environmental microbiology

Environmental Microbiology (2020) 22(4), 1370–1380 doi:10.1111/1462-2920.14951

Unusual deprivation of compatible solutes in *Acinetobacter baumannii*

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Summary

The opportunistic human pathogen *Acinetobacter baumannii* is one of the leading causes of nosocomial infections. The high prevalence of multidrug-resistant strains, a high adaptability to changing environments and an overall pronounced stress resistance contribute to persistence and spread of the bacteria in hospitals and thereby promote repeated outbreaks. Altogether, the success of *A. baumannii* is mainly built on adaptation and stress resistance mechanisms, rather than relying on 'true' virulence factors. One of the stress factors that pathogens must cope with is osmolarity, which can differ between the external environment and different body parts of the human host. *A. baumannii* ATCC 19606^T accumulates the compatible solutes glutamate, mannitol and trehalose in response to high salinities. In this work, it was found that most of the solutes vanish immediately after reaching stationary phase, a very unusual phenomenon. While glutamate can be metabolized, mannitol produced by MtlD is excreted to the medium in high amounts. First results indicate that *A. baumannii* ATCC 19606^T undergoes a rapid switch to a dormant state (viable but non-culturable) after disappearance of the compatible solutes. Resuscitation from this state could easily be achieved in PBS or fresh medium.

Introduction

In the past few years, the opportunistic human pathogen *Acinetobacter baumannii* has increasingly attracted attention due to the emergence of multidrug resistant strains and the rising danger of hospital-acquired infection with this Gram-negative bacterium. In 2017, the WHO emphasized the need for development of new antibiotics against carbapenem-resistant *A. baumannii* (World Health Organization, 2017; Tacconelli *et al*., 2018). Several factors contributing to the multifactorial virulence of the pathogen have already been identified (for a recent review, see Wong *et al*. (2017)), but nevertheless, researchers are far from overlooking the crucial traits enabling its success. However, it is unquestioned that the pronounced desiccation resistance of *A. baumannii* is of relevance, which enables the bacterium to persist for long periods of time in hospitals (Jawad *et al*., 1998; Zeidler and Müller, 2019a). Furthermore, adaptation to and persistence in the human host is of vital importance for pathogenic bacteria, which requires resistance mechanisms against several stress factors (Chowdhury *et al*., 1996).

One of those stress factors encountered in the host is osmotic stress, and, therefore, osmoadaptation mechanisms are essential for virulence (Sleator and Hill, 2002). In our previous studies (Zeidler *et al*., 2017; Zeidler *et al*., 2018), we observed that *A. baumannii* ATCC 19606^T responds to high salt concentrations in the environment by accumulation of compatible solutes, a widespread strategy to counterbalance the outer osmolarity and stabilize biomolecules (Kempf and Bremer, 1998; Roeßler and Müller, 2001; Empadinhas and da Costa, 2008). If present, glycine betaine can be taken up as an osmoprotectant, but in nonsupplemented minimal medium, compatible solutes must be synthesized de novo (Zeidler *et al*., 2017). Besides the common solute glutamate, *A. baumannii* ATCC 19606^T produces high amounts of mannitol, a polyol which is only rarely used as a compatible solute by bacteria (Kets *et al*., 1996; Zeidler *et al*., 2017). Mannitol is produced by a special bifunctional enzyme, a mannitol-1-phosphate dehydrogenase/phosphatase (MtlD), which is strictly regulated on the activity level in a way that it is completely inactive in the absence of salt (Zeidler *et al*., 2018). Additionally, transcription of *mtlD* is dependent on high osmolarity, and the same was observed for transcription of *otsBA*, the genes essential for biosynthesis of trehalose (Zeidler *et al*., 2017). Trehalose is the third compatible solute accumulated by *A. baumannii* ATCC 19606^T, which is only present in minor amounts, but nevertheless

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important for growth at high osmolarity in combination with elevated temperature. Furthermore, *otsB* is required for full virulence in a *Galleria mellonella* infection model (Gebhardt *et al*., 2015), highlighting the link between resistance against osmotic stress and pathogenicity. The response of *A. baumannii* to low water activities (osmotic stress and desiccation) has been reviewed recently (Zeidler and Müller, 2019a).

We were surprised by the apparent importance of trehalose despite its presence in only small amounts. As up to now, the solute pool in *A. baumannii* ATCC 19606^{T} had only been determined in late exponential growth phase, we supposed that the disaccharide might appear in higher concentrations to a later time point in growth of *A. baumannii*. This was reasoned by the fact that, for example, in *Escherichia coli*, the *otsB* promoter is induced in stationary growth phase even in the absence of salt (Hengge-Aronis *et al*., 1991). Furthermore, a growth phase-dependent switch in the proportion and the composition of the compatible solute pool can be observed in many bacteria, such as *Halobacillus halophilus* (Saum and Müller, 2008), *Salibacillus salexigens* (Bursy *et al*., 2007) or *Thermococcus litoralis* (Lamosa *et al*., 1998). Therefore, the aim of this study was the quantification of the compatible solutes accumulated by *A. baumannii* ATCC 19606^{T} in response to osmotic stress in different growth phases. Unexpectedly, this led to the finding that *A. baumannii* ATCC 19606^{T} loses nearly all the accumulated solutes upon entry in stationary growth phase, which was thereupon studied in more detail.

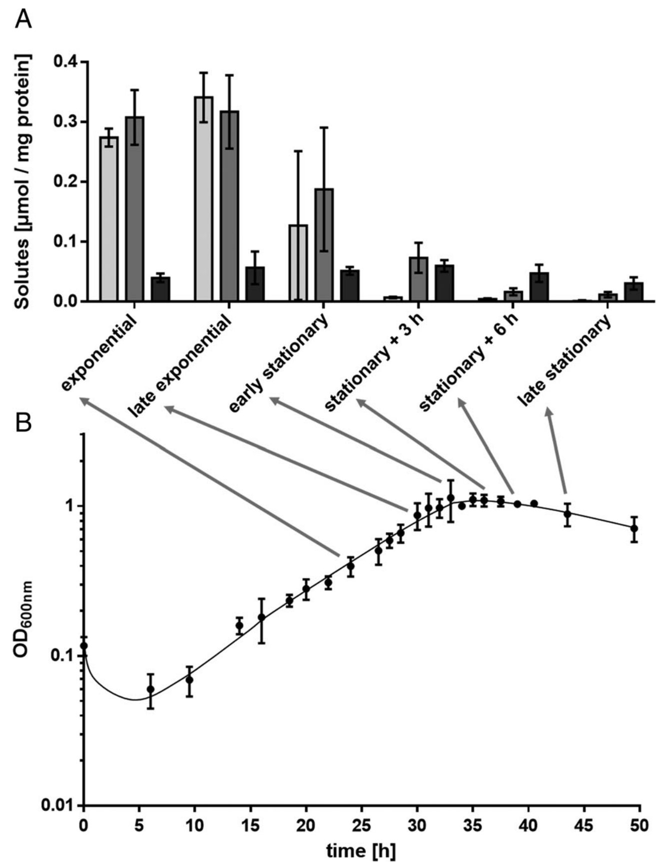

Fig. 1. Accumulation of compatible solutes in *Acinetobacter baumannii* ATCC 19606^{T} in different growth phases. Wild-type cells were grown in 100 ml mineral medium with 20 mM succinate in the presence of 500 mM NaCl, and solutes were extracted at different stages of growth, as indicated by the arrows.
A. Intracellular concentrations of glutamate (light grey), mannitol (dark grey) and trehalose (black) were determined at least in biological triplicates for each time point (mean values and standard deviations).
B. For the growth curve, several curves from individual experiments were combined and mean values were determined where possible (error bars represent standard deviations).

Results

Growth phase dependence of compatible solute accumulation

The pool of compatible solutes accumulated by *A. baumannii* ATCC 19606^{T} in response to high osmolarities had been analysed previously in late exponential growth phase (Zeidler *et al*., 2017). In the present study, the analyses were expanded to further time points earlier and later in the growth of *A. baumannii* ATCC 19606^{T} at high salinity (500 mM NaCl) (Fig. 1). In mid-exponential phase, nearly the same amounts of glutamate, mannitol and trehalose were detected as in the late exponential phase. Surprisingly, both main solutes, namely glutamate and mannitol, started to vanish upon entry into the stationary phase. At this time point, the data for both solutes were extremely variable, whereas the trehalose concentration remained unchanged. Already 3 hours later, the cells were completely deprived of glutamate, and only 0.07 μmol mannitol/mg protein were left, which is 23% of the value determined in late exponential phase. This concentration dropped further to 0.01 μmol/mg protein in late stationary growth phase. At that time point, also the trehalose concentration was nearly halved compared to the late exponential phase.

As the osmotic stress in the culture medium was still present, it could be assumed that *A. baumannii* ATCC 19606^{T} undergoes a growth phase-dependent switch in the osmolyte strategy and accumulates other compatible solutes in the stationary growth phase. In order to identify them, NMR analyses of late stationary phase cells grown in the presence of 500 mM NaCl were performed. Unexpectedly, no other solutes were detected. Despite the apparent discrepancy between outer and inner osmolarity, the bacteria could not be dead at this time point, as deprivation was already observed at the onset of

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stationary phase. Furthermore, intracellular trehalose could still be detected.

The fate of glutamate

The virtually complete loss of compatible solutes in stationary growth phase despite the lasting osmotic stress is very unusual and prompted further analyses to elucidate the basis for this observation. In principle, there are two possible reasons for the disappearance of the compatible solutes: the solutes are either degraded or they are excreted to the medium. As metabolization of the osmoprotectants only sounds reasonable in case the medium is depleted of succinate, the only carbon and energy source, the concentration of succinate in the cultures grown with 500 mM NaCl at various time points was determined (Fig. 2). Indeed, on the average less than 2 mM succinate of the initial 20 mM were left upon entry into stationary growth phase, indicating that the bacteria become stationary due to the lack of an energy source. Since *A. baumannii* ATCC 19606^T grows well in mineral medium with glutamate as the sole carbon source (Fig. 3A; growth rate in minimal medium without salt is $1.21 \pm 0.07\ h^{-1}$, which is higher than with succinate), it is tempting to speculate that the amino acid is metabolized. To finally exclude the possibility that glutamate is excreted to the medium, the culture supernatants were analysed for their glutamate content. A theoretical value for the expected glutamate concentration in case of a complete excretion can be calculated from the amount

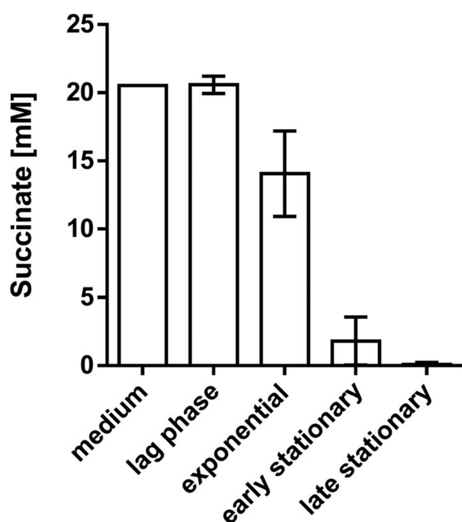

Fig. 2. Consumption of succinate during growth of *Acinetobacter baumannii* ATCC 19606^T. Bacteria were grown in mineral medium with 500 mM NaCl. The concentration of the sole carbon and energy source succinate in the medium was determined at various time points. The starting concentration of 20 mM was checked once, and all other data are mean values of three independent biological replicates with error bars representing standard deviations.

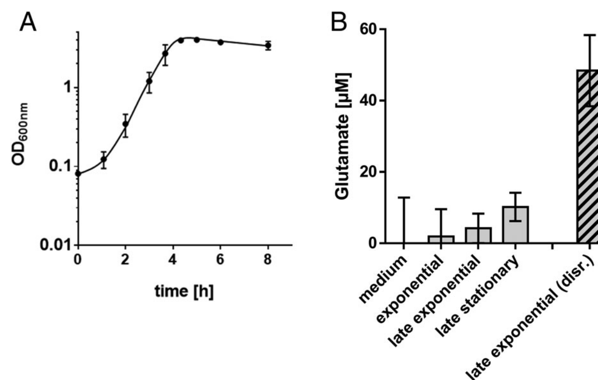

Fig. 3. Growth of *Acinetobacter baumannii* ATCC 19606^T on glutamate as carbon source and determination of glutamate in culture supernatants.

A. Growth of *A. baumannii* ATCC 19606^T in 100 ml mineral medium with 20 mM glutamate as sole carbon source.

B. Wild-type cells were grown in 100 ml mineral medium on succinate with 500 mM NaCl, aliquots of 1 ml were collected at different time points (exponential = OD_{600} 0.4–0.5; late exponential = OD_{600} 0.9–1.2; late stationary = ca. 50 h after inoculation, OD_{600} = 0.6–0.9), cells were removed and the glutamate concentration was measured in the supernatants. For comparison, cells in 1 ml aliquots from late exponential growth phase were disrupted by heat to release all solutes to the medium, and the supernatant was analysed (striped bar). Both experiments were performed in biological triplicates, shown are mean values and standard deviations.

of glutamate accumulated per mg dry weight and the approximate amount of dry weight received from a late exponential 100 ml culture (ca. 50 mg). The calculated value is around 50 μM glutamate, which fits perfectly to the experimental value obtained with heat-disrupted cells that have released all solutes to the medium (Fig. 3B). This is the glutamate concentration which could be expected in the medium in the case of excretion. However, the concentration of glutamate in the supernatant of nondisrupted cells from late stationary growth phase was only 10 μM, indicating that the bigger part of the amino acid had been metabolized by *A. baumannii* ATCC 19606^T.

The fate of mannitol

The theoretical value for the mannitol concentration in the medium in case of excretion was in the same range as for glutamate (see above). All the more it was unexpected to find mannitol concentrations between 250 and 850 μM in the different growth phases (Fig. 4), values ca. 10-fold higher than maximally calculated. In late exponential growth phase, the amount of mannitol in the medium was much higher than the amount accumulated intracellularly, so that no increase of the extracellular concentration could be measured upon disruption of the cells and the following release of intracellular solutes. The highest amount was reached in late stationary growth

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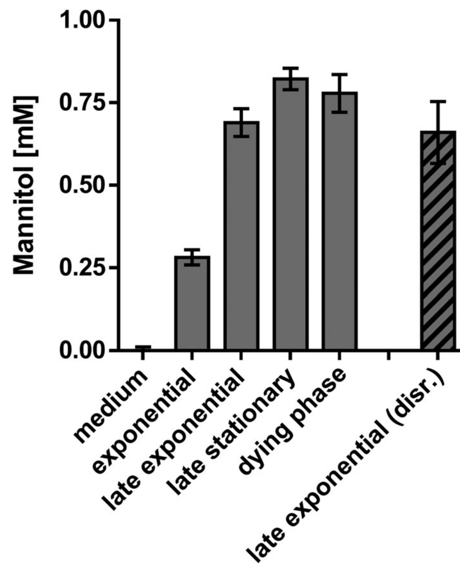

Fig. 4. Mannitol excretion by *Acinetobacter baumannii* ATCC 19606^T grown in the presence of 500 mM NaCl. Cells were grown on succinate in 100 ml mineral medium with 500 mM NaCl. The concentration of mannitol in 1 ml aliquots of supernatants of cells in different growth phases was determined in biological triplicates (non-striped bars), shown are mean values and standard deviations. Exponential = OD_{600} 0.4–0.5; late exponential = OD_{600} 0.9–1.2; late stationary = ca. 20 h later, OD_{600} 0.6–0.9; dying phase = 6 days after inoculation. The striped bar represents the supernatant of heat-disrupted cells (solutes released to the medium in 1 ml aliquot).

phase (ca. 2 days after inoculation), and the concentration did not decrease in the course of the next 4 days, indicating that mannitol was not taken up again and metabolized. This fits to the observation that mannitol, in contrast to glutamate, cannot be used as a sole energy and carbon source by *A. baumannii* ATCC 19606^T (data not shown). Mannitol could not be detected in the culture medium of a Δ*mtlD* mutant grown at high osmolarity (data not shown), proving that indeed the MtlD, which is essential for intracellular accumulation of mannitol (Zeidler *et al*., 2018), also synthesizes the mannitol present in the medium.

As expected, the appearance of mannitol was dependent on the osmolarity of the medium: no mannitol was present without salt, and with 300 mM NaCl, ca. 50% of the amount detected in the presence of 500 mM NaCl were found (Fig. 5A). The addition of 600 mM sucrose yielded the same extracellular amount of mannitol as 500 mM NaCl (Fig. 5A), indicating that the effect is not salt-specific. Also, mannitol enrichment in the medium was not specific for a certain carbon source, as concentrations in the same range could be observed after growth on arabinose or acetate (Fig. 5B). To elucidate whether mannitol accumulation in the medium is a characteristic for *A. baumannii* ATCC 19606^T or also occurs in other, nonpathogenic *Acinetobacter* species, supernatants of *A. baylyi* cells grown in the presence of 500 mM NaCl were analysed. In late stationary growth phase, *A. baylyi* excreted the same amount of mannitol as its pathogenic relative (Fig. 5C). Interestingly, in late exponential growth phase, only 78 μM mannitol could be detected in the medium, which equals 10% of the amount excreted by *A. baumannii* ATCC 19606^T in the same growth phase. This could be linked to the significant faster growth of *A. baylyi* at this osmolarity: late exponential growth phase was reached after 8 h (data not shown), in contrast to ca. 32 h in the case of *A. baumannii* ATCC 19606^T.

The reason for mannitol excretion remains elusive

The nearly complete loss of compatible solutes observed in *Acinetobacter* is very uncommon and seems to be absurd regarding the continuing osmotic stress. We hypothesized that mannitol might have a protective effect when present extracellularly in high concentrations. However, growth of *A. baumannii* ATCC 19606^T was not improved in medium with 500 mM NaCl supplemented with 1 mM mannitol (data not shown). The same was true for the Δ*mtlD* mutant (data not shown), which is defective in growth at high osmolarity due to the lack of mannitol (Zeidler *et al*., 2018). As mannitol is a known radical scavenger, a role in protection from oxidative stress was assumed, but the presence of 1 mM mannitol did neither enhance growth of wild-type cells stressed by H_2O_2 (2–4 mM) nor increase the number of surviving cells after 1 h when stressed with 20 mM H_2O_2 in exponential growth phase (data not shown).

Osmotic stress seems to induce a VBNC state in A. baumannii *ATCC* 19606^T

After the fate of the solutes had been investigated, we addressed the question how long the bacteria are able to withstand the osmotic stress in the absence of compatible solutes. Plating appropriate dilutions on LB agar plates revealed a very fast decrease of colony-forming units (CFU) in cultures grown in the presence of 300 or 500 mM NaCl, already shortly after reaching stationary phase (Fig. 6). Nearly, no culturable cells were left 6 days after inoculation. This was in sharp contrast to cells grown in the absence of NaCl, where the drop from 6.7×10^8 CFU/ml (early stationary phase) to 6.5×10^5 CFU/ml at day 6 was followed by nearly constant CFU numbers for the next 30 days (Fig. 6). As it seems irrational that the bacteria excrete all solutes even though they need them for survival, it was considered that they might not be dead after 1 week in medium with high NaCl concentrations but could have entered a dormant state where they are still alive, but not culturable on

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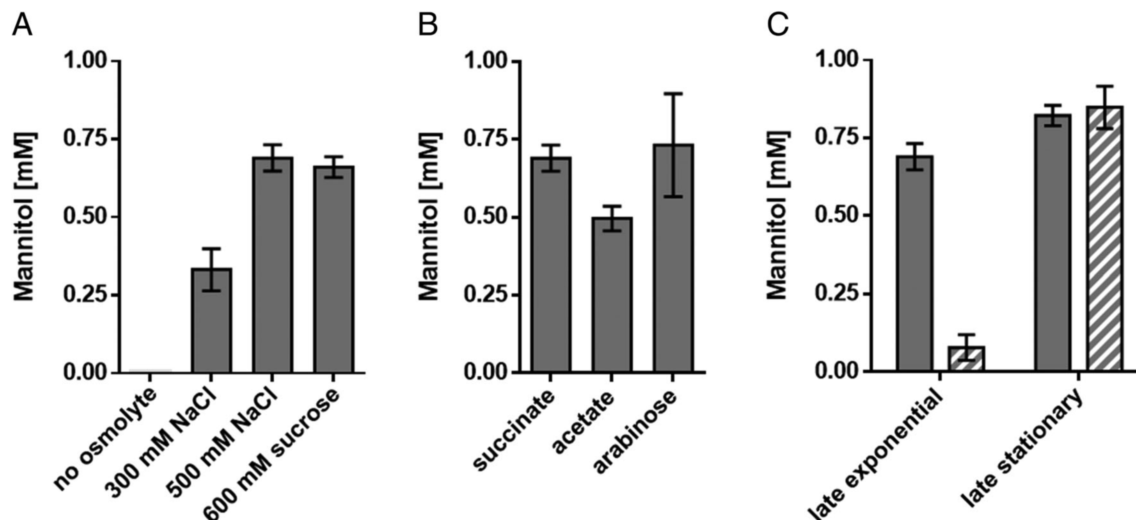

Fig. 5. Release of mannitol to the medium by *Acinetobacter baumannii* ATCC 19606^T and *A. baylyi* ADP1 grown under different conditions.
A. *Acinetobacter baumannii* ATCC 19606^T was grown in mineral medium without an additional osmolyte or with the addition of NaCl or sucrose as indicated. The mannitol concentration in the medium was determined in the late exponential growth phase (OD_{600} 0.9–1.2).
B. Supernatants of *A. baumannii* ATCC 19606^T grown in mineral medium with 20 mM succinate, acetate or arabinose as carbon source and the presence of 500 mM NaCl were analysed in late exponential growth phase.
C. *Acinetobacter baumannii* ATCC 19606^T (grey bars) and *A. baylyi* (striped bars) were grown in mineral medium with 500 mM NaCl. Mannitol concentrations were measured in supernatants of cells in two different growth phases as indicated (late stationary = ca. 20 h after late exponential, OD_{600} = 0.6–0.9 for *A. baumannii* ATCC 19606^T and OD_{600} 2.2 for *A. baylyi*). For all experiments, aliquots of 1 ml were taken, and supernatants were analysed after removal of cells. All bars represent the mean values of three independent biological replicates, error bars show standard deviations.

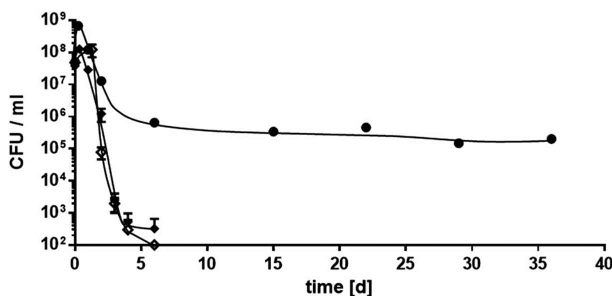

Fig. 6. Long-term survival of *Acinetobacter baumannii* ATCC 19606^T in mineral medium with different osmolarities. Wild-type cells were grown in 500 ml flasks containing 100 ml mineral medium with 20 mM succinate as carbon source and different NaCl concentrations (closed circle, no NaCl; closed diamond, 300 mM NaCl; open diamond, 500 mM NaCl) and further incubated at standard growth conditions (37°C, 130 rpm). At different time points, the CFU/ml were determined. Mean values of at least three independent biological replicates and standard errors of the mean are plotted.

standard solid medium (viable but non-culturable, VBNC state). If so, it can be hypothesized that the compatible solutes (especially mannitol) are excreted by *A. baumannii* to reach such a persisting state. In many bacteria that enter the VBNC state the unculturability can be relieved ('resuscitation'), and this can sometimes be achieved through removal of the stress factor that initially induced dormancy (Li *et al*., 2014). A successful resuscitation would be a good hint that *A. baumannii* persists in a VBNC state.

The experiment was performed with wild-type cells grown in mineral medium with succinate as carbon source and 300 or 500 mM NaCl on Day 3 after inoculation. A sample of these cultures was diluted 1:10 in mineral medium without osmotic stress. In both cases, the initial number of CFU/ml directly after inoculation was 0–10^3 and increased to nearly 10^7 after 3 days of incubation (Fig. 7A). This dramatic increase in CFU was very reproducible in cultures grown in the presence of 300 mM NaCl but could not always be observed with 500 mM NaCl, which is a very high osmotic stress for *A. baumannii*. Therefore, further experiments were conducted with 300 mM NaCl in the growth medium. First, time-dependent increase of CFU counts in the resuscitation medium was analysed. The dramatic increase was already observed after 1 day and then nearly remained constant after 2 and 3 days, leading to the conclusion that not more than 10^7 CFU/ml could be resuscitated (Fig. 7B). As this resuscitation medium was prepared without the addition of any carbon source (MM-C), growth can be excluded, which is reflected by the nearly unchanged total cell count, which was determined microscopically at time point zero (1.2×10^8 cells/ml) and after 2 days (1.4×10^8 cells/ml). As an additional control, a stationary culture grown in the absence of osmotic stress was diluted in mineral medium without carbon source to a starting concentration of 1.5–6 $\times 10^4$ CFU/ml. This value remained nearly constant for 3 days (Fig. 7C),

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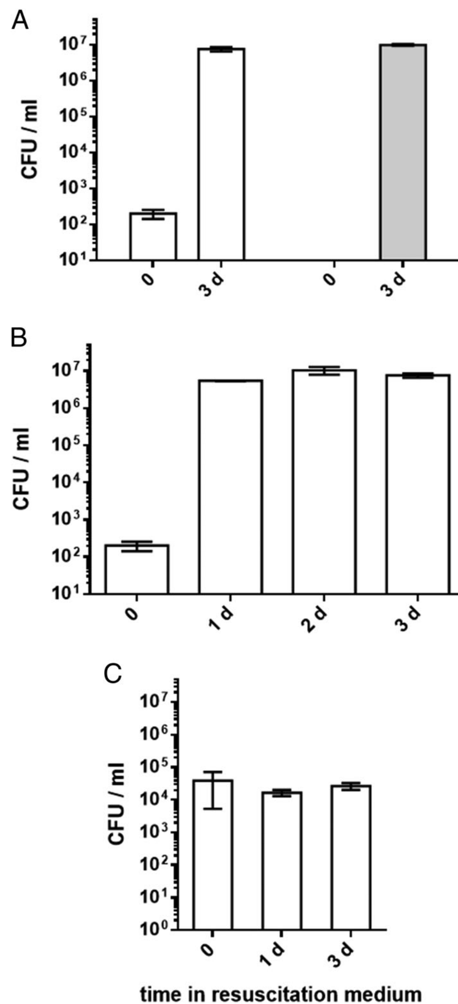

Fig. 7. *Acinetobacter baumannii* ATCC 19606^T enters a VBNC state upon salt stress and can be resuscitated.
A. Cultures grown in 100 ml mineral medium with succinate and 300 mM NaCl (white bars) or 500 mM NaCl (grey bars) were diluted 1:10 (2 ml in 18 ml in 100 ml flasks) in mineral medium without carbon source (MM-C) and further incubated at 37°C. CFU/ml were calculated directly after the dilution and after 3 days. Shown are mean values and standard errors of the mean of three biological replicates.
B. *Acinetobacter baumannii* ATCC 19606^T was grown in 100 ml mineral medium with succinate and 300 mM NaCl. After 3 days, the culture was diluted 1:10 in MM-C (as described in A) and the number of CFU/ml was determined after 1, 2, and 3 days of further incubation at 37°C.
C. As a growth control, MM-C was inoculated from a stationary overnight culture grown in mineral medium with 20 mM succinate. No growth was observed in two independent biological replicates (shown are mean values and standard deviations).

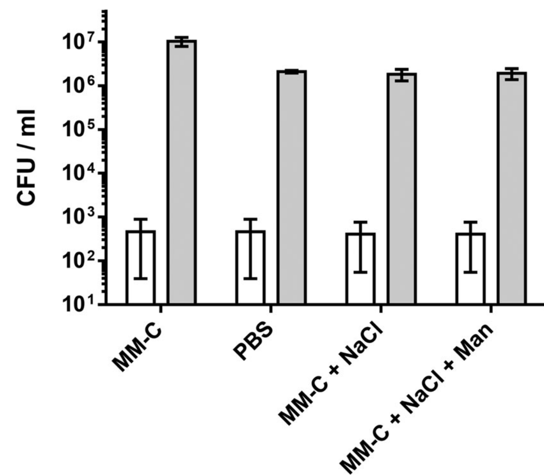

Fig. 8. *Acinetobacter baumannii* ATCC 19606^T can be resuscitated in different media and also in the presence of NaCl. After 3 days of growth on succinate in 100 ml mineral medium with 300 mM NaCl, bacteria were diluted 1:10 (2 ml in 18 ml in 100 ml flasks) in various resuscitation media, all of them without carbon source, and further incubated at 37°C. CFU/ml were determined directly after the dilution (white bars) and after 2 days (grey bars). Resuscitation was tested in three biological replicates in all media, shown are mean values and standard errors of the mean. Resuscitation media: mineral medium without C-source (MM-C); PBS; MM-C with 300 mM NaCl $\pm$ 1 mM mannitol (Man). The total number of cells (tested for MM-C) was 1.2–1.4 $\times$ 10^8 at both time points.

proving that *A. baumannii* ATCC 19606^T is not able to grow under the resuscitation conditions. Furthermore, the increase in culturable cells could similarly be observed when the 3-day-old culture was diluted in PBS, a buffer which does not support bacterial growth (Fig. 8). To exclude the presence of so-called injured cells (which do not grow due to intracellular production of radicals induced by an imbalance of catabolism and anabolism in stationary phase but are still metabolically active) instead of VBNC cells, the cultures were plated on LB agar with pyruvate, which degrades H_2O_2 (Pinto *et al*., 2015). No colonies were formed on this medium either. These results clearly indicate that *A. baumannii* ATCC 19606^T does not die but enters a VBNC state from which the bacteria can be resuscitated in liquid culture. It was assumed that removal of the osmotic stress led *A. baumannii* ATCC 19606^T to exit the VBNC state, but surprisingly culturability increased nearly to the same extent in mineral medium with 300 mM NaCl (Fig. 8). The addition of mannitol to the medium did not prevent resuscitation. Furthermore, preliminary results showed that a Δ*mtlD-otsB* mutant lacking the biosynthesis pathways for mannitol and trehalose (Zeidler and Müller, 2019b) also lost culturability after a few days in medium with 300 mM NaCl and could be resuscitated in MM-C comparable to wild-type cells (data not shown). This indicates that intracellular compatible solutes are not necessary for resuscitation. Anyway, the data presented are in line with the hypothesis that in late stationary phase solutes are excreted while cells enter a VBNC state.

Discussion

The accumulation of compatible solutes in response to osmotic stress is ubiquitous in all three domains of life and correspondingly well studied (Kempf and Bremer,

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1998; Roeßler and Müller, 2001; Bremer and Krämer, 2019). Thus, it is known from various bacteria that the pool of compatible solutes can vary, depending on the osmolyte concentration, temperature and growth phase. Nevertheless, the herein described almost complete loss of all compatible solutes in stationary growth phase of *A. baumannii* ATCC 19606^T is utterly unusual. Indeed, the reduction of the solute pool in stationary phase has been observed for other bacteria as well, but slower and not till the full deprivation (Lamosa *et al*., 1998; Silva *et al*., 1999). The fact that potassium glutamate is accumulated only transiently and vanishes after induction of the second stress response, i.e. the import or synthesis of true compatible solutes, is a general phenomenon (Booth and Higgins, 1990). We observed the same in *A. baumannii* ATCC 19606^T, where glutamate is probably metabolized. The use of compatible solutes as energy source in case of nutrient deprivation has been proposed earlier (Lamosa *et al*., 1998; Silva *et al*., 1999). Potentially, the bacteria reduce their compatible solute pool to a minimal level needed for persistence at the present osmolarity and wait for more favourable conditions which facilitate growth (Silva *et al*., 1999).

Also, excretion of compatible solutes is not per se unusual. *Bacillus subtilis* permanently releases proline, which is recaptured by the transporter OpuE (Hoffmann *et al*., 2012). Similarly, *Halomonas elongata* excretes ectoine which is immediately taken up again, whereas in *Halomonas salina*, there is a nonequilibrium between excretion and uptake so that the solute accumulates in the growth medium (Zhang *et al*., 2009; Gao *et al*., 2014). But still, the cells are not devoid of intracellular solutes as observed in *A. baumannii* ATCC 19606^T. In *E. coli*, the concentration of the main solute trehalose decreases in stationary growth phase (Welsh *et al*., 1991). Interestingly, it could be shown that trehalose is excreted (Styrvold and Strøm, 1991), but as trehalose is immediately degraded by a periplasmic trehalase only mutants with a defect in this enzyme actually accumulate trehalose in the growth medium. It is supposed that *E. coli* constantly overproduces trehalose due to a lacking feedback inhibition (Strøm and Kaasen, 1993). The same lack can be hypothesized in the case of mannitol excretion from *A. baumannii* ATCC 19606^T, as it was shown before that the presence of mannitol does neither inhibit activity of the *mtlD* promoter (when added to the medium) nor of MtlD itself (Zeidler *et al*., 2018). However, as mannitol cannot be reused as energy source, one would suspect that this should be too much of a fitness cost to be practiced without having any advantage. Regardless, up to now we could not determine any protective effect of extracellular mannitol.

Another hypothesis not validated so far is a physiological role of mannitol excretion in pathogenesis. At least in *Cryptococcus neoformans*, mannitol secretion is designated as a virulence factor (Buchanan and Murphy, 1998). The fungus does not only excrete the polyol in vitro to the growth medium but also in vivo (Wong *et al*., 1990). A low mannitol-producing mutant of *C. neoformans* had reduced pathogenicity (Wong *et al*., 1990; Chaturvedi *et al*., 1996a; Chaturvedi *et al*., 1996b). Moreover, in vitro studies showed that high concentrations of mannitol (and also of glucose, hyperglycaemia) can significantly reduce phagocytosis and oxidative burst (Otto *et al*., 2008).

Apart from its unknown function, the route of mannitol export is in the dark so far. Usually, compatible solutes are excreted via mechanosensitive channels. Homologues of *mscL* and *mscS* can also be found in the genome of *A. baumannii* ATCC 19606^T, but they normally respond to a sudden decrease in osmolarity and expel all solutes unspecifically (Berrier *et al*., 1992; Levina *et al*., 1999). As in the case of *A. baumannii*, the salt stress is still present and trehalose could still be detected in the bacteria even in late stationary phase, a role of those channels in mannitol secretion is unlikely. Specific transporters for mannitol export are not studied in bacteria, it is just assumed that they must exist, as heterofermentative lactic acid bacteria produce and export substantial amounts of mannitol (Wisselink *et al*., 2002).

Investigations on compatible solute deprivation in *A. baumannii* ATCC 19606^T led to the discovery of a putative VBNC state. Further studies will have to finally prove this, but so far, all experiments are in accordance with this assumption. Unexpectedly, high salinity alone does not seem to be the inducing signal, as the cells could be resuscitated in the presence of high NaCl concentrations. Whether some inhibitory substance, the presence of a signal molecule, the higher cell density (quorum sensing) or any other circumstance prevents culturability in the original culture shall be the subject of future investigations. Also, a possible connection to compatible solutes, accumulation and excretion should be analysed.

When hyperosmolarity or other stressful conditions last too long, many microorganisms can survive in a dormant state. A well-known strategy applied by some Gram-positive bacteria is spore formation ('true dormancy'), but there are other forms of dormancy where the vegetative cells themselves undergo morphological changes and gain the ability to persist under harsh conditions. One of them is the VBNC state, which is defined by the inability of bacteria to form colonies on standard solid medium usually supporting growth – even though they are still alive (Oliver, 2010; Li *et al*., 2014; Pinto *et al*., 2015). Among the various stress factors known as possible inducers of the VBNC state, high osmolarity is one of the most studied. The first discovery of cells in this dormant

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form was after incubating *E. coli* and *Vibrio cholerae* for 2 weeks in the presence of 2%–25% NaCl and following survival with culture- and non-culture-based methods (Xu *et al*., 1982). Since then, similar observations have been made with other organisms, such as *Salmonella enterica*, *Klebsiella pneumoniae* or *Enterobacter* sp. (Asakura *et al*., 2002; Sachidanandham and Gin, 2009). The two latter could easily be resuscitated in PBS at 37°C, comparable to the present study.

So far, only little is known about the VBNC state in *A. baumannii*. A recent study reported on the induction of VBNC and persister cell formation in *A. baumannii* ATCC 17978^T in response to cold shock in combination with nutrient starvation and high doses of the antibiotic ciprofloxacin (Nicol *et al*., 2018). Furthermore, the presence of a VBNC subpopulation in clinical isolates of *A. baumannii* was observed (Bravo *et al*., 2019). This was induced by starvation in saline as well as by desiccation on acetate cellulose filters, but only to a small degree due to very high overall survival rates. More pronounced was the switch to an unculturable state in response to disinfectants, which was achieved only 30 s after exposure to the agents (Bravo *et al*., 2019).

Regarding the sparse knowledge on the VBNC state in *A. baumannii*, further research is urgently needed. In general, many bacteria known to survive in a VBNC state are pathogens (Oliver, 2010; Li *et al*., 2014; Zhao *et al*., 2017). The prevalence of VBNC in pathogens poses a major threat to health care, and equally to food safety due to foodborne pathogens. These bacteria often remain undetected in samples of patients or food, or their number is underestimated, because routine detection methods are predominantly cultivation-based. As virulence can be retained in the VBNC state, or regained upon resuscitation, it is essential to know whether a certain pathogen can persist in a VBNC state to develop reliable detection methods (Li *et al*., 2014). Additionally, decontamination and treatment can be hindered, as dormant cells are often more resistant against antibiotics and harsh conditions in general (Oliver, 2010; Li *et al*., 2014; Ayrapetyan *et al*., 2018). In the case of *A. baumannii*, bacteria could undergo a switch to the VBNC state, for example in body fluids with elevated osmolarity, which would increase their persistence upon release to the hospital environment. Whether this is true for the type strain ATCC 19606^T as well as other strains of *A. baumannii* remains to be elucidated in the future.

Experimental procedures

Bacterial strains and culture conditions

Acinetobacter baumannii ATCC 19606^T wild-type and Δ*mtlD* (markerless deletion of the mannitol-1-phosphate dehydrogenase/phosphatase gene [Zeidler *et al*., 2018]) as well as *Acinetobacter baylyi* ADP1 were cultivated in mineral medium containing 50 mM phosphate buffer, different mineral salts (1 g/l NH_4Cl, 580 mg/l $MgSO_4 \times 7$ H_2O, 100 mg/l KNO_3, 67 mg/l $CaCl_2 \times 2$ H_2O, 2 mg/l $(NH_4)_6Mo_7O_{24} \times 4$ H_2O) and 1 ml/l of the trace element solution SL9 (12.8 g/l nitrilotriacetic acid (titriplex), 2 g/l $FeSO_4 \times 7$ H_2O, 190 mg/l $CoCl_2 \times 6$ H_2O, 122 mg/l $MnCl_2 \times 4$ H_2O, 70 mg/l $ZnCl_2$, 36 mg/l $MoNa_2O_4 \times 2$ H_2O, 24 mg/l $NiCl_2 \times 6$ H_2O, 6 mg/l H_3BO_3, 2 mg/l $CuCl_2 \times 2$ H_2O, modified after Tschech and Pfennig (1984)). If not stated otherwise, 20 mM sodium succinate were added as carbon source. For experiments with other carbon sources (20 mM sodium glutamate, sodium acetate or L-arabinose), those were also used for the pre-cultures. For growth under osmotic stress conditions, NaCl or sucrose was added to the medium in the concentrations indicated but not to the pre-cultures. Stock solutions of all components were autoclaved separately and mixed shortly before use. Standard growth conditions were 130 rpm and 37°C or 30°C for *A. baumannii* or *A. baylyi* respectively. Cultures were inoculated from overnight grown pre-cultures and growth was followed photometrically at 600 nm (OD_{600}).

Sample preparation for determination of compatible solutes

For analysis of intracellularly accumulated compatible solutes, bacteria were harvested from 100 ml cultures in different growth phases: exponential (OD_{600} 0.4–0.5) or late exponential growth phase (OD_{600} 0.9–1.2), at the onset of stationary phase as well as 3 or 6 h afterwards, and in late stationary growth phase (10 h afterwards). Cell pellets were lyophilized, and extraction of solutes with chloroform and methanol was performed as described before (Zeidler *et al*., 2017). For determination of solutes in the cultivation medium, 1 ml samples of cultures in different growth phases were centrifuged (14,000 rpm, 2 min), and the supernatant was incubated for 20 min in a heating block at 100°C. For comparison, 1 ml samples from late exponential growth phase were heated before centrifugation to disrupt the cells and release all intracellular compatible solutes into the medium. The supernatants were used directly for quantification of mannitol or concentrated three-fold for determination of glutamate.

Quantification of compatible solutes and succinate

Compatible solutes were measured as described previously (Zeidler *et al*., 2017). For detection of glutamate and trehalose, enzymatic kits from Megazyme (Bray, Ireland) were used, whereas mannitol was determined

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using a high-performance liquid chromatography (HPLC) method. Though, for supernatants we confirmed the presence of mannitol via HPLC but due to the high salt concentration which disturbed the quantification, we then switched to using an enzymatic kit as well (D-mannitol/ L-arabitol, Megazyme). This is based on the degradation of mannitol by a mannitol dehydrogenase. After confirming that the protein content of lyophilized cells did not change depending on the growth phase, the mean value obtained earlier (Zeidler *et al*., 2017) was used for quantification. Succinate concentrations in the medium were measured using the kit for succinic acid (succinate) by Megazyme based on a succinyl-CoA synthetase. All enzymatic tests were confirmed to be not affected by high amounts of NaCl. Cells from late stationary growth phase were also analysed for the presence of other compatible solutes via NMR as described earlier (Zeidler *et al*., 2017).

Survival experiments and resuscitation

To investigate the long-term survival of *A*. *baumannii* ATCC 19606^T in liquid cultures, bacteria were grown in 100 ml cultures under different conditions. At time points indicated, samples were diluted in saline (0.9% NaCl) and plated on LB agar (10 g NaCl, 10 g tryptone, 5 g yeast extract, H_2O_{VE} ad. 1 l, 18 g agar). Colonies were counted after 1 day at 37°C for determination of CFU/ml. For tests with sodium pyruvate, 5 g/l were added to LB medium. When *A*. *baumannii* ATCC 19606^T was cultivated in mineral medium with succinate and osmotic stress, the culturability on LB agar plates dramatically decreased after only a few days (see Results section). To check whether the bacteria are dead or in a dormant state (VBNC), resuscitation experiments were performed. For this purpose, *A*. *baumannii* ATCC 19606^T was inoculated to 100 ml mineral medium with 300 mM NaCl in 500 ml flasks and kept at 37°C and 130 rpm for 3 days. At this time point, the culture was diluted 1:10 in 100 ml flasks (18 ml resuscitation medium + 2 ml culture). As resuscitation medium, mineral medium without carbon source or PBS were used. Both media were inoculated from the same prospective VBNC culture, and this was done in biological triplicates. The CFU/ml in these dilutions were determined immediately, and again after incubating them for 2 days at 37°C and 130 rpm. Total cell counts in the dilutions were analysed using a Neubauer counting chamber, but this was only done for mineral medium (in biological triplicates). Chambers were loaded in two technical replicates, and four squares (8×10^{-4} mm^3) were counted each. Mean values were calculated from these eight values to determine the number of cells/ml.

Acknowledgements

The Deutsche Forschungsgemeinschaft funded this study through DFG Research Unit FOR 2251. We thank Prof. Helena Santos and her group (Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Oeiras, Portugal) for performing the NMR analysis.

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