Inhibition of the ATP Synthase Eliminates the Intrinsic Resistance of Staphylococcus aureus towards Polymyxins

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ABSTRACT Staphylococcus aureus is intrinsically resistant to polymyxins (polymyxin B and colistin), an important class of cationic antimicrobial peptides used in treatment of Gram-negative bacterial infections. To understand the mechanisms underlying intrinsic polymyxin resistance in S. aureus, we screened the Nebraska Transposon Mutant Library established in S. aureus strain JE2 for increased susceptibility to polymyxin B. Nineteen mutants displayed at least 2-fold reductions in MIC, while the greatest reductions (8-fold) were observed for mutants with inactivation of either graS, graR, vraF, or vraG or the subunits of the ATP synthase (atpA, atpB, atpG, or atpH), which during respiration is the main source of energy. Inactivation of atpA also conferred hypersusceptibility to colistin and the aminoglycoside gentamicin, whereas susceptibilities to nisin, gallidermin, bacitracin, vancomycin, ciprofloxacin, linezolid, daptomycin, and oxacillin were unchanged. ATP synthase activity is known to be inhibited by oligomycin A, and the presence of this compound increased polymyxin B-mediated killing of S. aureus. Our results demonstrate that the ATP synthase contributes to intrinsic resistance of S. aureus towards polymyxins and that inhibition of the ATP synthase sensitizes S. aureus to this group of compounds. These findings show that by modulation of bacterial metabolism, new classes of antibiotics may show efficacy against pathogens towards which they were previously considered inapplicable. In light of the need for new treatment options for infections with serious pathogens like S. aureus, this approach may pave the way for novel applications of existing antibiotics.

IMPORTANCE Bacterial pathogens that cause disease in humans remain a serious threat to public health, and antibiotics are still our primary weapon in treating bacterial diseases. The ability to eradicate bacterial infections is critically challenged by development of resistance to all clinically available antibiotics. Polymyxins constitute an important class of antibiotics for treatment of infections caused by Gram-negative pathogens, whereas Gram-positive bacteria remain largely susceptible towards this class of antibiotics. Here we performed a whole-genome screen among nonessential genes for polymyxin intrinsic resistance determinants in Staphylococcus aureus. We found that the ATP synthase is important for polymyxin susceptibility and that inhibition of the ATP synthase sensitizes S. aureus towards polymyxins. Our study provides novel insights into the mechanisms that limit polymyxin activity against S. aureus and provides valuable targets for inhibitors to potentially enable the use of polymyxins against S. aureus and other Gram-positive pathogens.

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Polymyxins (polymyxin B [PMB] and colistin) are lipopeptide antibiotics that consist of a peptide ring with a three-peptide side chain linked to a fatty acid tail. At physiological pH, polymyxins are polycationic, which in combination with the fatty acid tail makes them amphipathic. The amphipathic property of polymyxins promotes interaction with cell membranes, eventually leading to disruption of membrane integrity and cell death (1). The spectrum of activity of polymyxins is primarily confined to Gram-negative bacteria (2), where they increase the permeability of the outer membrane and the cytoplasmic membrane (3). Reduced susceptibility to polymyxins in Gram-negative bacteria can be mediated by reduction of the negative cell surface charge, which limits the electrostatic interaction between the positively charged polymyxins and negatively charged lipopolysaccharides (4).

Polymyxins are generally less active against Gram-positive bacteria (2), and Staphylococcus aureus is intrinsically resistant to PMB and colistin (5). The mechanisms conferring intrinsic resistance to polymyxins are not completely understood. However, the sensitivity of S. aureus to structurally different cationic antimicrobial peptides has been demonstrated to be affected through proteolytic degradation of the human cathelicidin LL-37 by the protease aureolysin, sequestration of human α-defensins by staphylokinase, alterations of cell surface charge, and active efflux of tMP-1 (thrombin-induced platelet microbicidal protein 1) by the efflux pump QacA (4, 6–8).

In S. aureus, two mechanisms have been demonstrated to alter cell surface charge in response to the presence of cationic antimicrobial peptides (9, 10). Incorporation of D-alanine on teichoic acids, mediated by the dltABCD operon, reduces the net negative charge of the cell surface and thereby reduces electrostatic interaction with cationic antimicrobial peptides (9). Similarly, incorporation of L-lysine to membrane phosphatidylglycerols by the enzyme MprF (FmtC) also reduces the net negative charge (10). Regulation of the dlt operon and mprF expression is mediated via the three-component system GraXSR (also known as ApsXSR), which together with the VraFG transporter system can sense and signal the presence of cationic antimicrobial peptides (6, 11). Inactivation of graR and vraG has previously been shown to increase the susceptibility of S. aureus to PMB (12), whereas degradation and sequestration have not been reported to affect polymyxin susceptibility in S. aureus.

S. aureus is an opportunistic human pathogen that can cause a variety of diseases ranging from skin infections to life-threatening systemic infections (13). The slow introduction of novel antimicrobial molecules to the clinic necessitates the understanding of the determinants that make S. aureus intrinsically resistant to polymyxins (14), an antimicrobial class that is extensively used against Gram-negative infections (1). Knowledge of intrinsic resistance mechanisms could provide targets for helper drugs to sensitize S. aureus to polymyxins. Therefore, we screened the Nebraska Transposon Mutant Library (NTML) of 1,920 single-gene inactivations in S. aureus JE2 for mutants (15), which were unable to grow at subinhibitory concentrations of PMB. The screen revealed multiple novel polymyxin intrinsic resistance genes, most importantly genes encoding subunits of the ATP synthase.

RESULTS

The polymyxin B intrinsic resistome. Staphylococcus aureus is intrinsically resistant to the clinically approved cationic antimicrobial peptides polymyxin B (PMB) and colistin (1, 16). To identify intrinsic resistance mechanisms in S. aureus, we screened the entire NTML for mutants that displayed lack of growth on agar plates supplemented with PMB equal to 0.5× the MIC of the wild type (WT). The MIC for PMB was subsequently determined using Etests for the identified mutants. Nineteen mutants were confirmed to be at least 2-fold more susceptible than the WT (Table 1). As expected, we identified transposon insertions in graS, graR, vraF, and vraG, corrob-
rating previous work on these determinants in *S. aureus* in relation to increased PMB susceptibility (12). Furthermore, transposon insertion in the potassium transporter gene *trkA* had a minor effect on PMB susceptibility, as previously observed in *Vibrio vulnificus* (17).

Interestingly, inactivation of multiple genes encoding subunits of the ATP synthase displayed increased sensitivity towards polymyxins: the genes included *atpA*, *atpB*, *atpG*, and *atpH*. The ATP synthase generates ATP from ADP and Pi at the F₁ domain with energy derived from proton movement through the F₀ domain (18). The F₁ domain is an assembly of five proteins with the stoichiometry α₃β₃γ₁δ₁ε₁ (18), where *atpA* encodes the α-subunit, *atpG* encodes the γ-subunit, and *atpH* encodes the δ-subunit. The gene *atpB* encodes the A-subunit of the F₀ domain. ATP catalysis proceeds at the β-subunits, whereas the functions of the α-subunits remain poorly understood, but have been shown to be important for attaining maximum activity of the ATP synthase (19). To the best of our knowledge, the ATP synthase has not previously been associated with PMB sensitivity in Gram-positive bacteria. However, in Gram-negative bacteria such as *Escherichia coli*, inactivation of *atpG* increased sensitivity towards colistin (20), in *Proteus mirabilis*, a mutant with inactivation of a gene with similarity to one of the ATP synthase genes displayed increased sensitivity to PMB (21), and in *Vibrio para-haemolyticus*, antimicrobial peptide-resistant mutants displayed upregulation of the ATP synthase F₁ α-subunit (22).

While we were unable to complement the *atpA*-inactivated mutant with a functional *atpA* gene on a plasmid, we successfully performed allelic exchange of the transposon insertion with the intact *atpA* gene, generating a strain displaying PMB sensitivity like that of the WT (data not shown).

The remaining mutants identified in the screen only displayed minor increases in PMB susceptibility (Table 1). For all of the mutants displaying increased susceptibility to PMB in the NTML, we additionally measured the susceptibility to colistin. Colistin was less effective against *S. aureus* JE2 than PMB; however, increased sensitivity to PMB correlated with increased sensitivity to colistin (Table 1).

**Medium composition affects the absolute MIC.** It has been reported that growth medium composition can affect polymyxin MIC (23): therefore, we also tested polymyxin B MICs of the WT and *atpA*, *graR*, *vraG*, and *vraF* mutants by employing the Etest on cation-adjusted Mueller-Hinton (MH) agar. Polymyxin B displayed greater activity against *S. aureus* on MH agar than on tryptic soy agar (TSA) plates; however, the fold

### TABLE 1 Intrinsic polymyxin B resistance determinants identified in the NTML and the corresponding MICs of polymyxin B and colistin

| Gene name          | Function                              | Gene no.   | MIC (µg/ml) |
|--------------------|---------------------------------------|------------|-------------|
| **Gene name**      | **Function**                          | **Gene no.**| **Polymyxin B** | **Colistin** |
| Wild type (S. aureus JE2) |                                    |            | 128         | >256         |
| *vraG*             | ABC transporter, permease protein     | SAUSA300_0648 | 16         | 24           |
| *vraF*             | ABC transporter, ATP-binding protein  | SAUSA300_0647 | 16         | 32           |
| *graR*             | DNA-binding response regulator        | SAUSA300_0645 | 16         | 32           |
| *graS*             | Sensor histidine kinase               | SAUSA300_0646 | 24         | 32           |
| *atpA*             | ATP synthase F₁ α subunit             | SAUSA300_2060 | 16         | 48           |
| *atpB*             | F₆F₇, ATP synthase subunit A          | SAUSA300_2064 | 16         | 48           |
| *atpG*             | F₆F₇, ATP synthase subunit γ          | SAUSA300_2059 | 16         | 48           |
| *atpH*             | F₆F₇, ATP synthase subunit δ          | SAUSA300_2061 | 16         | 48           |
| *cbiO*             | Cobalt transporter ATP-binding subunit| SAUSA300_2176 | 48         | 64           |
| *trkA*             | Potassium uptake protein              | SAUSA300_0988 | 48         | 128          |
| *vraS*             | Two-component sensor histidine kinase | SAUSA300_1866 | 64         | 256          |
| *yajC*             | Preprotein translocase subunit YajC   | SAUSA300_1594 | 64         | 256          |
| *lspA*             | Lipoprotein signal peptidase          | SAUSA300_1089 | 64         | 256          |
| *dgcg*             | Diacylglycerol glucosyltransferase    | SAUSA300_0918 | 64         | >256         |
| Hypothetical protein |                                    | SAUSA300_1802 | 64         | >256         |
| Hypothetical protein |                                    | SAUSA300_1254 | 48         | 192          |
| Hypothetical protein |                                    | SAUSA300_0980 | 48         | >256         |
| Hypothetical protein |                                    | SAUSA300_1495 | 64         | >256         |
changes between the WT and mutants largely remain identical (see Table S1 in the supplemental material). Interestingly, strains with inactivation of \textit{atpA}, \textit{graR}, \textit{vraG}, and \textit{vraF} are around the breakpoint level of being susceptible according to the guidelines of CLSI (1, 24). Polymyxin B breakpoints for \textit{Pseudomonas aeruginosa} are susceptible at a MIC of $\leq 2\, \mu g/ml$, intermediate at a MIC of $4\, \mu g/ml$, and resistant at a MIC of $\geq 8\, \mu g/ml$, whereas for \textit{Acinetobacter} spp., a MIC of $\geq 4\, \mu g/ml$ is considered resistant.

\textbf{D-Alanylation of teichoic acids and lysinylation of phosphatidylglycerols.} No mutants with inactivation of genes in the \textit{dltABCD} operon exist in the NTML (15); however, incorporation of D-alanine on teichoic acids mediated by the \textit{dltABCD} operon has previously been revealed to affect susceptibility to cationic antimicrobial peptides (9). Therefore, we examined PMB susceptibility of an isogenic strain pair, namely, an SA113 $\Delta$\textit{dltA} mutant (PMB MIC, $48\, \mu g/ml$) relative to the WT parent strain, SA113 (PMB MIC, $512\, \mu g/ml$) (Table S2 in the supplemental material). The result shows that D-alanylation of teichoic acids also is mediating resistance to PMB.

By screening the NTML, the mutant with inactivation of \textit{mprF} did not display increased sensitivity to PMB, and to confirm this result, we tested PMB susceptibility of the SA113 $\Delta$\textit{mprF} mutant (PMB MIC, $384\, \mu g/ml$) relative to ancestral WT strain SA113 (PMB MIC, $512\, \mu g/ml$) (Table S2). This suggests that lysinylation of phosphatidylglycerol is not an intrinsic PMB resistance mechanism, in contrast to other classes of cationic antimicrobial peptides (10, 25). Lysinylation of phosphatidylglycerols therefore seems to mediate selective protection against certain cationic antimicrobial peptides.

\textbf{Inactivation of \textit{atpA} confers hypersusceptibility to gentamicin.} We assessed whether an impaired ATP synthase affected susceptibility to other antimicrobial peptides (Table 2) and conventional antimicrobial agents (Table 3) by comparing the \textit{atpA} mutant with the WT, as well as the established determinants \textit{vraG}, \textit{vraF}, and \textit{graR}. Only marginal changes in susceptibility to the antimicrobial peptides bacitracin, gallidermin, and nisin, were detected for all of the mutants (Table 2). The \textit{vraF} and \textit{graR} mutants displayed at least 2-fold increased sensitivity to the human cathelicidin LL-37, whereas \textit{atpA} was indistinguishable from the WT (Table 2). For conventional antimicrobial agents, minor reductions in vancomycin MIC were observed for \textit{vraG}, \textit{vraF}, and \textit{graR} mutants, but not for the \textit{atpA} mutant. The \textit{vraG}, \textit{vraF}, and \textit{graR} mutants displayed increased sensitivity to gentamicin (3- to 6-fold), whereas \textit{atpA} displayed a 16-fold increased sensitivity (Table 3). Contrarily, no differences in sensitivities between all the mutants and the WT were detected for ciprofloxacin, linezolid, oxacillin, and daptomycin (Table 3). Increased susceptibility of the \textit{atpA} mutant was restricted to polymyxins and aminoglycosides, demonstrating that the ATP synthase is not generally involved in reducing antimicrobial activity of cationic antibiotics or antimicrobial peptides.

\begin{table}[h]
\centering
\caption{MICs of antimicrobial peptides for the wild type and selected mutants}
\begin{tabular}{|c|c|c|c|c|c|}
\hline
Peptide & Charge & MIC ($\mu g/ml$) & WT & \textit{vraG} & \textit{atpA} & \textit{vraF} & \textit{graR} \\
\hline
Gallidermin & Cationic & 16 & 8 & 16 & 8 & 8 & \\
Nisin & Cationic & 512 & 256 & 256 & 256 & 256 & \\
LL-37 & Cationic & $>128$ & 128 & $>128$ & 64 & 64 & \\
Bacitracin & Neutral & 256 & 256 & 128 & 256 & 128 & \\
\hline
\end{tabular}
\end{table}

\begin{table}[h]
\centering
\caption{MICs of conventional antibiotics for the wild type and selected mutants}
\begin{tabular}{|c|c|c|c|c|c|}
\hline
Antibiotic & Charge & MIC ($\mu g/ml$) & WT & \textit{vraG} & \textit{atpA} & \textit{vraF} & \textit{graR} \\
\hline
Ciprofloxacin & Neutral & 32 & 32 & 32 & 32 & 32 & \\
Oxacillin & Anionic & 0.5 & 0.50 & 0.500 & 0.50 & 0.50 & \\
Linezolid & Neutral & 2 & 2 & 2 & 2 & 2 & \\
Gentamicin & Cationic & 1.5 & 0.25 & 0.094 & 0.38 & 0.50 & \\
Vancomycin & Cationic & 1.5 & 1 & 1.500 & 0.75 & 1 & \\
Daptomycin & Cationic & 0.25 & 0.25 & 0.250 & 0.25 & 0.19 & \\
\hline
\end{tabular}
\end{table}
The *atpA* mutant displays hyperpolarization of the membrane. The magnitude of the membrane potential can have a large effect on the activity of antimicrobial peptides against different bacterial species (26). It has been hypothesized that due to the negative orientation of the membrane potential, cationic antimicrobial peptides are electrophoretically drawn into the nonpolar membrane (26). Furthermore, uptake of gentamicin into the cell is dependent on membrane potential, where hyperpolarization of the membrane increases uptake, while depolarization reduces uptake (27).

We therefore hypothesized that the *atpA* mutant was more susceptible to PMB due to hyperpolarization of the membrane in the absence of ATP synthase activity. Hence, we assessed the membrane potential for the *atpA* mutant using the fluorescent dye DiOC$_2$ (3), and indeed, the *atpA* mutant displayed hyperpolarization of the membrane (Fig. 1). This corroborates a previous study on an ATP synthase-deficient ΔFoF$_1$ mutant strain of *Corynebacterium glutamicum*, which also displayed increased membrane potential relative to the wild type (28).

Cell surface charge remains unchanged for the *atpA* mutant. A change toward a less negative cell surface charge has previously been correlated with a decrease in susceptibility to cationic antimicrobial peptides (9, 29–31). To assess the potential correlation between cell surface charge and sensitivity to PMB in our mutants, we measured the zeta potential of the *atpA*, *vraG*, *graR*, and *vraF* mutants and the WT (Fig. 2). No significant changes in zeta potentials were detected. Furthermore, we could not detect any significant differences between the *atpA* mutant and the WT for...
Inhibition of the ATP synthase increases efficacy of polymyxin B. The ATP synthase is a well-described protein complex, and multiple inhibitors have been identified that interfere with its function—e.g., the macrolide oligomycin A (32). To demonstrate the potential of the ATP synthase as a target for potentiating the efficacy of polymyxins against S. aureus, we assessed the killing efficacy of PMB in the presence or absence of the ATP synthase inhibitor oligomycin A (Fig. 4). At a concentration of PMB equal to 0.25 × the MIC of the WT, the combination therapy (PMB plus oligomycin A) reduced the colony-forming units (CFU) 60-fold after 4 h for the WT, whereas continued growth was observed for WT with treatment with PMB alone. The combinatory efficacy of PMB and oligomycin A is similar to the observed killing efficacy of PMB against the atpA mutant. Oligomycin A alone at the provided concentration (8 μg/ml) did not display any killing efficacy against S. aureus.

DISCUSSION
The limited availability of effective and well-tolerated therapies for antibiotic-resistant S. aureus has led to a search for inhibitors to improve the efficacy of existing antibiotic compounds by targeting acquired and intrinsic resistance mechanisms (33–36). Inhibition of wall teichoic acid synthesis restored β-lactam efficacy against methicillin-resistant S. aureus (33) and fluoroquinolone efficacy was increased by inhibition of the efflux pump NorA (35, 36). These studies, however, have focused on potentiating the efficacy of antibiotics that are normally used against staphylococcal infections and have not included antibiotics that S. aureus is intrinsically resistant against.

The present study provides the first whole-genome overview of intrinsic polymyxin resistance genes in S. aureus. Most importantly, we identified the ATP synthase as a novel target for potentiating the efficacy of polymyxins against S. aureus. Inhibition of the ATP synthase potentiates equally well the efficacy of polymyxins against S. aureus as inactivation of the previously established two-component system GraSR and the VraFG transporter system (Table 1).

The bacterial ATP synthase has been validated as an antimicrobial target with the recent approval of the antituberculosis agent bedaquiline (14). Bedaquiline selectively targets the subunit c of the ATP synthase in most mycobacteria, while displaying limited or no activity against other bacterial pathogens, including S. aureus (37). Derivatives of the diarylquinoline scaffold of bedaquiline have been generated to increase the activity towards other important Gram-positive pathogens (e.g., S. aureus), while still displaying limited or no activity against Gram-negative bacteria (38). Chemical inhibition of the ATP synthase with oligomycin A significantly increased the...
antistaphylococcal activity of PMB (Fig. 4). However, oligomycin A is nonselective and therefore also inhibits the mitochondrial ATP synthase (38), rendering it inappropriate for human use. Numerous other compounds have been identified that interact with ATP synthases (32), which can be explored as potentiators of polymyxins and aminoglycosides in S. aureus for human use. The ATP synthase also constitutes a potential target for potentiation of polymyxins against Gram-negative bacteria, as inactivation of atpG in E. coli increased sensitivity towards colistin (20).

Inactivation of the ATP synthase conferred hyperpolarization of the membrane (Fig. 1), and we propose this as a potential mechanism for the improved activity of polymyxins. Increased membrane potential may correlate with increased activity of other cationic antimicrobial peptides (26). Furthermore, deletion of the gene phoP in E. coli conferred hyperpolarization of the membrane and a concomitant increase in activity of PMB, while collapsing the proton gradient with m-chlorophenyl carbonyl cyanide hydrozone (CCCP) abrogated this effect (39).

The spectrum of activity of polymyxins also indicates the interrelatedness of the electron transport chain with polymyxin activity, as polymyxins generally display bactericidal activity against Gram-negative bacteria, except against anaerobic Gram-negative bacteria (23, 40, 41). The killing efficiency of polymyxins against P. aeruginosa has been reported to be diminished under anaerobic compared to aerobic conditions (42); however, another study could not confirm this (43).

The interrelatedness of the ATP synthase and membrane potential with polymyxin susceptibility is not yet completely understood; however, we have demonstrated that the ATP synthase is a potential target for sensitizing S. aureus towards polymyxins. The ATP synthase may also be targeted for potentiating the efficacy of aminoglycosides and potentially other cationic antimicrobial peptides not tested in this study.

Taken together, a greater understanding of the mechanisms conferring intrinsic resistance can provide novel targets for development of inhibitors to potentiate the efficacy of polymyxins and thereby potentially broaden the spectrum of activity of this class of antibiotics to important Gram-positive pathogens. With the need for new treatment options for infections with serious pathogens like S. aureus, targeting intrinsic resistance mechanisms may pave the way for novel applications of existing antibiotics.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and MIC determination. The strains used in this study include S. aureus strain JE2 (plasmid-cured derivative of USA300 LAC) and all derivative strains within the Nebraska Transposon Mutant Library (NTML), consisting of 1,920 unique transposon mutants with inactivation of nonessential genes (15). The bursa aurealis transposon used to create the collection contains the resistance cassette ermB, which confers resistance to erythromycin (15). Additionally we used S. aureus SA113 and two derivatives, SA113 ΔdltA (9) and SA113 ΔmprF (10). All bacterial strains

![Graph showing improved killing efficacy of PMB upon inhibition of the ATP synthase.](image)
were cultured at 37°C in tryptic soy broth (TSB) or on tryptic soy agar (TSA), with antimicrobial agents added as indicated. Two methods have been employed to determine MICs to various antimicrobial agents. (i) A 2-fold broth microdilution assay in TSB (100 μl) with an initial inoculum of approximately 5 × 10^8 cells/ml was employed to determine the MICs of polymyxin B sulfate (Sigma), gallidermin (Santa Cruz Biotechnology), nisin (Sigma), bacitracin (Sigma), and LL-37 (Isca Biochemicals). (ii) An Etest (BioMérieux) performed on TSA plates was employed to determine MICs of polymyxin B, colistin, ciprofloxacin, oxacillin, linezolid, gentamicin, vancomycin, and daptomycin. The MIC was determined upon incubation at 37°C for 22 h. When indicated, the Etest (BioMérieux) was performed on Mueller-Hinton agar plates (cation adjusted for calcium and magnesium).

Screening for increased polymyxin B susceptibility. The NTML is stored in glycerol at −80°C in 20 96-well microtiter plates. Material from the frozen stock was transferred directly with a Deutz 96 cryoprecipitate (44) from the 96-well microtiter plates onto TSA plates supplemented with 5 μg/ml erythromycin (as all the strains in the NTML are resistant to erythromycin [15]) and 64 μg/ml polymyxin B (0.5× the MIC). The plates were incubated at 37°C for 24 h and visually inspected for lack of growth of individual mutants.

Zeta potential. Overnight cultures were incubated at 37°C with orbital shaking at 180 rpm, harvested by centrifugation at 4,600 × g for 10 min, and suspended in phosphate-buffered saline (PBS: 10 mM phosphate buffer, 2.7 mM potassium chloride, 137 mM sodium chloride) to a density of 2.8 × 10^8 to 4.6 × 10^8 cells/ml. Zeta potentials were measured at 25°C with a Zetasizer Nano ZS (Malvern Instruments) using folded capillary cells (Malvern Instruments). Six measurements were taken for each sample, and zeta potentials were calculated using the Smoluchowski equation with Zetasizer software (v7.02).

Assessment of membrane potential measurements using flow cytometry. Membrane potential was assessed using a flow cytometry assay based on the BacLight bacterial membrane potential kit (Life Technologies). Cells from overnight cultures were inoculated in 10 ml TSB in 100-ml Erlenmeyer flasks and grown to an optical density at 600 nm (OD_{600}) of 0.2. Fifteen microtiter plates of culture was transferred to 1 ml filtered PBS. To each cell solution, 10 μl of the fluorescent membrane potential indicator dye DiOC_{2} (3) was added and cells were stained for 5 min at room temperature. Data were recorded on a BD Biosciences Accuri C6 flow cytometer (Becton, Dickinson and Company), with emission filters suitable for detecting red and green fluorescence. Settings on the flow cytometer were as follows: 50,000 recorded events at a forward scatter (FSC) threshold of 15,000 and medium flow rate. Gating of the stained cell population and analysis of flow cytometry data were performed in CFlow (BD Accuri). As an indicator of membrane potential, the ratio of red to green fluorescence intensity was calculated. The assay was verified with the NTML mutant containing a transposon insertion in menD (NE1345), which displays depolarization of the membrane (45).

Chromosomal reconstruction of atpA. Chromosomal reconstruction of the atpA mutant was achieved by use of the temperature-sensitive shuttle vector pBASE6 (46). A chromosomal region encompassing atpA was PCR amplified from WT S. aureus JE2 chromosomal DNA using primer pair 5′-ATATGACCTGCAAGAGTTAGATAAGATTGTCAAACTAG-3′ and 5′-GATACAAGATCTGATGGTTTGTATTGCTACTGTC-3′ and cloned into pBASE6 via SacI/BglII. This plasmid was purified from IM08B (47) and transformed directly into JE2 atpA::kanZ (NE592) at 30°C followed by chromosomal integration by plating on TSA (10 μg/ml chloramphenicol) at 44°C overnight. Plasmid cross-out was performed by passage at 30°C followed by plating on TSA (500 ng/ml anhydrotetracycline), and successful allelic exchange of the transposon insertion with the intact atpA gene was selected for by replica plating of colonies and screening for sensitivity toward erythromycin and chloramphenicol. Reconstruction of the atpA locus was verified by PCR amplification using primers 5′-CAAGATGCTAAGCATTATTGACGCGTGTC-3′ and 5′-CGTAATTTCGTCTTGCTCGTCTG-3′ positioned outside the chromosomal region used for homologous recombination.

Kill curve experiment assessing polymyxin B efficacy upon inhibition of the ATP synthase. From overnight cultures of S. aureus JE2 and the derivative atpA mutant, 100 μl was diluted into 900 μl fresh TSB medium in a Falcon tube and grown for 1 h, the cultures were diluted into 10 ml fresh TSB medium in 100-ml Erlenmeyer flasks, reaching an initial cell count of approximately 10^8 cells/ml. Oligomycin A (Sigma) was added to flasks as indicated at a concentration of 8 μg/ml. After 30 min of growth, polymyxin B was added to flasks as indicated at a concentration equal to 0.25× the MIC. CFU were determined on TSA plates before addition of oligomycin A (time zero [T_{0}]), before addition of polymyxin B (time 30 min [T_{30}]), and every hour for the following 4 h.

Analysis of α-alaninylate of the S. aureus cell envelope. S. aureus JE2 and the mutant strains were grown to the early stationary phase (6 h), washed with ammonium acetate buffer (20 mM), and adjusted to an OD_{600} of 30 in a total volume of 1 ml. Cells were taken up in NaOH to a final volume of 100 μl and were incubated for 1 h of shaking at 37°C to hydrolyze the α-alanine esters. The reaction was stopped with 100 μl of HCl, and the precipitated cell debris was removed by centrifugation and sterile filtration. α-Alanine was derivatized with ortho-phthalaldehyde (OPA), similar to previous experiments (48). Five microtiter plates were mixed for 120 s, and the reaction was stopped by adding 3 μl 100% acetic acid. The sample was then separated via ultraperformance liquid chromatography (UPLC) with an Acquity H class UPLC system from Waters. Five microtiter sample was run on a gradient in 24 min from 100% buffer A (25 mM sodium phosphate buffer, pH 7.2) to 100% buffer B (45% acetonitrile, 45% methanol, 10% H₂O) in a stepwise manner. The column temperature was 23°C, and the flow rate was 0.32 ml/min. Fluorescence was detected at 338 nm.
Isolation and quantification of polar lipids. Polar lipids were isolated from S. aureus cultures grown to the logarithmic phase (OD600 of 0.8) and extracted with chloroform-methanol-sodium acetate buffer (20 mM) (1:1:1 by volume) by the Bligh-Dyer method (49), vacuum dried, and dissolved in chloroform-methanol (2:1 by volume). Amino group- or phosphate group-containing lipids were detected by ninhydrin or molybdenum blue staining, respectively. Aminoacyl phospholipids were quantified in relation to total phospholipid content by determining lipid spot intensities of molybdenum blue-stained methanol

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TABLE S1, DOCX file, 0.1 MB.
TABLE S2, DOCX file, 0.1 MB.

SUPPLEMENTAL MATERIAL

TABLE S1, DOCX file, 0.1 MB.
TABLE S2, DOCX file, 0.1 MB.

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