glyoxylate cycle

As illustrated in figure 1, the part of the EMCP that does not overlap with the TCA or the serine cycle consists of 12 different CoA-ester intermediates (including acetyl-CoA), of which the ethyl- and methylmalonyl-CoA enantiomers, (2*S*)-methylsuccinyl-CoA, mesaconyl-CoA and beta-methylmalyl-CoA are particularly interesting as precursors for various products (Ochsner 2014). Thus, genes encoding the enzymes which convert the mentioned CoA-esters were each knocked out using the pCM184 plasmid system followed by removal of the integrated kanamycin marker after verification of the gene deletion (see material and methods). As expected, deletion strains Δ*epi*, Δ*ecm*, Δ*msd*, Δ*mcd*, Δ*mclA1A2* and Δ*mcmA* were not able to grow on methanol as carbon source, whereas strain Δ*mclA1* showed minor growth (see figure 2). These observations are consistent with literature data describing that the MclA1 homologue MclA2 can partially take over the beta-methylmalyl-CoA lyase function of MclA1 (Okubo 2010). Also the inability of strains Δ*ecm*, Δ*msd*, Δ*mcd* and Δ*mcmA* to grow on methanol confirms previous observations (Korotkova 2002; Korotkova 2005; Smith 1996) which were, at least in part, obtained with transposon mutants.

One discrepancy to literature data is the inability of our Δ*epi* strain to grow on methanol whereas the Δ*epi* mutant constructed by Korotkova (2002) was described to show normal growth on methanol. To check if methylotrophy of the EMCP gene deletions strains is restorable by a heterologous glyoxylate shunt, isocitrate lyase *aceA* alone or in combination with malate synthase A (*aceB*) or G (*msG*) from *E. coli* were cloned into pCM80 (constructs see table 1). As *M. extorquens* contains a malate synthase reaction catalyzed by two enzymatic steps (L-malyl-CoA lyase and malyl-CoA thioesterase, Okubo 2010), expression of isocitrate lyase should be theoretically feasible to introduce a glyoxylate shunt. However, a heterologous malate synthase might provide more metabolic flexibility and was therefore also included in the experiment.

No EMCP gene deletion strain recovered growth on methanol upon the constitutive expression of isocitrate lyase or isocitrate lyase in combination with malate synthase A or G, independent of the gene arrangement in pCM80 (see fig.1). Growth of

Δ*mclA1* expressing *aceA* was slightly improved, which is consistent with previous observations, showing that introduction of isocitrate lyase is only capable of improving growth of cells whose metabolism is limited by flux through the EMCP (Kiefer 2009) but did not restore growth of mutants with a complete lack of EMCP flux, as e.g. the EMCP gene deletion strain Δ*phaA* (Korotkova and Lidstrom 2001).

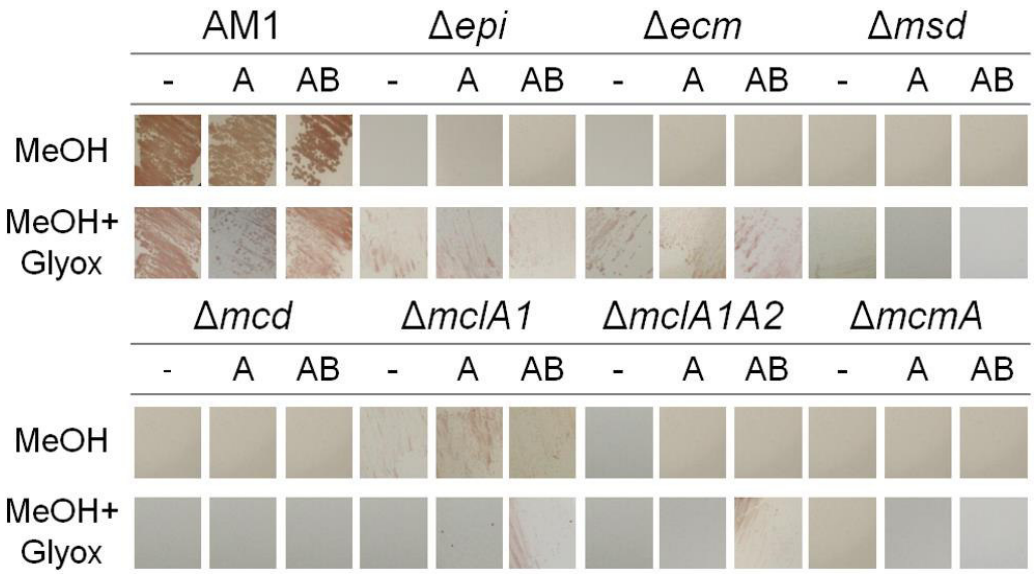

Figure 2: Growth of *M. extorquens* AM1 wildtype and EMCP-negative strains on solid methanol minimal medium without (MeOH) or with additional glyoxylate (MeOH+Glyox). Wildtype and deletion strains contained isocitrate lyase *aceA* (A) or isocitrate lyase/malate synthase *aceB* (AB) expression constructs based on pCM80. The AB columns additionally represent the results obtained with the constructs pCM80-*aceBA* and pCM80-*aceAG* which were identically to pCM80-*aceAB*. Emtpy vector controls are shown in the "-" columns.

To ensure, that glyoxylate regenerated by the heterologous glyoxylate shunt is sufficient for restoring growth on methanol, glyoxylate was added externally at a non-toxic concentration of 5 mM which has been shown as suitable for C1 growth recovery of Δ*ecm*, Δ*msd* and Δ*mcm* (Korotkova 2002). Glyoxylate addition restored growth of deletion strains *Δepi* and *Δecm* clearly and that of Δ*msd* slightly, independent of the existence of a heterologous glyoxylate cycle (see fig.1). Strains harboring deletion of genes Δ*mcd* and Δ*mcmA* were neither restorable by glyoxylate feeding nor by a combination of glyoxylate feeding and *aceA*/*B* expression. Surprisingly, strains Δ*mclA1* and Δ*mclA1A2* required *aceAB* expression grow on methanol with glyoxylate which raises the question if the added amount of glyoxylate is toxic for these strains and the heterologous malate synthase catalyzes the degradation of glyoxlyate. Note that glyoxylate alone is no sole carbon source for

AM1 wildtype or any deletion strain (data not shown). Hence, addition of glyoxylate does not restore methylotrophic growth of all respective deletion strains. The phenomenon that the severity of phenotypes caused by the absence of an EMCP enzyme increases with increasing distance to the PHB cycle has already been shown in another context for growth of *M. extorquens* AM1 on oxalate (Schneider 2012b).

As verification for sufficient expression of isocitrate lyase and malate synthase, the specific activities of both enzymes in crude cell extract of methanol-grown *M. extorquens* harboring pCM80-*aceA* /-*aceBA*/ -*aceAB* and –*aceAG* were measured and compared to cells containing pCM80. Figure 3 illustrates that *M. extorquens* has no native icl activity and expression of *E. coli aceA* leads to an activity of at least 0.6 U/mg, depending on the gene arrangement in the construct. That is more than five-fold above the minimum icl acitivity necessary for growth of icl+ methylotrophs on C1 carbon sources calculated by Anthony (1982). Although the used assay for malate synthase activity measurement has a higher background than the icl assay (see pCM80 values in figure 3), malate synthase activity was clearly detectable with specific activities between 0.6 and 1.8 U/mg which is in the range of the icl activities or above.

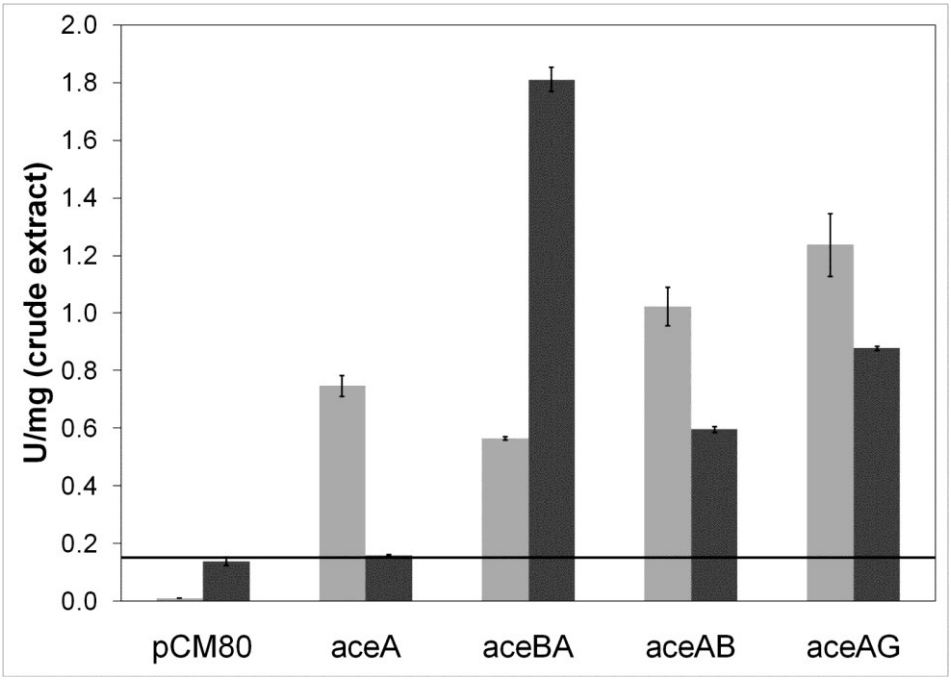

Figure 3: Enzyme activities of isocitrate lyase (light grey bars) and malate synthase (dark grey bars) measured in crude cell extracts of *M. extorquens* AM1 grown in methanol minimal medium. Genes encoding isocitrate lyase (*aceA*) or malate synthase (*aceB* or *aceG*) or combinations of both were expressed via pCM80 containing the strong constitutive *pmxaF* promoter. Necessary activity of isocitrate lyase for growth of icl+ methylotrophs on C1 carbon sources (Anthony 1982) is indicated by the black horizontal line. n=3; error bars represent standard deviations

Taken together, heterologous glyoxylate shunt enzymes are expressed actively in *M. extorquens* showing specific activities that clearly exceed the theoretical requirements for functional methylotrophy in serine+ icl+ methylotrophs. It is conceivable, that the affinity of the expressed isocitrate lyase is too low for the *M. extorquens* isocitrate pool present *in vivo* and the icl therefore catalyzes the condensation of glyoxylate and succinate to isocitrate instead of cleaving isocitrate. Even though flux through the TCA from oxaloacetate to isocitrate is low during growth of *M. extorquens* on methanol (Peyraud 2011), the low K_m of the *E. coli* icl, which is about 7 to 30 µM at a pH of 6.8 to 7.5 (MacKintosh and Nimmo 1988) and thus close to *M. extorquens* intracellular pH of 6.5 when growing on methanol (Crowther 2008), is a strong indication against this hypothesis. Also the previously described growth improving effects of icl expression for non-lethal EMCP flux reduction (Kiefer 2009; Korotkova and Lidstrom 2001) suggest a functional icl expression in *M. extorquens*. However, without information on the size of AM1s native isocitrate and glyoxylate pools, which are unfortunately lacking in literature, it is not possible to exclude that the icl reaction proceeds in the non-desired direction.
In summary, EMCP knockout strains unable to grow on methanol cannot be rescued by the integration of a heterologous glyoxylate cycle although enzyme activities are theoretically suitable for methylotrophy.

Quantification of NAD(P)H/NAD(P)$^+$ levels under standard and EMCP flux-limited growth conditions

In methanol-grown serine cycle methylotrophs using the EMCP for glyoxylate regeneration, four molecules methanol (C1) and three molecules CO_2 (assimilated via PEP, crotonyl-CoA and propionyl-CoA carboxylases) are used to build up one C3 molecule and one molecule succinyl-CoA (Christoserdova and Lidstrom 2013). Thereby 5 molecules NADH, 2 molecules NADPH, 6 molecules ATP and one molecule FAD are used. The corresponding equation is as follows:

3 C1 + 4 CO_2 + 2 NADPH + 5 NADH + 1 FAD^+ + 6 ATP → 1 C3 + 1 succinate + 2 $NADP^+$ + 5 NAD^+ + 1 FADH + 6 ADP

Assuming the replacement of the EMCP by the glyoxylate shunt, the balance would change as follows:

3 C1 + 2 CO_2 + 3 NADH + 5 ATP → 1 C3 + 0.5 succinate + 3 NAD^+ + 5 ADP

Two CO_2 fixation steps (crotonyl- and propionyl-CoA carboxylase) and two NADPH consumption steps (acetoacetyl-CoA reductase and crotonyl-CoA carboxylase) of the EMCP would cease leading to a significantly changed carbon and cofactor balance. The reduced carbon fixation capacity decreases the maximum possible growth rate which was also proposed by a model simulation (Peyraud 2011) but is very likely not the reason for the general growth inability. In contrast, the altered reduction equivalent balance might be accompanied with serious problems as *M. extorquens* has low transhydrogenase activity (Korotkova and Lidstrom 2001) and is therefore probably not able to quickly adapt its NAD(P)(H) pools which is crucial because growth on methanol is highly dependent on reducing power (Ochsner 2014). Hence, analyzing the NAD(P)(H) pools in the EMCP-negative strains expressing icl would be useful to identify imbalances which might cause their inability of methylotrophic growth. However, as these strains are not able to grow on methanol, we used an approach to create a strong limitation of EMCP flux, thus simulating the absence of the EMCP.

Such an EMCP inhibition has been shown for strongly reduced cobalt concentrations in methanol minimal medium which leads to accumulation of EMCP intermediates due to inhibition of cobalamin-dependent mutases Ecm and Mcm (Kiefer 2009; Sonntag 2015).

M. extorquens precultures grown in methanol minimal medium with non-growth limiting cobalt concentrations (12 µM) were diluted by a factor of 30 into the new methanol medium without any cobalt. Thus, only minor amounts of cobalt (0.4 µM) remained in the medium of the main culture leading to severe growth reduction on methanol due to limited mutase activity.

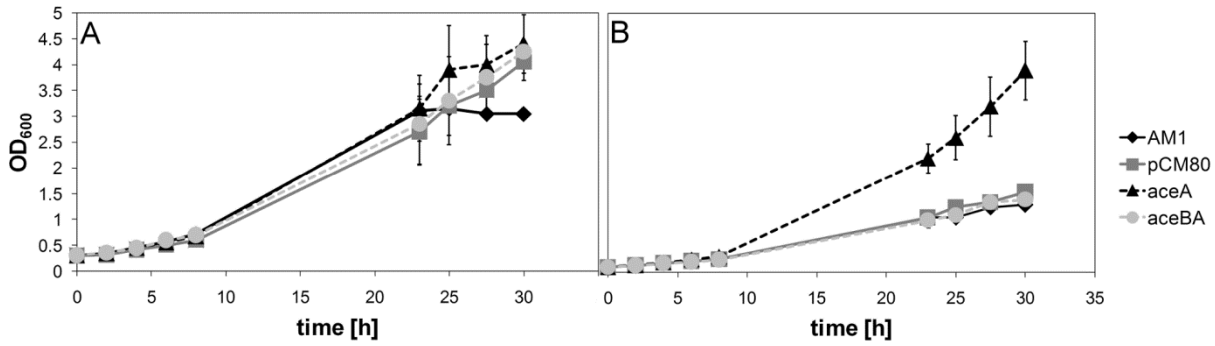

Figure 4: Growth of *M. extorquens* AM1 expressing *E. coli* isocitrate lyase alone (*aceA*) or in combination with malate synthase B (aceBA), empty vector control (pCM80) and AM1 without plasmid (AM1) in methanol minimal medium with 12 µM cobalt (A) and 0.4 µM cobalt after 1:30 preculture dilution (B). n=3; error bars represent standard deviations

As shown in figure 4, growth of *M. extorquens* harboring pCM80 or pCM80-*aceBA* was strongly reduced in medium containing only 0.4 µM Co^{2+}. Expression of isocitrate lyase without an additional malate synthase (pCM80-*aceA*) led to growth comparable to the growth behavior in medium with 12 µM cobalt (see Fig.4B). This confirms the previous observation that icl expression positively affects growth in case of a non-lethal EMCP inhibition. Suprisingly, growth of AM1_pCM80-*aceBA* expressing isocitrate lyase in combination with malate synthase was not improved in medium containing 0.4 µM Co^{2+} which might be a result of further glyoxylate conversion by malate synthase thereby reducing its availability in the serine cycle.

During growth under cobalt-limited and non-limited conditions, culture samples of AM1, AM1_pCM80-*aceA* and AM1_pCM80 were taken in the exponential growth phase and used for NAD(P)(H) quantifications.

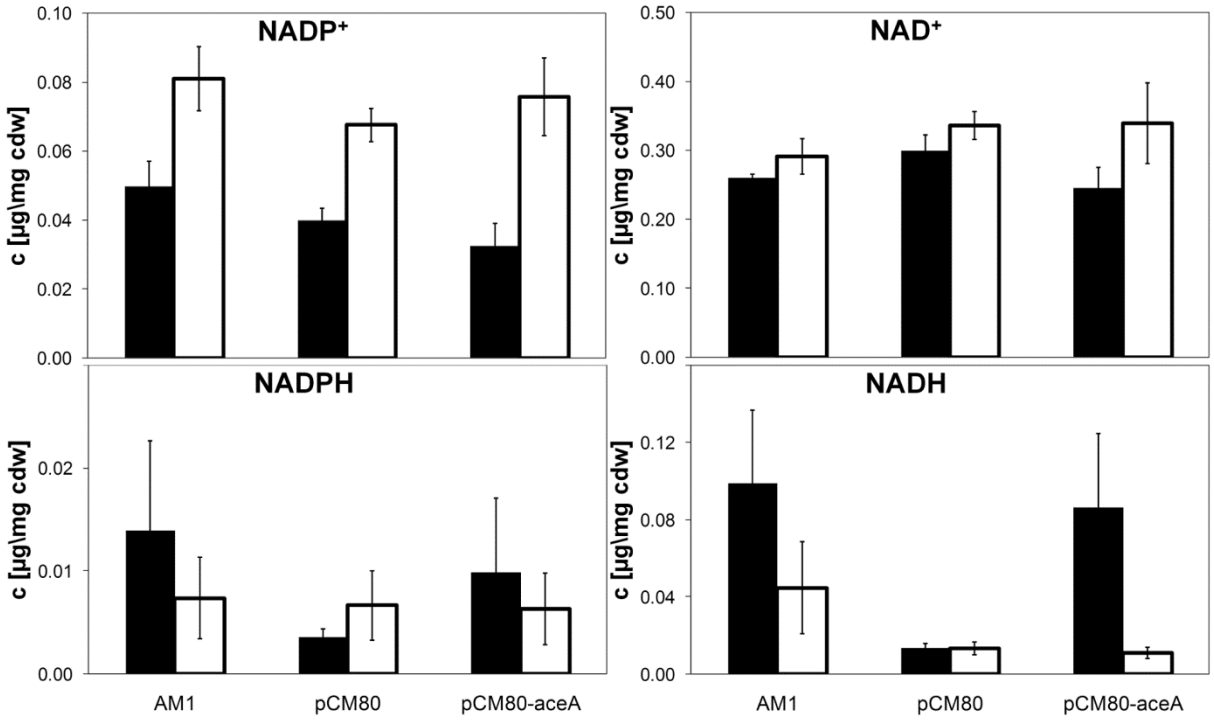

Figure 5: Intracellular concentrations of nicotinamide cofactors in *M. extorquens* AM1 without a plasmid (AM1), empty vector control (pCM80) and AM1 expressing heterologous isocitrate lyase (pCM80-*aceA*). Cells were grown in methanol medium containing non growth-limiting cobalt concentrations (12 µM, filled bars) or growth limiting cobalt concentrations (0.4 µM, empty bars) to create a strong reduction of flux through the EMCP. n=3; error bars represent standard deviations

$NADP^+$ pool sizes of AM1, AM1_pCM80 and AM1_pCM80-*aceA* increased about two-fold under cobalt-limited growth conditions compared to non-limited conditions (see Fig.5). NADPH and NAD^+ concentrations did not change significantly and concentrations of NADH decreased slightly for AM1_pCM80-*aceA* under cobalt-limited growth conditions. Expression of isocitrate lyase had no distinct influence on the pool sizes of NAD(P)(H) in normal and cobalt-limited medium compared to AM1 wildtype and the empty vector control. Hence, although NADP(H) pool sizes are changed, an inhibited/ blocked EMCP, expression of isocitrate lyase or both did not cause a total depletion or huge excess of a reduction equivalent as one might have expected from the changed balance discussed above. Therefore, strong shifts in the levels of distinct nicotinamide cofactor species or their ratios are probably not the reason for methylotrophic inability of EMCP-negative strains expressing isocitrate lyase.

Metabolomics reveal altered fluxes and pool sizes in case of EMCP inhibition with and without heterologous glyoxylate cycle

Several TCA cycle intermediates are important precursors of essential metabolites such as α-ketoglutarate for glutamate biosynthesis or succinyl-CoA for the synthesis of heme and lysine. Metabolomics performed by Peyraud (2011) showed that *M. extorquens* has a low flux from oxaloacetate to α-ketoglutarate and even no detectable flux from α-ketoglutarate to succinyl-CoA when growing on C1 carbon sources. Thus, succinyl-CoA is solely provided by flux from the EMCP (see Fig.1). A dysfunctional EMCP in combination with a heterologous isocitrate lyase could therefore lead to a scenario, where the already low flux from isocitrate to α-ketoglutarate is further decreased due to the consumption of isocitrate by the icl. In addition, succinyl-CoA might be totally depleted as neither flux from the TCA nor the EMCP can provide it under these conditions. Hence, EMCP-negative strains expressing *aceA* might suffer from insufficient biosynthesis of heme and amino acids glutamate, proline, arginine, glutamine and lysine.

To verify this hypothesis, metabolomic analysis by dynamic labeling experiments and measurements of the absolute intermediate concentrations were performed. As described in the last chapter, the absence of the EMCP was simulated by growth-limiting cobalt concentrations to enable growth of the cultures for metabolite analyses. AM1_pCM80 and AM1_pCM80-*aceA* were analyzed in their exponential growth phase in methanol medium containing 12 or 0.4 µM Co^{2+}.

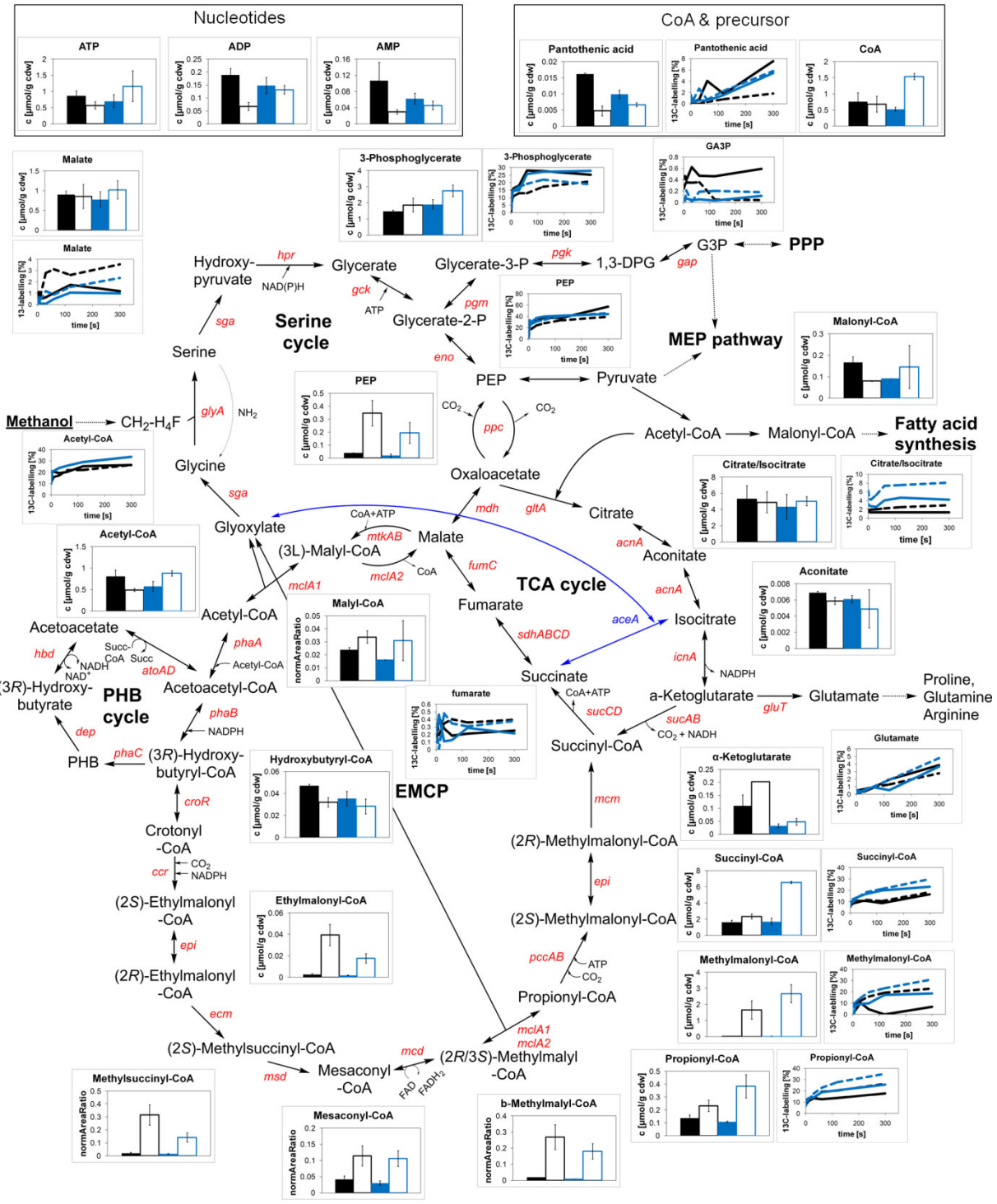

Figure 6: Pool sizes and ^{13}C-labelling of intermediates in the central metabolism of *M. extorquens* AM1 harboring pCM80 (black) or pCM80-*aceA* (blue) grown in methanol minimal medium with 12 µM Co^{2+} (filled columns) or 0.4 µM Co^{2+} (open columns). Gene names are given in red. Pathway names are written in bold letters. Glyoxylate, glycine, serine, hydroxypyruvate, glycerate, succinate and oxaloacetate were not detectable. Heterologous isocitrate lyase (*aceA*) reactions are shown in blue. Pathways: PHB cycle: Polyhydroxybutyrate cycle, TCA cycle: Tricarbonic acid cycle, EMCP: Ethylmalonyl-CoA pathway, MEP pathway: Methylerythrol-phosphate pathway, PPP; pentose phosphate pathway; Intermediates: PEP: Phosphoenolpyruvate, 1,3-DPG: 1,3-Diphosphoglycerate, G3P: Glycerinaldehyde-3-phosphate.

Dynamic labeling and concentrations of *M. extorquens* central metabolism intermediates are shown in figure 6. Confirming recent results (Sonntag 2015),

concentrations of intermediates following the first mutase step (ethylmalonyl-CoA mutase) increased by a factor of 3 (mesaconyl-CoA) to 32 (methylmalonyl-CoA) during growth in medium with 0.4 µM cobalt, which shows that the flux through the EMCP is considerably decreased under these conditions. Thereby, combined icl expression lowered accumulation of intermediates in the upper part of the EMCP such as ethylmalonyl-CoA and (2*S*)-methylsuccinyl-CoA but increased accumulation of propionyl- or methylmalonyl-CoA which are in the lower part of the EMCP. Comparing the dynamic labeling and pool sizes of the other pathway intermediates under conditions of reduced EMCP flux, icl expression or combination of both, following statements can be made: i) neither free coenzyme A nor nucleotide pools are decreased or highly increased during cobalt-limited growth and/or icl expression, ii) pentose phosphate pathway (PPP) precursors 3-phosphoglycerate and glycerinaldehyde-3-phosphate (GA3P) as well as PPP intermediates such as ribulose-5-phosphate, sedoheptulose-7-phosphate, 6-phosphogluconate, glucose-6-phosphate did not vary distinctly between the different conditions (data not shown), iii) PEP concentration increased in case of reduced EMCP flux which was less pronounced in combination with icl expression, whereas labeling of PEP was comparable under all conditions, iv) while citrate/iscoitrate (not separable in the used LC method) and aconitate pool sizes were similar, labeling of citrate/isocitrate was faster with icl expression, v) α-ketoglutarate pool sizes were decreased during growth in medium with 0.4 µM cobalt, but glutamate labeling was comparable under all conditions, vi) the succinyl-CoA pool increased slightly (1.3x) during growth in medium with 0.4 µM cobalt but very distinctly (3.5x) in case of EMCP flux reduction combined with icl expression, vii) other central intermediates such as fumarate, malate and acetyl-CoA did not vary significantly in concentrations or labeling speed under the four different conditions.

The obtained results clearly disprove the assumption that EMCP-negative strains expressing an icl suffer from a depleted succinyl-CoA pool as the measured succinyl-CoA concentration was strongly increased. This might be caused by two different mechanisms: succinyl-CoA synthetase (*sucCD*) catalyzes the condensation of succinate and CoA or succinyl-CoA synthesis is accomplished by a CoA-transferase. Three CoA-transferases are encoded in the genome of *M. extorquens* AM1 (Vuilleumier 2009): an acetyl-CoA::acetoacetyl-CoA transferase catalyzes the synthesis of acetoacetyl-CoA in the PHB cycle (Skovran 2010), a formyl-CoA

transferase which transfers CoA from oxalyl-CoA to formate which is required during growth on oxalate (Schneider 2012b) and an enzyme annotated as ketoacid succinyl-CoA transferase which has an unknown function in *M. extorquens* (Chistoserdova 2003) and is the most likely candidate that could take over the synthesis of succinyl-CoA. However, the definite reason for the succinyl-CoA accumulation in case of a reduced EMCP flux and expressed icl remains unknown.
Interestingly, the α-ketoglutarate pool size is considerably reduced in case of icl expression which strengthens the hypothesis of a lowered flux from isocitrate to α-ketoglutarate due to icl-catalyzed isocitrate consumption. The strongly reduced α-ketoglutarate concentrations might negatively influence the available amounts of glutamate and amino acids derived from glutamate (see fig.6). Hence additional provision of α-ketoglutarate or glutamate might help to enable growth of EMCP-negative strains expressing icl.

The observed increase of PEP under cobalt-limited growth conditions is surprising as neither synthesis nor conversion of PEP is related to any vitamin B12, i.e. cobalt, dependent enzyme activity. Large (1962) and coworkers clearly showed that *M. extorquens* AM1 PEP carboxylase (*ppc*) is not able to use cobalt as metal ion which excludes the possibility that a reduced Ppc activity as direct result of the decreased cobalt concentration leads to the accumulation of PEP. Thus, a reason for the observation remains unknown.
Metabolomics of *M. extorquens* AM1 with reduced EMCP flux and heterologous isocitrate lyase showed that its growth inability on C1 carbon sources is not a result of depleted succinyl-CoA but might be caused by reduced synthesis of α-ketoglutarate, which is an important amino acid precursor.

Attempts for compensation of reduced α-ketoglutarate availability in EMCP-negative strains containing a heterologous glyoxylate cycle

Two different approaches were followed to compensate the reduced availability of α-ketoglutarate in the EMCP-negative strains expressing icl. In the first attempt, glutamate as conversion product of α-ketoglutarate was externally added in amounts sufficient to provide cells with glutamate, proline, arginine and glutamine (see material and methods). Direct addition of α-ketoglutarate would not be feasible as

M. extorquens AM1 is able to use it as sole carbon source (Sonntag 2014). Furthermore, acetate was used as sole carbon source or as co-substrate in addition to methanol which should provide a higher flux through the TCA hopefully concomitant with a higher concentration of α-ketoglutarate. In contrast to methanol-grown cells, *M. extorquens* AM1 cells grown on acetate as sole carbon source have a high flux through the TCA because acetate directly enters the TCA as substrate for citrate synthase (Schneider 2012a)

Table 2: Growth of *M. extorquens* AM1 and deletion strains Δ*epi*, Δ*ecm*, Δ*msd*, Δ*mcd*, Δ*mclA1A2* and Δ*mcmA* summarized as Emcp$^-$, harboring no plasmid, plasmid pCM80 or pCM80-*aceA* after 4 days on solid medium containing methanol (MeOH), glutamate (Glu), acetate (Ac) or combinations of them as carbon sources. +++: growth similar to AM1 wt, ++: growth slower than AM1 wt; +: minimal growth; -: no growth

strain	MeOH	Glu	MeOH +Glu	Ac	MeOH +Ac	MeOH +Ac +Glu
AM1	+++	+	+++	+++	+++	+++
AM1_pCM80	+++	+	+++	+++	+++	+++
AM1_pCM80-*aceA*	+++	+	+++	+++	+++	+++
Emcp$^-$	-	-	-	-	-	-
Emcp$^-$_pCM80	-	-	-	-	-	-
Emcp$^-$_pCM80-*aceA*	-	-	-	-	-	-

To test the hypotheses described above, growth experiments with EMCP-negative strains unable to grow on methanol (Δ*epi*, Δ*ecm*, Δ*msd*, Δ*mcd*, Δ*mclA1A2*, Δ*mcmA* summarized as EMCP$^-$) with and without heterologous icl were performed on solid medium containing methanol, methanol and glutamate, acetate, methanol and acetate or methanol in combination with acetate and glutamate (see table 2). The comparable growth of AM1, AM1_pCM80 and AM1_pCM80-*aceA* on all media showed that neither glutamate nor acetate alone or in combination with methanol has a toxic effect. Glutamate itself is a suitable growth substrate which proves the ability of *M. extorquens* AM1 to import the amino acid. As shown in table 2, neither glutamate nor acetate supplementation restored growth of any tested EMCP-negative (+icl) strain on methanol. Also sole acetate was not sufficient for growth which is obvious as *M. extorquens* requires a functional EMCP for utilization of C2 carbon sources (Schneider 2012a). If *M. extorquens* would be unable to co-consume methanol and acetate, our strategy of using both substrates as mixture would probably not lead to an increased flux through the TCA. However, the shown co-consumption of methanol and succinate (Peyraud 2012) and the high substrate

flexibility of *M. extorquens* (Ochsner 2014) make it likely that also methanol and acetate can be co-utilized even though it has not been proven directly.

In summary, external feeding of glutamate or acetate did not restore methylotrophy of *M. extorquens* with an absent EMCP and heterologous glyoxylate cycle. This observation is a strong hint, that glutamate biosynthesis in EMCP-negative (+icl) strains is still sufficient for growth or that the flux reduction towards α-ketoglutarate/glutamate is at least not the only reason for their growth inability on C1 and C2 carbon sources.

Genome based search for serine+ icl+ methylotrophs without additional EMCP, CBB or RuMP

The results achieved so far show that methylotrophy of *M.extorquens* strains without functional EMCP is not restorable by a heterologous glyoxylate cycle although the activity of the isocitrate lyase is theoretically high enough. Rational examination of possible reasons revealed that neither a strongly shifted NAD(P)H/NAD(P)$^+$ balance nor a depletion of essential central metabolism intermediates occurs as result of this new pathway combination. However, one must consider, that the deletion of an essential EMCP gene was only simulated by limitation of cobalt ion availability and thereby activity of the two EMCP mutases in the respective experiments.

In a complementary approach we sought to identify natural serine+ icl+ methylotrophs without additional glyoxylate regeneration and CO_2 fixation pathways to possibly get hints for the apparently complex task of glyoxylate regeneration engineering in *M. extorquens* AM1. An organism using the serine cycle and glyoxylate shunt without EMCP, CBB or RuMP might contain specialized TCA cycle enzymes, isocitrate lyase or further regulatory mechanisms to sustain methylotrophy. Serine cycle icl+ methylotrophs were already described by Anthony (1982), amongst others *Pseudomonas* MA, Organism 5H2, *Pseudomonas* MS and *Pseudomonas aminovorans*. Interestingly, all of them are able to utilize methylamine, but not methanol as sole carbon source and the genome of none of them has been sequenced to date. Therefore the existence of an additional EMCP or CBB in these organisms cannot be excluded.

Hence, we analyzed all annotated bacterial genomes available at the Microbial Nucleotide Blast homepage for the presence of respective pathway marker genes. The details are described in Material and Methods.

Table 3: Bacterial strains harboring a combination of serine cycle+ ICL+ EMCP- CBB- RuMP- *moxF*+ xoxF+ pathways/ genes identified by BLAST analysis. Capability of methylotrophy is estimated from the presence of *moxF* and xoxF and experimental investigations described in the respective references.

Serine cycle+ ICL+ EMCP- CBB- RuMP- *xoxF+ moxF+*			
Strain	Methylotrophy	Description	Reference
Granulibacter bethesdensis CGDNIH1	Yes, confirmed	Acetobacterium; Human pathogen for chronic granulomatosis	Greenberg (2006)
Marinobacter sp. BSs20148	unknown	γ-Proteobacterium; not experimentally investigated	Song (2013)
Pusilimonas sp. T7-7	unknown	Alkane degrader: alkane oxygenase accepts methane sulfonic acid and nitromethane	Cao (2011) Li (2013)
Sphingobium sp. SYK-6	improbable	C1-THF produced by demethylation of lignin catabolism intermediates	Sonoki (2009)
Candidatus puniceispirillum marinum IMCC1322	probable, not confirmed	Genes for formaldehyde/formiate oxidation, DMSP demethylase and DMSO oxidase encoded in genome	Oh (2010)
Burkholderia sp. 383	unknown	β-Proteobacterium; contains DMSO oxidase homologue; not experimentally investigated	Vanlaere (2009)
Psychrobacter cryohalolentis K5 & *arcticus* 273-4	unknown	unable to utilize sugars; growth on acetate, lactate and glutamate; C1 growth not investigated	Bakermans (2006)
Thermaerobacter marianensis DSM 12885	improbable	Extreme thermophilic deap see organism; only weak acetate and succinate utilization	Takai (1999)
Lysinibacillus sphaericus C3-41	improbable	Formerly *Bacillus sphearicus*; growth on acetate but no utilization of formate	Sneath (1986)
Rubrobacter xylanophilus DSM 9941	very improbable	Thermophilic; growth on variety of C-sources such as sugars, complex sugars like xylan and a variety of organic acids	(Carretto 1996; Ferreira 1999)
Caldilinea aerophila DSM 14535	very improbable	Facultative aerobic thermophilic; uses sugars and organic acids like acetate and succinate; no growth on formate or ethanol	Sekiguchi (2003)
Beijerinckia indica subsp. indica ATCC 9039	very improbable	Acidophilic aerobic non-methylotrophic; growth on organic acids, sugars	Kennedy (2005)

In total, only 13 bacteria out of about 1500 were identified that contained the serine and glyoxylate cycle but not the EMCP, CBB cycle or RuMP (see Table 3). Furthermore, only 4 of the 13 microorganisms additionally harbor a MoxF and a XoxF homologue as likely indicators for methylotrophy and only one of these bacteria has been unequivocally experimentally proven to grow on C1 carbon sources, namely *Granulibacter bethesdensis* CGDNIH1 (Greenberg 2006). This Gram-negative, rod-shaped, aerobic acetic acid bacterium was isolated from lymph node cultures from a

chronic granulomatosis patient and is able to use methanol as well as acetate, ethanol, glutamate and glucose as sole carbon source. Other identified candidates such as *Marinobacter* sp. BSs20148, *Pusilimonas* sp. T7-7 or *Candidatus puniceipspirillum marinum* IMCC1322 might also be able to utilize C1 carbon sources but have not been investigated experimentally (see Table 3).

To verify whether *G. bethesdensis* growth on methanol is dependent on isocitrate lyase activity, growth on complex medium containing glucose as main carbon source and minimal medium 569 containing methanol as sole carbon source was analyzed with and without the addition of 3-nitropropionate (3-NP). 3-NP has a general toxic effect on many bacteria in higher concentrations but specifically inhibits isocitrate lyase in lower concentrations (Schloss and Cleland 1982). Addition of 3-NP didn´t negatively affect growth of *G. bethesdensis* on solid complex medium but no colonies appeared on minimal medium 569 containing methanol and 1 mM 3-NP even after 8 days of incubation (data not shown). This observation is a strong hint, that methylotrophy of *G. bethesdensis* is indeed dependent on the activity of icl and supports the BLAST analysis results regarding the lack of other glyoxylate regeneration pathways such as the EMCP in *G. bethesdensis*.

Detailed identification and analysis of the factors and mechanisms essential for methylotrophy in *G. bethesdensis* might provide insights into differences between the two types of serine cycle-dependent metabolism including pool size, flux balances and regulatory mechanisms which could help to establish a glyoxylate cycle in an EMCP-negative *M. extorquens*.

Furthermore, analysis of already described serine cycle icl+ bacteria such as *Pseudomonas* MA would be interesting to further elucidate the metabolic network of the pathways required for methylotrophy in *G. bethesdensi*s.

Conclusions

In this study, we aimed at the substitution of the native glyoxylate regeneration pathway, the EMCP, in *M. extorquens*, by a heterologous glyoxylate pathway by expressing isocitrate lyase in EMCP-negative *M. extorquens* mutants. Although isocitrate lyase was expressed in theoretically feasible activities, methylotrophy in EMCP-negative strains was not restorable by a heterologous glyoxylate cycle.

Metabolomics and consecutive feeding experiments performed with EMCP-negative (+icl) strains showed that neither depletion nor strong accumulation of a primary pathway intermediate or a redox imbalance seems to be the reason for the methylotrophic inability of EMCP-negative (+icl) strains. Inspection of the complete NCBI microbial genome database revealed that only one verified methylotroph, *Granulibacter bethesdensis*, unambiguously harbors the combination of a serine cycle and icl without an EMCP or additional C1 assimilation pathways such as the CBB cycle. Further analysis of *G. bethesdensis* metabolism and pathway regulations might help to understand the setup of this barely analyzed pathway combination.

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Eidesstaatliche Versicherung

Ich erkläre hiermit an Eides Statt, dass ich die vorgelegte Dissertation selbständig angefertigt und mich anderer Hilfsmittel als der in ihr angegebenen nicht bedient habe, insbesondere, dass alle Entlehnungen aus anderen Schriften mit Angabe der betreffenden Schrift gekennzeichnet sind.

Ich versichere, die Grundsätze der guten wissenschaftlichen Praxis beachtet, und nicht die Hilfe einer kommerziellen Promotionsvermittlung in Anspruch genommen zu haben.

Frankfurt am Main, den .....

Publikationsliste

Sonntag F., Müller J.E.N., Kiefer P., Vorholt J.A., Schrader J., Buchhaupt M. (2015). „High-level production of ethylmalonyl-CoA pathway derived dicarboxylic acids by *Methylobacterium extorquens* under cobalt-deficient conditions and by polyhydroxybutyrate negative strains." Applied Microbiology and Biotechnology, DOI 10.1007/s00253-015-6418-3 (In press: available online)

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