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K^+ and its role in virulence of *Acinetobacter baumannii*

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ABSTRACT

Acinetobacter baumannii is an opportunistic human pathogen that has become a global threat to healthcare institutions worldwide. The success of *A. baumannii* is based on the rise of multiple antibiotic resistances and its outstanding potential to persist in the human host and under conditions of low water activity in hospital environments. Combating low water activities involves osmoprotective measures such as uptake of compatible solutes and K^+. To address the role of K^+ uptake in the physiology of *A. baumannii* we have identified K^+ transporter encoding genes in the genome of *A. baumannii* ATCC 19606. The corresponding genes (*kup*, *trk*, *kdp*) were deleted and the phenotype of the mutants was studied. The triple mutant was defective in K^+ uptake which resulted in a pronounced growth defect at high osmolarities (300 mM NaCl). Additionally, mannitol and glutamate synthesis were strongly reduced in the mutant. To mimic host conditions and to study its role as an uropathogen, we performed growth studies with the K^+ transporter deletion mutants in human urine. Both, the double (Δ*kup*Δ*trk*) and the triple mutant were significantly impaired in growth. This could be explained by the inability of Δ*kup*Δ*trk*Δ*kdp* to metabolize various amino acids properly. Moreover, the reactive oxygen species resistance of the triple mutant was significantly reduced in comparison to the wild type, making it susceptible to one essential part of the innate immune response. Finally, the triple and the double mutant were strongly impaired in *Galleria mellonella* killing giving first insights in the importance of K^+ uptake in virulence.

1. Introduction

In their environment living cells are constantly challenged by fluctuations in water availability and changes in the environmental osmolarity (Kempf and Bremer, 1998; Ventosa et al., 1998; Wood, 1999). Since biological membranes are permeable to water, an increase in the environmental osmolarity will drag water from the cell which leads to shrinkage and finally cell death (Galinski and Trüper, 1994; Roeßler and Müller, 2001). The countermeasure taken by living cells is a simultaneous increase of the osmolarity of the cell water, ideally above the environmental osmolarity to keep up the turgor. Living cells have developed different strategies to increase cellular osmolarity (Da Costa et al., 1998; Oren, 1999; Roeßler and Müller, 2001). One is to accumulate compatible solutes, small and highly soluble organic molecules that can be amassed by the cell in molar concentration (Bremer and Krämer, 2019; Roeßler and Müller, 2001; Santos and Da Costa, 2002). Compatible solutes are either synthesized or taken up from the environment (Pflüger and Müller, 2004), while uptake is preferred over synthesis for energetic reasons (Oren, 1999). Synthesis of compatible solutes is, in the model organisms *Escherichia coli* (Dinnbier et al., 1988) and *Bacillus subtilis* (Whatmore et al., 1990), preceded by an uptake of K^+, the same was observed in *A. baumannii* recently (König et al., 2020) and it is discussed whether the increase in internal K^+ concentrations signals low water activity conditions to the cell (Galinski and Trüper, 1994; Roeßler and Müller, 2001).

Recently, we have shown that growth of *A. baumannii* is K^+ dependent and that K^+ is accumulated transiently and replaced by compatible solutes after an osmotic upshock (König et al., 2020). We have followed up these observations and addressed the role of K^+ transport systems in osmostress resistance of *A. baumannii* by mutational analyses. We describe here that K^+ uptake is not only important for osmoadaptation of *A. baumannii* but also for growth in human urine and virulence in *G. mellonella*. Thus, this work provides novel insights into the role of K^+ uptake in osmoadaptation and in pathobiology of *A. baumannii*.

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2. Results

2.1. K^+ transporter genes in A. baumannii ATCC 19606

The genome of *A. baumannii* ATCC 19606 encodes for different K^+ transport systems. In addition to unspecific outer membrane porins (OmP, HMPREF0010_00698) and a mechanosensitive channel (HMPREF0010_01639), the genome harbors a gene for a voltage-gated K^+ channel (HMPREF0010_00214) and an annotated K^+/H^+ antiporter (HMPREF0010_01792). Additionally, it encodes the well-known secondary K^+ transporter Kup (HMPREF0010_02461) and Trk (HMPREF0010_00412−00413) and the primary active, ATP-driven K^+ transport system Kdp (HMPREF0010_00200−00204). The genes encoding the Kdp-ATPase are preceded by two genes whose products are very similar to the Kdp-two component system, KdpDE (HMPREF0010_00203−00204). The potential transport systems in *A. baumannii* ATCC 19606 are depicted in Fig. 1; they are quite similar to the homologous transporter of *E. coli* (Table 1). Moreover, the same set of genes is found in the non-pathogenic *A. baylyi*, and the non-pathogen does not contain additional K^+ transporter genes.

2.2. Markerless deletion of all three K^+-specific importers

To address the function of the K^+ transporter Kdp, Kup and Trk, the encoding genes were deleted from the genome *via* an established markerless mutagenesis system (Stahl et al., 2015) based on homologous recombination, described in experimental procedures. In addition

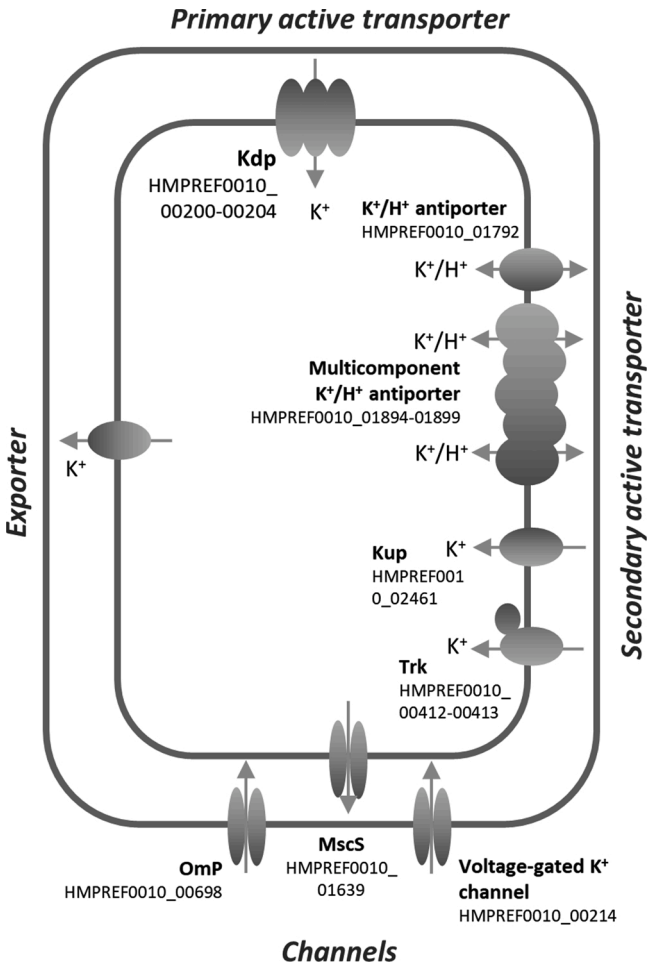

Fig. 1. Potential K^+ transporter in *A. baumannii* as delineated from the genome sequence.

Δ*kup*Δ*trk*, a double mutant, defect in both secondary active transporter, and a Δ*kup*Δ*trk*Δ*kdp* triple mutant were generated. To verify the deletion of the desired gene, control PCRs with specific primers listed in Supporting Information 1 were performed. The successful removal of the genes from the chromosome was verified by PCR analysis (Fig. S 1). Using the markerless mutagenesis system polar effects could be excluded by the genetic orientation of the corresponding genes.

2.3. K^+ transporter mutants are impaired in K^+ uptake

Recently, we have shown that K^+ is transiently accumulated after an osmotic upshock (König et al., 2020). To analyze the impact of K^+ transporter deletion on K^+ uptake we determined intracellular K^+ levels of the triple mutant. Indeed, accumulation of K^+ in the cytoplasm was largely impaired in the Δ*kup*Δ*trk*Δ*kdp* strain (Fig. 2). While the wild type increased its K^+ level up to 3-fold after the first 2−3 min, the mutant took up only 40 % of K^+ transiently. 25 min after the upshock, the K^+ concentration in the Δ*kup*Δ*trk*Δ*kdp* cells in the absence and presence of an osmotic upshock was identical.

2.4. K^+ transporter genes are involved in osmoregulation

Since a tight correlation between K^+ availability and compatible solute production was already shown, the solute pool of the triple mutant grown in minimal medium with 26.5 mM K^+ (standard minimal medium, (Zeidler et al., 2017)) and 300 mM NaCl was determined (Fig. S 2 A). Mannitol production was only about 50 % while the glutamate concentration was only 29 % compared to wild type levels. Only trehalose concentrations were similar, which suggests that only the mannitol and glutamate pools are dependent on K^+ transport. At limiting K^+ concentrations (Fig. S 2 B), the effect was even more dramatic. Mannitol and glutamate decreased to 22 and 26 % of wild type levels. These data reinforce our suggestion (König et al., 2020) that the internal K^+ concentration is critical for solute synthesis.

Having shown that K^+ accumulation and compatible solute production is largely impaired in the Δ*kup*Δ*trk*Δ*kdp* mutant we analyzed the individual role of the three K^+ transporter in osmoadaptation. Therefore, the K^+ transporter mutants were tested for their ability to adapt to 300 mM NaCl in minimal medium (Fig. S 3). Deletion of *kdp*, *kup* or *trk* had no effect on growth of non-stressed cells at normal K^+-concentrations (26.5 mM K^+) (data not shown). When the NaCl concentration was increased to 300 mM NaCl, deletion of *kdp* had no effect on growth (Fig. S 3) which is expected since the Kdp system is only active at low K^+ concentration (Roe et al., 2000). At high K^+ concentration, the secondary active transporter are produced and active (Epstein, 2003). Deletion of Kup resulted in a slight lag phase, and deletion of Trk had a more pronounced effect on growth at normal K^+ and high NaCl (Fig. S 3). Deletion of both *kup* and *trk* led to a severe impairment in growth with a lag phase of about 10 h (Fig. S 3), suggesting that the individual low affinity transporter can compensate for each other's functions.

For further characterization of the high affinity Kdp system produced only at low K^+, Δ*kdp* cells were depleted of K^+ by multiple transfers into K^+-free medium. When cells were transferred twice in K^+-free medium and then used to inoculate K^+-free medium again, growth of the Δ*kdp* strain was severely impaired (data not shown). This was even more pronounced at high NaCl. At 300 mM NaCl, the culture had a lag phase of 26 h before growth resumed with a growth rate reduced by 47 % (Fig. S 4). Addition of 10 mM KCl fully restored growth of Δ*kdp* at high salt (data not shown). In summary, these data are in line with the hypothesis that the K^+ transport systems are required for osmoadaptation, the Kdp system under limiting K^+ concentration and the secondary active transporter Kup and Trk at saturating K^+ concentrations. The Δ*kup*Δ*trk*Δ*kdp* strain in which all major K^+ transporter genes are deleted, did not grow in K^+-free minimal medium (Fig. 3 A). Stepwise addition of K^+ to the medium restored growth of the triple mutant.

Table 1
Potential K^+ transporter in *A. baumannii* ATCC 19606.

locus tag	encoded protein	gene length [bp]	molecular mass [kDa]	identity* [%]	similarity* [%]
HMPREF0010_00200	KdpA	1710	63.3	37.8	55.8
HMPREF0010_00201	KdpB	2034	75.3	55.8	73.8
HMPREF0010_00202	KdpC	606	22.4	36.6	52.3
HMPREF0010_00203	KdpD	2655	98.2	33.3	51.6
HMPREF0010_00204	KdpE	717	26.5	42.8	60.5
HMPREF0010_00412	TrkH	1338	49.5	19.5	35.6
HMPREF0010_00413	TrkA	651	24.1	10.6	20.3
HMPREF0010_02461	Kup	1878	69.5	48.4	66.4

*Identity and similarity values apply to the homologous *E. coli* proteins.

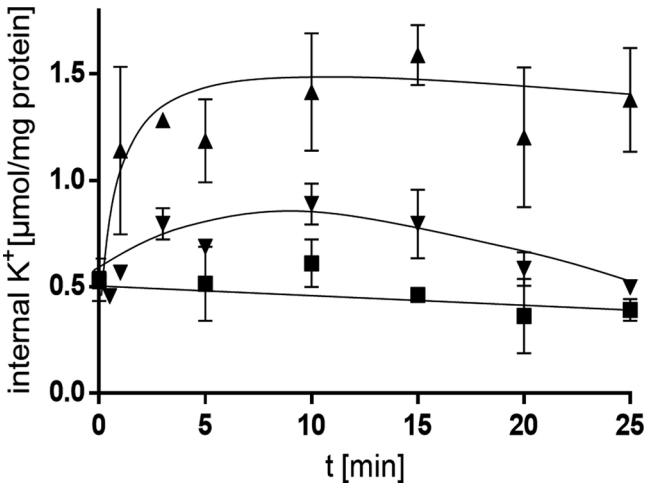

Fig. 2. The triple mutant is defective in K^+ accumulation as a response to osmostress. Wild type and Δ*kup*Δ*trk*Δ*kdp* cells were grown in minimal medium to mid-exponential growth phase. One mutant culture (■) did not receive NaCl, the other 300 mM NaCl (▼). Wild type cells shocked with 300 mM NaCl served as a control (▲). Cells were harvested *via* filtration at time points indicated after the addition of NaCl and the intracellular K^+ concentration was determined. Mean values and standard deviations of four independent biological replicates are shown.

Growth rates similar to the wild type were obtained in the presence of at least 20 mM K^+ (Fig. 3 B). Addition of 300 mM NaCl to minimal medium with saturating K^+ concentrations increased the growth defect of Δ*kup*Δ*trk*Δ*kdp* strain (Fig. 4). Exponential growth started after a lag phase of about 13 h (Fig. 4 A). Transfer of Δ*kup*Δ*trk*Δ*kdp* cells to K^+-limited conditions and osmotic stress (300 mM NaCl) resulted in an extended lag phase of 40 h after which growth resumed (Fig. 4 B).

2.5. Deletion of K^+ transporter affects the adaptation to alkaline and acidic pH

A. baumannii faces a broad spectrum of different pH values inside the human host. While acidic pH values of 1–4 dominate the gastric mucosa, alkaline pH are reached predominantly in the intestine (Evans et al., 1988). To test the ability to withstand changing pH conditions all K^+ transporter deletion mutants were grown in minimal medium of either pH 4.8 or 9.8 (Fig. 5). Since consumption of succinate leads to alkalization of the medium (increase from pH 7 to pH 9) succinate was unsuitable as energy source for this experiment. In contrast, consumption of arabinose did not change the pH of the medium, therefore it was used as carbon and energy source instead. Acidic conditions resulted in a slight growth defect of Δ*trk* and Δ*kdp*, while growth of the triple mutant was completely abolished (Fig. 5 A). The double mutant and Δ*kup* were not affected. A converse behavior was observed at alkaline pH (Fig. 5 B). Here, growth of both the triple and double mutant started immediately and was not inhibited under these conditions at all. In contrast, wild type, Δ*trk* and Δ*kdp* cells were unable to grow. The single mutant Δ*kup* had a prolonged lag-phase before resuming growth after 8 h. Taken together, K^+ transporter are important for pH regulation in *A. baumannii* and apparently they are specific for growth at different pH values.

2.6. Growth of ΔkupΔtrkΔkdp is impaired in human urine

Besides causing respiratory tract infections and bacteremia *A. baumannii* can cause urinary tract infections (Bergogne-Berezin and Towner, 1996) and is able to grow in human urine (Di Venanzio et al., 2019). An important trait of human urine is its high salt concentration (up to 400 mM NaCl) with a pH of 6. Cells of the wild type and all mutant strains were pre-cultivated on LB, washed in sterile saline and then transferred to human urine (Fig. 6). The wild type started to grow immediately with a rate of 0.54 h^{-1} until the final OD_{600nm} of 0.79 was reached after 7 h. The triple mutant resumed growth after an extended

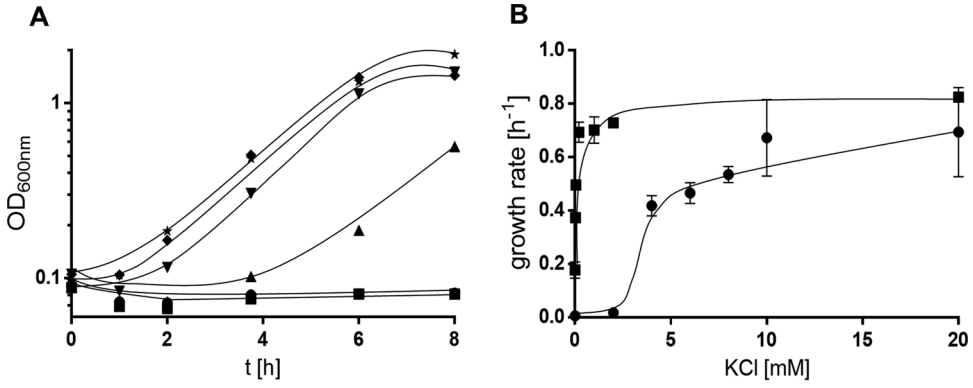

Fig. 3. Growth of *A. baumannii* Δ*kup*Δ*trk*Δ*kdp* is K^+ dependent. (A) Δ*kup*Δ*trk*Δ*kdp* cells were grown to stationary growth phase in minimal medium containing 20 mM succinate and washed with K^+-free minimal medium before inoculation of the main culture to an OD_{600nm} = 0.1 containing different K^+ concentrations (0 mM: ■; 1 mM: ●; 5 mM: ▲; 10 mM: ▼; 15 mM: ◆; 26.5 mM: ★). **(B)** The K^+ concentrations of the growth medium varied between 0 and 26.5 mM K^+ and corresponding growth rates were determined. Growth of wild type (■) and Δ*kup*Δ*trk*Δ*kdp* cells (●) was monitored by measuring the optical density at 600 nm over 8 h. Cells were incubated on a rotary shaker in 100 mL media in a 500 mL flask at 37 °C. Mean values and standard deviations of three independent biological replicates are shown.

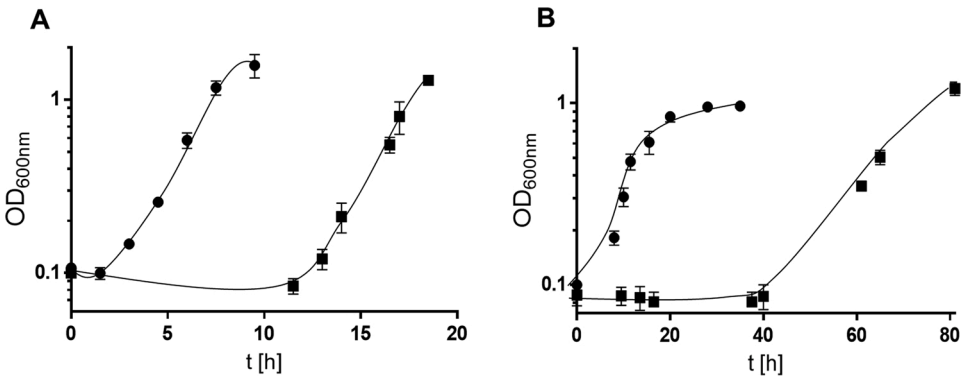

Fig. 4. Growth of the triple mutant Δ*kup*Δ*trk*Δ*kdp* under high salt. Wild type (●) and Δ*kup*Δ*trk*Δ*kdp* cells (■) were grown in minimal medium containing 20 mM succinate and were then transferred to **(A)** minimal medium containing 300 mM NaCl (26.5 mM K^+, 20 mM succinate) or to **(B)** K^+-free minimal medium containing 300 mM NaCl (trace amounts, 20 mM succinate). Cells were incubated on a rotary shaker in 100 mL media in a 500 mL flask at 37 °C and growth was monitored by measuring the optical density at 600 nm. Mean values and standard deviations of three independent biological replicates are shown.

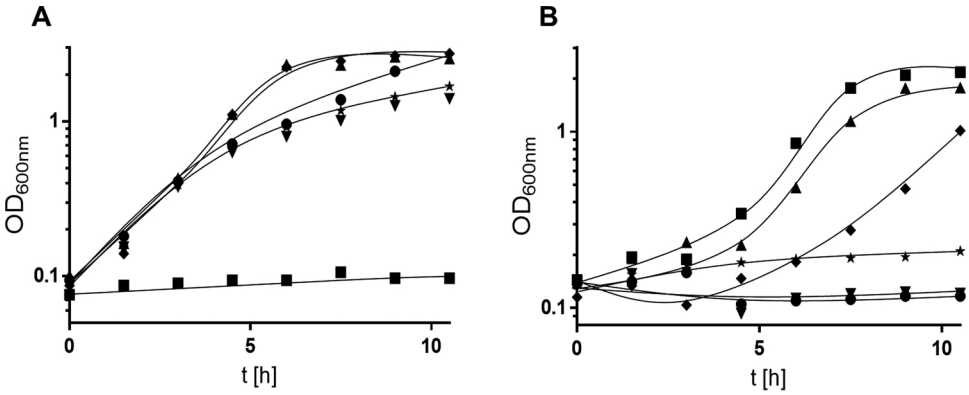

Fig. 5. K^+ transporter are crucial for adaptation to different pH values. Wild type (●) and mutant cells (Δ*kup*Δ*trk*Δ*kdp*: ■; Δ*kup*Δ*trk:* ▲; Δ*kup:* ◆; Δ*trk:* ▼; Δ*kdp:* ★*)* cells were grown in minimal medium (20 mM arabinose, 26.5 mM K^+) to stationary growth phase. Growth medium was buffered to either pH 4.8 with citrate phosphate **(A)** or to pH 9.8 with CHES **(B)**. Cells were incubated on a rotary shaker in 100 mL media in a 500 mL flask at 37 °C and growth was monitored by measuring the optical density at 600 nm. One representative out of at least three experiments is shown.

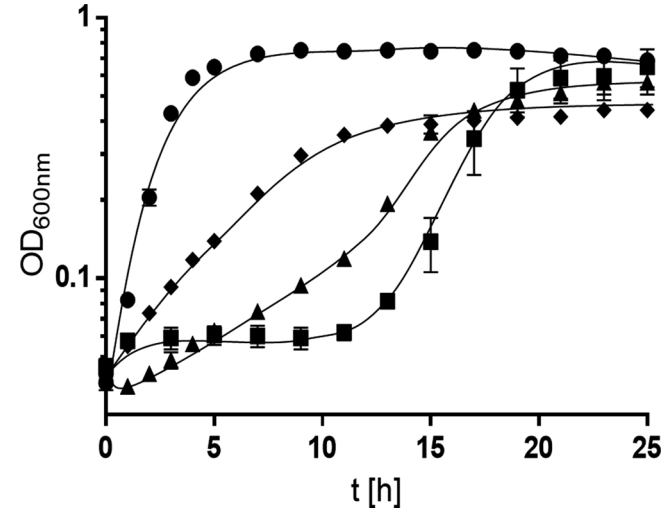

Fig. 6. Growth in human urine is impaired in the K^+ transporter deletion mutants. Wild type (●), Δ*kup*Δ*trk*Δ*kdp* (■), Δ*kup*Δ*trk* (▲) an Δ*kup* (◆) cells were grown in LB to stationary growth phase, harvested and washed two times in sterile saline. First void human urine was pooled from 12 donors (mixed male and female) and sterile filtered. Cells were incubated on a rotary shaker in 100 mL media in a 500 mL flask at 37 °C and growth was monitored by measuring the optical density at 600 nm. Mean values and standard deviations of three independent biological replicates are shown.

lag phase of 15 h whereas the double mutant started after 11 h after inoculation. Δ*kup* is the only single deletion mutant showing any defect in growth under these conditions.

2.7. ΔkupΔtrkΔkdp is defective in amino acid utilization

Amino acids are the most abundant carbon source for bacteria in human urine (Culham et al., 2001; Guyton and Hall, 2006; Putnam, 1971). To elucidate whether the triple mutant has a defect in amino acid utilization, drop dilution assays on Bacto™ Casamino acids and on tryptone medium were performed (Fig. 7). Surprisingly, growth of the triple mutant was completely abolished in CAS medium, while Δ*kup*Δ*trk* had only a slight growth defect under these conditions. Consistently,

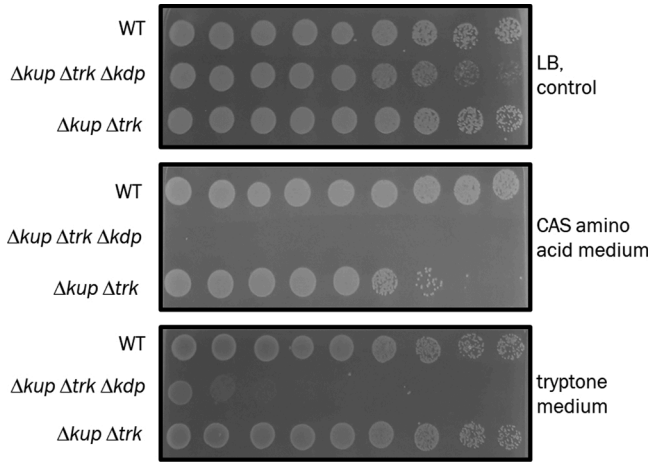

Fig. 7. Δ*kup*Δ*trk*Δ*kdp* is defective in the utilization of amino acids. Wild type, Δ*kup*Δ*trk*Δ*kdp* and Δ*kup*Δ*trk* cells were grown in minimal medium (26.5 mM K^+, 20 mM succinate) to stationary growth phase, harvested and washed three times in sterile saline. Cell suspensions were adjusted to $OD_{600nm} = 1$ and 10 µl of serial dilutions were spotted on LB, CAS and tryptone medium agar plates each. Plates were incubated over night at 37 °C.

there was only little growth of the triple mutant in tryptone medium. Next, we tested growth on individual amino acids. Therefore, minimal medium containing either alanine & leucine (neutral), aspartate & glutamate (negatively charged), histidine (positively charged) or phenylalanine (aromatic) as sole carbon source were used (Fig. 8). Independent of the amino acid used, the triple mutant had a clear growth defect in comparison to the wild type, indicating that it is not only defective in osmoadaptation but also in utilizing one of the main carbon sources in the human host. Whereas growth on alanine, phenylalanine and aspartate resumed after some time, it did not with histidine. Interestingly, the triple mutant performed better when grown on leucine. This effect is based on a further spontaneous mutation within the triple mutant (data not shown). Isoleucine and valine were tested as well but neither of them was utilized as sole carbon and energy source (data not shown).

2.8. Reactive oxygen species resistance is diminished in the triple mutant

Bacteria invading humans have to combat defense mechanisms of the host. One strategy to overcome bacterial infections and to eliminate pathogens in the bloodstream is the accumulation of reactive oxygen species (ROS) by phagocytes. Therefore, bacteria have evolved several functions to neutralize ROS. To test the role of K^+ transporter in ROS resistance, serial dilutions of the wild type, Δ*kup*Δ*trk*Δ*kdp* and Δ*kup*Δ*trk* were spotted on ROS generating agar plates (Fig. 9). Although growth of both strains was affected by the presence of reactive oxygen species, the triple mutant showed a clear defect in ROS resistance in comparison to the wild type. As a control all strains were spotted on LB agar showing only a minor growth deficit of the triple mutant.

2.9. Δkup Δtrk Δkdp and Δkup Δtrk are less effective in killing Galleria mellonella larvae

Since the triple mutant performed poorly in ROS resistance, its ability to infect a complex model organism was tested. Therefore, *G. mellonella* larvae were infected with 10 µl (~ 1 * 10^6 CFU) of wild type, Δ*kup*Δ*trk*Δ*kdp* and Δ*kup*Δ*trk* cells grown on LB and incubated at 37 °C for six days (Fig. 10). The wild type killed 60 % of the larvae within the first 24 h, while only 20 % were killed by the triple mutant. Over the course of time both deletion mutants were less effective in killing *G. mellonella* larvae, although the wild type killed the whole batch after four days. Additionally, several larvae which were injected with the deletion strains could recover from their infection, showing that the innate immune system could clear the bacteremia caused by Δ*kup*Δ*trk*Δ*kdp* and Δ*kup*Δ*trk*. The single mutants were not impaired in virulence in the *Galleria* model (data not shown).

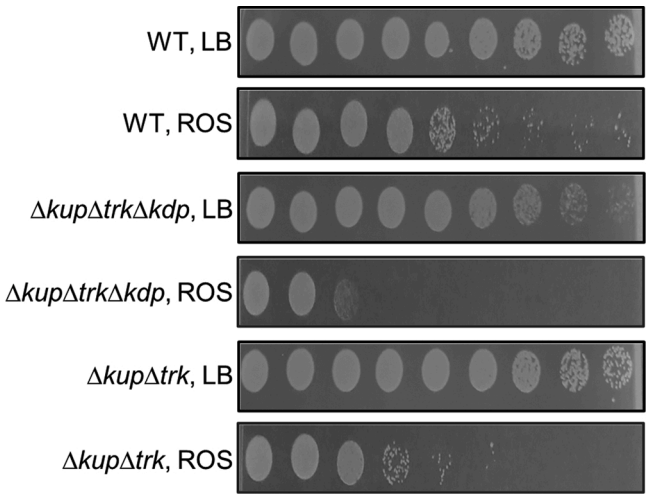

Fig. 9. Δ*kup*Δ*trk*Δ*kdp* and Δ*kup*Δ*trk* are sensitive to ROS inactivation. Wild type, Δ*kup*Δ*trk*Δ*kdp* and Δ*kup*Δ*trk* cells were grown in minimal medium (26.5 mM K^+, 20 mM succinate) to stationary growth phase, harvested and washed three times in sterile saline. Cell suspensions were adjusted to OD_{600nm} = 1 and 10 µl of serial dilutions were spotted on LB and ROS generating agar plates (containing 100 µM H_2O_2, 10 µM $FeSO_4$, 10 µM NaI) each. Plates were incubated over night at 37 °C.

3. Discussion

The major bacterial K^+ transporter Kup, Trk, Ktr and Kdp are well-

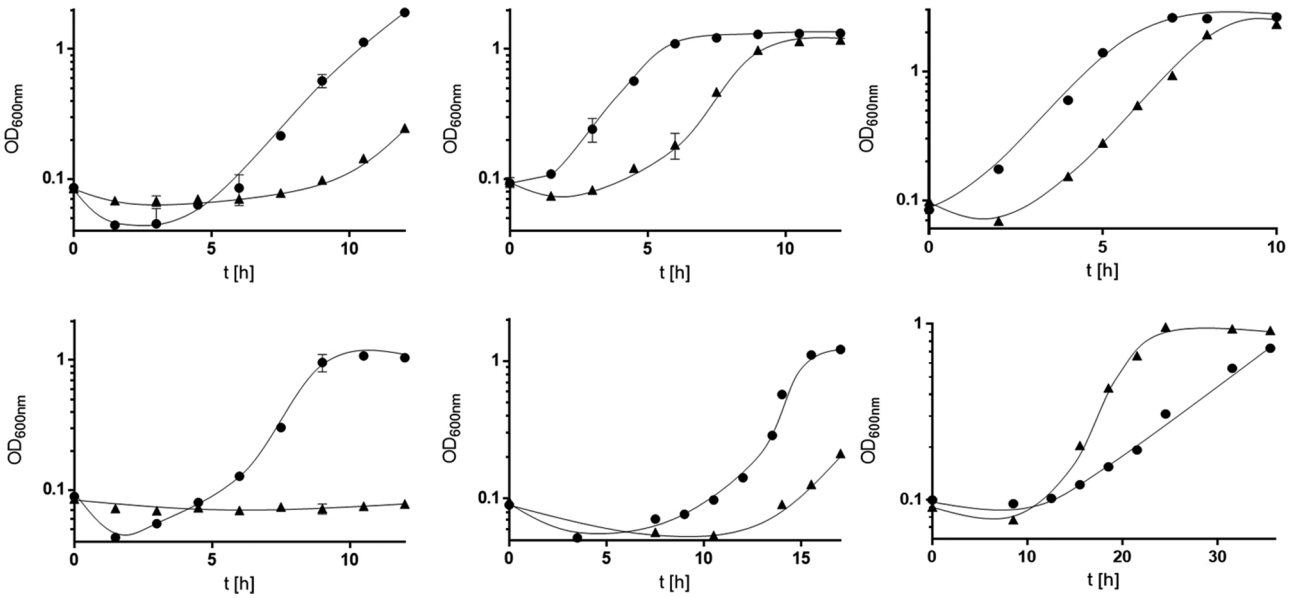

Fig. 8. Growth of Δ*kup*Δ*trk*Δ*kdp* on different amino acids. Wild type (●) and Δ*kup*Δ*trk*Δ*kdp* (▲) cells were grown in minimal medium containing 20 mM succinate and were then transferred to medium containing either 20 mM alanine (upper left corner), 15 mM aspartate (upper center), 10 mM histidine (lower left corner), 10 mM phenylalanine (lower center), 20 mM glutamate (upper right corner) or 5 mM leucine (lower right corner) as sole carbon source. Cells were incubated on a rotary shaker in 100 mL media in a 500 mL flask at 37 °C and growth was monitored by measuring the optical density at 600 nm. Mean values and standard deviations of three independent biological replicates are shown.

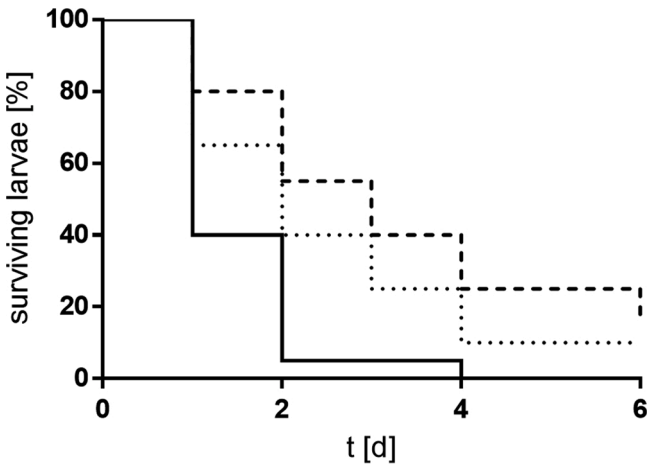

Fig. 10. K^+ transporter deletion mutants are less effective in killing *Galleria mellonella* larvae. Wild type (solid line), Δ*kup*Δ*trk*Δ*kdp**** (dashed line) and Δ*kup*Δ*trk*** (dotted line) cells were grown to stationary growth phase in LB, harvested and washed three times in sterile PBS. 10 µl (~$1*10^6$ CFU) were injected in *Galleria mellonella* larvae each. Pre- and post-selection was performed as describes in Experimental procedures. One representative out of at least three experiments is shown. Significance is assigned as: p < 0001 = ***, p < = 001 = **.

described in many different organisms, including pathogens and their involvement in general osmostress resistance is well analyzed (Epstein and Kim, 1971; Su et al., 2009; Xue et al., 2011). In this study, we showed that K^+ surpasses its role as an osmoprotectant drastically by generating a K^+ transporter triple mutant and revealing its importance in ROS resistance, growth in human urine, amino acid utilization and virulence.

While *A. baumannii* is most commonly associated with pneumoniae or blood stream infections its relevance as an uropathogen is increasing (Di Venanzio et al., 2019). Therefore, growth of the K^+ transporter deletion mutants in human urine was analyzed. Both, the triple and double mutant showed an extended lag phase while Δ*kup* grew immediately, but only with a highly reduced growth rate. This demonstrates that K^+ transporter play an important role in persistence in human urine of *A.baumannii* ATCC 19606 thereby promoting infections of the urogenital tract. Since urine harbors sufficient K^+ concentrations the defect is based on other parameters (Kirchmann and Pettersson, 1994; Putnam, 1971). Human urine imposes a variety of stresses on bacterial pathogens including osmotic stress and restricted carbon sources which makes it an unfavorable environment for many bacteria. Depending on nutrition and water intake osmolarity can increase up to 400 mM NaCl which could be one reason for the extreme growth phenotype of the triple and double mutant, but not for Δ*kup* which is evidentially not affected by high osmolarities. Next to glucose, the main energy source in human urine are different amino acids (Culham et al., 2001; Guyton and Hall, 2006; Putnam, 1971). Since *A. baumannii* is not able to utilize glucose we tested the triple and double mutant for efficient amino acid utilization. Interestingly, growth was abolished in the triple mutant, revealing a direct correlation between K^+ uptake and the general amino acid metabolism. One explanation for this phenotype would be the loss of proper pH regulation. Deletion of K^+ uptake systems abolishes the establishment of the K^+ gradient preventing efficient outbalancing of imported amino acid charges (Booth, 1985; Epstein, 2003; Harold, 1977). Further, K^+ is a known co-factor of many proteins and some transporters therefore it may have a direct or indirect involvement in amino acid metabolism (Halpern et al., 1973; Sleator and Hill, 2002). Besides high concentrations in human urine, amino acids are prominent in the vertebrate lungs as well, another colonization target of *A. baumannii* (Lonergan et al., 2020). It is also known that pathogenic species of *A. baumannii* have acquired multiple amino acid catabolization pathways in comparison to their environmental counterparts, which makes amino acid metabolism a striking advantage in colonization and infection of the human host (Lonergan et al., 2020). Since sufficient K^+ supply is a necessity for efficient amino acid utilization K^+ transport systems become important candidates for alternative drug targets.

Evading the innate immune response is a major prerequisite for human pathogens. One common strategy in combating invading bacteria is the production of ROS by phagocytes, the so called "respiratory burst" (Fang, 2004; Yang et al., 2013). In this study we observed that the triple and the double mutant are defective in ROS resistance implying a reduced survival rate in the human host. Pathogenic bacteria have evolved numerous mechanisms for ROS resistance making it difficult to identify the specific role of K^+ in this context. Besides exporting reactive oxygen species or suppressing phagosomal ROS production, their enzymatic detoxification is of great importance. As already stated, K^+ is a common co-factor of proteins making the loss of ROS resistance an indirect side effect of reduced K^+ uptake (Halpern et al., 1973; Sleator and Hill, 2002). Another important way to avoid damage by ROS are scavenging processes *e.g.* mediated by mannitol production as it is reported for candida species (Chaturvedi et al., 1996). Having shown that the triple mutant is defective in mannitol production under high salt conditions, we hypothesize that mannitol synthesis is probably stimulated under host conditions making the reduced ROS resistance a consequence of the malfunction in compatible solute production.

The triple and double deletion mutant were less efficient in killing *G. mellonella* larvae in comparison to the wild type. These findings underline the necessity of a functional K^+ transport in the infection process of *A. baumannii* ATCC 19606. In search for virulence determinants in *A. baumannii* Gebhardt et al. found a single *trk* insertion mutant to be less virulent in a *Galleria* infection model (Gebhardt et al., 2015). As stated in the results single K^+ transporter mutants of *A. baumannii* ATCC 19606 were not impaired in virulence in *Galleria mellonella*. This differs from the results obtained with *A. baumannii* AB5075 (Gebhardt et al., 2015). This difference could be due to the much higher virulence of strain AB5075 responsible for the more pronounced effect of a single K^+ transporter mutation in *Galleria* infection.

In conclusion, we have identified K^+ as an universal factor promoting virulence and persistence in *A. baumannii* ATCC 19606. By deleting all major K^+ transporter we could underline the importance of general osmostress resistance in host-bacteria interactions and supported the assumption of a multifactorial involvement of K^+ in the (patho-)physiology of *A. baumannii*. Our findings give rise to new approaches in identifying and selecting K^+ transporter in bacteria as a drug target in the future.

4. Material and methods

4.1. Bacterial strain and culture conditions

A. baumannii strain ATCC 19606^T was either grown in complex medium (LB (Bertani, 1951)) or in minimal medium with 20 mM succinate (Zeidler et al., 2017). Culture conditions and medium composition were as described before (Zeidler et al., 2017). For growth on human urine pooled sterile filtered first void urine was used.

4.2. Markerless mutagenesis

The markerless deletion mutant of all K^+ transporter (*kdp*: HMPREF0010_00200−00204, *trk*: HMPREF0010_00412−00413, *kup*: HMPREF0010_02461) in *A. baumannii* ATCC 19606^T was created as described before (Stahl et al., 2015). All primers used are listed in the Supporting Information (Tab. S 1). ~ 1500 bp up- and downstream of the gene to be deleted were amplified from genomic DNA using the primer pairs listed in the Supporting Informations. Up- and downstream regions contained 30–50 bp of the gene of interest. The PCR fragments

were cloned into pBIISK_*sacB/kanR* using PstI, BamHI and SpeI in case of *kdp* deletion, PstI, BamHI and NotI for deletion of *trk* and *kup*. The resulting plasmids pBIISK-*sacB/kanR_kdp*_up_down_nw, pBIISK-*sacB/-kanR_kup*_up_down_nw and pBIISK-*sacB/*kanR_*trk*_up_down_nw were used for transformation of electrocompetent *A. baumannii* ATCC 19606. For generating the double and triple mutant electrocompetent cells of either Δ*trk* or Δ*kup*Δ*trk* were used. Electroporation was performed at 2.5 kV, 200 Ω and 25 mF. Transformants were selected on LB-agar 150 mg/mL kanamycin and screened for integration of the plasmid into the genome *via* single homologous recombination performing colony PCR (primer for *kdp* deletion: ctrl_*kdp*_for & *kdpE*_down_nw_rev and ctrl_*kdp*_rev & *kdpA*_up_nw_for; primer for *kup* deletion: ctrl_*kup*_for & *kup*_down_nw_rev and ctrl_*kup*_rev & *kup*_up_nw_for; primer for *trk* deletion: ctrl_*trk*_for & *trk*_down_nw_for and ctrl_*trk*_rev & *trk*_up_nw_rev). Counter selection for segregation of the plasmid was done using sucrose. Integrants were grown overnight in LB + 10 % sucrose, plated on LB-agar + 10 % sucrose and single colonies were checked to have lost their ability to grow on kanamycin. The clones that had lost the plasmid were analyzed for deletion of the gene in a colony PCR with the control primers (for *kdp* deletion: ctrl_*kdp*_for and ctrl_*kdp*_rev; primer for *kup* deletion: ctrl_*kup*_for and ctrl_*kup*_rev; primer for *trk* deletion: ctrl_*trk*_for and ctrl_*trk*_rev). The correct locus of the deletion was confirmed by sequencing the PCR product.

4.3. Determination of intracellular K^+ concentrations

Determination of intracellular K^+ concentrations was performed by atomic absorption spectroscopy according to an established protocol (König et al., 2020).

4.4. Drop dilution assay

To study the effect of reactive oxygen species (ROS) and amino acid utilization, drop dilution assays were performed. Therefore, cells were grown in minimal medium (26.5 mM K^+, 20 mM succinate) to stationary growth phase, washed three times in sterile saline and adjusted to $OD_{600nm} = 1$. 10 µl of serial dilutions were spotted on LB agar plates each. Plates were incubated over night at 37 °C. To study ROS resistance a hydroxyl radical generating system was used (Nguyen et al., 2019). Therefore, LB agar plates contained 100 µM H_2O_2, 10 µM $FeSO_4$ and 10 µM NaI. To test for amino acid utilization either Bacto™ Casamino acids (2.5 g NaCl, 5 g CAS amino acids ad. 1 L) or tryptone medium (2.5 g NaCl, 5 g tryptone ad. 1 L) was used.

4.5. Galleria mellonella infection assay

Galleria mellonella larvae were obtained by a local provider. Caterpillars were preselected by melanization, size and movement in response to touch. Larvae were weighed and only larvae meeting the criteria of 350 ± 50 mg were utilized. Larvae were stored at 4 °C. *A. baumannii* wild-type and K^+ transporter mutant cells were grown on LB medium or minimal medium (20 mM succinate) until late exponential growth phase, washed with phosphate buffered saline (PBS) and adjusted to an OD_{600nm} of 2. In each set of infection, 20 caterpillars were used to test each bacterial strain in one test. 10 µl (~ 1 * 10^6 CFU) of the bacterial suspension were injected into one of the last prolegs. As control served a set of untreated caterpillars as well as a set of caterpillars in which 10 µl of PBS were injected. Caterpillars were incubated at 37 °C in the dark for 6 days. They were considered as dead if they did not respond to gentle probing. All experiments were repeated at least 4 times and experiments in which 2 or more caterpillars in one of the control groups died were not considered. Significance of survival differences was assessed with the *t*-test.

Author's contributions

Conceptualization and design: PK, BA and VM. Acquisition of data and conduction of experiments: PK. Analysis and interpretation of data: PK, BA and VM. Writing original draft: PK, BA and VM. Reviewing and editing: PK, BA and VM.

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Declaration of Competing Interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijmm.2021.151516.

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