Trehalose-6-phosphate-mediated phenotypic change in Acinetobacter baumannii

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Summary

The stress protectant trehalose is synthesized in Acinetobacter baumannii from the OtsA/OtsB pathway. Previous studies proved that deletion of otsB led to a decreased virulence, the inability to grow at 45°C and a slight reduction of growth at high salinities indicating that trehalose is the cause of these phenotypes. We have questioned this conclusion by producing ΔotsA and ΔotsBA mutants and studying their phenotypes. Only deletion of otsB, but not deletion of otsA or otsBA, led to growth impairments at high salt and high temperature. The intracellular concentrations of trehalose and trehalose-6-phosphate were measured by NMR or enzymatic assay. Interestingly, none of the mutants accumulated trehalose any more but the ΔotsB mutant with its defect in trehalose-6-phosphate phosphatase activity accumulated trehalose-6-phosphate. Moreover, expression of otsA in a ΔotsB background under conditions where trehalose synthesis is not induced led to growth inhibition and the accumulation of trehalose-6-phosphate. Our results demonstrate that trehalose-6-phosphate affects multiple physiological activities in A. baumannii ATCC 19606.

Introduction

Members of the genus Acinetobacter are Gram-negative rods thriving in diverse ecosystems and even extremophilic strains growing at high salinity and/or UV light are known (Albarracin et al., 2012; Kurth et al., 2015). Environmental strains are well known for their metabolic diversity and, in particular, for their ability to oxidize aromatic compounds by a specialized pathway, the ß-ketoadipate pathway (Stainer and Ornston, 1973). The biochemistry and regulation of this pathway have been well studied in Acinetobacter baylyi (Stainer and Ornston, 1973; Neidle and Ornston, 1986; Brzostowicz et al., 2003). Some Acinetobacter species such as Acinetobacter baumannii are opportunistic human pathogens, whose natural habitat is still unknown (Antunes et al., 2014). They have gained much attraction since the Iraq and Afghanistan war 2002–2004 when many soldiers were infected with A. baumannii (Centers for Disease Control and Prevention, 2004). Since then, A. baumannii has started a triumphal march and has become a major threat in health care institutions worldwide (Dijkshoorn et al., 2007; Averhoff, 2015). Over the last decade, infections with multidrug-resistant (MDR) A. baumannii have steadily increased (Dijkshoorn et al., 2007; Perez et al., 2007; Peleg et al., 2008) and, therefore, the World Health Organization has placed carbapenem-resistant A. baumannii on position 1 (together with carbapenem-resistant Pseudomonas aeruginosa, and carbapenem-resistant, ESBL-producing Enterobacteriaceae) at its ‘critical’ level in February 2017, to prioritize research and development efforts for new antimicrobial treatment (World Health Organization, 2017).

Unlike other pathogens, A. baumannii does not produce toxins (Peleg et al., 2008; Antunes et al., 2014; Harding et al., 2018). Instead, A. baumannii possesses virulence factors that confer adaptation to the human host. These virulence factors mediate cell adhesion and invasion, iron uptake, acquisition of multiple carbon and energy sources, capsule or biofilm formation (Farrugia et al., 2013; Stahl et al., 2015; Chapartegui-González et al., 2018; Ramirez et al., 2019; Runci et al., 2019; Singh et al., 2019; Weidensdorfer et al., 2019). Apart from the adaptation to the human host A. baumannii withstands dry conditions for a long time and is able to grow at high salt concentrations and high osmolarities in general (Zeidler et al., 2017; Chiang et al., 2018; Farrow et al., 2018; Zeidler et al., 2018; Zeidler and
Müller, 2019b). The unravelling of the molecular basis of desiccation- and osmo-resistance has just begun. Life in high salt, high osmolarity and dry environments has a common feature: a low water activity of the environment (Vriezen et al., 2007; Zeidler and Müller, 2019a). Since biological membranes are permeable to water, water is pulled out of the cells at low water activities in the environment, cells shrink and die, if no countermeasures are taken (Roeßler and Müller, 2001; Bremer and Krämer, 2019). The action that Acinetobacter species such as A. baumannii or A. baylyi take is the accumulation of compatible solutes inside the cell (Sand et al., 2013; Zeidler et al., 2017; Zeidler et al., 2018). Compatible solutes such as glycine betaine or its precursor choline are taken up from the environment, or synthesized de novo such as glutamate or mannitol (Sand et al., 2013; Scholz et al., 2016; Zeidler et al., 2017; Zeidler et al., 2018). The pathways involved and their regulation has been reviewed recently (Zeidler and Müller, 2019a).

In addition to glutamate and mannitol, A. baumannii synthesizes trehalose as a response to salt and heat stress, albeit in very low amounts (Zeidler et al., 2017) and therefore, trehalose has been overlooked for some time. Trehalose is synthesized by A. baumannii by a two-step process. First, OtsA, a trehalose-6-phosphate synthase, condenses glucose-6-phosphate and UDP-glucose to trehalose-6-phosphate (Tre-6-P), which is dephosphorylated by OtsB, a trehalose-6-phosphate phosphatase to trehalose and inorganic phosphate (Zeidler et al., 2017). Expression of otsB is salt- and heat-stimulated and a ΔotsB mutant no longer accumulates trehalose and no longer grows at high temperatures (Zeidler et al., 2017). Growth at high salinities is marginally affected, which is consistent with the observation that trehalose is only a minor compatible solute. Trehalose is not only known to protect bacteria against osmotic stresses, but also it is a multifunctional molecule that protects bacteria against various stresses like desiccation, temperature stress and confers drug resistance (Welsh and Herbert, 1999; Elbein et al., 2003; Reina-Bueno et al., 2012; Lee et al., 2019). Most interestingly, a ΔotsB mutant is no longer able to infect Galleria mellonella, a moth model to study infection of eukaryotes by A. baumannii (Gebhardt et al., 2015). One of the obvious open questions was whether the lack of trehalose or a possible accumulation of Tre-6-P is the cause of the observed phenotypes. To address this important question, we have constructed a ΔotsA mutant and a ΔotsB/otsA double mutant and studied their phenotypes in comparison to the ΔotsB mutant.

Results

Intracellular trehalose concentrations are increased in arabinose-grown cells

Since the amount of trehalose produced during growth on succinate at 37°C was very small, we aimed to find a substrate leading to higher trehalose yields. Arabinose is a known carbon and energy source for A. baumannii ATCC 19606 and growth on succinate or arabinose in mineral medium was comparable (Fig. 1). At 500 mM NaCl, growth on arabinose was much faster than growth on succinate at 37°C, indicating that arabinose-grown cells can cope better with high salinities than succinate-grown cells. Indeed, the intracellular concentration of trehalose increased up to 0.12 μmol mg⁻¹ protein and was fourfold higher than in succinate-grown cells (0.03 μmol mg⁻¹ protein in the late stationary phase). Moreover, even in the absence of additional NaCl, arabinose-grown cells produced trehalose, albeit in very small amounts and only in the late stationary phase (0.008 μmol mg⁻¹ protein). We assume that cells grown on the sugar arabinose may have an energetic benefit over succinate-grown cells. This benefit seems to be used for trehalose synthesis in the late stationary phase.

Construction of ΔotsA and ΔotsBA mutants

Acinetobacter baumannii ATCC 19606 accumulates trehalose via the OtsAB pathway. As described previously deletion of otsB results in the inability to accumulate trehalose under osmotic stress and heat (Zeidler et al., 2017). To address the role of OtsA, a markerless
\( \Delta \text{otsA} \) and \( \Delta \text{otsA/otsB} \) double mutant was generated using the established insertion-duplication procedure (Stahl \textit{et al.}, 2015). Deletion of \( \text{otsA} \) or \( \text{otsBA} \) was confirmed by sequencing of the PCR product obtained with the primer pair \( \text{otsBA}_{\text{locus}} \) (Fig. 2).

A \( \Delta \text{otsB} \) mutant accumulates Tre-6-P and is impaired in growth at high salt and high temperature, whereas \( \Delta \text{otsA} \) and \( \Delta \text{otsBA} \) mutants do not exhibit these two phenotypes.

In previous experiments with cells grown on succinate at 37°C, little trehalose was produced upon osmotic upshift and a \( \Delta \text{otsB} \) mutant growing at 500 mM NaCl had only a slightly reduced growth rate of 0.08 h\(^{-1} \) (td = 8.3 h) compared to 0.13 h\(^{-1} \) of the wild type (td = 5.2 h). However, the phenotype of the \( \Delta \text{otsB} \) mutant was much more pronounced during growth on arabinose. Deletion of \( \text{otsB} \) caused a prolonged lag phase during the adaptation on mineral medium with arabinose as sole energy and carbon source and the addition of 300 mM NaCl largely impaired growth (Fig. 3). In contrast, the \( \Delta \text{otsA} \) and the \( \Delta \text{otsB/otsA} \) mutants had no growth phenotype, neither at low nor at high NaCl (Fig. 3). To complement the \( \Delta \text{otsB} \) mutant, \( \text{otsB} \) was integrated into the genome by the use of a RecAB-recombineering system. In addition, upstream of \( \text{otsB} \) we inserted the putative \( \text{otsBA} \) promoter. The complemented strain \( \Delta \text{otsB}_{\text{P}_{\text{otsBA}}}, \text{otsB} \) exhibited a growth rate at high salt comparable to the wild-type strain (Fig. 3). This provides evidence that the impaired growth rate of the \( \Delta \text{otsB} \) mutant at high salt is due to the \( \text{otsB} \) deletion.

To determine trehalose and Tre-6-P concentrations, solutes were extracted from the cells with ethanol-chloroform, identified by NMR and quantified. As seen before, wild-type cells accumulated trehalose, whereas neither the \( \Delta \text{otsB} \) nor the \( \Delta \text{otsA} \) and \( \Delta \text{otsB/otsA} \) mutants did (Fig. 4A). The \( \Delta \text{otsB} \) mutant accumulated Tre-6-P, however the \( \Delta \text{otsA} \) or \( \Delta \text{otsB/otsA} \) double mutant did not (Fig. 4B). Notably, the Tre-6-P concentration in the \( \Delta \text{otsB} \) mutant was 25-fold higher than the trehalose concentration in wild-type cells. When the \( \Delta \text{otsB} \) mutant was complemented with \( \text{otsB} \) trehalose was again accumulated (Fig. 4C) and Tre-6-P accumulation was nearly abolished (Fig. 4D). If the impaired growth phenotype of the \( \Delta \text{otsB} \) mutant at high salt is caused by accumulation of Tre-6-P, breakdown of Tre-6-P by another enzyme should restore the wild type phenotype. To address this question, \( \text{treC} \), encoding a trehalose-6-phosphate hydrolase from \textit{Escherichia coli} (Rimmele and Boos, 1994), was cloned into the \( \Delta \text{otsB} \) mutant by using the RecAB-recombineering system. The \( \Delta \text{otsB}_{\text{P}_{\text{otsBA}}}, \text{treC} \) mutant grew like the wild type strain on arabinose (Fig. 3) and Tre-6-P was no longer

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**Fig 2.** Genetic organization of the otsBA locus in \( A. \text{baumannii} \) ATCC 19606 (A) the \( \Delta \text{otsB} \) mutant (B) and the strains \( \Delta \text{otsB}_{\text{P}_{\text{otsBA}}}, \text{otsB} \) (C) and \( \Delta \text{otsB}_{\text{P}_{\text{otsBA}}}, \text{treC} \) (D). The otsBA locus was amplified in \( A. \text{baumannii} \) ATCC 19606 and the \( \Delta \text{ots} \) mutants via PCR using the primer pair \( \text{otsBA}_{\text{locus}} \) (E). [Color figure can be viewed at wileyonlinelibrary.com]
accumulated (Fig. 4D). This suggests that indeed the growth phenotype of the ΔotsB mutant was due to the accumulation of Tre-6-P.

As originally described, the ΔotsB mutant did not grow at 45°C in mineral medium with arabinose and increasing NaCl concentrations. A. baumannii ATCC19606 (○), ΔotsA (○), ΔotsBA (▽), ΔotsB (△), ΔotsB_PotsBA_otsB (○) and ΔotsB_PotsBA_treC (○) were inoculated from a preculture grown in mineral medium with arabinose into mineral medium with arabinose without (A) or with the addition of 300 mM NaCl (B). All cultivations were performed at 37°C. The standard error of the mean was calculated from three independent experiments.

Fig 3. Effect of deletion of the ots genes in A. baumannii ATCC 19606 on growth in mineral medium with arabinose and increasing NaCl concentrations. A. baumannii ATCC19606 (○), ΔotsA (○), ΔotsBA (▽), ΔotsB (△), ΔotsB_PotsBA_otsB (○) and ΔotsB_PotsBA_treC (○) were inoculated from a preculture grown in mineral medium with succinate as sole carbon and energy source. The precultures were used to inoculate fresh mineral medium with arabinose as sole energy and carbon source and 300 mM NaCl. After 10 h of growth the intracellular trehalose and Tre-6-P concentration of the strains was analysed via NMR (A + B) or with an enzymatic assay (C + D). The standard deviation was calculated from three independent experiments.

Fig 4. Intracellular accumulation of trehalose and Tre-6-P in A. baumannii ATCC 19606 and the ots strains during growth in mineral medium with arabinose and salt stress. A. baumannii ATCC 19606, the Δots mutants, ΔotsB_PotsBA_otsB and ΔotsB_PotsBA_treC were grown overnight in mineral medium with succinate as sole carbon and energy source. The precultures were used to inoculate fresh mineral medium with succinate and with the addition of 300 mM NaCl. After 10 h of growth the intracellular trehalose and Tre-6-P accumulation were analysed (Fig. 6B). As expected, complementation with otsB restored the ability to synthesize trehalose while integration of treC abolished Tre-6-P accumulation but did not restore trehalose accumulation (Fig. 6A). In addition, these experiments clearly demonstrate that the ΔotsB mutant accumulates Tre-6-P and provides evidence that the impaired growth phenotype of the ΔotsB mutant at high salt or high temperature is not due to the lack of trehalose but due to the accumulation of Tre-6-P.
Overexpression of otsA leads to accumulation of Tre-6-P and growth inhibition

The data presented so far led to the conclusion that accumulation of Tre-6-P causes growth inhibition. To further underline this conclusion, we generated strains that conditionally express the ots genes. We assumed that expression of otsA should result in Tre-6-P production. Therefore, the ΔotsB mutant was transformed with the plasmids pVRL2_P_ara_otsA, pVRL2_P_ara_otsB and pVRL2_P_ara_otsBA. These plasmids encode the ots genes (otsA, otsB or otsBA) under transcriptional control of an arabinose-inducible promoter. This resulted in three strains producing OtsA, OtsB or OtsBA upon induction by arabinose. The strains were grown overnight in mineral medium with succinate at 37°C and low NaCl to avoid expression of ots genes. These precultures were then used to inoculate mineral medium containing 1, 2 or 4% arabinose. Growth of the ΔotsB mutant was only marginally affected under these conditions, since the inducer of gene expression (heat or high NaCl) was lacking (Fig. 3A). However, the strains expressing otsA from the arabinose inducible promoter had a pronounced growth phenotype. At 1% the growth rate was 0.24 h⁻¹ compared with 0.58 h⁻¹ for the wild type grown on arabinose at low NaCl. Higher arabinose concentrations inhibited growth even more (μ = 0.034 h⁻¹ at 2% and μ = 0.024 h⁻¹ at 4%) (Fig. 7). Expression of otsA in the ΔotsB mutant led to the synthesis of Tre-6-P and the level of Tre-6-P increased with the arabinose concentration. There was no Tre-6-P accumulated in the strains ΔotsB + pVRL2_P_ara_otsB and ΔotsB + pVRL2_P_ara_otsBA expressing otsB or otsBA upon induction by arabinose (Fig. 8). These data are in line with our conclusion that accumulation of Tre-6-P leads to growth inhibition and is the cause of the phenotype of the ΔotsB mutant.

Discussion

In this study, we have provided compelling evidence that the growth-impaired phenotype at high temperature or high salt of the ΔotsB mutant of A. baumannii ATCC 19606 is not caused by the lack of trehalose accumulation but instead by the accumulation of Tre-6-P. For a long time, Tre-6-P was thought to only be an intermediate of a metabolic pathway without any further physiological implications. However, genetic studies revealed that Tre-6-P plays a regulatory role in plants (Figueroa and Lunn, 2016) as well as in fungi and nematodes (Borgia et al., 1996; Kormish and McGhee, 2005; Deroover et al., 2016; Thammahong et al., 2017). Furthermore, there is a report of Tre-6-P-mediated regulation in bacteria, in the human pathogen Mycobacterium tuberculosis (Korte and Alber, 2016). Regulation by Tre-6-P can occur either through the absence of Tre-6-P or its hyperaccumulation. In plants and yeast Tre-6-P is
Tre-6-P in A. baumannii

thought to be a signal molecule and a global regulator during development and sugar metabolism (Derroover et al., 2016; Figueroa and Lunn, 2016). Arabidopsis thaliana, for example, uses Tre-6-P to sense the availability of sucrose within the cell (Lunn et al., 2006). Deletion of the trehalose-6-phosphate synthase gene – and thus depletion of Tre-6-P – causes dramatic effects and interferes with growth and development (Zaragoza et al., 1998; van Vaeck et al., 2001; Eastmond et al., 2002; Gómez et al., 2006; Figueroa and Lunn, 2016; Thammahong et al., 2017; Vicente et al., 2018). In A. baumannii ATCC 19606, this is obviously different. We could not observe any growth defects of the ΔotsA or ΔotsBA mutant – both lacking the ability to synthesize Tre-6-P - providing evidence that Tre-6-P is not used as an essential signalling molecule during growth and development in A. baumannii ATCC 19606 or at least not under the tested conditions. In contrast, hyperaccumulation of Tre-6-P causes growth defects in A. baumannii ATCC 19606. A very similar observation was made in fungi. Deletion of the trehalose-6-phosphate phosphatase genes in Saccharomyces cerevisiae and Aspergillus nidulans caused a heat shock-dependent accumulation of Tre-6-P which was accompanied with growth inhibition (Piper and Lockheart, 1988; Borgia et al., 1996). Homozygote deletion of the two Tre-6-P phosphatase genes (Δtps2Δtps2) in Candida albicans also caused a heat sensitive phenotype and sensitivity against oxidative stress (Martinez-Esparza et al., 2009) and it was postulated that the heat sensitive phenotype was caused by Tre-6-P accumulation (Martinez-Esparza et al., 2009). Most of these studies argue for a growth inhibition due to Tre-6-P toxicity alone. This is also reported for M. tuberculosis, where the Tre-6-P phosphatase gene (otsB2) is essential and already very low amounts of Tre-6-P are highly toxic (Korte and Alber, 2016). In contrast, disruption of the Tre-6-P phosphatase gene (otrA) in A. nidulans causes Tre-6-P accumulation already at low temperature while growth was only inhibited at elevated temperatures (42°C) (Borgia et al., 1996). These findings pointed out that at least in the case of A. nidulans a simple toxicity of Tre-6-P cannot explain the temperature-dependent phenotype of the mutant. A similar observation was made in A. baumannii ATCC 19606 upon osmotic stress with regard to the carbon and energy source. Deletion of otsB caused no effect on growth in mineral medium with succinate and the addition of high amounts of sodium chloride (500 mM) caused only slight reduction of growth in comparison to the wild type (Zeidler et al., 2017). In contrast, the ΔotsB mutant was slightly impaired during growth on mineral medium with arabinose and the addition of sodium chloride (300 mM) abolished growth. These differences can be explained by a toxic effect of Tre-6-P that is dependent on the presence of special interaction partners, for example, enzymes that are involved in arabinose degradation.
The molecular mechanism behind Tre-6-P regulations is poorly understood. In *M. tuberculosis*, gene expression is strongly changed upon Tre-6-P accumulation. Silencing of *otsB2* in *M. tuberculosis* resulted in Tre-6-P accumulation and global changes in expression (877 upregulated and 37 downregulated genes) (Korte and Alber, 2016). Accumulation of the toxic sugar phosphate maltose-1-phosphate (Mal-1-P) in *M. tuberculosis* caused by deletion of the maltosyltransferase gene (*glgE*) was also associated with alterations in gene expression (Kalscheuer et al., 2010). Surprisingly, the overlap in alterations of gene expression dependent on Tre-6-P or Mal-1-P was small and restricted to upregulation of only a few genes involved in arginine synthesis and DNA damage-inducible genes (Kalscheuer et al., 2010; Korte and Alber, 2016). This comparison provides evidence that alterations in gene expression caused by Tre-6-P are quite specific (Korte and Alber, 2016).

However, there are also reports of Tre-6-P interaction with enzymes that are not involved in gene expression. The major hexokinase from *S. cerevisiae* is reported to be competitively inhibited by Tre-6-P *in vitro*. Even though *A. baumannii* ATCC 19606 does not encode a hexokinase a comparable effect could also be possible. Inhibition of enzymes that convert glucose-6-P or derivatives could also cause growth inhibition and could explain differences during growth on different carbon and energy sources.

In the human pathogen, *Aspergillus fumigatus* deletion of the Tre-6-P phosphatase gene abolished virulence (Puttikamonkul et al., 2010). Up to now the only studies that revealed an effect of trehalose genes on infection by *A. baumannii* AB5075 where those of Gebhardt et al. (2015). They observed that *otsB* belongs to the genes that are required for growth in *G. mellonella* larvae and that deletion of *otsB* results in an avirulent phenotype in *G. mellonella*. Interestingly, *otsA* did not belong to the genes that are required for growth in *G. mellonella* larvae (Gebhardt et al., 2015), arguing for a role of Tre-6-P in infection. Unfortunately, no infection studies with an *otsA* mutant were performed so far and the effect of Tre-6-P and trehalose during infection has to be studied in further experiments.

### Experimental procedures

**Bacterial strain and culture conditions**

Bacterial strains used in this study are listed in Table 1. Bacteria were either grown in LB-media (Bertani, 1951) or in phosphate-buffered mineral medium (Zeidler et al., 2017) at 37°C or 45°C as specified in the experiments. Succinate or arabinose (20 mM each) was added as sole carbon and energy source. Growth experiments were performed in 500 ml Erlenmeyer flasks filled with 100 ml medium. Sodium chloride was added as indicated. Overnight cultures were then used to inoculate fresh media to an initial OD₆₀₀ₙ₉ of 0.1. Growth was monitored photometrically by measuring the optical density at 600 nm. The *ots* expression strains were grown in presence of 100 µg ml⁻¹ gentamicin.

### Markerless mutagenesis

Markerless deletion of *otsA* (HMPREF0010_01305) or *otsBA* (HMPREF0010_01305 + HMPREF0010_01306) was performed using a *sacB*kanR cassette as described before (Stahl et al., 2015). A 1500 bp DNA fragment spanning the region upstream the gene of interest and a 1500 bp fragment downstream of the gene of interest was amplified via PCR and cloned into pBIISK_ sacB_kanR vector using Gibson assembly, according to the instructions of the manufacturer (Gibson Assembly Master Mix, New England Biolabs, Ipswich, MA, USA) (primers used are listed in the Supporting Information Table S1). The resulting recombinant plasmids were used to transform *A. baumannii* ATCC 19606 via electroporation (2.5 kV, 200 Ω and 25 μF). Transformants were selected on LB agar (1.8%) containing 50 µg ml⁻¹ kanamycin. Counterselection was achieved by growing the strains in LB + 10% sucrose. Clones were screened for the loss of kanamycin resistance and deletion mutants were verified via PCR. The resulting PCR products were sequenced.

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**Table 1. Bacterial strains used in this study.**

| Strain | References |
|--------|------------|
| *Escherichia coli* DH5α | Invitrogen™, USA |
| *Escherichia coli* K12 | Invitrogen™, USA |
| *Acinetobacter baumannii* ATCC 19606 | ATCC, USA |
| *Acinetobacter baumannii* ATCC 19606 ∆otsB | (Zeidler et al., 2017) |
| *Acinetobacter baumannii* ATCC 19606 ∆otsA | This study |
| *Acinetobacter baumannii* ATCC 19606 ∆otsBA | This study |
| *Acinetobacter baumannii* ATCC 19606 ∆otsB + PAT04 | This study |
| *Acinetobacter baumannii* ATCC 19606 ∆otsB_P_vRL2_P_vRL2_otsB | This study |
| *Acinetobacter baumannii* ATCC 19606 ∆otsB_P_vRL2_P_vRL2_otsA | This study |
| *Acinetobacter baumannii* ATCC 19606 ∆otsA_P_vRL2_P_vRL2_otsA | This study |
| *Acinetobacter baumannii* ATCC 19606 ∆otsB | This study |
| *Acinetobacter baumannii* ATCC 19606 ∆otsB_P_vRL2_P_vRL2_otsB | This study |
| *Acinetobacter baumannii* ATCC 19606 ∆otsB_P_vRL2_P_vRL2_otsA | This study |
| *Acinetobacter baumannii* ATCC 19606 ∆otsA_P_vRL2_P_vRL2_otsA | This study |
Generating the inserts for RecAB-mediated gene editing

Insertion of otsB or treC into the genome of the ΔotsB mutant was done in cis using the RecAB-recombineering system (Tucker et al., 2014). Therefore, two recombinant plasmids pBIISK_up_P_otsBA_otsB_kanR_down and pBIISK_updO_P_otsBA_treC_kanR_down were generated.

To generate pBIISK_updO_P_otsBA_otsB_kanR, we amplified otsB and 338 bp preceding to otsB start codon (coding for the native promoter P_otsBA) from the genome of A. baumannii ATCC 19606 using the primer pair otsB_inkl_upstream. A kanamycin resistant marker was amplified from the plasmid pKD4 with the primer pair Kan_comp_fwd. The 300 bp flanking the distinct insertion site (393 bp upstream of otsA) for recombineering were amplified from the genome of A. baumannii ATCC 19606 using the primer pairs Upstream_comp and Downstream_comp. The resulting PCR products were cloned into the vector pBIISK using Gibson assembly (Gibson Assembly Master Mix, New England Biolabs, Ipswich, MA, USA) resulting in the plasmid pBIISK_up_P_otsBA_otsB_kanR_down.

To generate pBIISK_updO_P_otsBA_treC_kanR_down, we amplified the plasmid pBIISK_updO_P_otsBA_otsB_kanR without otsB using the primer pair pBIISK_P_otsB_otsB. TreC was amplified from genomic DNA from E. coli K12 using the primer pair treC. The resulting fragments were assembled using Gibson assembly (Gibson Assembly Master Mix, New England Biolabs) resulting in the plasmid pBIISK_up_P_otsBA_treC_kanR_down.

The two recombinant plasmids pBIISK_up_P_otsBA_otsB_kanR_down and pBIISK_updO_P_otsBA_treC_kanR_down were used as templates in a PCR using the primer pair Linear PCR fragment. The PCR fragments (P_otsBA_otsB_kanR or P_otsBA_treC_kanR) harbouring the otsBA promoter, otsB (or treC) and the kanamycin-cassette in a row – flanked by 125 bp upstream and downstream of the insertion region (393 bp upstream of otsA). The PCR fragments were then recombined into the genome of A. baumannii ΔotsB using the RecAB system.

RecAB-mediated recombineering for gene editing

We used recombineering as genetic tool not for gene deletion as originally described (Tucker et al., 2014) but for gene insertion. To this end, we inserted P_otsBA_otsB_kanR (or P_otsBA_treC_kanR) into the genome of the ΔotsB mutant. First, we transformed the ΔotsB mutant with the pAT04_RecAB plasmid via electroporation (2.5 kV, 200 Ω and 25 μF) following the selection on LB agar containing 30 μg ml⁻¹ tetracycline. The resulting strain ΔotsB + pAT04_RecAB is capable to facilitate recombineering. Recombineering was done according to Tucker et al. (2014). Briefly, 5 μg of either P_otsBA_otsB_kanR or P_otsBA_treC_kanR were transformed in the RecAB producing ΔotsB mutant via electroporation (2.5 kV, 200 Ω and 25 μF). Transformants were selected on LB agar containing kanamycin (7.5, 10 or 15 μg ml⁻¹). Integration of the PCR fragments was verified via PCR.

Generation of ots expressing strains

For the generation of ots expression strains, we used the ΔotsB mutant as parental strain and the E. coli Acinetobacter shuttle plasmids pVRL2 (Lucidi et al., 2018) as backbone for the expression plasmids. Plasmids were designed according to Lucidi et al. (2018) with slight modifications. Briefly, otsA, otsB or otsBA were amplified via PCR from the genome of A. baumannii ATCC 19606. The resulting PCR products were cloned into the SalI and SacI restriction sites of pVRL2 (primers are listed in the Supporting Information Table S1). The resulting plasmids harbour either otsA, otsB or otsBA under the control of an arabinose-inducible promoter. The plasmids were verified by DNA sequencing. To generate the expression strains, A. baumannii ΔotsB was transformed with the recombinant pVRL2 plasmids via electroporation (2.5 kV, 200 Ω and 25 μF) following a selection of transformants on LB agar with 100 μg ml⁻¹ gentamycin. The resulting expression strains ΔotsB + pVRL2_Para_otsA, ΔotsB + pVRL2_Para_otsB and ΔotsB + pVRL2_Para_otsBA were grown overnight in mineral medium with succinate, 100 μg ml⁻¹ gentamycin and in the absence of arabinose. For expression studies, the overnight cultures were transferred in mineral medium with 1, 2 or 4% arabinose as sole carbon source and inducer.

NMR analyses

For NMR analyses, precultures were grown overnight at 37°C in mineral medium with succinate as carbon source. The precultures were used to inoculate mineral medium containing 20 mM arabinose as carbon source and 300 mM NaCl. Cells were grown at 37°C in 500 ml of the medium filled in 2 l erlenmeyer flasks. A total cell culture volume of 1.5 l was harvested in the case of A. baumannii ATCC 19606, ΔotsA and ΔotsBA mutant. In case of the ΔotsB mutant, 4.5 l of cell culture was harvested (4700 rpm, 4°C, 20 min) 10 h after inoculation. To generate a low phosphate background for NMR analyses, cells were washed two times with 300 mM NaCl (50 ml). Cell pellets were frozen in liquid nitrogen, lyophilized and stored at −65°C. Solutes were extracted using an ethanol-based extraction as previously described (Martins and Santos, 1995; Sand et al., 2011; Zeidler et al., 2017). 1H-NMR spectra were acquired on a Bruker Avance III 800 spectrometer (Bruker, Rheinstetten, Germany).
Quantification of trehalose and Tre-6-P using enzymatic assays

Solutions were extracted as described before (Zeidler et al., 2017) and the pellet was dissolved in 212.5 μl H2O. For quantification of trehalose and Tre-6-P, samples were divided into two portions, with a total volume of 85 μl each. To both samples 10 μl 10x FastAP Buffer (Thermo Fisher Scientific, Waltham, MA, USA) was added. In addition, 5 μl of H2O was added to sample 1 for quantification of trehalose. The other part of the sample was treated with 5 μl FastAP (alkaline phosphatase; Thermo Fisher Scientific) for dephosphorylation of Tre-6-P. Both samples were incubated for 2 h at 37°C for complete dephosphorylation. The trehalose content of sample 1 and 2 was quantified with the trehalose assay kit ‘K-TREH’ (Megazyme, Bray, Ireland). Sample 1 reflects the amount of trehalose of the cell. Sample 2 reflects the trehalose and Tre-6-P amount of the cells. The subtraction of sample 1 from sample 2 gives the amount of Tre-6-P in the cells. The enzyme based dephosphorylation assay and quantification of Tre-6-P using NMR led to the same results.

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**Supporting Information**

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

**Table S1** Supplementary Information.