environmental microbiology reports

Environmental Microbiology Reports (2022) **14**(1), 170–178 doi:10.1111/1758-2229.13042

Brief report

The ß-ketoadipate pathway of *Acinetobacter baumannii* is involved in complement resistance and affects resistance against aromatic antibiotics

Jennifer Breisch,[1] Lisa Sophie Huber,[1] Peter Kraiczy,[2] Josephine Hubloher[1] and Beate Averhoff[1]*

[1]*Department of Molecular Microbiology & Bioenergetics, Institute of Molecular Biosciences, Goethe-University Frankfurt am Main, Frankfurt, Germany.*

[2]*Institute of Medical Microbiology and Infection Control, University Hospital Frankfurt, Goethe-University Frankfurt am Main, Frankfurt, Germany.*

Summary

Acinetobacter baumannii **can thrive on a broad range of substrates such as sugars, alcohols, lipids, amino acids and aromatic compounds. The latter three are abundant in the human host and are potential candidates as carbon sources for the metabolic adaptation of *A. baumannii* to the human host. In this study we determined the biodegradative activities of *A. baumannii* AYE with monocyclic aromatic compounds. Deletion of genes encoding the key enzymes of the ß-ketoadipate pathway, the protocatechuate-3,-4-dioxygenase (Δ*pcaHG*) and the catechol-1,-2-dioxygenase (Δ*catA*), led to a complete loss of growth on benzoate and *p*-hydroxybenzoate, suggesting that these substrates are metabolized via the two distinct branches (*pca* and *cat*) of this pathway. Furthermore, we investigated the potential role of these gene products in host adaptation by analyzing the capability of the mutants to resist complement-mediated killing. These studies revealed that the mutants exhibit a decreased complement resistance, but a dramatic increase in survival in normal human serum in the presence of *p*-hydroxybenzoate or protocatechuate. These results indicate that the ß-ketoadipate pathway plays a role in adaptation of *A. baumannii* to the human host. Moreover, the single and double mutants exhibited increased antibiotic resistances indicating a link between the two dioxygenases and antibiotic resistance.**

Received 29 September, 2021; accepted 29 December, 2021. *For correspondence. E-mail averhoff@bio.uni-frankfurt.de; Fax +49 69 79829306; Tel. + 49 69 79829509.

Introduction

Acinetobacter baumannii can colonize different niches in the human host causing soft tissue, bloodstream, ventilator-associated lung and urinary tract infections leading to pneumonia, meningitis, osteomyelitis, or even sepsis (Dijkshoorn *et al*., 2007; Peleg *et al*., 2008; Antunes *et al*., 2011; Doughari *et al*., 2011; Harding *et al*., 2018). Also, animals were found to be a reservoir of *A. baumannii* and moreover, these pathogens have also been isolated from different soil samples (Müller *et al*., 2014; van der Kolk *et al*., 2019; Wareth *et al*., 2019). The ability to colonize different hosts and to thrive in different host niches but also outside the human host such as in soil environments suggests that this pathogen must be able to adapt to substantial environmental changes through modification of its metabolism and nutritional needs (Mitchell *et al*., 2009; Bleuven and Landry, 2016). Indeed, *A. baumannii* exhibits a high metabolic flexibility and distinct systems for nutrient acquisition and homeostasis (Dijkshoorn *et al*., 2007; Peleg *et al*., 2008; Fiester and Actis, 2013; Harding *et al*., 2018). It is able to metabolize a broad range of carbon sources including different sugars (Hubloher *et al*., 2020), amino acids (König *et al*., 2021), alcohols (Camarena *et al*., 2010), fatty acids (Stahl *et al*., 2015), trimethyl ammonium compounds (Breisch *et al*., 2018) and aromatic amino acids (Bouvet and Grimont, 1986). This metabolic diversity together with its high desiccation resistance (Jawad *et al*., 1996; Wendt *et al*., 1997; Guardabassi *et al*., 1998; Zeidler and Müller, 2019) and multidrug or even pan-drug resistance (Allen and Green, 1987; Dijkshoorn *et al*., 2007; Peleg *et al*., 2008; Towner, 2009; Roca *et al*., 2012; Lee *et al*., 2017) plays an important role in the emergence of *A. baumannii* as very notorious opportunistic human pathogen.

The utilization of aromatic carbon sources is a clear metabolic benefit in the human host, due to the high

abundance of aromatics, such as amino acids, hormones and signal substances (Teufel *et al*., 2010; Cerqueira *et al*., 2014). The three most common pathways for the degradation of aromatic compounds are the phenylacetic acid pathway (Teufel *et al*., 2010), the homogentisate pathway (Arias-Barrau *et al*., 2004) and the *ß*-ketoadipate pathway (Harwood and Parales, 1996). The latter consists of two parallel branches, the catechol and protocatechuate branch which converge at the enol-lactone of *ß*-ketoadipate (Harwood and Parales, 1996). A broad range of aromatic and hydroaromatic substrates, such as benzoate, *p*-hydroxybenzoate, vanillate, mandelate, coumarate, ferulate, salicylate are metabolised via the *ß*-ketoadipate pathway to succinyl-CoA or acetyl-CoA which enter the central metabolism via the tricarboxylic acid cycle (Sze and Dagley, 1987; Vasudevan and Mahadevan, 1992; Bleichrodt *et al*., 2010). The full chemistry, genetic organization and regulation of this pathway has been known for decades and has been studied in detail in the soil bacterium *Acinetobacter baylyi* (D'Argenio *et al*., 1999; Trautwein and Gerischer, 2001; Dal *et al*., 2002; Brzostowicz *et al*., 2003; Dal *et al*., 2005; Young *et al*., 2005; Siehler *et al*., 2007; Fischer *et al*., 2008; Williams and Kay, 2008). However, information with respect to the role of the *ß*-ketoadipate pathway in *A. baumannii* is very scarce. Here we report on the role of the *ß*-ketoadipate pathway in aromatic degradation, complement resistance and antibiotic resistance of *A. baumannii*.

Results and discussion

A. baumannii *ATCC 19606 and AYE use different aromatic compounds as sole carbon and energy source*

To address the question of whether *A. baumannii* exhibits a functional *ß*-ketoadipate pathway, growth studies of the *A. baumannii* type strain ATCC 19606 and the genetically modified clinical isolate AYE comMR, the latter is devoid of the large resistance island AbaR, containing up to 25 putative antibiotic resistance genes against mainly aminoglycosides, tetracycline and sulfonamides (Godeux *et al*., 2020), with potential substrates of the *ß*-ketoadipate pathway such as *p*-hydroxybenzoate, benzoate, benzyl acetic ester, benzyl alcohol, quinate, kynurenic acid, ferrulate, styrene, toluol, salicylic acid, mandelic acid, cinnamic acid and folic acid were performed in mineral medium [MM; (Zeidler *et al*., 2017)]. Both strains were able to grow on benzoate, *p*-hydroxybenzoate, quinate and kynurenic acid as sole carbon and energy source with comparable growth rates (Table 1). The growth of both strains on *p*-hydroxybenzoate and benzoate suggests that both strains exhibit a functional *ß*-ketoadipate pathway. The growth on kynurenic acid indicates that both strains exhibit a kynureninase which catalyzes the conversion of kynurenic acid to anthranilate (Stanier *et al*., 1951; Kurnasov *et al*., 2003). The latter can be further degraded by different pathways such as via catechol funnelled either into the *ß*-ketoadipate pathway (Stanier *et al*., 1951) or the meta cleavage pathway (Costaglioli *et al*., 2012), the gentisate pathway (Cain, 1968), the 3-hydroxyanthranilate pathway (Liu *et al*., 2010) or the 2-aminobenzoyl-CoA pathway (Schühle *et al*., 2001). The growth on quinate suggests the presence of the genes encoding QuiA, QuiB and QuiC feeding quinate into the protocatechuate branch of the *ß*-ketoadipate pathway (Smith, 1990; Elsemore and Ornston, 1995).

Table 1. Growth of *A. baumannii* ATCC 19606 and AYE on different aromatic substrates.

Substrate[a]	ATCC 19606 (t_d) (h)	AYE (t_d) (h)
Without carbon source	–	–
Na-acetate	0.4	0.4
Kynurenic acid	0.61	0.5
Quinate	0.55	0.46
Phenylacetic acid	–	–
Phenylalanine	0.68	0.6
Tyrosine	0.8	0.5
Tryptophan	–	–
Benzoate	1	0.9
p-hydroxybenzoate	1.1	0.98
Benzyl alcohol	–	–
Benzyl acetate	–	–
Ferrulate	–	–
Indole-3-acetate	0.68	0.61
Styrene	–	–
Toluol	–	–
Salicylic acid	–	–
Mandelic acid	–	–
Cinnamic acid	–	–
Folic acid	–	–

A. baumannii ATCC 19606 and AYE was grown in 5 ml MM (Zeidler *et al*., 2017) with 20 mM Na-acetate as carbon source overnight at 37°C. Fresh MM with 20 mM Na-acetate was inoculated to a final OD_{600} of 0.01 and incubated over 14 h shaking at 37°C. Afterwards, 100 ml fresh MM with the given carbon sources are inoculated to a final OD_{600} of 0.1 and incubated shaking at 37°C. OD_{600} was determined every hour and the doubling time (t_d) was determined with GraphPad Prism 6.00 for Windows (GraphPad Software, La Jolla, CA, USA).

[a]20 mM Na-acetate and 5 mM of all other carbon sources were used; -: no growth; t_d: doubling time.

Identification of ß*-ketoadipate pathway genes*

The finding that *A. baumannii* AYE comMR can use benzoate and *p*-hydroxybenzoate as sole carbon and energy source raised the question, whether this strain employs the *ß*-ketoadipate pathway to oxidize the aromatic substrates. We screened the genome of *A. baumannii* AYE for the presence of potential *pca* and *cat* genes encoding the enzymes of the two branches of this pathway and

identified all potential *pca* and *cat* genes (Fig. 1A). Eight *cat* and nine *pca* genes were organized in two distinct gene clusters spanning 7501 and 7914 kbp respectively (Fig. 1B). Sequence alignments of the deduced proteins revealed that the proteins exhibit highest similarities to the *ß*-ketoadipate pathway enzymes of *A. baylyi*, such as similarities and identities in the range of 65%–92% and 86%–99% were found respectively (Fig. 1B). Interestingly, *pcaU*, which encodes an IclR-type transcriptional regulator of the *pca* genes in *A. baylyi* (Gerischer *et al*., 1998) was absent in the *pca* gene cluster of *A. baumannii* AYE (Fig. 1B).

The pcaHG *and* catA *genes are essential for the degradation of* p*-hydroxybenzoate and benzoate*

To address the role of the *ß*-ketoadipate pathway in benzoate and *p*-hydroxybenzoate degradation single and double mutants exhibiting markerless deletions of either one or both key enzyme genes such as *pcaHG* (encoding protocatechuate-3,4-dioxygenase) or *catA* (encoding catechol-1,2-dioxygenase) were generated in *A. baumannii* AYE comMR (Godeux *et al*., 2020). To generate markerless single or double mutants the RecET-based recombination-mediated genetic engineering (recombineering) was used (Tucker *et al*., 2014). Therefore, the plasmid pAT04 expressing the Rec_{AB}-recombination system and a PCR product exhibiting the up- and downstream region of the gene flanking a kanamycin resistance cassette were transformed into *A. baumannii* AYE comMR (for primer information see supporting information Table S1). The mutants were selected on LB-agar containing kanamycin and verified by PCR (primer in supporting information Table S1). Excision of the resistance cassette was performed using the flippase (FLP) recombinase target sites flanking the cassette which are substrate of a FLP recombinase encoded by pAT03 with a tetracycline antibiotic resistance cassette instead of the ampicillin resistance gene (Tucker *et al*., 2014). Both single and double markerless mutants were verified by PCR (primer in supporting information Table S1).

Analyses of the growth phenotype of the Δ*pcaHG* mutant revealed that growth with *p*-hydroxybenzoate was completely abolished, whereas growth with benzoate was unaffected. Moreover, the Δ*pcaHG* mutant was not able to grow with quinate, as expected. The Δ*catA* mutant was completely defective in growth with benzoate but unaffected in growth with *p*-hydroxybenzoate. Furthermore, the Δ*catA* mutation did not abolish growth with kynurenic acid leading to the conclusion that kynurenic acid is not funnelled into the catechol branch of the *ß*-ketoadipate pathway. A Δ*pcaHG*/Δ*catA* double mutant grew with neither benzoate nor *p*-hydroxybenzoate. The growth defects of the single mutants were complemented with the *pcaHG* or *catA* gene encoded by the *E. coli*/*Acinetobacter* shuttle vector pVRL1 [(Lucidi *et al*., 2018), primer pairs for amplification are in the supporting information Table S1]. Taken together these results provide clear evidence that *pcaHG* and *catA* encode the two dioxygenase key enzymes of the two branches of the *ß*-ketoadipate pathway which initialize the degradation of benzoate and *p*-hydroxybenzoate, respectively.

A ΔpcaHG/ΔcatA *mutant is impaired in complement resistance*

The fact that aromatic compounds are abundant in the human host, such as amino acids or hormones and that pathways important for the degradation of aromatic compounds play a role in virulence of *A. baumannii* such as the phenyl acetic acid pathway (Cerqueira *et al*., 2014), prompted us to analyze the susceptibility of *A. baumannii* AYE comMR to complement-mediated killing in normal human serum (NHS) and compared the resulting phenotype with those of the Δ*pcaHG* and Δ*catA* mutants. Therefore, *A. baumannii* AYE comMR and the mutants were incubated for 2 h in 25% or 30% NHS. The number of colony-forming units (CFU) was determined after plating on LB agar. The percentage of CFU was calculated with 100% CFU corresponding to the CFUs in a control assay with phosphate-buffered saline containing 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$ ($PBS^{+/+}$). Incubation of *A. baumannii* AYE comMR in 25% NHS resulted in nearly a 300% increase in CFUs in comparison to the control incubated in $PBS^{+/+}$, whereas incubation of *A. baumannii* AYE comMR in 30% NHS led to a 13% decrease in CFUs (Fig. 2). This finding leads to the conclusion that growth of *A. baumannii* AYE comMR is not affected by NHS below 25% but is inhibited in the presence of higher serum concentrations. Potential carbon sources could be the different amino acids in NHS. In contrast to *A. baumannii* AYE comMR all mutants exhibited a decrease in CFUs after incubation in 25% NHS. Just around 60% of the cells of the Δ*catA* (63%), Δ*pcaHG* (61%) and the Δ*pcaHG*/Δ*catA* (51%) mutants survived in 25% NHS (Fig. 2). Applying 30% NHS even lower CFUs could be detected with the Δ*pcaHG* and Δ*catA* mutants such as a decrease of around 70% in CFUs (Δ*catA* 73%, Δ*pcaHG* 79% and Δ*pcaHG*/Δ*catA* 70%) was found (Fig. 2). These results suggest that the *ß*-ketoadipate pathway plays a role in mediating serum resistance of *A. baumannii* AYE comMR. Furthermore, our finding that the CFUs of the two single mutants and the Δ*pcaHG*/Δ*catA* double mutant were comparable after incubation in NHS suggests that both branches are essential for complement resistance.

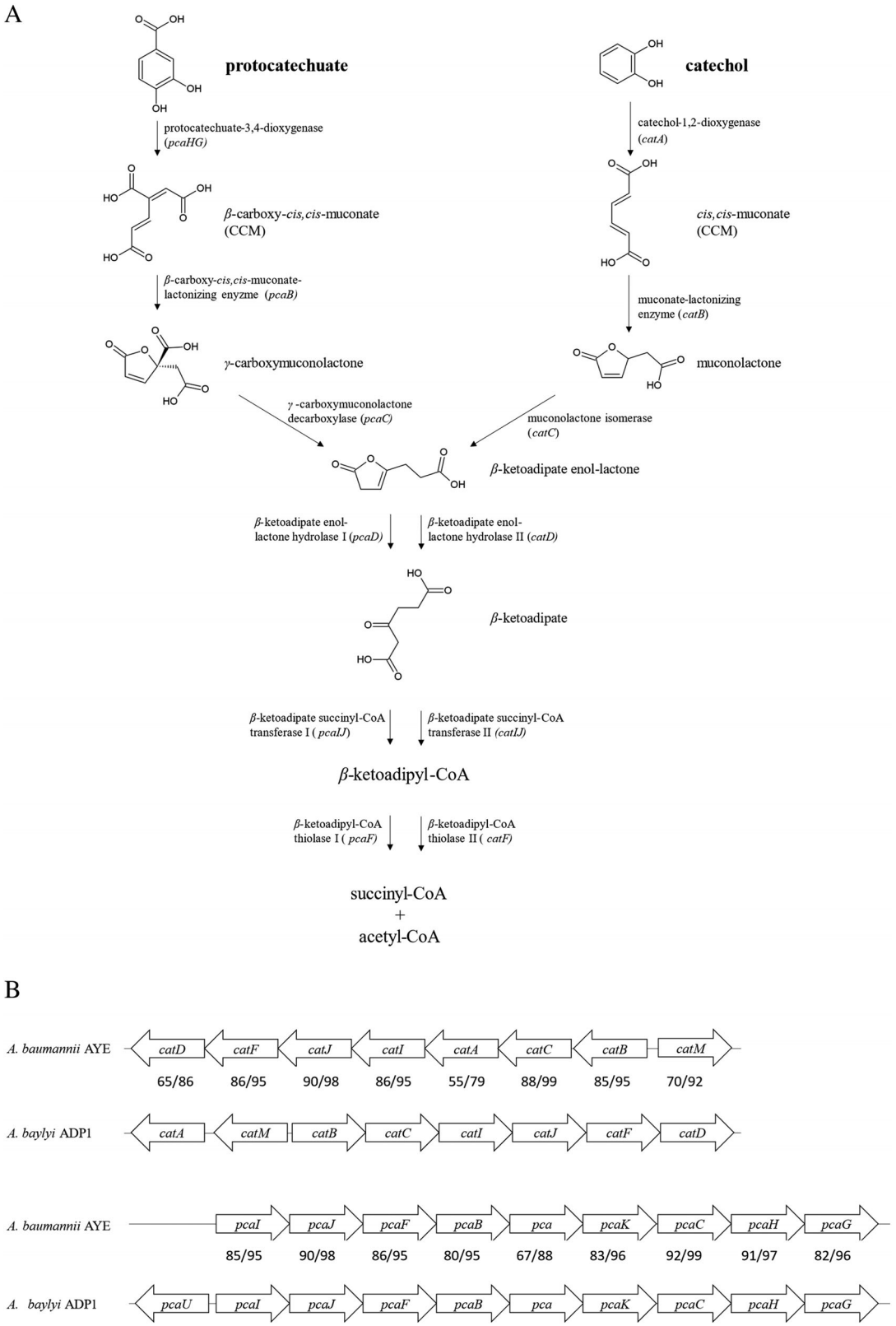

Fig. 1. The ß-ketoadipate pathway (A) and genetic organization of the *cat* and *pca* gene clusters in *A. baumannii* AYE and *A. baylyi* ADP1 (B). The genes responsible for catechol degradation (*cat*) and for protocatechuate degradation (*pca*) are organized in two gene clusters. Similarities/identities are stated underneath the genes.

Growth of the A. baumannii *AYE comMR strain and the* ΔpcaHG *and* ΔcatA *mutants in inactivated NHS*

To address the question of whether NHS impaired growth of the generated mutants, growth studies with the Δ*pcaHG* and the Δ*catA* single mutant, the Δ*pcaHG*/Δ*catA* double mutant, and *A. baumannii* AYE comMR were performed by incubating the bacterial cells in heat-inactivated NHS (100%). As shown in Fig. 3, growth of all three mutants and the *A. baumannii* AYE comMR strain was unaffected in heat-inactivated NHS and no differences could be observed after a 5 h incubation period indicating that the reduced survival of the cells after incubation in 25% or 30% NHS is not due to an impaired growth of the mutants.

Complement resistance of the ΔpcaHG/ΔcatA *mutant is stimulated in the presence of* p*-hydroxybenzoate and protocatechuate*

To get deeper insights into the role of aromatic compounds in complement resistance of *A. baumannii* AYE comMR, the *A. baumannii* AYE comMR strain and the Δ*pcaHG*/Δ*catA* double mutant were incubated for 2 h in 30% NHS in the presence or absence of 5 mM *p*-hydroxybenzoate or in the presence of 5 mM Na-acetate as control. The CFUs were determined after plating on LB agar. As shown in Fig. 4A, incubation of the *A. baumannii* AYE comMR strain in 30% NHS alone or in 30% NHS supplemented with 5 mM Na-acetate led to 15% and 25% reduced CFUs respectively, in comparison to $PBS^{+/+}$. However, the addition of 5 mM *p*-hydroxybenzoate to 30% NHS led to an increase of the CFUs by 175% of the *A. baumannii* AYE comMR strain.

Even more dramatic was the effect with the Δ*pcaHG*/Δ*catA* double mutant. Incubation of the Δ*pcaHG*/Δ*catA* double mutant in 30% NHS supplemented with 5 mM *p*-hydroxybenzoate resulted in a 15-fold increase in CFUs in comparison to the CFUs obtained after incubation in $PBS^{+/+}$ and an even 20-fold increase in CFUs in comparison to the CFUs obtained after incubation in 30% NHS alone. Furthermore, the CFUs after the incubation of the Δ*pcaHG*/Δ*catA* mutant in NHS as well as in NHS supplemented with Na-acetate were comparable to those obtained with the double mutant after incubation in NHS without Na-acetate. This leads to the conclusion that either *p*-hydroxybenzoate or the oxidation product protocatechuate which is not further oxidized in the Δ*pcaHG*/Δ*catA* double mutant might play a role in complement resistance.

To address the question whether *p*-hydroxybenzoate or the accumulation of protocatechuate led to the increased complement resistance, an *A. baumannii* AYE comMR Δ*pobA* deletion mutant was generated using the RecET recombineering system as described before (primers are given in supporting information Table S1). As expected, the Δ*pobA* mutant did not grow with *p*-hydroxybenzoate but with protocatechuate (data not shown). Investigating complement resistance of this particular mutant under the same conditions but in the presence or in the absence of *p*-hydroxybenzoate revealed that the Δ*pobA* mutant did not exhibit any increase in

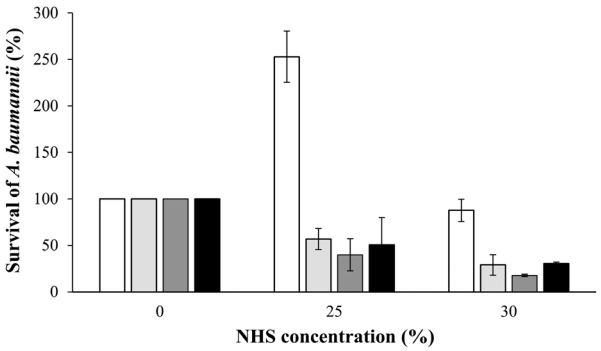

Fig. 2. Survival of *A. baumannii* AYE comMR, the Δ*catA* and Δ*pcaHG* single mutant and the Δ*pcaHG*/Δ*catA* double mutant in 25% or 30% NHS. *Acinetobacter baumannii* AYE comMR strains (wild type: white bars; Δ*catA*: light grey bars; Δ*pcaHG*: dark grey bars; Δ*pcaHG*/Δ*catA*: black bars) were grown in LB medium (5 g L^{-1} NaCl, 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract), harvested at an OD_{600} of 0.5–0.6, washed two times in $PBS^{+/+}$ (2.7 mM KCl, 1.5 mM KH_2PO_4, 137.9 mM NaCl, 8.1 mM Na_2HPO_4, 0.9 mM CaCl, 0.5 mM MgCl) and resuspended to a final OD_{600} of 0.4 in $PBS^{+/+}$. The frozen NHS was thawed on ice and diluted to a final concentration of 30% or 25% with $PBS^{+/+}$. 10 µl of the respective cell culture were given to 200 µl $PBS^{+/+}$, 200 µl 25% NHS or 30% NHS and incubated over 2 h at 37°C. 790 µl $PBS^{+/+}$ were given to the assay mixtures followed by several dilutions. 100 µl of the 10^{-2}, 10^{-3} and 10^{-4} dilutions were plated onto LB agar and CFUs were counted. The CFUs are given in percentage in comparison to the CFUs of the cultures incubated with $PBS^{+/+}$. Each value is the mean of ±SEM of at least three independent measurements.

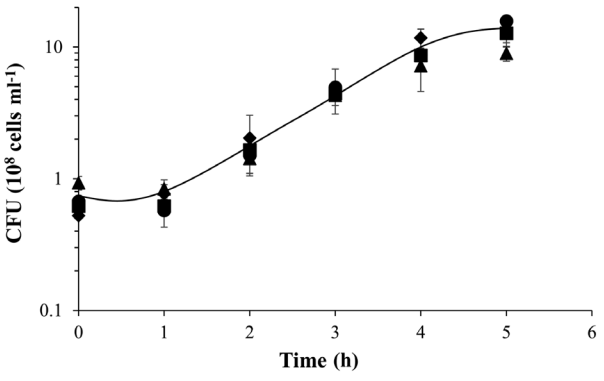

Fig. 3. Growth of *A. baumannii* AYE comMR in heat-inactivated NHS. *A. baumannii* AYE comMR strains (wild type: ■; Δ*catA*: ♦; Δ*pcaHG*: ▲; Δ*pcaHG*/Δ*catA*: ●) were cultivated overnight in LB medium at 37°C and inoculated to a final OD_{600} of 0.1 in 100% heat-inactivated NHS. Growth was monitored by counting CFUs at given time points. Each value is the mean of ±SEM of at least three independent measurements.

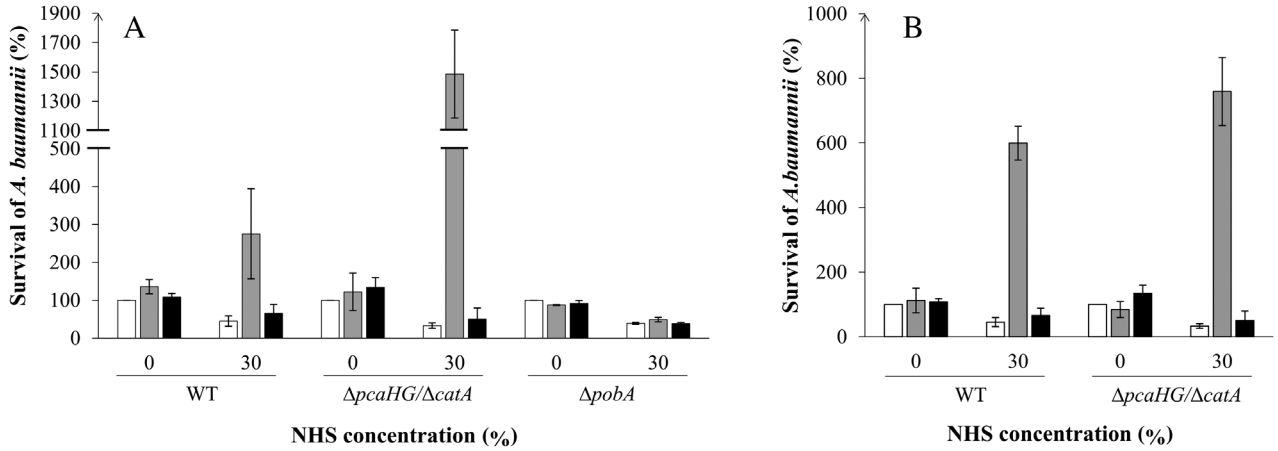

Fig. 4. *p*-hydroxybenzoate (A) and protocatechuate (B) induce serum resistance in *A. baumannii* AYE comMR. *A. baumannii* AYE comMR and the Δ*pcaHG*/Δ*catA* double mutant were grown in LB medium, harvested at an OD_{600} of 0.5–0.6, washed two times in $PBS^{+/+}$ and resuspended to a final OD_{600} of 0.4 in $PBS^{+/+}$. The frozen NHS was thawed on ice and diluted to a final concentration of 30% with $PBS^{+/+}$. 10 µl of the cell cultures were added to 200 µl $PBS^{+/+}$ or 30% NHS both without addition of any carbon source or supplemented with 5 mM *p*-hydroxybenzoate, 5 mM protocatechuate or 5 mM Na-acetate. After incubation over 2 h at 37°C, 790 µl $PBS^{+/+}$ were added to the reaction mixtures and several dilutions were prepared. 100 µl of the 10^{-2}, 10^{-3} and 10^{-4} dilutions were plated on LB agar and CFUs were counted. The CFUs are given in percentage in comparison to the CFUs of the cultures incubated with $PBS^{+/+}$ without any additional carbon source. A. CFUs of cultures which were incubated in the absence of an additional substrate are shown in white bars, cultures which were incubated in the presence of 5 mM *p*-hydroxybenzoate are shown in grey bars and those which were incubated in the presence of 5 mM Na-acetate are shown in black bars. B. CFUs of cultures which were incubated in the absence of an additional substrate are shown in white bars, cultures which were incubated in the presence of 5 mM protocatechuate are shown in grey bars and those which were incubated in the presence of 5 mM Na-acetate are shown in black bars. Each value is the mean of ±SEM of at least three independent measurements.

CFUs in the presence of *p*-hydroxybenzoate. Moreover, addition of *p*-hydroxybenzoate had no effect on the complement resistance of the deletion mutant, which leads to the conclusion that the accumulation of protocatechuate in the Δ*pcaHG*/Δ*catA* double mutant plays a role in complement resistance.

To test the direct effect of protocatechuate on the complement resistance of *A. baumannii* AYE comMR, we analyzed the complement resistance of *A. baumannii* AYE comMR and the Δ*pcaHG*/Δ*catA* double mutant in the presence of 5 mM protocatechuate (Fig. 4B). The presence of protocatechuate also significantly stimulated the complement resistance. However, the *A. baumannii* AYE comMR strain showed a decrease in CFU after incubation in 30% NHS alone or in 30% NHS plus 5 mM Na-acetate by 55% and 35% respectively, the addition of 5 mM protocatechuate to 30% NHS led to an increase of CFU by 500%. The same holds true for the Δ*pcaHG*/Δ*catA* double mutant, such as after incubation with 30% NHS alone or with 30% NHS supplemented with 5 mM Na-acetate the CFUs of the double mutant decreased by 75% and 50% respectively. After incubation in 30% NHS supplemented with 5 mM protocatechuate the CFUs of the double mutant increased by 650%. These results lead to the conclusion that protocatechuate indeed stimulates the complement resistance of *A. baumannii* AYE comMR. It is tempting to speculate that this increased complement resistance is due to regulatory effects, where protocatechuate is a potential signal molecule, but this has to be addressed in future studies.

The ß-ketoadipate pathway is important for antibiotic resistance or susceptibility

To get insights into the role of the *ß*-ketoadipate pathway in antibiotic resistance, the Δ*pcaHG* and the Δ*catA* single mutant, the Δ*pcaHG*/Δ*catA* double mutant and *A. baumannii* AYE comMR were subjected to antibiotic resistance assays using aromatic and non-aromatic antibiotics. The strains were cultivated in MM with 20 mM Na-acetate as carbon source to the late stationary phase and diluted with saline (0.9% NaCl) to a final OD_{600} of 1. Several dilutions were prepared and 5 µl per dilution were dropped onto MM with 20 mM Na-acetate, 5 mM benzoate and 5 mM *p*-hydroxybenzoate agar or onto MM agar containing the same carbon sources plus antibiotics such as 5 µg ml^{-1} novobiocin, 15 µg ml^{-1} benzalkonium chloride, 20 µg ml^{-1} chlorhexidine or 2 µg ml^{-1} kanamycin. Interestingly, *A. baumannii* AYE comMR showed a higher susceptibility against novobiocin than the deletion mutants (Fig. 5). However, the Δ*pcaHG* deletion mutant showed growth up to a dilution of 10^{-3} and the Δ*catA* and Δ*pcaHG*/Δ*catA* mutants even to a dilution of 10^{-5}, growth of *A. baumannii* AYE comMR was only detectable up to the 10^{-1} dilution. It is tempting to speculate that the aromatic antibiotic novobiocin is modified by the

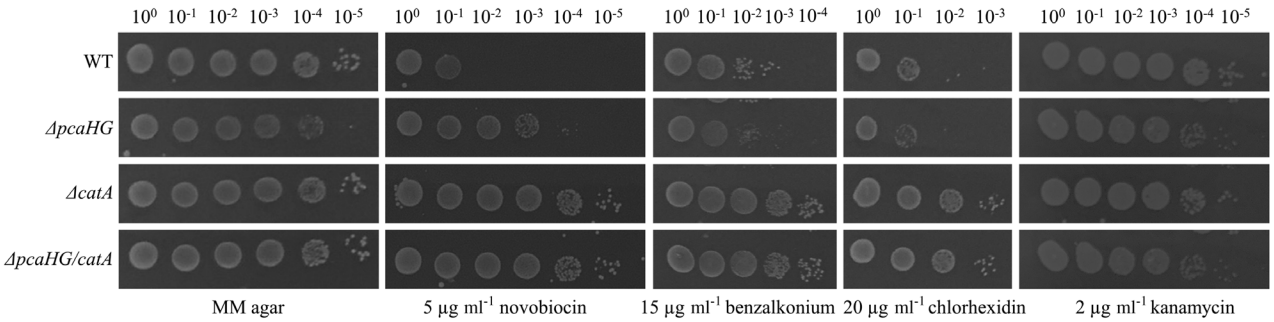

Fig. 5. Effect of Δ*pcaHG* and Δ*catA* deletions on antibiotic resistance. The *A. baumannii* AYE comMR wild type strain and the single and double mutants were incubated overnight at 37°C in MM with Na-acetate as carbon source and 5 mM benzoate and *p*-hydroxybenzoate to induce the *ß*-ketoadipate pathway. Bacterial cells were harvested and diluted in saline (0.9% NaCl) to a final OD_{600} of 1. Several dilutions were prepared and 5 µl of each dilution was dropped onto MM agar with Na-acetate as carbon source and 5 mM benzoate and *p*-hydroxybenzoate (Zeidler *et al*., 2017) containing different antibiotics followed by incubation overnight at 37°C. The experiments were repeated three times and one representative result is shown.

protocatechuate-3,4-diooxygenase (PcaHG) and the catechol-1,2-dioxygenase (CatA) leading to even higher toxic effects than novobiocin itself. The Δ*catA* deletion mutant and the Δ*pcaHG*/Δ*catA* double mutant showed a higher resistance against the aromatic antibiotics benzalkonium chloride and chlorhexidine than *A. baumannii* AYE comMR and the Δ*pcaHG* single mutant, such as growth was detectable up to a dilution of 10^{-4} in the case of benzalkonium and 10^{-3} in the presence of chlorhexidine, whereas *A. baumannii* AYE comMR and the Δ*pcaHG* single mutant showed growth only up to a dilution of 10^{-2} or 10^{-1} in the presence of both benzalkonium and chlorhexidine, respectively (Fig. 5). In the presence of the glycoside antibiotic kanamycin, no growth difference between *A. baumannii* AYE comMR and the mutants was detectable. Analogous results were obtained during growth of the strains on LB-agar plates with the given antibiotics or on MM agar with Na-acetate as sole carbon source in the presence of the given antibiotics (data not shown).

Conclusions

This work provides clear evidence that *pcaHG* and *catA* encode a functional protocatechuate-3,4-dioxygenase and catechol-1,2-dioxygenase, the two key enzymes of the two branches of the *ß*-ketoadipate pathway in *A. baumannii* AYE. Deletion of these two genes results in a decrease in complement resistance. It is tempting to speculate that aromatic substrates in the blood serum might be degraded to signal compounds via the *ß*-ketoadipate pathway and, thus lead to an increased survival of this bacterium in human serum. In the absence of these dioxygenases, the aromatic substances in the blood serum, such as hormones or aromatic amino acids are possibly not converted into such signal compounds and complement resistance is reduced. Interestingly, the Δ*pcaHG*/Δ*catA* mutant exhibits a dramatically increased complement resistance in the presence of *p*-hydroxybenzoate. This finding along with the finding that protocatechuate also significantly increased complement resistance together with the abolished increase in complement resistance of the Δ*pobA* mutant suggest that accumulation of protocatechuate enhances complement resistance of *A. baumannii* AYE comMR. The Δ*catA*, Δ*pcaHG* and Δ*pcaHG*/Δ*catA* mutants exhibited increased resistance against different aromatic antibiotics compared to the *A. baumannii* AYE comMR strain, whereas the resistance against the non-aromatic antibiotic was unaffected. This indicates that the key enzymes of the *ß*-ketoadipate pathway might modify the aromatic moieties of these aromatic antibiotics leading to increased toxicity. Whether this indeed is the case will be addressed in future studies.

Acknowledgements

This study was supported by a grant from the Deutsche Forschungsgemeinschaft through DFG Research Unit FOR2251 (DFG7-2). We thank Eleonora Mungo for generation of the Δ*pobA* mutant.

References

Allen, K.D., and Green, H.T. (1987) Hospital outbreak of multi-resistant *Acinetobacter anitratus*: an airborne mode of spread? *J Hosp Infect* **9**: 110–119.

Antunes, L.C., Imperi, F., Carattoli, A., and Visca, P. (2011) Deciphering the multifactorial nature of *Acinetobacter baumannii* pathogenicity. *PLoS One* **6**: e22674.

Arias-Barrau, E., Olivera, E.R., Luengo, J.M., Fernandez, C., Galan, B., Garcia, J.L., *et al*. (2004) The homogentisate pathway: a central catabolic pathway involved in the degradation of L-phenylalanine, L-tyrosine, and 3-hydroxyphenylacetate in *Pseudomonas putida*. *J Bacteriol* **186**: 5062–5077.

Bleichrodt, F.S., Fischer, R., and Gerischer, U.C. (2010) The *beta*-ketoadipate pathway of *Acinetobacter baylyi* undergoes carbon catabolite repression, cross-regulation and vertical regulation, and is affected by Crc. *Microbiology* **156**: 1313–1322.

Bleuven, C., and Landry, C.R. (2016) Molecular and cellular bases of adaptation to a changing environment in microorganisms. *Proc Biol Sci* **283**: 20161458.

Bouvet, P.J.M., and Grimont, P.A.D. (1986) Taxonomy of the genus *Acinetobacter* with the recognition of *Acinetobacter baumannii* sp. nov., *Acinetobacter haemolyticus* sp. nov., *Acinetobacter johnsonii* sp. nov., and *Acinetobacter junii* sp. nov. and emended descriptions of *Acinetobacter calcoaceticus* and *Acinetobacter lwoffii*. *Int J Syst Evol* **36**: 228–240.

Breisch, J., Waclawska, I., and Averhoff, B. (2018) Identification and characterization of a carnitine transporter in *Acinetobacter baumannii*. *Microbiologyopen* **8**: e00752.

Brzostowicz, P.C., Reams, A.B., Clark, T.J., and Neidle, E.L. (2003) Transcriptional cross-regulation of the catechol and protocatechuate branches of the *beta*-ketoadipate pathway contributes to carbon source-dependent expression of the *Acinetobacter* sp. strain ADP1 *pobA* gene. *Appl Environ Microbiol* **69**: 1598–1606.

Cain, R.B. (1968) Anthranilic acid metabolism by microorganisms. formation of 5-hydroxyanthranilate as an intermediate in anthranilate metabolism by *Norcardia opaca*. *Antonie Van Leeuwenhoek* **34**: 17–32.

Camarena, L., Bruno, V., Euskirchen, G., Poggio, S., and Snyder, M. (2010) Molecular mechanisms of ethanol-induced pathogenesis revealed by RNA-sequencing. *PLoS Pathog* **6**: e1000834.

Cerqueira, G.M., Kostoulias, X., Khoo, C., Aibinu, I., Qu, Y., Traven, A., and Peleg, A.Y. (2014) A global virulence regulator in *Acinetobacter baumannii* and its control of the phenylacetic acid catabolic pathway. *J Infect Dis* **210**: 46–55.

Costaglioli, P., Barthe, C., Claverol, S., Brözel, V.S., Perrot, M., Crouzet, M., *et al*. (2012) Evidence for the involvement of the anthranilate degradation pathway in Pseudomonas aeruginosa biofilm formation. *Microbiology* **1**: 326–339.

D'Argenio, D.A., Segura, A., Coco, W.M., Bunz, P.V., and Ornston, L.N. (1999) The physiological contribution of *Acinetobacter* PcaK, a transport system that acts upon protocatechuate, can be masked by the overlapping specificity of VanK. *J Bacteriol* **181**: 3505–3515.

Dal, S., Steiner, I., and Gerischer, U. (2002) Multiple operons connected with catabolism of aromatic compounds in *Acinetobacter* sp. strain ADP1 are under carbon catabolite repression. *J Mol Microbiol Biotechnol* **4**: 389–404.

Dal, S., Trautwein, G., and Gerischer, U. (2005) Transcriptional organization of genes for protocatechuate and quinate degradation from *Acinetobacter* sp. strain ADP1. *Appl Environ Microbiol* **71**: 1025–1034.

Dijkshoorn, L., Nemec, A., and Seifert, H. (2007) An increasing threat in hospitals: multidrug-resistant *Acinetobacter baumannii*. *Nat Rev Microbiol* **5**: 939–951.

Doughari, H.J., Ndakidemi, P.A., Human, I.S., and Benade, S. (2011) The ecology, biology and pathogenesis of *Acinetobacter* spp.: an overview. *Microbes Environ* **26**: 101–112.

Elsemore, D.A., and Ornston, L.N. (1995) Unusual ancestry of dehydratases associated with quinate catabolism in *Acinetobacter calcoaceticus*. *J Bacteriol* **177**: 5971–5978.

Fiester, S.E., and Actis, L.A. (2013) Stress responses in the opportunistic pathogen *Acinetobacter baumannii*. *Future Microbiol* **8**: 353–365.

Fischer, R., Bleichrodt, F.S., and Gerischer, U.C. (2008) Aromatic degradative pathways in *Acinetobacter baylyi* underlie carbon catabolite repression. *Microbiology* **154**: 3095–3103.

Gerischer, U., Segura, A., and Ornston, L.N. (1998) PcaU, a transcriptional activator of genes for protocatechuate utilization in *Acinetobacter*. *J Bacteriol* **180**: 1512–1524.

Godeux, A.S., Svedholm, E., Lupo, A., Haenni, M., Venner, S., Laaberki, M.H., and Charpentier, X. (2020) Scarless removal of large resistance Island AbaR results in antibiotic susceptibility and increased natural transformability in *Acinetobacter baumannii*. *Antimicrob Agents Chemother* **64**: e00951-00920.

Guardabassi, L., Petersen, A., Olsen, J.E., and Dalsgaard, A. (1998) Antibiotic resistance in *Acinetobacter* spp. isolated from sewers receiving waste effluent from a hospital and a pharmaceutical plant. *Appl Environ Microbiol* **64**: 3499–3502.

Harding, C.M., Hennon, S.W., and Feldman, M.F. (2018) Uncovering the mechanisms of *Acinetobacter baumannii* virulence. *Nat Rev Microbiol* **16**: 91–102.

Harwood, C.S., and Parales, R.E. (1996) The *ß*-ketoadipate pathway and the biology of self-identity. *Annu Rev Microbiol* **50**: 553–590.

Hubloher, J.J., Zeidler, S., Lamosa, P., Santos, H., Averhoff, B., and Müller, V. (2020) Trehalose-6-phosphate-mediated phenotypic change in *Acinetobacter baumannii*. *Environ Microbiol* **22**: 5156–5166.

Jawad, A., Heritage, J., Snelling, A.M., Gascoyne-Binzi, D. M., and Hawkey, P.M. (1996) Influence of relative humidity and suspending menstrua on survival of *Acinetobacter* spp. on dry surfaces. *J Clin Microbiol* **34**: 2881–2887.

König, P., Averhoff, B., and Müller, V. (2021) K^+ and its role in virulence of *Acinetobacter baumannii*. *Int J Med Microbiol* **311**: 151516.

Kurnasov, O., Jablonski, L., Polanuyer, B., Dorrestein, P., Begley, T., and Osterman, A. (2003) Aerobic tryptophan degradation pathway in bacteria: novel kynurenine formamidase. *FEMS Microbiol Lett* **227**: 219–227.

Lee, C.R., Lee, J.H., Park, M., Park, K.S., Bae, I.K., Kim, Y. B., *et al*. (2017) Biology of *Acinetobacter baumannii*: pathogenesis, antibiotic resistance mechanisms, and prospective treatment options. *Front Cell Infect Microbiol* **7**: 55.

Liu, X., Dong, Y., Li, X., Ren, Y., Li, Y., Wang, W., *et al*. (2010) Characterization of the anthranilate degradation pathway in *Geobacillus thermodenitrificans* NG80-2. *Microbiology* **156**: 589–595.

Lucidi, M., Runci, F., Rampioni, G., Frangipani, E., Leoni, L., and Visca, P. (2018) New shuttle vectors for gene cloning and expression in multidrug-resistant *Acinetobacter* species. *Antimicrob Agents Chemother* **62**: e02480-02417.

Mitchell, A., Romano, G.H., Groisman, B., Yona, A., Dekel, E., Kupiec, M., *et al*. (2009) Adaptive prediction of environmental changes by microorganisms. *Nature* **460**: 220–224.

Müller, S., Janssen, T., and Wieler, L.H. (2014) Multidrug resistant *Acinetobacter baumannii* in veterinary medicine-emergence of an underestimated pathogen? *Berl Munch Tierarztl Wochenschr* **127**: 435–446.

Peleg, A.Y., Seifert, H., and Paterson, D.L. (2008) *Acinetobacter baumannii*: emergence of a successful pathogen. *Clin Microbiol Rev* **21**: 538–582.

Roca, I., Espinal, P., Vila-Farres, X., and Vila, J. (2012) The *Acinetobacter baumannii* oxymoron: commensal hospital dweller turned pan-drug-resistant menace. *Front Microbiol* **3**: 148.

Schühle, K., Jahn, M., Ghisla, S., and Fuchs, G. (2001) Two similar gene clusters coding for enzymes of a new type of aerobic 2-aminobenzoate (anthranilate) metabolism in the bacterium *Azoarcus evansii*. *J Bacteriol* **183**: 5268–5278.

Siehler, S.Y., Dal, S., Fischer, R., Patz, P., and Gerischer, U. (2007) Multiple-level regulation of genes for protocatechuate degradation in *Acinetobacter baylyi* includes cross-regulation. *Appl Environ Microbiol* **73**: 232–242.

Smith, M.R. (1990) The biodegradation of aromatic hydrocarbons by bacteria. *Biodegradation* **1**: 191–206.

Stahl, J., Bergmann, H., Göttig, S., Ebersberger, I., and Averhoff, B. (2015) *Acinetobacter baumannii* virulence is mediated by the concerted action of three phospholipases D. *PLoS One* **10**: e0138360.

Stanier, R.Y., Hayaishi, O., and Tsuchida, M. (1951) The bacterial oxidation of tryptophan. I. A general survey of the pathways. *J Bacteriol* **62**: 355–366.

Sze, I.S., and Dagley, S. (1987) Degradation of substituted mandelic acids by *meta* fission reactions. *J Bacteriol* **169**: 3833–3835.

Teufel, R., Mascaraque, V., Ismail, W., Voss, M., Perera, J., Eisenreich, W., *et al*. (2010) Bacterial phenylalanine and phenylacetate catabolic pathway revealed. *Proc Natl Acad Sci U S A* **107**: 14390–14395.

Towner, K.J. (2009) *Acinetobacter*: an old friend, but a new enemy. *J Hosp Infect* **73**: 355–363.

Trautwein, G., and Gerischer, U. (2001) Effects exerted by transcriptional regulator PcaU from *Acinetobacter* sp. strain ADP1. *J Bacteriol* **183**: 873–881.

Tucker, A.T., Nowicki, E.M., Boll, J.M., Knauf, G.A., Burdis, N.C., Trent, M.S., and Davies, B.W. (2014) Defining gene-phenotype relationships in *Acinetobacter baumannii* through one-step chromosomal gene inactivation. *mBio* **5**: e01313-01314.

van der Kolk, J.H., Endimiani, A., Graubner, C., Gerber, V., and Perreten, V. (2019) *Acinetobacter* in veterinary medicine, with an emphasis on *Acinetobacter baumannii*. *J Glob Antimicrob Resist* **16**: 59–71.

Vasudevan, N., and Mahadevan, A. (1992) Degradation of non-phenolic *beta*-o-4 lignin substructure model compounds by *Acinetobacter* sp. *Res Microbiol* **143**: 333–339.

Wareth, G., Neubauer, H., and Sprague, L.D. (2019) *Acinetobacter baumannii* - a neglected pathogen in veterinary and environmental health in Germany. *Vet Res Commun* **43**: 1–6.

Wendt, C., Dietze, B., Dietz, E., and Rüden, H. (1997) Survival of *Acinetobacter baumannii* on dry surfaces. *J Clin Microbiol* **35**: 1394–1397.

Williams, P.A., and Kay, C.M. (2008) The catabolism of aromatic compounds by *Acinetobacter*. In *Acinetobacter Molecular Biology*. Norfolk, UK: Caister Academic Press, pp. 99–118.

Young, D.M., Parke, D., and Ornston, L.N. (2005) Opportunities for genetic investigation afforded by *Acinetobacter baylyi*, a nutritionally versatile bacterial species that is highly competent for natural transformation. *Annu Rev Microbiol* **59**: 519–551.

Zeidler, S., Hubloher, J., Schabacker, K., Lamosa, P., Santos, H., and Müller, V. (2017) Trehalose, a temperature- and salt-induced solute with implications in pathobiology of *Acinetobacter baumannii*. *Environ Microbiol* **19**: 5088–5099.

Zeidler, S., and Müller, V. (2019) Coping with low water activities and osmotic stress in *Acinetobacter baumannii*: significance, current status and perspectives. *Environ Microbiol* **21**: 2212–2230.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1: Supporting information