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2   **Graphical Abstract**

Methanol and methyl group conversion in acetogenic bacteria:

Biochemistry, physiology and application

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Running title: value-added chemicals from methanol

One sentence summary: This review describes the transfer of methyl groups to the central metabolism of acetogenic bacteria by three-component methyltransferase systems and the further conversion to biochemicals of high value, thereby emphasizing the biochemistry and physiology of model organisms.

Keywords: biocommodities, acetogen, methyltransferase, value-added chemicals, biofuel, biotechnology

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28   **Abstract**

29   The production of bulk chemicals mostly depends on exhausting petroleum sources and leads

30   to emission of greenhouse gases. Within the last decades the urgent need for alternative sources

31   has increased and the development of bio-based processes received new attention. To avoid the

32   competition between the use of sugars as food or fuel, other feedstocks with high availability

33   and low cost are needed, which brought acetogenic bacteria into focus. This group of anaerobic

34   organisms uses mixtures of CO_2, CO and H_2 for the production of mostly acetate and ethanol.

35   Also methanol, a cheap and abundant bulk chemical produced from methane, is a suitable

36   substrate for acetogenic bacteria. The microorganisms with the ability to convert methanol (or

37   methyl groups from other sources) are summarized in the group of methylotrophs that includes

38   aerobic as well as anaerobic organisms like acetogens. In methylotrophic acetogens the methyl

39   group is transferred to the Wood-Ljungdahl pathway, a pathway to reduce CO_2 to acetate *via* a

40   series of C_1-intermediates bound to tetrahydrofolic acid. Here we describe the biochemistry and

41   bioenergetics of methanol conversion in the biotechnologically interesting group of anaerobic,

42   acetogenic bacteria. Further, the bioenergetics of biochemical production from methanol is

43   discussed.

44

45   **Introduction**

46   Bulk chemicals are mainly produced from crude oil. Under the prospect of future crude oil

47   scarcity and given the release of polluting greenhouse gases (CO, CO_2 and CH_4) during

48   industrial bulk chemical production, it becomes important to open new and bio-based

49   production schemes. Bio-based production of industrially relevant chemicals using low cost

50   sources like bagasse, sugar cane molasses and corn starch hydrolysates has been proposed

51   (Zhang *et al.* 2018). However, to avoid the competition between the use of sugars as food or

52   fuel, it would be pertinent to focus on additional high availability and low cost materials.

53   Synthesis gas might be particularly interesting for this purpose. Syngas (mainly consisting of

54   CO_2, CO and H_2), is produced as waste gas in e.g. steel mills by the gasification of coal and is

55   available in high amounts. Syngas is a potential source of electrons (H_2, CO) and carbon (CO_2,

56   CO), which can be used by acetogenic bacteria under the production of acetate and ethanol

57   (Abrini *et al.* 1994, Buschhorn *et al.* 1989, Köpke *et al.* 2010, Maddipati *et al.* 2011). In

58   addition, traces of butyrate, 2,3-butanediol and butanol are formed by some acetogens (Dürre

59   2016, Köpke *et al.* 2011, Krumholz and Bryant 1985). Acetate, 2,3-butanediol and butanol are

60   important platform chemicals, whereas butanol and ethanol can also be used as biofuels.

61   Besides syngas, also methanol is a highly available, low cost substrate. As such, it has been

62   discussed as an attractive feedstock for microbial production of high-value biochemicals

63   (Cotton *et al.* 2020, Müller 2019, Satanowski and Bar-Even 2020). Methanol is currently

64   produced from reformed natural gas in quantities up to 110 million metric tons per year

65   (https://www.methanol.org/the-methanol-industry/). Methanol is readily available and with a

66   price of 250-350 US dollar per metric ton represents a low price feedstock. Conversion of CO,

67   CO_2 and CH_4 to methanol would open additional avenues for the biotechnological use of C_1

68   substrates as building block for biocommodities.

69   Anaerobic methylotrophic microorganisms like methanogenic archaea and acetogenic bacteria

70   cleave the C-O bond of methanol heterolytically (Hagemeier *et al.* 2006). In methanogens the

71   methyl group is then transferred to 2-mercaptoethanesulfonate by a methyltransferase system

72   (Coenzyme M/CoM) (McBride and Wolfe 1971, Taylor and Wolfe 1974), whereas in acetogens it

73   is transferred to tetrahydrofolate (THF) (Kreft and Schink 1994, Stupperich and Konle 1993).

74   This circumvents the formation of highly reactive formaldehyde, which is the major cause of

75   methanol toxicity. In general, methanol is used as a feedstock in concentrations not higher than

76   100 mM and not much is known about methanol tolerance of acetogens and methanogens, but

77   adaptation of acetogens to higher methanol concentrations (~500 mM) by forced laboratory

78   evolution is reported (Tremblay *et al.* 2015).

79   Methyltransferase systems consist of three components: a substrate specific methyltransferase

80   I (MTI) which cleaves the C-O bond of the methylated substrate, a cobalamin or cobamide

81   binding corrinoid protein CoP, which serves as an intermediate methyl group acceptor, and

82   thirdly, a methyltransferase II (MTII), which transfers the methyl group to the final acceptor

83   CoM or THF (Kaufmann *et al.* 1997, Kreft and Schink 1994, Shapiro and Wolfe 1980, van der

84   Meijden *et al.* 1983a, van der Meijden *et al.* 1983b, van der Meijden *et al.* 1984), respectively (Fig.

85   1). Note that "cobamide" is the superordinate term for e.g. cobalamin, 5-

86   hydroxybenzimidazolyl- and *p*-cresolylcobamide etc. which differ by their glycosidic-bound

87   compound (Stupperich *et al.* 1990b). Corrinoid is a more universal term referring to the

88   tetrapyrrole macrocycle which, in case of cobamides, coordinates cobalt. For simplicity reasons

89   we will use cobalamin and cobamide synonymously in this review. Methyltransferase

90   components may be differently denoted, for example MTI is sometimes denoted as MtxB, CoP

91   as MtxC and MTII as MtxA (x denotes the substrate specificity). In some cases MtxB and MtxC

92   build a stable complex, which has also been designated as MTI (Sauer *et al.* 1997, van der

93   Meijden *et al.* 1984). Sometimes the substrate specificity of the methyltransferase system is

94   indicated by a subscription of MTI (e.g. MTI_{van} for vanillate, MTI_{ver} for veratrol). Since

95   corrinoids are very reactive in their active Co(I)-state, they autooxidize spontaneously to an

96   inactive Co(II)-state. The reduction of the Co(II)P to its superreduced cob(I)amide state is

97   catalysed by ATP-dependent enzymes (Fig. 1). These activating enzymes (AE) usually belong

98   to the cluster of orthologous groups COG3894 of metal binding proteins and have been given

99   different names such as reductive activator for cobalamin enzymes (RACE), reductive activator

100   of methyl transfer (Ram) or methyltransferase activating proteins (MAP) (Daas *et al.* 1993,

101   Ferguson *et al.* 2009, Schilhabel *et al.* 2009). MAP were reported not to have a prosthetic group

102   and therefore might not be a member of COG3894 (Daas *et al.* 1996). The products of the

103   methyl group transfer, namely methyl-CoM and methyl-THF, are intermediates of the Wood-

104   Ljungdahl pathway (WLP) and thus can be further used for the generation of cell mass and

105   conservation of energy.

106   This review focuses on the biochemistry and bioenergetics of methyl group conversion by

107   acetogenic organisms with a special focus on the biotechnological relevant methyl group donor

108   methanol. Based on structural information of methanogenic methyltransferases, we first outline

109   the mechanism of methyltransferase systems to illustrate the methyl group transfer to the WLP.

110   We then underline the similarities and differences of the methanogenic and acetogenic

111   methyltransferase systems. Whereas the MTI of acetogens and methanogens are highly similar

112   and most probably use the same mechanism to transfer the methyl group to the CoP, the MTII

113   of acetogens is homologous to CH_3-THF binding enzymes (Das *et al.* 2007, Visser *et al.* 2016)

114   and may use a mechanism similar to these enzymes. After we outlined the methyl group transfer

115   from methylated substrates to THF, we provide metabolic models on the further conversion of

116   methyl-THF to acetate, which are based on biochemical studies and *in silico* analysis of the

117   respective enzymes. Thereby, the ATP gain, which is strongly influenced by the redox

118   equivalents used by chosen model organisms, is emphasized. We then expand these models to

119   show the theoretical limits of biocommodity production from methanol, which would be

120   beneficial compared to their production from H_2+CO_2 in terms of bioenergetics. Finally, we

121   discuss factors which influence the product formation from methanol and may give the

122   opportunity to steer the carbon and electron flow into the product of interest.

123

124   **The cobamide-dependent three component methyltransferase systems**

125   **Methanol-converting methyltransferase system in methanogens, a paradigm for**

126   **methyltransferases in acetogens**

127   Metabolism of methanol by anaerobic microorganisms was first reported for methanogenic

128   archaea first (Schnellen 1947). Purification of high amounts of MTI and MTII from

129   *Methanosarcina barkeri* paved the road for functional and structural characterization of the

130   single subunits and the elucidation of the methyl transfer mechanism (Harms and Thauer 1996,

131   Sauer *et al.* 1997, Sauer and Thauer 1999). The group of Thauer identified Zn^{2+} as essential for

132   the activity of MTI and MTII (Sauer and Thauer 1997), which led to the assumption that

133   methanol was activated by Zn^{2+} acting as an electrophilic Lewis acid. Sequence comparison of

134   MtaB homologs allowed for the definition of an MtaB Zn^{2+} recognition motif: **E**-X$_2$-GGK-X$_3$-

135   D-X$_{55}$-**C**-X$_2$-AN-X-A-X$_{40}$-GA-X-GP-X-KD**C**GYE, which is conserved in related proteins of

136   acetogens (Hagemeier *et al.* 2006). His136 acts as the α-axial ligand in the active site of the

137   CoP (Sauer *et al.* 1997). This so called "base-off/his-on" configuration of the cobamide was

138   first found in the CFeS-protein involved in acetyl-CoA synthesis of *M. thermoacetica* (formerly

139   *Clostridium thermoaceticum*). Histidine as ligand is now known as a common feature of

140   corrinoid-binding proteins (Ragsdale *et al.* 1987). In 2006 the crystal structure of the MtaBC

141   complex was solved (Hagemeier *et al.* 2006), which led to a deeper understanding of the

142   structural components and their importance for the cleavage of the CH$_3$-OH bond. The

143   methanol:cobamide methyltransferase consists of two MtaBC heterodimers building a

144   (MtaBC)$_2$ heterotetramer. MtaB forms a TIM-barrel surrounded by 7 alpha-helices. MtaC is

145   composed of a cobamide-binding Rossman fold, a helical domain and an N-terminal extension,

146   which interact with the helical domain of MtaB, leading to the formation of a stable MtaBC

147   complex. Corrinoid proteins lacking the N-terminal arm do not form a stable complex with their

148   corresponding methyl-X:cobamide methyltransferases (Burke and Krzycki 1997, Ferguson *et*

149   *al.* 2000, Ferguson *et al.* 1996, Hao *et al.* 2002). The N-terminal extension is missing in

150   acetogenic CoPs as well, which do not form a complex with MtaB (MTI) in acetogens.

151   The mechanism of methyl transfer from methanol to CoM was already proposed in 1983 to be

152   a two-step S$_N$2 reaction (van der Meijden *et al.* 1983a). This was supported by stereochemical

153   studies, which showed a net retention (two times inversion) during methyl transfer (Zydowsky

154   *et al.* 1987). Methanol is proposed to bind between the coordinated Zn^{2+} of MtaB and the bound

155   cobamide of MtaC, which reaches into the deep funnel of the TIM-barrel of MtaB. Thereby the

156   Zn^{2+} and the corrinoid cobalt are aligned, further supporting the proposed S_N2 mechanism

157   (Hagemeier *et al.* 2006). Whereas the Zn^{2+} ligates and activates the hydroxyl group of methanol,

158   the methyl group is attacked by the supernucleophile Co(I) causing a polarization and, hence, a

159   heterolytic cleavage of the otherwise very inert C-O bond. In the model of Hagemeier *et al.*

160   (2006), the flexible cobamide-binding Rossmann domain of the CoP rearranges after methyl

161   group binding. This facilitates presenting the methylcob(III)amide to MtaA, which catalyses

162   the last step of methanol:CoM methyltransfer. In 2012, also the structure of MtaA from

163   *Methanosarcina mazei* was crystallized by Hoeppner *et al.* (2012). MtaA forms a C-terminal

164   TIM-barrel structure and an N-terminal domain consisting of three alpha helices and a beta

165   hairpin. The interface between the MtaBC complex and MtaA is most likely formed by the core

166   complex of MtaBC and the N-terminal domain of MtaA. Like in MTI the substrate (CoM)

167   activation is supported by a catalytic zinc ion in MTII. Finally the methylcobamide of the CoP

168   is directed to the active site inside the TIM-barrel like fold of MTII where the methyl transfer

169   is catalysed (Hoeppner *et al.* 2012, Sauer and Thauer 2000). It should be noted that Zn^{2+}

170   dependent activation of the substrate is only one of at least two mechanisms to weaken the

171   methyl-X bond. For instance, mono-, di- and trimethylamine methyltransferases activate their

172   substrate in a different way. A pyrrolysine residue, encoded in the active site of MTI, binds the

173   substrate covalently, which results in a positive charge of the amine, enabling nucleophilic

174   attack of the cob(I)amide (Hao *et al.* 2002, Soares *et al.* 2005). In contrast, quaternary amines,

175   such as glycine betaine and proline betaine, already harbour a positive charge and hence do not

176   need electrophilic activation. Consequently, there no pyrrolysine in the glycine-/proline

177   betaine:cobamide methyltransferases MTI$_{GB}$ and MTI$_{PB}$ (Picking *et al.* 2019, Ticak *et al.* 2014).

178

179   **Methanol-converting methyltransferase systems in acetogenic bacteria**

180   Since acetogenic bacteria share the Wood-Ljungdahl pathway for conversion of C_1-substrates

181   with methanogens, it is not surprising that acetogens can use methanol and other methyl group

182   containing substrates as carbon and energy source. Growth on methanol by an acetogen was

183   first reported in 1969 with *Eubacterium limosum* (*Butyribacterium rettgeri*) (Hamlett and

184   Blaylock 1969). From 1980 onwards, further reports on methanol utilizing acetogens appeared,

185   such as *Acetobacterium woodii*, *Butyribacterium methylotrophicum*, *Clostridium magnum*,

186   *Sporomusa ovata*, the thermophilic acetogenic model organism *Moorella thermoacetica*, and

187   many others accumulated (Table 1). In 2007 Das and coworkers described a methanol

188   methyltransferase system from acetogens (Das *et al.* 2007). They identified a cluster consisting

189   of three genes whose gene products resembled the MTI, CoP and MTII proteins. Two of those

190   (MTI and CoP) had high similarities to the genes known to code for the methanol:CoP

191   methyltransferase MtaBC complex of methanogens and previous studies with *Holophaga*

192   *foetida* (formerly strain TMBS 4) and *Sporomusa ovata* had already shown that CoPs not only

193   play a crucial role in methyl group transfer in methanogens but also in acetogens (Kreft and

194   Schink 1993, Kreft and Schink 1994, Stupperich *et al.* 1992, Stupperich and Konle 1993).

195   Similar as in the methanogenic system the MTI transfers the methyl group to a cobamide-

196   containing methyl group acceptor. The acceptor CoP can differ by the nature of the cobamide

197   bound, e.g. in *M. barkeri* the methyl group is transferred to a

198   5-hydroxybenzimidazolylcobamide- (factor III-) carrying CoP typical for archaea, whereas in

199   *M. thermoacetica* the CoP binds 5-methoxybenzimidazolylcobamide (factor IIIm). Also in

200   *Holophaga foetida* factor III was identified as the predominant cobamide (Kreft and Schink

201   1993). The most abundant cobamide in *A. woodii* and *Clostridium formicaceticum* are 5,6-

202   dimethylbenzimidazolylcobamide (Vitamin B_{12}, cobalamin) and 5-methoxy-6-

203   methylbenzimidazolylcyanocobamide, respectively. In *Sporomusa ovata* the major cobamides

204   are phenolyl- and *p*-cresolylcobamide (factor Ib), which lack the axial benzimidazolyl but have

205 aromatic compounds bound instead. Hence, they only occur in base-off state (Stupperich *et al.*

206 1990a, Stupperich *et al.* 1988, Stupperich *et al.* 1989, Stupperich *et al.* 1990b). Further studies

207 identified *p*-cresolylcobamide to be bound by the methanol-induced CoP of *S. ovata*

208 (Stupperich *et al.* 1992). So far, *S. ovata* is unique in preferring the above mentioned cobamides

209 (Mok and Taga 2013).

210 Despite different cobamides, MTI and CoP of acetogens and methanogens are very similar (e.g.

211 37% identity and 95% length coverage between MtaB and 34% identity and 96% length

212 coverage between MtaC of *M. barkeri* and *M. thermoacetica*) and their reaction mechanism is

213 most probably the same. In contrast, the product of the third gene of the methanol

214 methyltransferase operon from *M. thermoacetica* did not show significant similarity to the

215 methanogenic methylcobamide:CoM methyltransferase but it showed 34% identity (97%

216 length coverage) to the CH_3-THF:CFeS methyltransferase (AcsE) of *M. thermoacetica*. and it

217 is similar to the N-terminal amino acid sequence of the cobalamin-dependent methionine

218 synthase (MetH) of several bacteria (Das *et al.* 2007). In 2016, Visser and colleagues discovered

219 a further methanol-specific methyltransferase system by a proteomic approach in *Sporomusa*

220 strain An4 (a *Sporomusa ovata* strain) (Visser *et al.* 2016). They also observed an AcsE/MetH

221 homolog encoded downstream of MtaC and MtaB rather than a methanogenic MtaA homolog.

222 Due to the high abundance of the three components during growth on methanol, they concluded

223 that the AcsE/MetH-homolog is the MTII of the acetogenic methanol:THF methyltransferase

224 system. This conclusion seems reasonable, since in contrast to methanogens, acetogens use DL-

225 tetrahydrofolate as the final acceptor of the methyl group yielding methyl-THF as a product

226 (Berman and Frazer 1992, el Kasmi *et al.* 1994, Kreft and Schink 1994, Meßmer *et al.* 1993).

227 Another methanol:THF methyltransferase system was identified in a study addressing the

228 microbial conversion of secoisolariciresinol diglucoside to enterodiol in the human intestine.

229 This demethylation reaction is catalysed by *E. limosum* ZL-II. In order to identify the

230 methyltransferase responsible for this reaction, several potential methyltransferases of *E.*

231   *limosum* ZL-II were expressed heterologously. This led to the identification of three genes

232   (*Eli_2003-Eli_2005*) which comprise the methyltransferase catalysing not only the

233   demethylation of secoisolariciresinol but also methanol. In addition, Eli_0370 was identified as

234   the activating enzyme responsible for the reduction of the corrinoid (Chen *et al.* 2016). In 2019,

235   Keller and colleagues identified a methanol induced methyltransferase system in

236   *Thermoacetogenium phaeum* (Tph_c03590-Tph_c03610) by transcriptional studies (Keller *et*

237   *al.* 2019). The methanol- as well as the glycine betaine:THF methyltransferase systems of *A.*

238   *woodii* were identified by transcriptional analysis as well (Kremp *et al.* 2018, Lechtenfeld *et al.*

239   2018). Comparison of the derived amino acid sequences of the single components showed that

240   isoenzymes of CoP and MTII generally share high similarity. In contrast, MTI$_{MeOH}$ and MTI$_{GB}$

241   differed significantly on protein level (22% identity, 13% length coverage) indicating structural

242   differences between the enzymes, which are necessary for recognizing different substrates.

243   Based on the fact that the MTI$_{MeOH}$ differs strongly from MTIs with a different substrate

244   specificity, the amino acid sequence of MtaB from *A. woodii* was used as marker to identify

245   potential methanol:THF methyltransferase systems in other acetogens (Table 1, Fig. 2).

246   *Clostridium methoxybenzovorans* produces acetate from H_2+CO_2 and is therefore considered

247   as acetogenic bacterium (Mechichi *et al.* 1999b), but its genome is missing an acetyl-CoA

248   synthase encoding gene. Also growth of *C. methoxybenzovorans* on methanol is reported, but

249   in contrast to the acetogens listed in Table 1 no MtaB homolog is encoded in its genome.

250   Therefore the question arises how methanol is degraded by *C. methoxybenzovorans*. One

251   possibility is methanol oxidation by a methanol dehydrogenase, which would be uncommon for

252   acetogens.

253

254   **Methyl-X-converting methyltransferase systems in acetogenic bacteria**

255   As mentioned before, acetogens do not only utilize methyl groups from methanol but also from

256   methylated substrates like glycine betaine, methyl chloride, methylamines and also methyl

257   groups of phenyl methyl ethers such as veratrole, (iso-) vanillate, syringate, sinapinate, a variety

258   of methoxybenzoates and methoxycinnamates (Daniel *et al.* 1991). Since the O-, N-, Cl-methyl

259   cleaving part of the methyltransferase system (MTI) harbours the substrate binding site and

260   since binding requires specificity, a given organism can have a variety of different, inducible

261   MTI encoding genes. For example, the model acetogen *A. woodii* encodes 23 different MTI

262   homologs (Fig. 3) and only the substrate of a few is known to date (Kremp *et al.* 2018,

263   Lechtenfeld *et al.* 2018).

264   Although growth on methanol was already reported 50 years ago, the discovery of cobamide-

265   dependent methyltransferase systems in acetogens started in 1991 with the isolation of

266   *Acetobacterium dehalogenans* DSM 11527 (formerly strain MC) from a sewage treatment plant

267   in Stuttgart-Möhringen (Traunecker *et al.* 1991). Subsequently, a broad range of

268   methyltransferase systems was discovered in *A. dehalogenans*. Substrates which are converted

269   by *A. dehalogenans* include methylchloride (Meßmer *et al.* 1993, Traunecker *et al.* 1991),

270   vanillate, syringate (Kaufmann *et al.* 1997, Kaufmann *et al.* 1998) and veratrol (1,2-

271   Diemethoxybenezene) (Engelmann *et al.* 2001). Also in *M. thermoacetica* a syringate-specific

272   *O*-demethylase was identified (el Kasmi *et al.* 1994). Knowing that the methyl group is

273   transferred *via* a cobamide to THF as the final methyl group acceptor, enzymatic coupling of

274   the methyl transfer to $NADP^+$ reduction by a methylene-THF reductase (MTHFR) and a

275   methylene-THF- dehydrogenase (MTDH) was possible and the methyltransferase activity

276   became spectrophotometrically detectable. This new enzymatic assay allowed for fast

277   purification and characterization of the methyltransferase systems (Kaufmann *et al.* 1997,

278   Kaufmann *et al.* 1998, Meßmer *et al.* 1996). As expected, the first isolation of a vanillate-

279   specific *O*-demethylase system of *A. dehalogenans* resulted in a three-component system which

280   consisted of MTI, MTII and a corrinoid protein. Additionally, an activating enzyme, responsible

281   for cobamide activation, was found which required ATP and electrons for its functionality

282   (Kaufmann *et al.* 1997, Kaufmann *et al.* 1998). In 2001, a second *O*-demethylase system was

283   purified from *A. dehalogenans*, showing to be veratrol-specific (Engelmann *et al.* 2001).

284   Besides vanillate or veratrol, MTI_{van} and MTI_{ver} also demethylated other, structural similar

285   substrates with altering activities. Engelmann and colleagues further observed that the CoP of

286   the MTI_{van} system could serve as a methyl group acceptor for MTI_{ver}, indicating an universality

287   of the CoP. Whereas the isoenzymes MTI_{van} and MTI_{ver} both bound Zn^{2+} by novel zinc binding

288   motifs (**E**-X_{14}-**E**-X_{20}-**H** for MTI_{van} and **D**-X_{27}-**C**-X_{39}-**C** for MTI_{ver}), the AE was shown to

289   contain a [2Fe-2S] cluster (Schilhabel *et al.* 2009, Studenik *et al.* 2011). Very recently, ATP

290   hydrolysis by the AE was demonstrated to increase the midpoint potential of the protein bound

291   cobamide, which enables electron transfer from the low potential electron carriers ferredoxin

292   or flavodoxin hydroquinone *via* the [2Fe-2S] cluster in the AE to inactive Co(II) (Dürichen *et*

293   *al.* 2019, Kißling *et al.* 2020). By mutation studies, the N-terminus of MTI was found to be

294   responsible for the selection of substrates whereas the TIM-barrel forming C-terminus had no

295   influence on substrate specificity (Kreher *et al.* 2010).

296   In contrast to the MTI_{van} of *A. dehalogenans*, the MTI_{van} of *M. thermoacetica* lacks the expected

297   zinc and addition of Zn^{2+} to enzymatic assays did not stimulate its activity (Naidu and Ragsdale

298   2001). Recently, a so far unique proline betaine (stachydrine):THF methyltransferase system

299   was discovered in *E. limosum* (Picking *et al.* 2019). Demethylation of proline betaine stops at

300   the level of N-methyl proline, which is excreted by *E. limosum*. The incomplete demethylation

301   seems to be a waste of energy, but is also known for *A. woodii*, which takes up and demethylates

302   glycine betaine and excretes dimethylglycine (Lechtenfeld *et al.* 2018). Besides the glycine

303   betaine importer and the methyltransferase system, a second transporter of the

304   betaine/choline/carnitine transporter (BCCT) family is encoded in the gene cluster of glycine

305   betaine utilization in *A. woodii*, but its substrate specificity and its role in the metabolism

306   remains to be established.

307

308 **Putative mechanism of the methanol:THF methyltransferase system - similarities and**

309 **differences to the methanol:CoM methyltransferase system**

310 Up to date, there is no atomic structure of an acetogenic methyl-X:THF methyltransferase

311 system. Only the single CoP (MtaC) of the methanol:THF methyltransferase system from *M.*

312 *thermoacetica* has been purified and was crystallized in 2007 by Das and colleagues.

313 Unfortunately the N-terminus (amino acids 1-84), which forms the helical layer in the structure

314 of MtaC from *M. barkeri*, was truncated during crystallization (Das *et al.* 2007). Since sequence

315 and structure alignments of MtaC clearly show the absence of the N-terminal extension of

316 methanogenic MtaC, which was shown to be responsible for the formation of a stable MtaBC

317 complex in *M. barkeri* (Hagemeier *et al.* 2006), it is not surprising that MtaC of *M.*

318 *thermoacetica* was not copurified with MtaB. Also for the vanillate- and veratrol-dependent

319 MT systems of acetogenic bacteria no copurification of CoP and MTI was observed (Naidu and

320 Ragsdale 2001, Engelmann *et al.* 2001).

321 Sequence alignments of methanogenic and acetogenic MtaB showed high similarities within

322 but also between the clades. The amino acids participating in Zn^{2+}-polarization and -binding

323 are highly conserved, therefore in acetogens the methanol activation seems to follow the same

324 mechanism as in the methanogenic MtaBC complex. In addition, all so far characterized

325 acetogenic MTIs (apart from MTI_{van} of *M. thermoacetica*) have been described as Zn^{2+}

326 containing proteins with Zn^{2+}-binding motifs, indicating a similar, Lewis acid-catalysed

327 activation of the methoxylated substrates for a heterolytic cleavage (Naidu and Ragsdale 2001).

328 A major difference between methanogenic and acetogenic methyltransferase systems is the

329 final methyl group acceptor. CoM and THF do not only differ in their molecular mass (164 Da

330 and 445 Da respectively), but the methyl group is transferred to the thiolate of CoM in

331 methanogens and builds a C-S bond, whereas in acetogens the methyl group is transferred to an

332 N^5 of THF. Furthermore, CoM-SH is activated for the nucleophilic attack on methylcobamide

333 by the Zn^{2+}-containing MTII, but the MTII of acetogens does not bind zinc ions (Hoeppner *et*

334   *al.* 2012, Naidu and Ragsdale 2001, Schilhabel *et al.* 2009). In sum, the acetogenic MTII is

335   more similar to the methyl-THF binding module of the cobalamin-dependent methionine

336   synthase, which has been studied extensively in a number of organisms like *Thermotoga*

337   *maritima* and *E. coli* (Bandarian *et al.* 2002, Datta *et al.* 2008, Dixon *et al.* 1996, Drennan *et*

338   *al.* 1994, Goulding *et al.* 1997, Koutmos *et al.* 2009). Moreover, the MTII is similar to the

339   methyl-THF:CFeSP methyltransferase, which has been particularly studied in *M.*

340   *thermoacetica* (Roberts *et al.* 1994, Seravalli *et al.* 1999a). With respect to the similarities

341   mentioned above, the mechanism of MTII might be similar to that of MetH or AcsE, which will

342   be described below.

343

344   **Two alternative mechanisms for cobalamin-dependent THF-binding methyltransferases**

345   MetH is a multimodular protein which transfers a methyl group from methyl-THF to

346   homocysteine in order to synthesize methionine (Goulding *et al.* 1997). In analogy to the

347   methyltransferase systems the first module (with the so called folate domain) builds a TIM-

348   barrel and transfers the methyl group from methyl-THF to cobalamin, which is bound to a

349   Rossmann domain in the second module (Evans *et al.* 2004). Since the modules are connected

350   *via* flexible linkers, rearrangement of the cobalamin module enables the exposure and the

351   transfer of the methyl group to homocysteine in the TIM-barrel forming third module. The

352   fourth module binds S-adenosylmethionine, which is needed for reductive reactivation of

353   oxidized cob(II)alamin (Dixon *et al.* 1996, Drummond *et al.* 1993, Koutmos *et al.* 2009).

354   Whereas in *E. coli* all modules belong to one polypeptide, in *T. maritima* the fourth module

355   consists of a single polypeptide. The methyl-THF binding module of *T. maritima* was

356   cocrystallized with the homocysteine binding domain and the amino acid residues, responsible

357   for THF binding were identified (Evans *et al.* 2004). Sequence alignments of the folate domain

358   of MetH from *T. maritima* and *E. coli*, the methyl-THF CFeSP methyltransferase AcsE from

359   *M. thermoacetica* and methyl-cobamide:THF methyltransferases of acetogens show that the

360   THF binding residues (**GE**-X$_2$-**N**-X$_{66-69}$-**D**-X$_{20-21}$-**NS/T**-X$_{58-66}$-**D**-X$_{34-39}$-**SN**-X$_{7-11}$-**R**) are

361   conserved among the THF-binding methyltransferases. Therefore at least the structure of the

362   active site of MTII might be similar to that of the methyl-THF binding module of MetH and

363   AcsE. Biochemical studies using transient kinetics, pH dependencies and ^{13}C NMR showed

364   that methyl-THF becomes protonated, but the nature of the proton delivering general acid

365   remains unsolved. Although the crystal structures of the AcsE and MetH revealed that the

366   conserved Asn199 (in AcsE) is brought into a network of H-bonds and in a distance from which

367   H-bond formation to N^5 of methyl-THF/THF is possible (Doukov *et al.* 2007, Evans *et al.*

368   2004), it is not suitable as direct proton donor and may have a role in stabilizing the transition

369   state rather than in protonation. Further, different catalytic mechanisms have been proposed for

370   AcsE and MetH (Seravalli *et al.* 1999b, Smith and Matthews 2000). There is evidence for AcsE

371   that protonation occurs after formation of a binary enzyme-methyl-THF complex. This

372   destabilizes the methyl-N^5 bond and makes the methyl group a better target for the nucleophilic

373   attack of the highly active Co(I)-CFeSP, which is the rate limiting step in this proposed S$_N$2

374   mechanism. In contrast, the protonation of THF in MetH is proposed to occur after methyl-N^5

375   cleavage and the cobalamin cofactor is thought to be important for methyl-THF activation.

376   Therefore, a ternary enzyme-methyl-THF-cob(I)amide complex has to be formed first and

377   oxidative addition of Co(I) to the N^5-methyl bond was proposed as an alternative mechanism

378   of methyl transfer by cobalamin-dependent methyltransferases (Evans *et al.* 2004, Smith and

379   Matthews 2000).

380

381   **Methanol metabolism in acetogenic bacteria**

382   **General methanol metabolism**

383   In general, the methyl groups of methanol (and other methyl group donors) are transferred to

384   the methyl branch of the WLP by the methanol:THF methyltransferase system to yield methyl-

385   THF. One molecule methyl-THF is oxidized to CO_2 thus generating one ATP and six reducing

386   equivalents, catalysed by the MTHFR, the MTDH and finally by the formate dehydrogenase

387   (FDH). The resulting six reducing equivalents are then used for the reduction of three mol of

388   CO_2 to CO by the CO dehydrogenase/acetyl-CoA synthase (CODH/ACS) in the carbonyl

389   branch (Roberts *et al.* 1992). Since only one mol CO_2 is produced in the methyl branch,

390   acetogenesis from methanol requires two additional mol of CO_2 (Figs. 5, 6, 8, 9). The resulting

391   three mol of CO are condensed with three mol methyl-THF (resulting from three additional mol

392   of methanol) and CoA by the CODH/ACS to form three mol of acetyl-CoA. The acetyl group

393   is then transferred to a phosphate group by the phosphotransacetylase and in the following

394   acetate kinase reaction, one acetate and one mol of ATP is generated per mol acetate. In sum,

395   1.33 ATP are produced per mol of acetate by substrate level phosphorylation (SLP).

396   Depending on the organism the electron carriers involved in methyl group oxidation and the

397   catalysing enzymes differ, which, in the end, greatly influences the overall ATP yield. One of

398   the biggest current uncertainties is the energetics of the methylene-THF reductase reaction. The

399   redox potential of the methylene-/methyl-THF couple of -200 mV (Wohlfarth and Diekert

400   1990) does not allow a direct reduction of NAD^+. One solution to this dilemma is electron

401   bifurcation: Cooxidation of reduced ferredoxin (Fd^{2-}) by a possible electron bifurcating

402   MTHFR could solve the problem. In most acetogens the core subunits of MTHFR are MetV

403   and MetF (Bertsch *et al.* 2015; Clark and Ljungdahl 1984, Hess *et al.* 2014, Jeong *et al.* 2015,

404   Mock *et al.* 2014, Visser *et al.* 2016). For *M. thermoacetica* it was proposed that MetVF build

405   a complex with HdrCBA and MvhD, which are encoded upstream of *metV* and *metF* (Fig. 4).

406   This potential complex would then transfer electrons from NADH to methylene-THF and an

407   additional, unidentified electron acceptor in the reductive path (Mock *et al.* 2014). Please note

408   that ferredoxin, which is used by many electron bifurcating enzymes as a second, low potential

409   electron acceptor, was not reduced. Whether or not the methylene-THF reductase of *M.*

410   *thermoacetica* uses electron bifurcation and if so, what the second electron acceptor might be,

411   still has to be elucidated. Since the MTHFR of *S. ovata* has the same genetic organization as

412   the one in *M. thermoacetica* (Kremp *et al.* submitted, Visser *et al.* 2016) it is reasonable that *S.*

413   *ovata* oxidizes methyl-THF by the same, still not fully understood, mechanism. The MTHFR

414   of *E. limosum* does not have any Hdr encoding genes surrounding the gene locus (Jeong *et al.*

415   2015), but possible electron bifurcation could be conveyed by an interaction of MetVF with

416   electron transfer flavoproteins (EtfAB) (Jeong *et al.* 2015). Experimental evidence for the role

417   of EtfAB in this context is currently missing but they are known to be subunits of several

418   electron bifurcating enzymes (Bertsch *et al.* 2013, Chowdhury *et al.* 2014, Li *et al.* 2008). In

419   contrast, the MTHFR of *A. woodii* does not use the mechanism of electron bifurcation, but

420   employs an additional RnfC-like subunit (RnfC2) to use NADH as electron donor in the

421   reductive pathway. In the oxidative direction, removal of the endproduct (CO_2) from the cell

422   makes oxidation feasible (Bertsch *et al.* 2015, Kremp *et al.* 2018). In the following, the

423   biochemistry and bioenergetics of some of the acetogens, which are reported to grow on

424   methanol (Table 1), are discussed in more detail.

425

426   **Methanol metabolism in *Acetobacterium woodii***

427   In *A. woodii*, NAD^+ (Bertsch *et al.* 2015, Ragsdale and Ljungdahl 1984) and protons

428   (Schuchmann and Müller 2013) are reduced during methyl group oxidation (Fig. 5). However,

429   in the carbonyl-branch reduction of CO_2 to CO requires reduced ferredoxin, and thus, electrons

430   from NADH and H_2 have to be transferred to ferredoxin. Reduction of ferredoxin ($E_0' = -450$

431   mV – -500 mV) with NADH ($E_0' = -320$ mV) or H_2 ($E_0' = -414$ mV) is an endergonic reaction

432   and *A. woodii* employs two energy-coupled redox-balancing modules, one soluble and one

433   membrane-bound. First, the electrons from H_2 are shuttled to NAD^+ and ferredoxin by the

434   electron bifurcating hydrogenase (Awo_c27010-Awo_c26970). Then 0.5 mol of NADH plus

435   the two mol of NADH from methyl group oxidation are fed into the membrane-bound Rnf

436   complex (Awo_c22010-Awo_c22060) that uses the electrochemical Na^+ potential established

437 by ATP hydrolysis to drive electron flow from NADH to ferredoxin (Hess *et al.* 2013). In *A.*

438 *woodii* 3.3 Na^+ are pumped by hydrolysis of one ATP (Matthies *et al.* 2014) and electron flow

439 from NADH to Fd is endergonic with $\Delta G_0{}' = 25 - 35$ kJ/mol. Hence, translocation of 2 ions is

440 assumed to drive electron transfer (Müller and Hess, 2017). Thus, 1.5 mol of ATP have to be

441 invested for reverse electron transport and altogether 2.5 mol ATP are generated during

442 acetogenesis from four mol methanol according to equation 1:

443       $4\ CH_3OH + 2\ CO_2 + 2.5\ ADP + 2.5\ P_i \rightarrow 3\ CH_3COOH + 2\ H_2O + 2.5\ ATP$ (eq. 1).
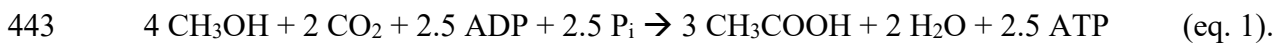
444

445 **Methanol metabolism in *Moorella thermoacetica***

446 In contrast to *A. woodii*, methyl group oxidation in *Moorella thermoacetica* delivers electrons

447 in form of NADPH by a trimeric formate dehydrogenase (Ljungdahl and Andreesen 1975,

448 Yamamoto *et al.* 1983) and a bifunctional formyl-THF cyclohydrolase–methenyl-THF

449 dehydrogenase (O'Brien *et al.* 1973). An electron bifurcating MTHFR (Moth_1196-Moth1191)

450 was proposed to be present in *M. thermoacetica* which oxidizes methyl-THF and a second, so

451 far unknown, electron donor simultaneously to reduce NAD^+ (Mock *et al.* 2014). For reasons

452 of simplicity we assume that the MTHFR uses Fd^{2-} as a second donor in our metabolic model

453 (Fig. 6). Again, CO_2 reduction requires reduced ferredoxin and *M. thermoacetica* uses three

454 redox-balancing modules to get the right amount of reduced ferredoxin: Two mol NADPH

455 transfer   their   electrons   *via*   an   electron   bifurcating   <u>N</u>ADH-dependent   reduced

456 <u>f</u>erredoxin:<u>N</u>ADP$^+$ oxidoreductase (Nfn, Moth_1517, Moth_1518) to one mol NAD^+ and one

457 mol ferredoxin, respectively (Huang *et al.* 2012). In combination with the bifurcating

458 hydrogenase HydABC (Moth_1717-Moth1719) (Wang *et al.* 2013) the electrons end up in form

459 of two mol H_2. As an alternative, an $NADP^+$-dependent hydrogenase (Moth_1883-Moth_1885)

460 could produce H_2 from NADPH directly. H_2 can then be used for the reduction of ferredoxin

461 by the <u>e</u>nergy <u>c</u>onverting <u>h</u>ydrogenase, the Ech complex (Moth_2184-2191). Since the electron

462 transfer of electrons from H_2 to Fd is energetically less unfavourable ($\Delta G_0{}' = 7-17$ kJ/mol) than

463   Fd reduction with NADH ($\Delta G_0' = 25\text{-}35$ kJ/mol), translocation of only one H^+ is assumed to

464   drive the process. To build up the H^+ gradient, ATP is hydrolysed by the F_1F_O-ATP synthase,

465   working as an ATPase (Moth_2377-Moth_2384). Assuming a ratio of 4 H^+ translocated per

466   ATP hydrolysed, 0.5 ATP have to be hydrolysed to translocate two H^+ across the membrane.

467   The resulting two mol Fd^{2-} and the remaining two mol NADH from methyl group oxidation

468   are, again, used for the production of four mol H_2 *via* the electron bifurcating hydrogenase (Fig.

469   6). Hence, additional four mol Fd are reduced by the membrane-bound Ech complex and one

470   more ATP is invested for this uphill electron transport. Finally, the resulting Fd^{2-} fills the lack

471   of electrons in the methyl- as well as in the carbonyl branch. Acetogenesis from four mol

472   methanol by *M. thermoacetica* generates 2.5 mol ATP (0.63 ATP/methanol) according to

473   equation 1. If we assume that the MTHFR does not confurcate electrons from Fd^{2-} and methyl-

474   THF to NAD^+ but uses NAD^+ as sole cofactor, the ATP synthase has to hydrolyse 1 ATP only,

475   resulting in an ATP yield of 0.75 ATP per mol methanol converted. The plausibility of those

476   models can be tested by verifying that they would allow growth on H_2+CO_2. An adaptation of

477   the model depicted in Fig. 6 to acetogenesis from H_2+CO_2 would give a balanced redox balance

478   and a net ATP gain (Fig. 7A). However, without an electron bifurcating methylene-THF

479   reductase, the redox balance is balanced but the ATP yield would be zero (Fig. 7B).

480

481   **Methanol metabolism in *Sporomusa ovata***

482   In *Sporomusa ovata*, the methylene-THF dehydrogenase (SOV_1c07570) was shown to be

483   NADP(H)-dependent (Kremp *et al.* submitted) and proteome studies with strain An4 showed

484   that a potential electron bifurcating FDH (SpAn4DRAFT_2935-2937/SOV_1c07740-

485   SOV_1c07760) was highly abundant during growth on methanol (Visser *et al.* 2016). Recently,

486   this potential FDH (SOV_1c07740-SOV_1c07760) was found to encode a novel type of

487   electron bifurcating transhydrogenase (*Sporomusa* type Nfn, or short Stn) rather than an FDH

488   (Kremp *et al.* submitted). This suggested that electrons are transferred from NADPH to NAD^+

489   and Fd simultaneously in *S. ovata*. However, the proteomics of Visser *et al.* (2016) showed that

490   another potential, selenocysteine containing FDH (SpAn4DRAFT_2944/SOV_1c07830-

491   SOV_1c07840) was highly abundant as well. The latter FDH shows high sequence similarity

492   to the FDH of *Clostridium pasteurianum*, which is why we assume ferredoxin dependency of

493   the enzyme (Liu and Mortenson 1984, Scherer and Thauer 1978). The genes encoding the

494   MTHFR of *S. ovata* (SOV_1c07680-SOV_1c07730) are organized in a similar genetic context

495   to that of *M. thermoacetica* (Fig. 4). Therefore, it might be that *S. ovata* uses a similar Hdr

496   associated, bifurcating mechanism for methyl-THF oxidation as *M. thermoacetica*. Assuming

497   Fd-dependent FDH and an electron bifurcating MTHFR, methyl group oxidation would deliver

498   1 mol NADPH and 2 mol NADH (Fig. 8). Electrons are transferred from NADPH to NAD^+ and

499   Fd by the Stn. To enable the reduction of three mol CO_2 in the carbonyl branch, the resulting

500   NADH plus the left over NADH from the methyl branch are then oxidized by the Rnf complex

501   (SOV_1c08080-SOV_1c08130) to transfer their electrons to Fd. Since the F_1F_O-ATP synthase

502   (SOV_3c04150- SOV_3c04070) does not show a specific Na^+-binding site, the driving force

503   for the endergonic electron transport is most likely a H^+-gradient. Assuming a ratio of 4

504   H^+/ATP, 1.25 ATP have to be invested to enable reduction of three ferredoxin at the Rnf

505   complex. In sum, acetogenesis from four mol methanol produces three mol acetate and 2.75

506   mol ATP resulting from substrate level phosphorylation (eq. 2).

507       $4\ CH_3OH + 2\ CO_2 + 2.75\ ADP + 2.75\ P_i \rightarrow 3\ CH_3COOH + 2\ H_2O + 2.75\ ATP$ (eq. 2).

508   Assuming that the MTHFR is not electron bifurcating only 0.75 ATP have to be invested for

509   ferredoxin reduction at the Rnf complex and the overall ATP gain from four mol methanol

510   would be enhanced to 3.25 ATP, but this setup would not allow for growth on H_2+CO_2.

511

512   **Methanol metabolism in *Eubacterium limosum***

513   Based on the genome sequence of *E. limosum* ATCC 8486 (Song *et al.* 2017) and the metabolic

514   model of Jeong *et al.* (2015) of *E. callanderi* KIST612 (formerly *E. limosum* KIST612) the

515   electron carrier of the methylene-THF dehydrogenase (Eli_0374) is most likely NADH. In

516   contrast, the cofactors of the MTHFR and FDH are uncertain. Due to the lack of a hydrogenase

517   coding gene the FDH (Eli_0994) is most likely not an HDCR as known from *A. woodii* but it

518   is similar to Fd-dependent FDH of *C. pasteurianum*. For this reason, we display Fd as suitable

519   electron acceptor in our model (Fig. 9). If we assume that the MTHFR (Eli_0375, Eli_0376)

520   uses NAD^+ as sole cofactor, acetogenesis from methanol is possible, but the energetics would

521   not allow for growth on H_2+CO_2. Since *E. limosum* is able to grow on H_2+CO_2, we include an

522   electron bifurcating MTHFR (Fig. 9). Thus, the oxidation of one methyl group delivers three

523   NADH, which are subsequently used for ferredoxin reduction at the Rnf complex (Eli_2638-

524   Eli_2643). Like in *A. woodii* an electrochemical Na^+-gradient has to be established by the ATP

525   synthase (Eli_2184-Eli_2192) to enable this uphill electron transport (Jeong *et al.* 2015, Litty

526   and Müller 2020). Assuming that the ATP synthase uses 4 Na^+/ATP, 1.5 ATP have to be

527   invested to enable the transfer of six electrons from NADH to Fd. Hence, the reduction of three

528   mol CO_2 can be catalysed and in sum four mol of methanol lead to the synthesis of 2.5 ATP

529   (eq. 1). It should be noted, that the model presented here only shows homoacetic acid

530   fermentation from methanol, whereas it is well known that *E. limosum* runs mixed acid

531   fermentation from methanol with butyrate as a second product. The ratio of butyrate to acetate

532   is dependent on several factors and even homobutyric acid fermentation can be achieved

533   (Loubiere and Lindley 1991, Pacaud *et al.* 1986b), as will be discussed more in detail below.

534

535   **Production of value-added chemicals from methanol**

536   The production of value-added chemicals from H_2+CO_2 by acetogens and the bioenergetic

537   constraints are well known (Bertsch and Müller 2015a, Schiel-Bengelsdorf and Dürre 2012). In

538   the following, we focus on the bio-based production of ethanol, butyrate, butanol, acetone and

539   lactate from methanol. Whereas ethanol and butanol are used as biofuels, butyrate and lactate

540   are used for the production of bioplastics and acetone is needed in various chemical syntheses.

541   The production of these compounds *via* the WLP inevitable produces acetyl-CoA as the central

542   intermediate. In *A. woodii* all redox reactions of the WLP are known (Müller 2019) and acetyl-

543   CoA production under autotrophic conditions needs an expense of 0.7 ATP/acetyl-CoA

544   (Bertsch and Müller 2015a), whereas only 0.17 ATP/acetyl-CoA (0.5 ATP/3 mol of acetyl-

545   CoA, Fig. 5) are invested using methanol as substrate. Therefore, the production of compounds

546   from methanol could be beneficial compared to H_2+CO_2 utilization. We already showed

547   metabolic models of the conversion of methanol to acetate in detail, in the following these

548   models are expanded by the production pathways of the above mentioned biochemicals starting

549   from acetyl-CoA. These models will reflect the theoretical limits of product formation with

550   respect to the enzymatic equipment and ATP gain of the respective organism. Nevertheless, to

551   steer the carbon and electron flow into the compound of interest and to prevent side product

552   formation, strain optimization through genetic modifications may be necessary (Bourgade *et al.*

553   submitted).

554

555   **Ethanol production**

556   Ethanol is used as platform chemical in the global industry and it is a component of alcoholic

557   beverages. It is also deployed as fuel additive (10-15%) and in some countries like Brazil, the

558   Czech Republic, the US and Sweden up to 85% ethanol are added to gasoline. The production

559   of ethanol by acetogens can start from two intermediates, namely acetyl-CoA and acetate.

560   Ethanol production from acetate includes the acetate kinase (Ack) -catalysed reaction, which

561   produces additional ATP *via* SLP (Hitschler *et al.* 2018, Bertsch and Müller 2015a, Abubackar

562   *et al.* 2016). The first of two steps in ethanol production is acetaldehyde formation by reduction

563   of either acetyl-CoA or acetate catalysed by an aldehyde dehydrogenase (Aldh) or an

564   aldehyde:ferredoxin oxidoreductase (Aor), respectively. Whereas Aldh can use NADH as

565   electron donor (Bertsch *et al.* 2016, Goodlove *et al.* 1989, Peng *et al.* 2008), the redoxpotential

566   of the NADH/NAD$^+$ couple is too positive (E_0' = -320 mV) for the reduction of acetate to

567   acetaldehyde (E_0' = -580 mV). Therefore, a low potential electron donor such as Fd^{2-} is needed

568   (Heider *et al.* 1995, Nissen and Basen 2019). The resulting acetaldehyde is then further reduced

569   to ethanol by an NAD(P)H-dependent alcohol dehydrogenase (Adh) (Bertsch *et al.* 2016,

570   Goodlove *et al.* 1989, Peng *et al.* 2008).

571   With regard to our metabolic models, there is a need for 12 more reducing equivalents to convert

572   three mol of acetyl-CoA or acetate to ethanol. As we explained methanol metabolism produces

573   six reducing equivalents by oxidation of one methyl group, hence two further methyl groups

574   have to be oxidized to enable ethanol formation. Depending on the redox-carriers of the methyl

575   branch the ATP yield will vary between different organisms. In case of *A. woodii* the production

576   of three mol acetyl-CoA from four mol methanol comes along with an expense of 0.5 ATP (Fig.

577   5). The oxidation of two additional methyl groups combined with the action of the electron

578   bifurcating hydrogenase supplies the cell with five NADH, one Fd^{2-} and two ATP (Fig. 10). In

579   the scenario using Aldh and Adh six NADH are needed, thus Fd^{2-} is oxidized by the Rnf

580   complex for the reduction of one NAD$^+$ and additional 0.6 ATP are gained by the ATP synthase.

581   In contrast, reduction of acetate to acetaldehyde needs Fd^{2-}, so 1.2 ATP have to be invested for

582   the electron transfer from two NADH to two Fd. Ethanol formation from acetate includes the

583   Ack reaction and therefore three ATP are gained *via* SLP. Ethanol production in *A. woodii* was

584   already reported in 1989 (Buschhorn *et al.* 1989), but there is no gene encoding an Aor in the

585   genome of *A. woodii*. However, *A. woodii* has an NADH-dependent, bifunctional ethanol

586   dehydrogenase AdhE (with Aldh- and Adh-domain) and many additional putative Adh

587   encoding genes (Bertsch *et al.* 2016), hence ethanol production in *A. woodii* runs *via* the

588   Aldh/Adh pathway. In sum, ethanol production from methanol by *A. woodii* should be possible,

589   supplying the cell with 0.7 ATP/ethanol produced according to equation 3:

590        $6 \; CH_3OH + 2.1 \; ADP + 2.1 \; P_i \rightarrow 3 \; CH_3CH_2OH + 3 \; H_2O + 2.1 \; ATP$ (eq. 3).
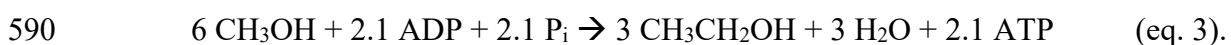
591   Expressing a functional Aor pathway in a genetically modified strain of *A. woodii* would

592   increase the ATP gain up to 3.3 ATP (1.1 ATP/ethanol) (Fig. 10). In comparison, complete

593   conversion of H_2+CO_2 to ethanol would end up in an ATP demand of 0.1 ATP/ethanol

594   (Aldh/adh) or an ATP yield of 0.3 ATP/ethanol (Aor), respectively and, hence, is not possible

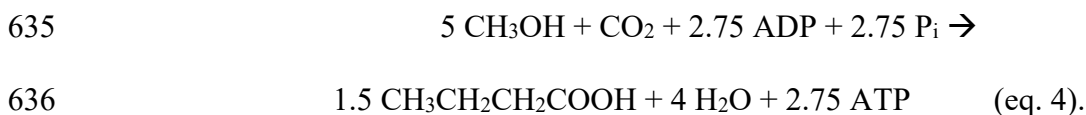595   without genetic modifications (Bertsch and Müller 2015a).

596

Butyrate and butanol production

598   Butyrate is used for the production of cellulose-based bioplastics like cellulose acetate butyrate-

599   polymers and esters of butyrate such as methyl butyrate are used as flavour in the food industry

600   (de Souza *et al.* 2017). Furthermore, butyrate is an important precursor of butanol, which is less

601   corrosive and has a higher energy density than ethanol and, hence, is an even better biofuel

602   (Dürre 2007). In addition, butanol is needed in high amounts as a solvent in the chemical

603   industry. For the production of butyrate and butanol from the intermediate acetyl-CoA six

604   reactions are necessary. Two mol acetyl-CoA are condensed to acetoacetyl-CoA by a thiolase

605   (Thl) and reduced by an NAD(P)H-dependent 3-hydroxybutyryl-CoA dehydrogenase (Hbd)

606   (Boynton *et al.* 1996, Madan *et al.* 1973, Shen *et al.* 1996) (Fig. 11). Water is split off by a

607   crotonase (Crt) and crotonyl-CoA is reduced to butyryl-CoA by a butyryl-CoA dehydrogenase

608   (Bcd). Due to the high redox potential of the crotonyl-CoA/butyryl-CoA couple (E_0' = -10 mV),

609   NADH (E_0'= -320 mV) is sufficient for the reduction of crotonyl-CoA and in some organisms

610   like *Clostridium kluyveri* and *E. limosum* this exergonic reaction is coupled to the endergonic

611   reduction of ferredoxin by the mechanism of flavin-based electron bifurcation (Jeong *et al.*

612   2015, Li *et al.* 2008). For the following production of butyrate usually a phosphotransbutyrylase

613   (Ptb) and a butyrate kinase (Buk) are employed and one ATP is generated by SLP per butyrate,

614   but other pathways are possible.

615   Starting from three acetyl-CoA there is a lack of six reducing equivalents, which is, again, filled

616   by the oxidation of one methyl group from methanol. *E. limosum* is known for butyrate

617   production from methanol (Pacaud *et al.* 1985) and the production of three mol acetyl-CoA

618   from four mol methanol has a demand of 0.5 ATP (Fig. 9). The oxidation of one methyl group

619   yields three NADH and one ATP (Fig. 11). The Hbd of *E. limosum* is most likely NADH-

620   dependent, as it is reported for the closely related methylotroph *B. methylotrophicum* and the

621   Bcd of *E. limosum* was shown to be electron bifurcating (Jeong *et al.* 2015, Shen *et al.* 1996).

622   Altogether the conversion of three acetyl-CoA to butyrate needs 4.5 NADH and delivers 1.5

623   Fd^{2-} by the Hbd and the electron bifurcating Bcd (Fig. 11). The resulting lack of 1.5 NADH is

624   compensated by the ferredoxin:NAD^+ oxidoreductase (Rnf), thereby pumping Na^+ across the

625   membrane, sufficient for the synthesis of 0.75 ATP. The genome of *E. limosum* does not code

626   for a Buk and CoA is supposed to be transferred to acetate by a butyryl-CoA:acetate CoA

627   transferase (Kelly *et al.* 2016) (Fig. 11). If so, an additional "catalytic" acetate has to serve as

628   acceptor for CoA of butyryl-CoA and butyrate is released. The "catalytic" acetate is rebuilt by

629   Pta and Ack and one ATP is produced per butyrate released. On the other hand, acyl-kinases

630   are known to have a multi-substrate affinity (Ichikawa *et al.* 1985, Schaupp and Ljungdahl

631   1974) and butyrate kinase activity has been measured in cell free extracts of *E. limosum*

632   (Lindley *et al.* 1987), therefore also the Ptb/Buk pathway might be involved in butyrate

633   formation. Independently of that, butyrate production from methanol yields 1.83 ATP/butyrate

634   (Fig. 11, eq. 4):

635                    $5\ CH_3OH + CO_2 + 2.75\ ADP + 2.75\ P_i \rightarrow$

636            $1.5\ CH_3CH_2CH_2COOH + 4\ H_2O + 2.75\ ATP$ (eq. 4).

637   In contrast, coming from H_2+CO_2, an expense of 0.5 ATP/acetyl-CoA is needed and the transfer

638   of electrons from H_2 to the electron carriers required results in an ATP gain of only 1

639   ATP/butyrate.

640   Analogous to the production of ethanol, the production of butanol can start from butyryl-CoA

641   and butyrate, employing a combination of Aldh and Adh or Aor and Adh. The electron donor

642   of only a few butyraldehyde- and butanol dehydrogenases is known, for example the AdhE2 of

643   *C. acetobutylicum* uses NADH for both reduction steps (Fontaine *et al.* 2002) as well as the

644   butyraldehyde dehydrogenase (Bldh) and butanol dehydrogenase (Bdh) of *B. methylotrophicum*

645   (Nguyen *et al.* 2013, Shen *et al.* 1999). Butanol production has been extensively studied in *B.*

646   *methylotrophicum* (Grethlein *et al.* 1991, Shen *et al.* 1996, Worden *et al.* 1991), which is closely

647   related to *E. limosum*. Therefore, we assume the metabolism of *B. methylotrophicum* to be

648   similar to that of *E. limosum* (Figs. 9 and 11). In either case the conversion of 1.5 butyryl-CoA

649   to butanol needs six additional reducing equivalents from methyl group oxidation. As

650   mentioned before, oxidation of one methyl group reduces three NAD^+ which are subsequently

651   used for the production of butanol (Fig. 11). In total 2.25 ATP are gained during production of

652   1.5 butanol from six mol of methanol by *B. methylotrophicum* giving a ratio of 1.5 ATP/butanol

653   (eq. 5).

$$6\ CH_3OH + 2.25\ ADP + 2.25\ P_i \rightarrow$$

$$1.5\ CH_3CH_2CH_2CH_2OH + 4.5\ H_2O + 2.25\ ATP \quad (eq.\ 5).$$
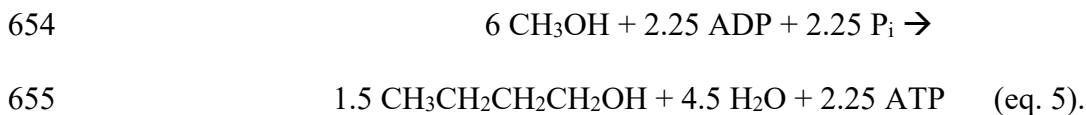
656   A functional Aor would increase the ATP yield up to 3 ATP. The conversion of $H_2 + CO_2$ to

657   butanol is also feasible but with a lower ATP gain of 0.5 ATP/butanol (Aldh) or 1 ATP/butanol

658   (Aor) produced. *A. woodii* does not code for the butyrate/butanol production pathway.

659   However, due to the differences between the ATP synthases and the formate dehydrogenases

660   of *A. woodii* and *E. limosum*/*B. methylotrophicum*, the energetics for butyrate/butanol

661   production from methanol would be even better (2.13 ATP/butyrate and 2 ATP/butanol), if this
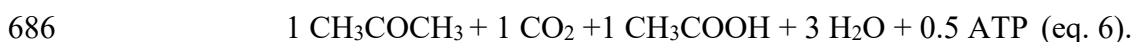
662   pathway was implemented into *A. woodii*.

663   Besides butyrate and ethanol, *E. limosum* produces the C_6-compound caproate in presence of

664   high concentrations of butyrate (Genthner *et al.* 1981). Caproate is the precursor of hexanol,

665   which is considered as an alternative biofuel in diesel engines (de Poures *et al.* 2017). For chain

666   elongation butyryl-CoA is condensed with acetyl-CoA by a second Thl resulting in the

667   formation of 3-ketohexanoyl-CoA. Even if the responsible enzymes are not known yet, further

668   conversion is thought to be catalysed by a subset of enzymes, similar to that of butyrate

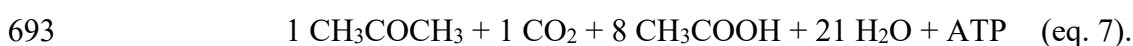669   synthesis from 3-hydroxybutyryl-CoA.

670

671   **Acetone production**

672   Acetone is a bulk chemical used for many chemical syntheses but the main application is its

673   use for the production of polymethylmethacrylat (acrylic glass). Acetone production from

674   acetyl-CoA requires three steps. A thiolase performs chain elongation condensing two mol of

675   acetyl-CoA. The resulting acetoacetyl-CoA transfers its CoA to acetate by the CoA-transferase

676   (Ctf). Subsequently, CO_2 is released by acetoacetate decarboxylase (Acd), which leaves acetone

677   as product (Fig. 12A). This pathway is known and acetone production was observed in the non-

678   methylotrophic organism *Clostridium acetobutylicum*. Since acetone production does not need

679   additional reducing equivalents, in theory 1.5 acetone could be produced from four methanol if

680   the ATP gain from acetyl-CoA production was already positive. For example, *A. woodii* 0.17

681   ATP/acetyl-CoA (0.5 ATP/3 acetyl-CoA) have to be invested, therefore it is not possible to

682   reach an entire transfer of carbons from methanol and CO_2 to acetone. For a positive ATP yield

683   one acetate has to be produced from three acetyl-CoA leaving four carbons for the production

684   of one acetone and one CO_2 according to equation 6 (Fig. 12A).

685   $$4 \ CH_3OH + 2 \ CO_2 + 0.5 \ ADP + 0.5 \ P_i \rightarrow$$

686   $$1 \ CH_3COCH_3 + 1 \ CO_2 + 1 \ CH_3COOH + 3 \ H_2O + 0.5 \ ATP \quad (eq. \ 6).$$

687   Recently, the acetone production pathway from *C. acetobutylicum* was expressed in *A. woodii*

688   and acetone production from H_2+CO_2 was indeed observed (Hoffmeister *et al.* 2016).

689   Production of the same amount of acetyl-CoA during autotrophic growth needs even more (0.7

690   ATP/acetyl-CoA) to be hydrolysed (Bertsch and Müller 2015a), hence, even more carbon has

691   to end up in acetate (Fig. 12B) according to equation 7:

692   $$20 \ CO_2 + 40 \ H_2 + ADP + P_i \rightarrow$$

693   $$1 \ CH_3COCH_3 + 1 \ CO_2 + 8 \ CH_3COOH + 21 \ H_2O + ATP \quad (eq. \ 7).$$

694   With respect to equations 6 and 7, 50% and 33% of carbon from methanol + CO_2 end up in

695   acetone and acetate, respectively. In contrast, acetone production from H_2+CO_2 transfers only

696   15% carbon to acetone and 80% to acetate, which fits quite well to the observed yields of 15.6%

697   and 81.4% from Hoffmeister *et al.* (2016). Therefore methanol is a better substrate for acetone

698   production.

699

700   **Lactic acid production**

701   Lactic acid is commonly used in cosmetic-, pharmaceutical-, and textile industry. Further it is

702   the monomer of polylactic acid, a biodegradable alternative to plastics (Haider *et al.* 2019).

703   Lactic acid is produced from acetyl-CoA by two reactions. First a pyruvate:ferredoxin

704   oxidoreductase (Pfor) carboxylates and reduces a molecule acetyl-CoA to form pyruvate,

705   thereby releasing CoA. Afterwards pyruvate is further reduced with NADH by a lactate

706   dehydrogenase (Ldh). In *A. woodii* the Ldh is electron bifurcating and reduces pyruvate and

707   ferredoxin with NADH, simultaneously (Fig. 13). Production of three acetyl-CoA from

708   methanol needs an investment of 0.5 ATP in *A. woodii*. Further conversion needs three mol

709   CO_2 and six mol NADH, delivered by oxidation of two methyl groups. Using the electron

710   balancing modules described above, three mol of lactate can be produced from six mol

711   methanol and three mol CO_2 with an ATP gain of 2.1 ATP (0.7 ATP/lactic acid) according to

712   equation 8:

713                     $6\ CH_3OH + 3\ CO_2 + 2.1\ ADP + 2.1\ P_i \rightarrow$

714                     $3\ CH_3HCOHCOOH + 3\ H_2O + 2.1\ ATP$ (eq. 8).

715   In contrast, production of lactic acid from H_2+CO_2 would cause a negative ATP yield of -0.1

716   ATP/lactic acid, thus it is not feasible (Bertsch and Müller 2015a).

717

718   **Product formation is influenced by several factors**

719   The metabolic models describe the potential of to produce valuable biochemicals from

720   methanol with respect to the bioenergetics and the enzymatic equipment. In the following

721   section, two parameters, which redirect the carbon and electron flow, are discussed, especially

722   for the examples *E. limosum* and *B. methylotrophicum*, whose methanol metabolism has been

723   studied extensively (Kerby and Zeikus 1987, Lebloas *et al.* 1996, Lebloas *et al.* 1994, Lindley

724   *et al.* 1987, Loubiere and Lindley 1991, Loubiere and Lindley 1994, Lynd *et al.* 1982, Lynd

725   and Zeikus 1983, Pacaud *et al.* 1986a, Pacaud *et al.* 1986b, Pacaud *et al.* 1985). We further

726   used to formulate suggestions regarding the use of methanol converting acetogens in

727   combination with other substrates of biotechnological interest (mixotrophy), in combination

728   with other organisms (syntrophy) or with bioelectrochemical systems (microbial fuel cell,

729   microbial electrosynthesis).

730

731   **Redox conditions/electron supply**

732   Acetogens occupy the same ecologic niche as methanogenic archea. Acetogenic growth on

733   methanol results in intermediary H_2 production, which delivers electrons for CO_2 reduction in

734   the carbonyl branch, and consumes 4 methanol per 3 acetate produced in pure culture (Cord-

735   Ruwisch and Ollivier 1986, Cord-Ruwisch *et al.* 1988, Heijthuijsen and Hansen 1986). Methyl

736   group oxidation to CO_2+H_2 is thermodynamically unfavourable (ΔG_0' = 23.5 kJ/mol) (Thauer

737   *et al.* 1977), however, in coculture with methanogens that lower the H_2 pressure, methylotrophic

738   acetogens can grow by methanol oxidation to CO_2+H_2 (Cord-Ruwisch and Ollivier 1986, Cord-

739   Ruwisch *et al.* 1988, Heijthuijsen and Hansen 1986). In sewage sludge plants this may be a

740   dominant role for acetogens (Stams 1994). *Vice versa*, supplementation of the culture with H_2

741   makes methyl group oxidation even less favourable. Under these conditions, methyl group

742   oxidation is impaired and carbon flow is redirected to the production of highly reduced

743 compounds. At least in the CO-fermenting acetogen *Clostridium autoethanogenum* availability

744 of excess H_2 is known to trigger the formation of highly reduced products like ethanol (Diender

745 *et al.* 2019). The effect of excess H_2 on methanol conversion by acetogens has never been

746 described, but the influence of other electron (and carbon-) sources like formate on the

747 conversion of methanol is known to enhance the production of reduced compounds as well

748 (Kerby *et al.* 1983). Therefore, the supply of additional electron (and carbon-) sources like H_2

749 or formate may be considered as a method, giving the opportunity to steer the bioconversion of

750 methanol towards the production of valuable compounds. Since *S. sphaeroides* and *A. woodii*

751 are known for acetogenesis from CO_2 and electrons from a cathodic source (e.g. F(0), graphite

752 cathode), adding electrons *via* a reversed microbial fuel cell, which is basically supplementation

753 of H_2 (Philips 2020, Philips *et al.* 2019, Tremblay *et al.* 2015), might be another possibility to

754 produce a more reduced compound. The latter approach could allow for parallel usage of an

755 organic one carbon source (methanol) and renewable electricity (stemming from e.g. solar or

756 wind generated energy). In off-peak times excess electricity could be stored in form of organic

757 compounds (Satanowski and Bar-Even 2020).

758

759 **Feed-back inhibition**

760 As mentioned, addition of acetate to methanol (+ CO_2) consuming *E. limosum*/*B.*

761 *methylotrophicum* cultures caused a shift of mixed acid fermentation to homobutyric

762 fermentation at specific acetate concentration (Loubiere and Lindley 1991, Lynd and Zeikus

763 1983, Pacaud *et al.* 1986b). This effect is explained by feed-back inhibition, which halts acetate

764 production and steers the carbon flow from acetyl-CoA to the butyrate production pathway (Fig.

765 11). Due to the requirement of additional reducing equivalents for butyrate production, the ratio

766 of methanol/CO_2 consumed increases with increasing butyrate/acetate ratio (Pacaud *et al.*

767 1986b). A similar effect can be achieved by deletion of the respective genes, which was shown

768 for *C. ljungdahlii* (Ueki *et al.* 2014). In the study of Pacaud *et al.* (1986a) acetate concentrations

769   above 200 mM led to a second increase of butyrate yield. The explanation for this was that

770   acetate was consumed and fed to the WLP, which, in fact, enhanced the availability of electrons

771   in the system. In the same manner, other organic acids (butyrate and propionate) were converted

772   to caproate and valerate respectively by methanol metabolising *E. limosum* cultures (Lindley *et*

773   *al.* 1987). Thereby the concentration of the added organic acid was critical: Chain elongation

774   of the organic acids occurred usually only if the substrate concentration of the supplemented

775   acid was above 100 mM, but Pacaud and colleagues showed that concentrations between 90-

776   310 mM (depending on the organic acid) also led to growth inhibition of *E. limosum* (Pacaud

777   *et al.* 1986a, Pacaud *et al.* 1986b). This effect of chain elongation was also achieved by *in situ*

778   produced butyric acid in fed batch experiments (Lindley *et al.* 1987). Mixotrophic growth on

779   methanol + acetate/butyrate or formate pushed the metabolism in the direction of more reduced

780   products (Lindley *et al.* 1987, Loubiere and Lindley 1991), therefore other cosubstrates might

781   as well offer this opportunity. Particularly important here may be syngas as cosubstrate. One

782   major problem of syngas conversion is the inhibitory effect of low concentrations of CO to

783   hydrogenases (Purec *et al.* 1962). Therefore, acetogens growing on syngas firstly metabolize

784   CO, before H_2+CO_2 conversion takes place (Bertsch and Müller 2015b, Najafpour and Younesi

785   2006). Contrary, a CO-adapted strain of *Thermoanaerobacter kivui* was able to grow on syngas,

786   simultaneously utilizing CO and H_2 for the production of acetate, indicating the presence of an

787   hydrogenase which is CO-tolerant (or an adaptation mechanism, which protects the

788   hydrogenase from CO) (Weghoff and Müller 2016). *T. kivui* is no methylotrophic organism but

789   successful adaptation of methylotrophic acetogens like *A. woodii* or *B. methylotrophicum* to

790   100% CO has been reported (Kerby *et al.* 1983, Lynd *et al.* 1982). This suggests that adaptive

791   laboratory evolution might be the key to enable acetogens to use syngas as an additional

792   reducing power and carbon source for the bioconversion of methanol. Addition of syngas as a

793   cosubstrate might have a versatile function: Due to the reduced state of CO no methyl group

794   has to be oxidized in order to generate reducing equivalents for CO_2 reduction. As a result,

795   acetate production might be enhanced. The high availability of H_2 and CO_2 will further favour

796   acetate formation from the methyl group of methanol and CO, since H_2 and CO_2 formation is

797   thermodynamically unfavourable under these conditions. In addition, H_2 delivers excess

798   electrons to the system, which might lead to the formation of more reduced compounds from

799   acetate/acetyl-CoA (depending on the set of available enzymes).

800   Besides mixotrophic cultivation of methylotrophic acetogens, which give the opportunity to

801   steer the fermentation process into the direction of interesting products, syntrophic cultivation

802   of methanol converting acetogens and other biocommodity producing organisms represents a

803   possibility to expand the product spectrum of fermentation processes, which is very limited

804   otherwise (Zeng 2019). For example, butanol and hexanol, which are no native products of *C.*

805   *autoethanogenum*, were produced from CO in a stable coculture with *Clostridium kluyveri*

806   (Diender *et al.* 2016). In that case, *C. kluyveri* was responsible for fatty acid production and *C.*

807   *autoethanogenum* converted the fatty acid to their corresponding alcohols.

808   The production of bioelectricity by microbial fuel cells (MFC) is an upcoming technology and

809   is regarded as a renewable alternative for fossil-based power generation (Slate *et al.* 2019).

810   Myung *et al.* (2018) showed that consortia of methanotrophs, acetogens and exoelectrogens

811   produced electricity from methane. In a two staged MFC system methane was converted to

812   methanol by the methanotroph, further consumed by an acetogen (*Acetobacterium sp.*) to

813   produce acetate, which was used by *Arcobacter sp.* for the generation of an electric current.

814   Also pectin degradation of *Lachnospira multiparus* was coupled to methylotrophic acetogens

815   in a syntrophic coculture. Pectin was degraded by pectin methylesterases producing

816   polygalacturonic acid, which is further fermented with methanol as byproduct. Whereas pure

817   cultures of *L. multiparus* produced methanol, H_2, CO_2, lactate and formate as main products,

818   methanol was further converted to acetate and butyrate in a coculture with *E. limosum* (Rode *et*

819   *al.* 1981).

820

821   **Conclusion**

822   To calculate the feasibility of the production of valuable compounds, precise metabolic models

823   are needed. The metabolism of methanol in acetogens is quite well understood, nevertheless

824   there are still questions to answer. One major uncertainty in the acetogenic metabolism is the

825   MTHFR catalysed methyl-THF oxidation, which builds an energetic barrier. In literature there

826   are several theories discussed how to overcome this barrier. One of the most accepted theories

827   is the coupling of methyl-THF oxidation to simultaneous oxidation of a second electron donor

828   in an electron confurcating manner (Jeong *et al.* 2015, Köpke *et al.* 2010, Mock *et al.* 2014),

829   but currently no biochemical evidence is available for this hypothesis. Therefore, clarifying the

830   mechanism of the methyl-THF oxidation and the redox partners involved is a major task, which

831   should be addressed in the future.

832   Whereas in various studies acetogens are used as chassis for the production of valuable

833   biochemicals using gas mixtures of H_2+CO_2 and sometimes CO (Fernández-Naveira *et al.*

834   2016, Hoffmeister *et al.* 2016, Köpke *et al.* 2011, Ueki *et al.* 2014), not much is known about

835   biochemical production using methanol as substrate. The models provided in this review show

836   the theoretical bioenergetic limits of biocommodity production from methanol, but more effort

837   has to be put into this field to identify and bypass the bottlenecks, which limit or even prevent

838   the formation of valuable products.

839

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844

845 **Author contribution statement**

846 FK prepared the figures and tables. FK and VM conceptualized and wrote the manuscript.

847

848 **Conflict of interest statement**

849 The authors declare that the research was conducted in the absence of any commercial or

850 financial relationships that could be construed as a potential conflict of interest.

851

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852

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1373

1374   **Figure legends**

1375   **FIGURE LEGENDS**

1376   **Fig. 1. Mechanism of methyl group transfer catalysed by cobalamin-dependent**

1377   **methyltransferase systems.** The substrate CH_3-X is demethylated by MTI and further

1378   transferred to the CoP yielding CH_3-CoP (CoIII). To enter the Wood-Ljungdahl pathway the

1379   methyl group is transferred from CH_3-CoP to THF (acetogens) or CoM (methanogens) by the

1380   MTII. Accidently oxidized corrinoids (Co(I)$\rightarrow$Co(II)) are reductively activated by an activating

1381   enzyme (AE).

1382
1383   **Fig. 2. Methanol:THF methyltransferase systems in acetogenic bacteria.** A selection of

1384   genomes from acetogens capable to grow on on methanol (Tab. 1) was screened for MtaB using

1385   the amino acid of sequence of MtaB from *A. woodii* (Kremp *et al.* 2018) as template for the

1386   BLASTP search. MTI encoding genes (*mtaB*) are shown in yellow MTII encoding genes (*mtaA*)

1387   are shown in blue and CoP encoding genes (*mtaC*) are represented in red. The Integrated

1388   Microbial Genomes & Microbiomes system was used for sequence comparison (Chen *et al.*

1389   2019).

1390

1391   **Fig. 3. Clusters of methyltransferase encoding genes of *A. woodii*.** MTI encoding genes

1392   (*mtxB*) are shown in yellow, MTII encoding genes (*mtxA)* are shown in blue and CoP encoding

1393   genes (*mtxC*) are represented in red. Possible activating enzymes (*CoP act/regen*) are

1394   represented in green.

1395

1396   **Fig. 4. Methylene-THF reductase-encoding gene cluster.** The basic subunits of MTHFR are

1397   encoded by *metV* and *metF* (yellow). In *A. woodii rnfC2* (green) encodes the NADH-binding

1398   subunit of MTHFR. In *M. thermoacetica* Hdr-like and Mvh-like proteins are proposed to build

1399   an electron bifurcating complex with MetVF. The encoding genes *hdrCBA* and *mvhD* (blue),

1400 which are also found in *S. ovata*, are located upstream of *metVF*. *E. limosum* is neither

1401 surrounded by *rnfC2* nor *hdrCBA* and *mvhD*. Surrounding genes: *folD*, methylene-THF

1402 dehydrogenase; *lpdA*1, dihydrolipoyl dehydrogenase; *acsE*, CoFeSP methyltransferase; *nuoE*,

1403 NADH:ubiquinon oxidoreductase subunit E; *hyp*, hypothetical protein.

1404

1405 **Fig. 5. Biochemistry and bioenergetics of acetogenesis from methanol in *A. woodii*.**

1406 Abbreviations: MTI, methanol:cobamide methyltransferase; CoP, corrinoid protein; MTII,

1407 cobamide:THF methyltransferase; HDCR, hydrogen-dependent CO_2 reductase; bif. Hyd,

1408 electron bifurcating hydrogenase; Rnf, Rnf complex; CODH/ACS, CO dehydrogenase/Acetyl-

1409 CoA synthase complex.

1410

1411 **Fig. 6. Biochemistry and bioenergetics of acetogenesis from methanol in *M. thermoacetica*.**

1412 Methyl-THF is oxidized by an electron bifurcating methylene-THF reductase using Fd^{2-} as

1413 additional electron donor. A combination of Nfn and an electron bifurcating hydrogenase is

1414 employed for the transfer of electrons from NADPH to H_2, alternatively a NADP-reducing

1415 hydrogenase can reduce H^+ directly with NADPH (not shown). Abbreviations: MTI,

1416 methanol:cobamide methyltransferase; CoP, corrinoid protein; MTII, cobamide:THF

1417 methyltransferase; Nfn, NADH-dependent reduced ferredoxin:$NADP^+$ oxidoreductase; bif.

1418 Hyd, electron bifurcating hydrogenase; CODH/ACS, CO dehydrogenase/Acetyl-CoA synthase

1419 complex; Ech, energy converting hydrogenase.

1420

1421 **Fig. 7. Biochemistry and bioenergetics of acetogenesis from H_2+CO_2 in *M. thermoacetica*.**

1422 Different scenarios are shown. In (A) methylene-THF is reduced by an electron bifurcating

1423 methylene-THF reductase, using Fd^{2-} as additional electron donor, whereas (B) represents a

1424 model without an electron bifurcating MTHFR. A combination of Nfn and an electron

1425 bifurcating hydrogenase is employed for the transfer of electrons from H_2 to $NADP^+$,

1426   alternatively a NADP-reducing hydrogenase can reduce NADP$^+$ directly with H$_2$ (not shown).

1427   Abbreviations: MTI, methanol:cobamide methyltransferase; CoP, corrinoid protein; MTII,

1428   cobamide:THF methyltransferase; Nfn, NADH-dependent reduced ferredoxin:NADP$^+$

1429   oxidoreductase; bif. Hyd, electron bifurcating hydrogenase; CODH/ACS, CO

1430   dehydrogenase/Acetyl-CoA synthase complex; Ech, energy converting hydrogenase.

1431

1432   **Fig. 8. Biochemistry and bioenergetics of acetogenesis from methanol in *S. ovata.*** Methyl-

1433   THF is assumed to be oxidized by an electron bifurcating MTHFR. Methylene-THF oxidation

1434   is NADP$^+$ dependent and the FDH is assumed to be Fd-dependent. NADPH is oxidized by the

1435   Stn to reduce NAD$^+$ and Fd$^+$. Abbreviations: MTI, methanol:cobamide methyltransferase; CoP,

1436   corrinoid protein; MTII, cobamide:THF methyltransferase; bif. Hyd, electron bifurcating

1437   hydrogenase; NADP-dep. Hyd; NADP$^+$-dependent hydrogenase; Rnf, Rnf complex;

1438   CODH/ACS, CO dehydrogenase/Acetyl-CoA synthase complex; Stn, *Sporomusa* type Nfn.

1439

1440   **Fig. 9. Biochemistry and bioenergetics of acetogenesis from methanol in *E. limosum.***

1441   Methyl-THF is assumed to be oxidized by a confurcating enzyme using Fd^{2-} as additional

1442   electron donor. The methylene-THF dehydrogenase is assumed to be NAD(H)-dependent and

1443   the FDH is assumed to be Fd-dependent. Abbreviations: MTI, methanol:cobamide

1444   methyltransferase; CoP, corrinoid protein; MTII, cobamide:THF methyltransferase; Rnf, Rnf

1445   complex; CODH/ACS, CO dehydrogenase/Acetyl-CoA synthase complex.

1446

1447   **Fig. 10. Biochemistry and bioenergetics of ethanol formation from methanol by *A. woodii.***

1448   Grey arrows indicate the pathway which is not found in *A. woodii*. The ethanol production

1449   pathway valid for *A. woodii* is indicated by black arrows. The production of three acetyl-CoA

1450   needs an investment of 0.5 ATP. The ATP synthase of *A. woodii* synthesizes 1 ATP per 3.3 Na$^+$

1451   (Matthies *et al.* 2014). Pta, Phosphotransacetylase; Ack, Acetatkinase; Aor,

1452 aldehyde:ferredoxin oxidoreductase; Aldh, aldehyde dehydrogenase; Adh, alcohol

1453 dehydrogenase.

1454

1455 **Fig. 11. Biochemistry and bioenergetics of butyrate and butanol formation from methanol**

1456 **by *E. limosum* and *B. methylotrophicum*.** Whereas butyrate production is reported for *E.*

1457 *limosum* and *B. methylotrophicum*, only *B. methylotrophicum* is able to produce butanol. The

1458 Aor pathway (grey arrow) of butyrate reduction is not valid for *E. limosum* and *B.*

1459 *methylotrophicum*. The production of three acetyl-CoA needs an investment of 0.5 ATP. Thl,

1460 thiolase; Hbd, 3-hydroxybutyryl-CoA dehydrogenase; Crt, crotonase; Bcd, butyryl-CoA

1461 dehydrogenase; Etf, electron transfer flavoprotein; Ptb, phosphotransbutyrylase; Buk, butyrate

1462 kinase; Aldh, aldehyde dehydrogenase; Adh, alcohol dehydrogenase.

1463

1464 **Fig. 12. Biochemistry and bioenergetics of acetone formation from methanol by *A. woodii*.**

1465 The production of three acetyl-CoA from methanol needs an investment of 0.5 ATP, whereas

1466 2.1 ATP are needed from H_2+CO_2. In (A) acetone production from methanol is depicted,

1467 whereas (B) shows the production of acetone from H_2+CO_2. Acetone production pathway: Thl,

1468 thiolase; Ctf, CoA-transferase; Acd, acetoacetate decarboxylase.

1469

1470 **Fig. 13. Biochemistry and bioenergetics of lactate formation from methanol by *A. woodii*.**

1471 The production of three acetyl-CoA from methanol needs an investment of 0.5 ATP. Lactate

1472 production pathway: Pfor, pyruvate:ferredoxin oxidoreductase; Ldh, lactate dehydrogenase;
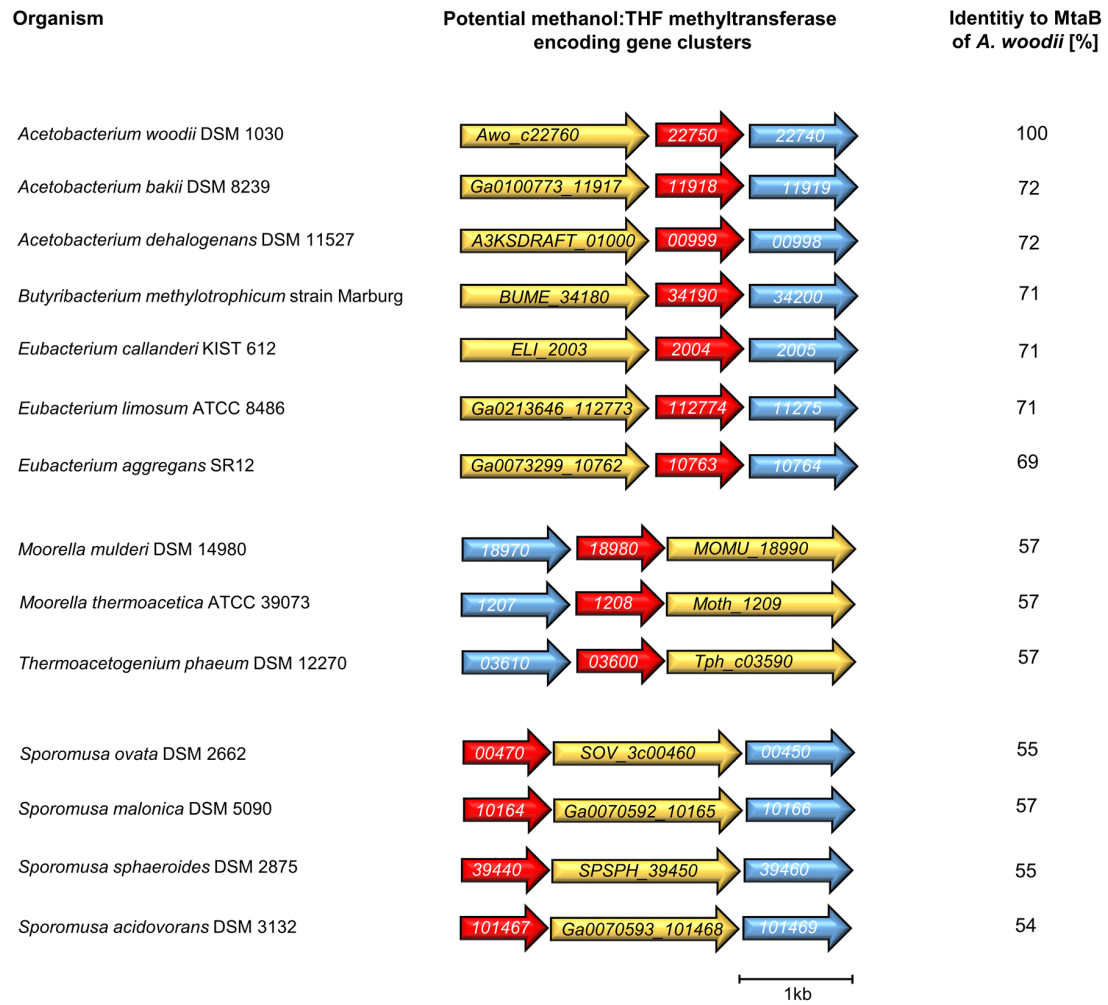
1473 Etf, electron transfer flavoprotein.

1474

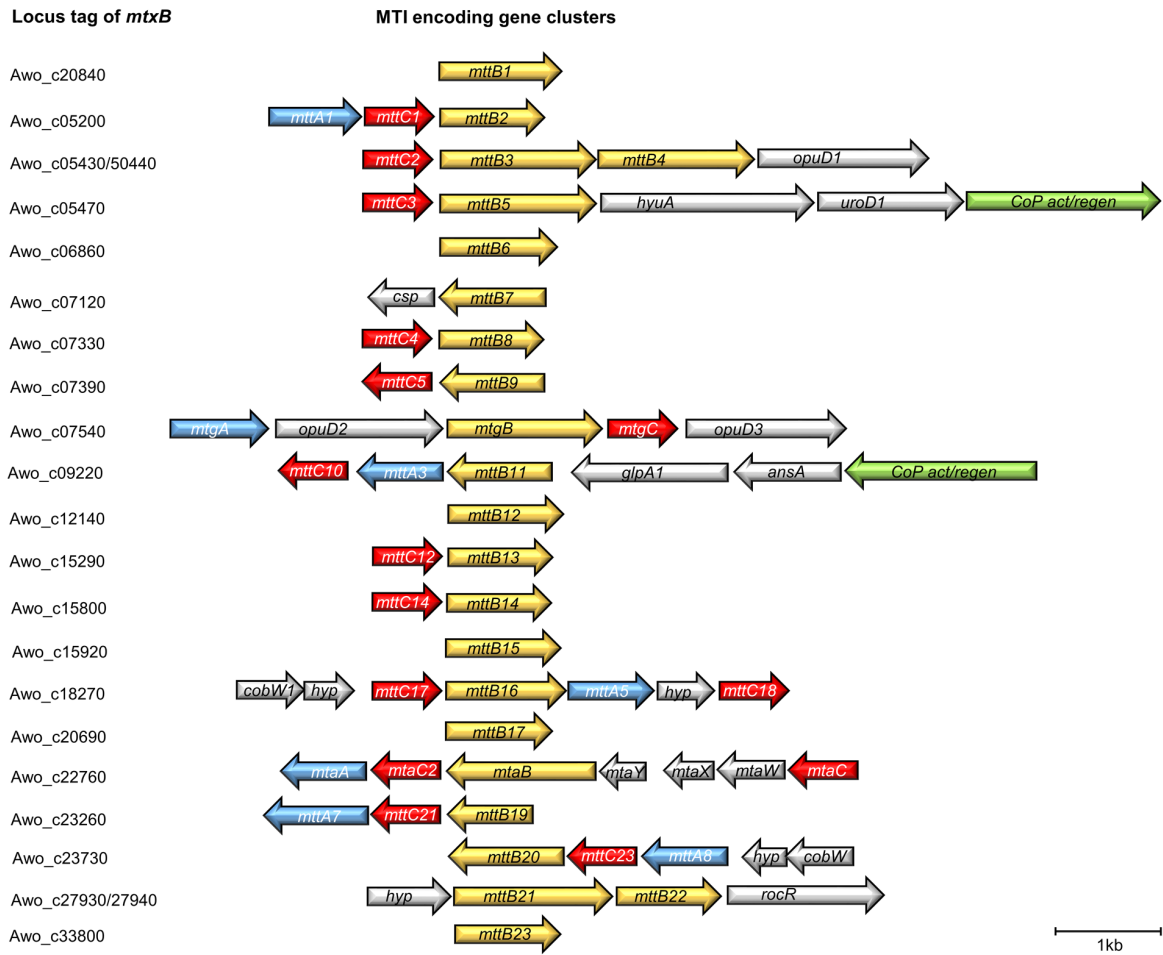1475   **Figures**
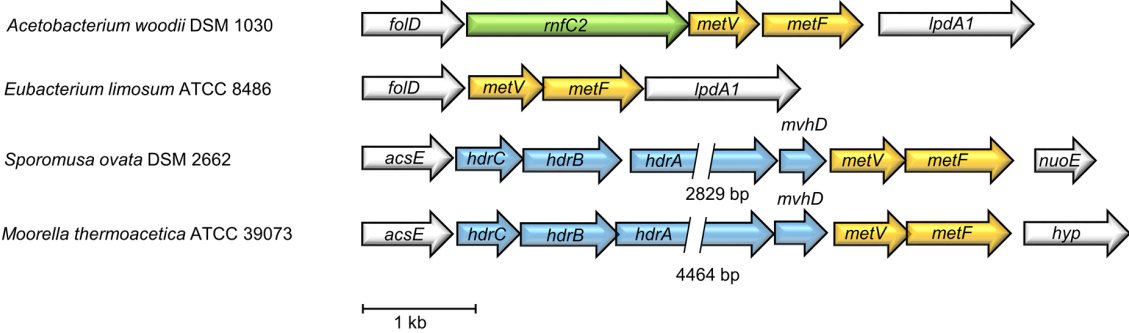

1476   Figure 1.
1477

Organism	Potential methanol:THF methyltransferase encoding gene clusters	Identitiy to MtaB of *A. woodii* [%]
Acetobacterium woodii DSM 1030		100
Acetobacterium bakii DSM 8239		72
Acetobacterium dehalogenans DSM 11527		72
Butyribacterium methylotrophicum strain Marburg		71
Eubacterium callanderi KIST 612		71
Eubacterium limosum ATCC 8486		71
Eubacterium aggregans SR12		69
Moorella mulderi DSM 14980		57
Moorella thermoacetica ATCC 39073		57
Thermoacetogenium phaeum DSM 12270		57
Sporomusa ovata DSM 2662		55
Sporomusa malonica DSM 5090		57
Sporomusa sphaeroides DSM 2875		55
Sporomusa acidovorans DSM 3132		54

1478
1479   Figure 2.
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Figure 3.

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1484
1485   Figure 4.
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1487
1488   Figure 5.
1489
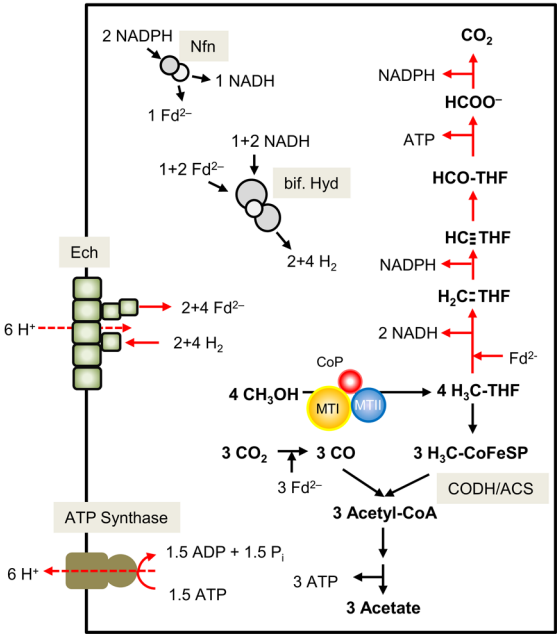
1490
1491   Figure 6.
1492

A
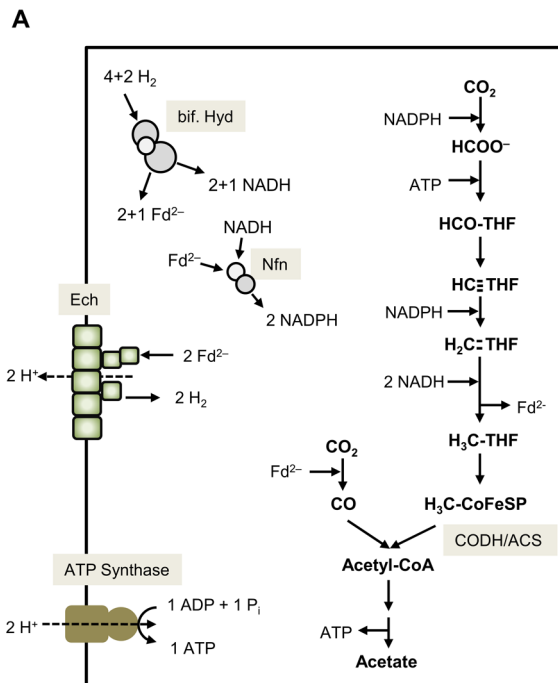
1493
1494   Figure 7a.
1495

B
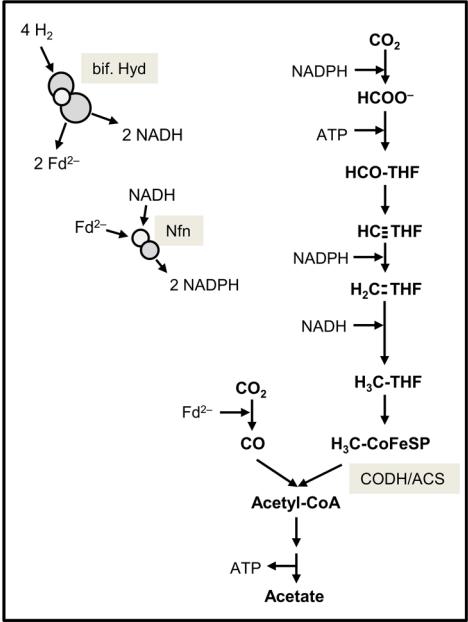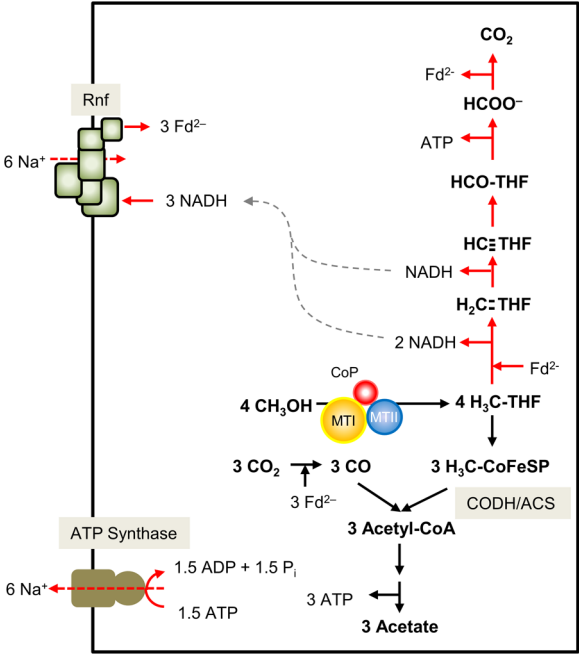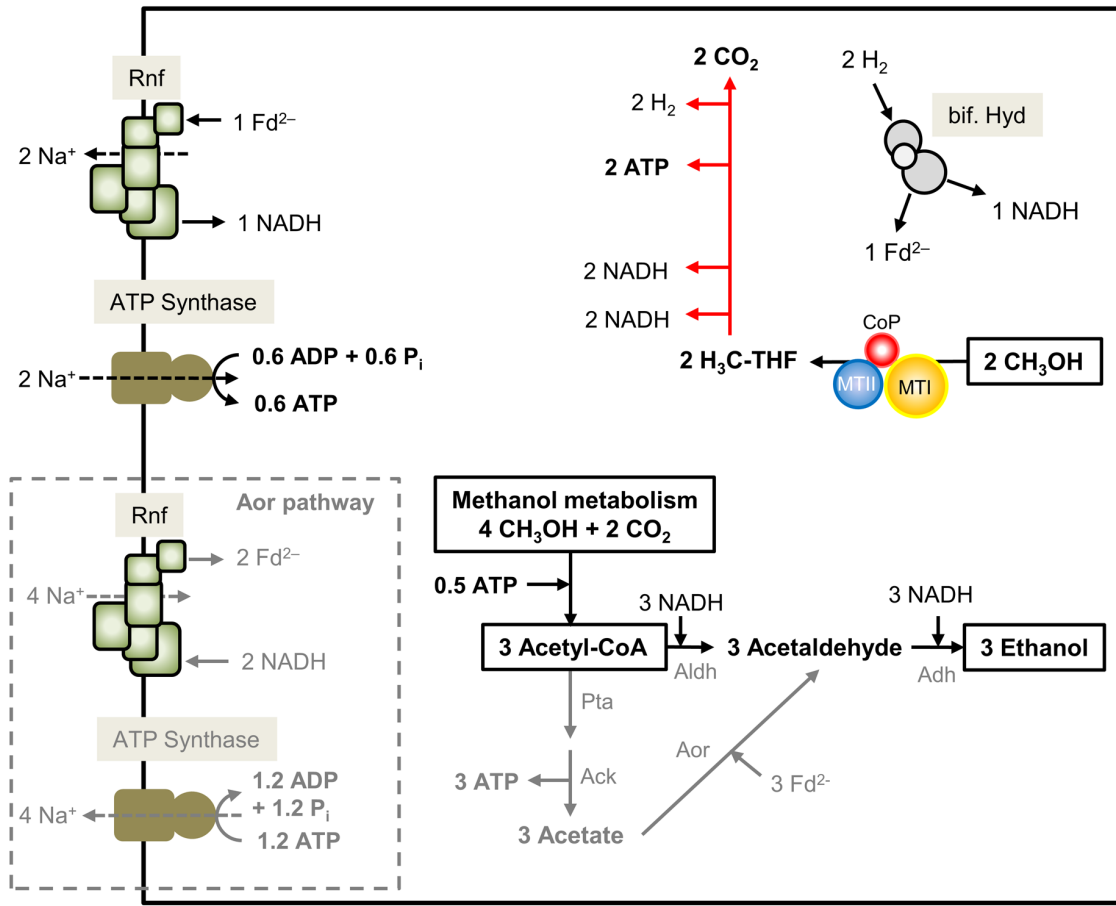

1496
1497   Figure 7b.
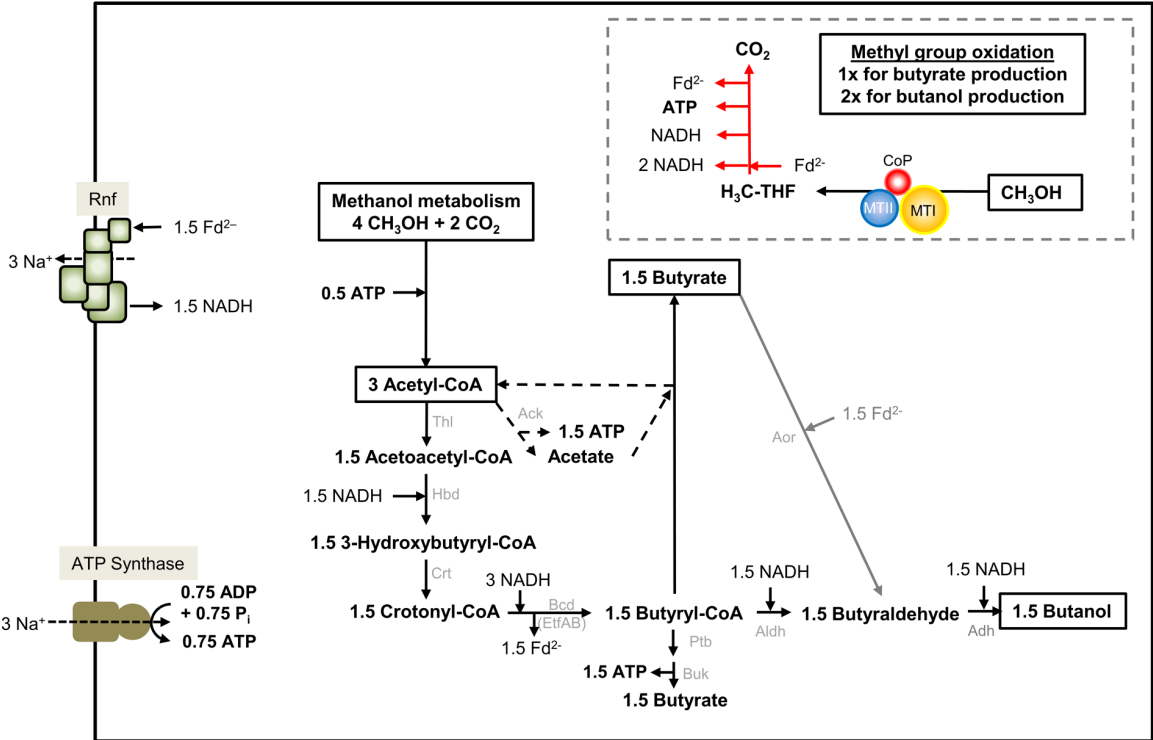1498
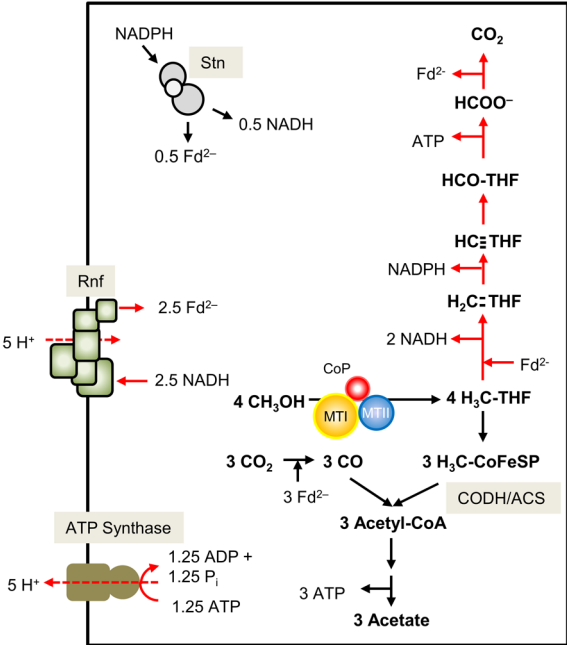
1499
1500   Figure 8.
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1503   Figure 9.
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1506   Figure 10.
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1509   Figure 11.
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A
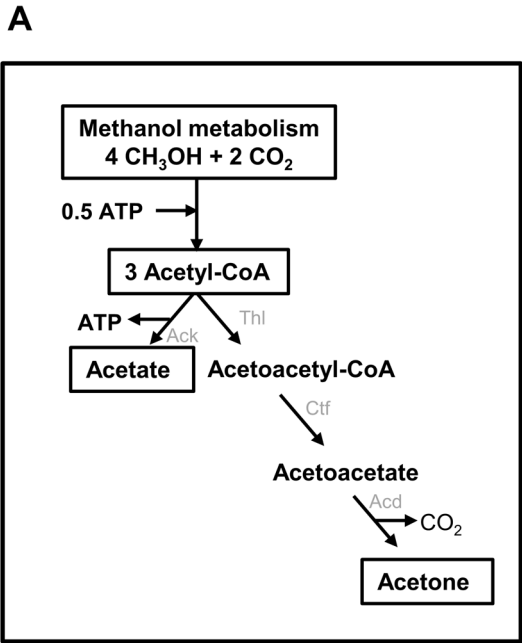
1511
1512   Figure 12a.
1513

B

Autotrophy
$40 H_2 + 20 CO_2$

7 ATP

10 Acetyl-CoA

8 ATP   Thl

Ack

8 Acetate   Acetoacetyl-CoA

Ctf

Acetoacetate

Acd

CO_2

Acetone
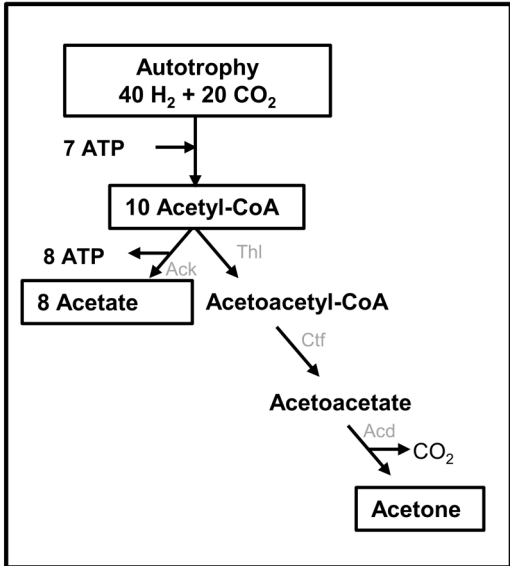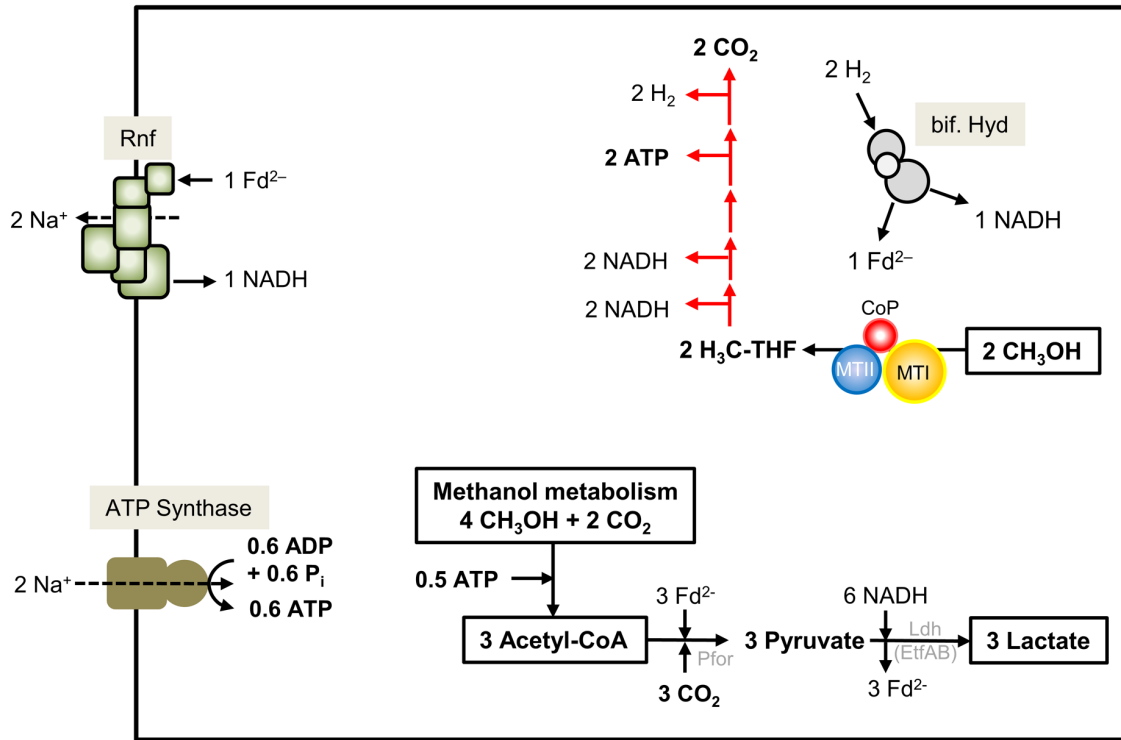
1514
1515   Figure 12b.
1516

1517
1518   Figure 13.
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1520   **Table 1. Methanol converting acetogens.**

Organism	Reference	(Draft) genome availability	Locus tag of MtaB (IMG/NCBI databases)
Acetobacterium bakii	Kotsyurbenko *et al.* (1995)	+	Ga0100773_11917
Acetobacterium carbinolicum	Eichler and Schink (1984)	-	
Acetobacterium dehalogenans	Traunecker *et al.* (1991)	+	A3KSDRAFT_01000
Acetobacterium paludosum	Kotsyurbenko *et al.* (1995), Ross *et al.* (2000)	+	WP_148566731
Acetobacterium tundrae	Simankova *et al.* (2000)	+	WP_148602035
Acetobacterium woodii	Bache and Pfennig (1981)	+	Awo_c22760
Alkalibaculum bacchii	Allen *et al.* (2010)	+	Ga0244545_10532
Butyribacterium methylotrophicum	Lynd and Zeikus (1983)	+	Bume_34180
Clostridium formicaceticum	Andreesen *et al.* (1970)	+	Ga0198698_113829
Clostridium magnum	Schink (1984)	+	EJ33DRAFT_01162
Clostridium methoxybenzovorans	Mechichi *et al.* (1999b)	+	**No MtaB present**
Clostridium **strain CV-AA1**	Adamse and Velzeboer (1982)	-	
Eubacterium aggregans	Mechichi *et al.* (1998)	+	Ga0073299_10762
Eubacterium callanderi **KIST612**	Chang *et al.* (1997), Chang *et al.* (1999)	+	Eli_2003
Eubacterium limosum	Genthner *et al.* (1981)	+	Ga0213646_112773
Moorella mulderi	Balk *et al.* (2003)	+	Momu_18990
Moorella thermoacetica	Daniel *et al.* (1988)	+	Moth_1209
Moorella thermoautotrophica	Wiegel *et al.* (1981)	+	Mtjw_12120 / Mtin_10780
Sporobacterium olearium	Mechichi *et al.* (1999a)	-	
Sporomusa acidovorans	Ollivier *et al.* (1985)	+	Ga0070593_101468
Sporomusa aerivorans	Boga *et al.* (2003)	-	
Sporomusa malonica	Dehning *et al.* (1989)	+	Ga0070592_10165
Sporomusa ovata	Möller *et al.* (1984)	+	Sov_3c00460
Sporomusa paucivorans	Hermann *et al.* (1987)	-	
Sporomusa silvacetica	Kuhner *et al.* (1997)	+	Ga0336821_5535
Sporomusa sphaeroides	Möller *et al.* (1984)	+	Spsph_39450
Sporomusa termitida	Breznak *et al.* (1988)	+	WP_144351108
Thermoacetogenium phaeum	Hattori *et al.* (2000)	+	Tph_c03590

1521