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Brief Report

A first response to osmostress in *Acinetobacter baumannii*: transient accumulation of K^+ and its replacement by compatible solutes

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Summary

The extraordinary desiccation resistance of the opportunistic human pathogen *Acinetobacter baumannii* is a key to its survival and spread in medical care units. The accumulation of compatible solute such as glutamate, mannitol and trehalose contributes to the desiccation resistance. Here, we have used osmolarity as a tool to study the response of cells to low water activities and studied the role of a potential inorganic osmolyte, K^+, in osmostress response. Growth of *A. baumannii* was K^+-dependent and the K^+-dependence increased with the osmolarity of the medium. After an osmotic upshock, cells accumulated K^+ and K^+ accumulation increased with the salinity of the medium. K^+ uptake was reduced in the presence of glycine betaine. The intracellular pools of compatible solutes were dependent on the K^+ concentration: mannitol and glutamate concentrations increased with increasing K^+ concentrations whereas trehalose was highest at low K^+. After osmotic upshock, cells first accumulated K^+ followed by synthesis of glutamate; later, mannitol and trehalose synthesis started, accompanied with a decrease of intracellular K^+ and glutamate. These experiments demonstrate K^+ uptake as a first response to osmostress in *A. baumannii* and demonstrate a hierarchy in the time-dependent accumulation of K^+ and different organic solutes.

Introduction

Fluctuation in the environmental osmolarity and water availability is one of the biggest challenges for living cells (Kempf and Bremer, 1998; Ventosa *et al*., 1998; Wood, 1999). This is also true for the opportunistic human pathogen *Acinetobacter baumannii*. Its natural habitat is still unknown but it is frequently found in health care institutions worldwide (Dijkshoorn *et al*., 2007; Peleg *et al*., 2008). One key to its success in the hospital environment is its enormous potential to withstand long times of dryness; *A. baumannii* is known to survive on abiotic surfaces for month or even years (Antunes *et al*., 2011). The unravelling of the molecular basis of drought- and osmo-resistance has just begun (Zeidler and Müller, 2019). High salinity and drought have in common a low water activity, although they have individual stress components. *Acinetobacter baumannii* takes up glycine betaine or its precursor choline from the environment (Zeidler *et al*., 2017; Breisch *et al*., 2019). In the absence of solutes in its environment, it synthesizes glutamate or mannitol, and, to a smaller extent, trehalose (Zeidler *et al*., 2017, 2018). Whether or not inorganic solutes such as the commonly taken up K^+ is also taken up by *A. baumannii* remained to be established and was addressed in this study.

Results and discussion

K^+-dependence of growth of A. baumannii

To elucidate the minimal K^+ concentration required for growth of *A. baumannii* and to deplete cells of K^+ for further experiments, cells were grown in minimal media with 20 mM succinate as carbon and energy source and subsequently transferred in K^+-free media (Fig. 1A). After the first transfer, the growth rate as well as final yield was not affected but after the second transfer, a drastic reduction in growth rate from 0.64 to 0.19 h^{-1} was observed. At the same time, the yield was reduced by 80%. To restore exponential growth, K^+ was added back to the medium. As can be seen in Fig. 1B, the growth rate increased exponentially with increasing

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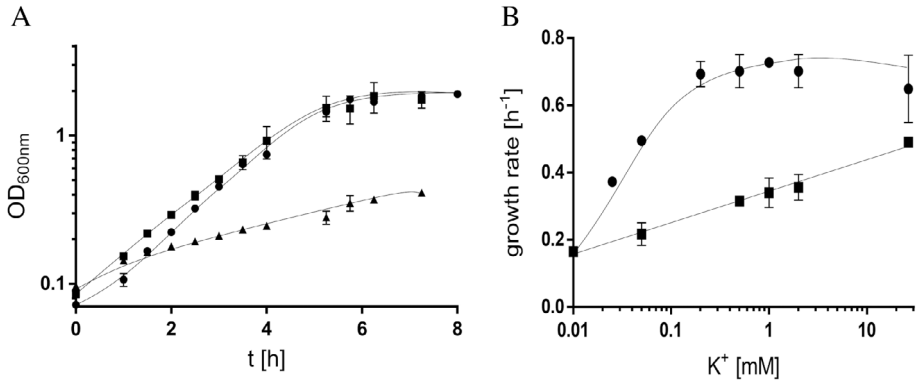

Fig 1. Effect of K^+ depletion and K^+-dependence of growth of *A. baumannii*. (A) *Acinetobacter baumannii* strain ATCC 19606^T was inoculated from a stationary preculture in minimal medium containing 20 mM succinate and 26.5 mM K^+ (●) and then transferred twice in K^+-free medium to an optical density (OD) of 0.1 each (■ first transfer, ▲ second transfer). (B) Cells were inoculated from a stationary preculture, grown twice in the absence of K^+, in minimal medium without NaCl into minimal medium with (■) and without (●) 300 mM NaCl and increasing amounts of K^+ to an OD of 0.1. One representative experiment out of at least three independent replicates is shown. Culture conditions and medium composition were as described before (Sand *et al*., 2013).

K^+ concentration and maximal growth was reached at 200 μM K^+.

Acinetobacter baumannii *accumulates K^+ as a response to osmotic stress*

When cells were transferred twice in K^+-free medium and then transferred to minimal medium containing 300 mM NaCl and increasing amounts of K^+, the growth rates were reduced by more than 50% compared to NaCl-free media (Fig. 1B). The amount of K^+ required for optimal growth increased fivefold to 1 mM K^+. The higher K^+ requirement for growth at high NaCl concentrations is in line with the hypothesis that K^+ is involved in osmostress response. To analyse the effect of NaCl on intracellular K^+ concentrations, cells were grown in minimal medium to mid-exponential growth phase and then subjected to an osmotic upshock by the addition of 300 mM NaCl. Cells were separated *via* filtration from excess media and washed to remove bound K^+, followed by cell lysis and determination of the K^+ concentration by atomic absorption flame spectroscopy. At low salinities (no NaCl added), the intracellular K^+ concentration was 0.56 ± 0.08 μmol mg^{-1} protein (Fig. 2A). If one assumes an internal volume of 2 μl mg^{-1} protein (Dinnbier *et al*., 1988), this would calculate roughly to 0.4 M intracellular K^+. This is plausible given the osmolarity of the minimal medium. After addition of NaCl to a final concentration of 300 mM, the internal K^+ concentration increased within 3–4 min threefold and then declined slowly over time. The transient increase in intracellular K^+ after an osmotic upshock has been seen before with other bacteria (Dinnbier *et al*., 1988; Whatmore *et al*., 1990; McLaggan *et al*., 1994). The intracellular K^+ concentration was a clear function of the external salinity (NaCl concentration) (Fig. 2B).

K^+ accumulation at high NaCl concentration may be the result of the action of an Na^+/K^+ antiport driven by the membrane potential to expel cytotoxic Na^+ from the cell or the result of a general osmotic effect. To address this question, cells were grown in minimal medium to mid-exponential growth phase; then the osmolarity in the medium was increased by addition of sucrose or glucose. As can be seen in Fig. 3A, non-ionic osmolytes also induced K^+ influx with the same rate and to the same extent as with 300 mM NaCl. This experiment clearly demonstrates that uptake of K^+ is a response to general osmotic stress.

If K^+ is taken up as an osmolyte in a first, fast response to osmostress, the presence of organic osmolytes taken up fast by the cells in response to osmostress such as glycine betaine should repress or reduce K^+ uptake. This was addressed by incubating cells in the presence of 1 mM glycine betaine prior to addition of NaCl. The presence of glycine betaine indeed resulted in a drastically reduced K^+ accumulation (Fig. 3B). While a minor amount of K^+ was taken up in the first minutes, K^+ was exported fast.

The intracellular pool of compatible solutes is a function of the external K^+ concentration

The experiments described so far are consistent with the notion that K^+ is accumulated by the cells as a first response to osmostress. To elucidate whether the K^+ concentration affects the pool size of compatible solutes, cells were grown in minimal medium on 20 mM succinate in the presence of 300 mM NaCl and different K^+ concentrations. Strict K^+ limitation lead to a significant reduction of intracellular glutamate (0.2 ± 0.02 μmol mg^{-1} protein)

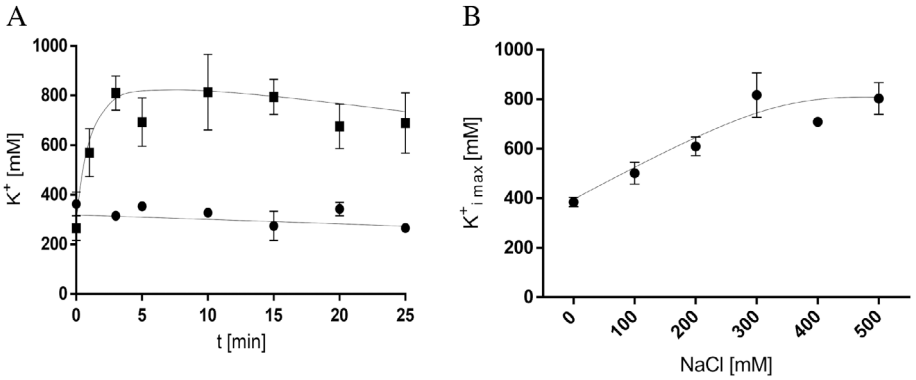

Fig 2. Accumulation of K^+ by *A. baumannii* as a response to osmostress. For determination of intracellular K^+ concentrations, cells were grown in minimal medium containing 20 mM succinate as carbon and energy source to mid-exponential growth phase. Then NaCl was added (time point 0) and samples were taken at the time points indicated. To separate cells from excess media, 1 ml cell culture was filtered *via* nitrocellulose filters (25 mm diameter, 0.2 μm pore size, Whatman, Maidstone, United Kingdom), followed by two washing steps with 1 ml K^+-free minimal medium for maximal 20 s. Filters were incubated in an acid mixture containing 0.75 ml of 3 M perchloric acid and 0.75 ml of 30% trichloracetic acid and 1 mg ml^{-1} CsCl. Samples were vigorously shaken for 20 s and stored for further use or at least over night at –20°C. Solutions were diluted five-fold in 5 mM CsCl (suppression of interferences) and K^+ was detected and quantified by flame spectroscopy (shortly AAS, AAnalyst 200, Perkin Elmer, Waltham, MA, USA). The signal output was linear in the range from 5 to 60 μM K^+ (potassium standard for AAS KNO_3, Sigma Aldrich, St. Louis, MO, USA). Protein concentrations were determined according to Schmidt (1963). (A) One culture (●) did not receive NaCl, the other 300 mM NaCl at time point 0 (■). (B) Different amounts of NaCl were added (0–500 mM) at time point 0. For the calculation of internal K^+ concentrations, an intracellular volume of 2 μl μg^{-1} protein was assumed (Dinnbier *et al*., 1988). Shown are mean and standard deviations from at least two independent experiments.

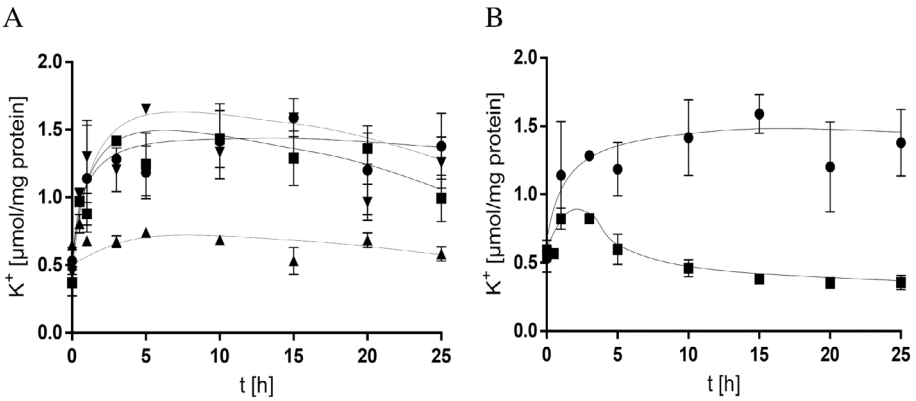

Fig 3. K^+ uptake as a response to ionic and non-ionic osmolytes and its reduction by the presence of glycine betaine. Intracellular K^+ concentrations were determined as described. Cells were grown in minimal medium to mid-exponential growth phase. Then cells were shocked with (A) 536 mM sucrose (●), 600 mM glucose (■) and 300 mM NaCl (▼) at time point 0. Different concentrations were calculated and used to obtain similar osmolarity values. The control did not receive any osmolytes (▲). (B) Cells were grown in minimal medium containing 1 mM glycine betaine to mid-exponential growth phase. Then, cells were shocked with 300 mM NaCl (■) at time point 0. As a control, cells grown in the absence of glycine betaine were shocked as well (●). Shown are mean and standard deviations from at least two independent experiments.

and mannitol (0.04 ± 0.01 μmol mg^{-1} protein) concentrations (Fig. 4). Increasing K^+ concentrations restored glutamate and mannitol levels. Surprisingly, the trehalose concentration was extraordinarily high at low K^+ concentrations and reached 0.26 ± 0.06 μmol mg^{-1} protein, which is roughly 13-fold than what was observed before (0.02 μmol mg^{-1} protein; Zeidler *et al*., 2017). With increasing K^+ concentrations, the trehalose content decreased and reached a minimum at 1 mM K^+; at this K^+ concentration, the trehalose concentration was only 0.02 μmol mg^{-1} protein, or 10% of the maximal value which is in the range of the trehalose concentration determined before (0.02 μmol mg^{-1} protein; Zeidler *et al*., 2017).

Dynamics of intracellular pools of K^+ and compatible solutes after osmotic upshock

To address the time-dependence of the accumulation of K^+ and organic solutes, cells were grown to mid-exponential growth phase. After the addition of NaCl to a final concentration of 300 mM, the intracellular K^+ concentration increased by 65% within 2–3 min (Fig. 5).

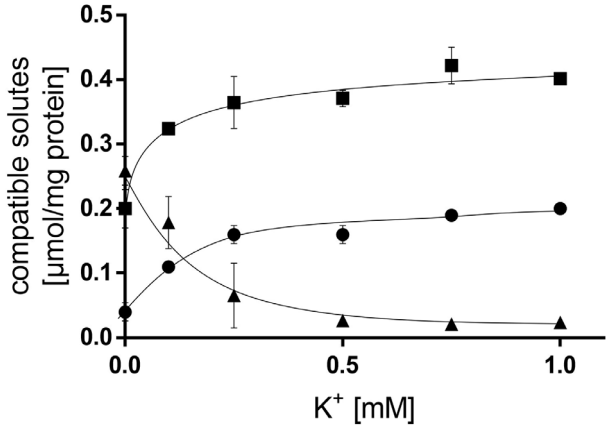

Fig 4. The amount of compatible solutes is dependent on the external K^+ accumulation. Cells were grown in minimal medium at 300 mM NaCl and harvested in mid-exponential growth phase. Glutamate (■), mannitol (●) and trehalose (▲) concentrations were determined as described before by enzymatic assays (Zeidler *et al*., 2017). Shown are mean and standard deviations from at least three independent experiments.

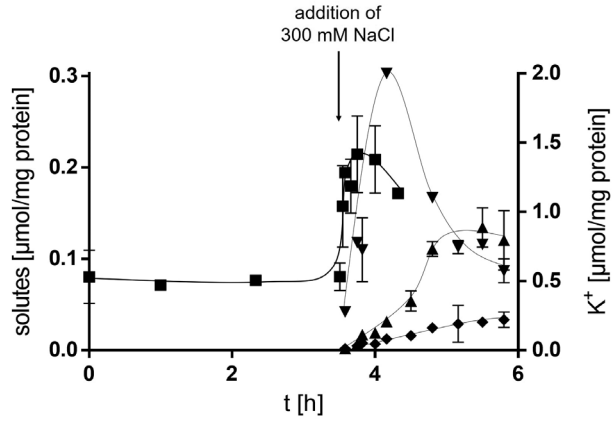

Fig 5. Dynamics of intracellular pool sizes of K^+ and compatible solutes after osmotic upshock. Cells were grown in minimal medium to mid-exponential growth phase. At the time point indicated by the arrow, NaCl was added to a final concentration of 300 mM. Glutamate (▼), mannitol (▲), trehalose (◆) and K^+ (■) concentrations were determined as described at the time points indicated (Zeidler *et al*., 2017). Shown are mean and standard deviations from at least two independent experiments.

Uptake of K^+ was accompanied by synthesis of glutamate, delayed by only a few minutes. Ten minutes after the upshock, the synthesis of mannitol and trehalose started. At the same time, K^+ and glutamate concentrations decreased again. This experiment clearly shows a temporal order of solute accumulation: K^+ → glutamate → mannitol and trehalose. The same has been observed before for *Escherichia coli* and *Bacillus subtilis*. In the former, trehalose is finally synthesized and in the latter, proline (Strøm *et al*., 1986; Larsen *et al*., 1987; Dinnbier *et al*., 1988; Whatmore *et al*., 1990).

Conclusion

Like other bacteria such as *E. coli* (Dinnbier *et al*., 1988; McLaggan *et al*., 1994) and *B. subtilis* (Whatmore *et al*., 1990), *A. baumannii* transiently accumulates K^+, followed by the synthesis of compatible solutes. Glutamate is produced to counterbalance the positive charges (Booth and Higgins, 1990; Kempf and Bremer, 1998). Uptake of K^+ can be mediated by one or more of the primary and secondary active transporter encoded in the genome of *A. baumannii*. The transiently high concentration of K^+ (and glutamate) is often discussed to induce the synthesis of compatible solutes (Sutherland *et al*., 1986; Gowrishankar and Manna, 1996; Gralla and Vargas, 2006) and there is good evidence for this hypothesis. On the other hand, other data (Balaji *et al*., 2005) are not in line with this hypothesis. Anyway, in the absence of K^+, synthesis of compatible solutes is largely reduced in *A. baumannii* and maximal growth is observed at 0.2 mM K^+, but this value is increased at high NaCl concentrations. This is of physiological significance. In the urinary tract, an ecological niche for *A. baumannii*, the K^+ concentration is 40–70 mM and the NaCl concentration is 400 mM (Culham *et al*., 2001; Meyer *et al*., 2019). In addition, the human blood (4 mM) and different types of host cells (100–160 mM) supply enough K^+ to sustain growth of *A. baumannii* (Su *et al*., 2009; Xue *et al*., 2011). To answer the question whether K^+ uptake is important for survival in or infection of the host remains a challenging task for future studies.

Acknowledgements

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Conflict of interest

The authors declare no conflict of interest.

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