



OPEN

Inhibition of the ATP synthase sensitizes *Staphylococcus aureus* towards human antimicrobial peptides

Liping Liu¹, Christian Beck², Katrine Nøhr-Meldgaard¹, Andreas Peschel², Dorothee Kretschmer², Hanne Ingmer¹✉ & Martin Vestergaard¹

Antimicrobial peptides (AMPs) are an important part of the human innate immune system for protection against bacterial infections, however the AMPs display varying degrees of activity against *Staphylococcus aureus*. Previously, we showed that inactivation of the ATP synthase sensitizes *S. aureus* towards the AMP antibiotic class of polymyxins. Here we wondered if the ATP synthase similarly is needed for tolerance towards various human AMPs, including human β -defensins (hBD1-4), LL-37 and histatin 5. Importantly, we find that the ATP synthase mutant (*atpA*) is more susceptible to killing by hBD4, hBD2, LL-37 and histatin 5 than wild type cells, while no changes in susceptibility was detected for hBD3 and hBD1. Administration of the ATP synthase inhibitor, resveratrol, sensitizes *S. aureus* towards hBD4-mediated killing. Neutrophils rely on AMPs and reactive oxygen molecules to eliminate bacteria and the *atpA* mutant is more susceptible to killing by neutrophils than the WT, even when the oxidative burst is inhibited. These results show that the staphylococcal ATP synthase enhance tolerance of *S. aureus* towards some human AMPs and this indicates that inhibition of the ATP synthase may be explored as a new therapeutic strategy that sensitizes *S. aureus* to naturally occurring AMPs of the innate immune system.

Bacterial pathogens that cause disease in humans remain a serious threat to public health and antibiotics are still our primary weapons in treating many bacterial diseases. The ability to eradicate bacterial infections is however challenged by development of resistance for every type of antibiotic introduced to the clinic¹. The majority of the new small molecule antibiotics in clinical development are however inhibiting the same targets as already marketed antibiotics². As an alternative to small molecule antibiotics, antimicrobial peptides (AMPs) are also explored in clinical trials, however most of the AMPs are only tested for topical applications due to toxicity issues and low metabolic stability³. Here we propose a new strategy to combat bacterial infections, namely to sensitize bacteria to the naturally occurring antimicrobial peptides of the human body and hence boosting the antibacterial capabilities of the innate immune system to eradicate bacterial infections.

Humans are continuously exposed to numerous, and potentially pathogenic, microorganisms, where the innate immune system provides the first line of defense. AMPs constitute an important defense mechanism of the innate immune system against invading microorganisms, due to their antimicrobial and immune stimulatory properties^{4,5}. In humans, several classes of AMPs have been identified, such as α - and β -defensins, the cathelicidin LL-37 and histatins⁵. The α -defensins consist of six members, which are divided into human neutrophil peptides (HNPI-4) and human α -defensin 5 and 6 (HD5 and HD6). HNPI-4 are highly concentrated in the granules of neutrophils, but are also expressed in monocytes, lymphocytes and natural killer cells. HD5 and HD6 are primarily expressed in Paneth cells of the small intestine⁴. The β -defensins consist of four members (hBD1-4) and are primarily secreted by mucosal surface epithelia, e.g. by keratinocytes in the human skin⁶. Histatins comprise a family of cationic, histidine-rich peptides that are present in human saliva and are important for maintaining

¹Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Stigbøjlen 4, 1870 Frederiksberg C, Denmark. ²Department of Infection Biology, Interfaculty Institute for Microbiology and Infection Medicine Tübingen (IMIT), Cluster of Excellence 'Controlling Microbes to Fight Infections', University of Tübingen, Auf der Morgenstelle 28, 72076 Tübingen, Germany. ✉email: hi@sund.ku.dk

oral health by limiting infections in the oral cavity⁵. Several histatins have been characterized, however histatin 5 displays the strongest antimicrobial activity⁵. LL-37 is an antimicrobial peptide that belongs to the cathelicidin family and is expressed in various epithelial- (e.g. keratinocytes) and immune cells (e.g. neutrophils and macrophages)⁷. The bactericidal activities of many AMPs have generally been attributed to pore formation in bacterial cytoplasmic membranes, however this mode of action may be too simplistic^{8,9}. The bactericidal activity of hBD3 has for example been associated with lipid II binding, leading to inhibition of cell wall biosynthesis¹⁰ and some AMPs also have intracellular targets¹¹. Many antimicrobial peptides display a net positive charge, which is important in the initial electrostatic attraction to negatively charged bacterial phospholipid membranes and negatively charged teichoic acids on the surface of Gram positive bacteria, e.g. *Staphylococcus aureus*¹¹.

S. aureus is a common colonizer of the human body⁶, where approximately 30–50% of healthy adults transiently carry this species and approximately 20% are persistently colonized¹². The skin, nose and intestinal tract are important ecological niches for *S. aureus* carriage¹³. Topical colonization with *S. aureus* imposes a risk for subsequent infections, if the skin or mucosal barriers are breached and enables transmission of *S. aureus* cells to the adjacent tissues or the bloodstream¹². *S. aureus* is an opportunistic pathogen that may cause life-threatening diseases, such as sepsis, endocarditis and pneumonia¹². Even though keratinocytes express various antimicrobial peptides, such as hBD1-4 and LL-37, *S. aureus* frequently colonizes human skin⁶. Among the human β -defensins, only hBD3 displays potent bactericidal activity against *S. aureus* at physiological conditions^{14–16}, and keratinocytes are dependent on hBD3 for killing of *S. aureus*¹⁷. However, it is incompletely understood, why the remaining β -defensins display limited anti-staphylococcal activity. This indicates that sensitizing *S. aureus* towards the innate immune system AMPs may potentially facilitate eradication of colonizing *S. aureus*.

Multiple factors affect bacterial susceptibility towards AMPs, such as cell membrane composition, cell surface charge and transmembrane potential⁸. The positive charge of many AMPs facilitates the interaction with negatively charged bacterial envelopes¹¹. A common resistance mechanism exploited by bacteria is to reduce the net negative charge of the cell envelope, for example by lysinylation of phospholipids¹⁸ and D-alanylation of teichoic acids in *S. aureus*¹⁹. Curiously, deficiency of wall teichoic acids selectively confers reduced susceptibility to hBD3, while not affecting susceptibility to LL-37 and HNP1-3²⁰. The transmembrane potential affects the ability of cationic AMPs to permeabilize membranes^{8,21}, where an inside-negative transmembrane potential facilitates insertion of some cationic AMPs into bacterial membranes⁸. Interference with the electron transport chain by inactivation of menaquinone- (*men* mutants) or hemin (*hem* mutants) biosynthesis pathways leads to membrane depolarization in *S. aureus* and in the appearance as small colony variants (SCVs) on agar plates²². Electron transport chain SCVs have been associated with reduced susceptibility towards multiple AMPs, including hBD2-3²³, thrombin-induced platelet microbicidal protein²⁴ and nisin²⁴. We recently reported that inactivation of genes encoding for multiple subunits of the ATP synthase sensitizes *S. aureus* towards polymyxins²⁵, a class of cationic AMPs that is used for treatment of Gram-negative infections²⁶. The ATP synthase basically serves two physiological functions, first being synthesis of ATP from ADP and inorganic phosphate by using energy from the proton motive force. Secondly, during conditions with a low proton-motive force the ATP synthase can work in reverse as an ATPase and thereby contributes to the establishment of a cross-membrane proton gradient through ATP hydrolysis²⁷. In *S. aureus*, the ATP synthase is primarily used to hydrolyze ATP for maintaining the cross-membrane proton gradient both under fermentative and respiratory conditions²⁸. ATP synthase inactivation in *S. aureus* leads to hyper-polarization of the membrane^{25,28}, which was hypothesized to be the mechanism for sensitizing ATP synthase mutants towards polymyxins²⁵.

Several molecules have been shown to inhibit the ATP synthase in different species²⁹. For example inhibition of the ATP synthase with oligomycin A sensitizes *S. aureus* towards polymyxin B²⁵ and aminoglycosides³⁰. However, oligomycin A displays similar 50% inhibitory concentration (IC₅₀) between *S. aureus* and human mitochondrial ATP synthases³¹, and cannot be used clinically due to toxicity issues³². Resveratrol is a widely used nutraceutical that has been shown to bind to the bovine ATP synthase in the F₁-domain³³ and also binds reversibly to the ATP synthase in *E. coli*, partially inhibiting both ATP hydrolysis and ATP synthesis³⁴. Co-administration of resveratrol sensitizes *S. aureus* towards aminoglycosides³⁰. However, resveratrol is readily metabolized following oral administration, which probably only enables topical use³⁵.

Since ATP synthase inactivation sensitizes *S. aureus* towards polymyxins, we hypothesize that this strategy also can sensitize *S. aureus* towards various human AMPs. Potentiation of human AMPs that are currently ineffective against *S. aureus* may potentially become a new therapeutic strategy, where treatment relies on deprivation of AMP resistance mechanisms and hence boosting of the naturally occurring AMPs of the innate immune system.

Materials and Methods

Bacterial strains, growth conditions and chemicals. The *Staphylococcus aureus* JE2 wild type (WT) strain and derivative mutants used in this study are highlighted in Table 1. Antimicrobial peptides used in this study included histatin-5 (Innovagen, Sweden), LL-37 (Isca Biochemicals, United Kingdom) and hBD1-4 (Innovagen, Sweden), as well as polymyxin B Etests (bioMérieux, France). We used the ATP synthase inhibitor resveratrol (Santa Cruz Biotechnology). Bacterial strains were routinely cultured at 37 °C in tryptic soy broth (TSB) or on tryptic soy agar (TSA).

Antimicrobial susceptibility assays. *Microdilution.* The minimum inhibitory concentration for resveratrol was determined using a two-fold broth microdilution assay in TSB (100 μ l) with an initial inoculum of approximately 5×10^5 cells/ml. MIC was determined upon incubation at 37 °C for 24 h.

Etest. The MIC for polymyxin B was determined using Etest (bioMérieux, France) in the absence and in the presence of sub-inhibitory concentrations (0.0625x–0.25x MIC) of resveratrol or menadione (1 μ g/ml, Sigma).

Organism	Description and genotype	Source
<i>S. aureus</i>	JE2, CA-MRSA USA300	
<i>S. aureus</i>	JE2 <i>menD</i> ::ΦNΣ	60
<i>S. aureus</i>	JE2 <i>atpA</i> ::ΦNΣ	60
<i>S. aureus</i>	JE2 <i>atpB</i> ::ΦNΣ	60
<i>S. aureus</i>	JE2 <i>atpG</i> ::ΦNΣ	60
<i>S. aureus</i>	<i>atpA</i> ⁺ —Strain with allelic exchange of the transposon insertion (<i>atpA</i> ::ΦNΣ) with the intact <i>atpA</i> gene	25

Table 1. Strains and mutants used.

From overnight cultures, strains were diluted to approximately 10^8 CFU/ml and then distributed on TSA plates using a sterile cotton swab. MIC was determined upon incubation at 37 °C for 24 h.

Bacterial cell survival assays. From overnight cultures of *S. aureus* JE2 and mutants, 10 µl was diluted into 990 µl fresh TSB medium in a falcon tube and grown for 2 h for the cells to reach early exponential phase. After 2 h the cultures were re-suspended in 10 mM sodium phosphate buffer (pH 7.4), termed NaPi (Medicago, Sweden). Cells were subsequently diluted in NaPi to approximately 5×10^5 CFU/ml, and combined with antimicrobial peptides and resveratrol when indicated, to a final volume of 100 µl and incubated in 96-well plates with shaking for 2 h at 37 °C. Bacteria were plated for CFU on TSA plates. Following overnight incubation at 37 °C for 24 h, viable cells were enumerated and relative cell survival was calculated as $CFU_{with\ peptide}/CFU_{without\ peptide}$ at 2 h post-exposure. Values provided are the mean \pm SEM derived from at least three independent biological replicates.

Isolation of PMNs from human blood. Blood was collected from healthy adult volunteers and written informed consent was given. Isolation of neutrophils was performed following the procedure described in³⁶. All methods were carried out in accordance with relevant guidelines and regulations. The institutional review board (IRB) of the University of Tübingen approved the study and all adult subjects provided informed consent. This study was done in accordance with the ethics committee of the medical faculty of the University of Tübingen that approved the study, Approval number 015/2014 BO2. Briefly, heparinized blood was diluted 1:1 (v/v) with PBS containing 0.5% BSA and 2 mM EDTA and layered onto a gradient of Biocoll (density, 1.077 g/ml; Biochrom) and Histopaque (density, 1.119 g/ml; Sigma). After centrifugation for 20 min at 380×g, neutrophils were collected from the Histopaque phase. Cells were subjected to a brief hypotonic shock with ultrapure-water containing 155 mM ammonium chloride, 1 mM potassium hydrogen carbonate and 0.1 mM EDTA at pH 7.4, washed, and suspended at 2.5×10^6 cells/ml in RPMI containing 200 mg/ml HSA, 2 mM glutamine, 2 mM sodium pyruvate and 10 mM HEPES.

Phagocytosis of *S. aureus* by neutrophils. Starter cultures of *S. aureus* JE2 and its respective *atpA* mutant were grown in TSB medium overnight. Main cultures were subsequently inoculated at an OD_{600} of 0.1 and grown to an OD of 1. 10^9 CFU of WT and *atpA* mutant cells were adjusted in PBS, stained with 10 µM carboxyfluorescein succinimidyl ester (CFSE) for 1 h at 37 °C and subsequently washed 3 times with PBS. 10^8 CFU/ml were opsonized with 10% normal human serum (NHS) in RPMI for 1 h at 37 °C. To check for correct CFUs, the dilution was plated onto TSA agar plates. 2.5×10^6 previously isolated PMNs were seeded in a 96 well round-bottom plate and challenged with opsonized WT and *atpA* mutant *S. aureus* JE2 at a MOI of 1. Incubation was performed for 20 min or 1 h at 37 °C. After incubation, the plate was centrifuged at 300×g for 10 min, and the pellet was fixed with 3.7% formaldehyde for 20 min in the dark. The fixed cells were then washed and suspended in PBS. The samples were analyzed with a BD FACSCalibur by measuring 5000 events for each sample.

Killing of *S. aureus* by neutrophils. Starter cultures of *S. aureus* JE2 and its respective *atpA* mutant were grown in TSB medium overnight. Main cultures were subsequently inoculated at an OD_{600} of 0.1 and grown to an OD of 1. 10^9 CFU of WT and *atpA* mutant cells were adjusted in PBS medium, then washed and resuspended in PBS. 10^8 CFU/ml were opsonized with 10% normal human serum (NHS) in RPMI for 1 h at 37 °C. 2.5×10^6 previously isolated PMNs were seeded in 96 well round-bottom plates and challenged with opsonized WT and *atpA* mutant *S. aureus* JE2 at a MOI of 1. Incubation was performed for 1 h at 37 °C in a 96 well round-bottom plate and inoculation controls were included. After incubation, the plate was centrifuged at 300×g for 10 min, the supernatants were collected, and the remaining neutrophil pellets were lysed using cold ddH₂O for 10 min on a rocker. The lysed neutrophils and remaining bacteria were resuspended and pooled with the previously collected supernatants. Dilutions of 10^{-2} were plated on TSA plates with an Eddy Jet 2 W and incubated overnight at 37 °C. Values provided are the mean \pm SEM derived from at least seven independent biological replicates.

Statistics. The data were analyzed in GraphPad Prism 7 (GraphPad Software Inc.) using paired t-tests or one-way analysis of variance (ANOVA) with a post hoc analysis of Dunnett's multiple comparison tests. Log-transformed data was used for bacterial survival. $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***)

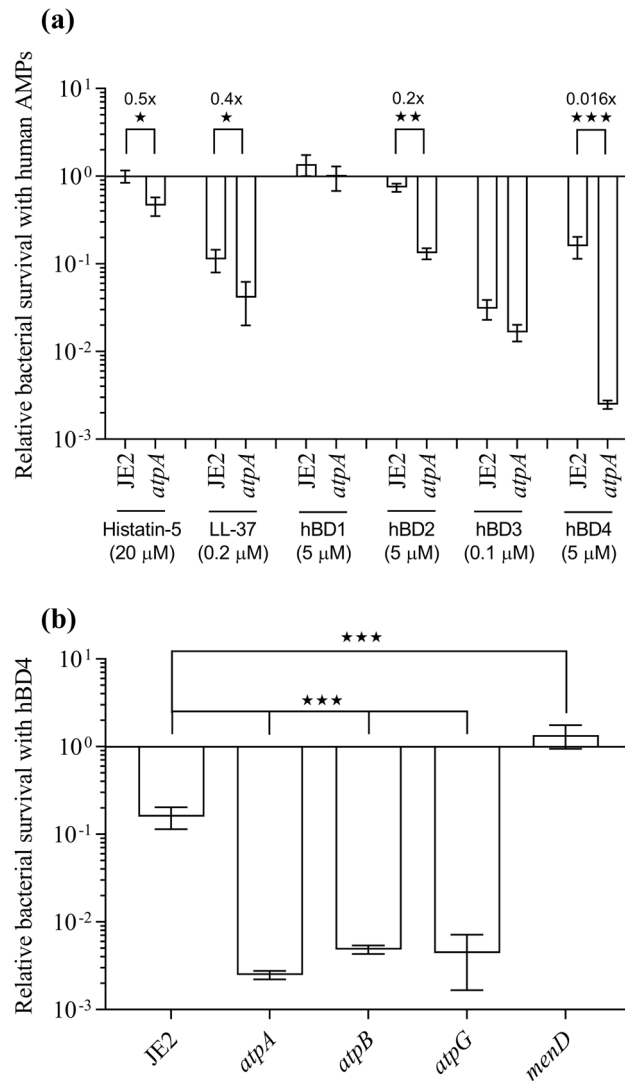


Figure 1. ATP synthase mutants are more susceptible to specific human AMPs than the WT. **(a)** The susceptibilities to the different AMPs assayed are presented as the relative survival following 2 h exposure at the indicated AMP concentrations for JE2 (WT) and *atpA* mutant. **(b)** Survival of ATP synthase mutants (*atpA*, *atpB* and *atpG*) and *menD* mutant following 2 h exposure to hBD4 (5 μM). Each survival value provided is the mean ± SEM derived from at least three independent measurements. ★*p* < 0.05, ★★*p* < 0.01 and ★★★*p* < 0.001.

Results

ATP synthase mutants are more susceptible to hBD2 and hBD4. Since inactivation of the ATP synthase increases susceptibility of *S. aureus* towards the antimicrobial peptides, polymyxins²⁵, we wondered if inactivation of the ATP synthase also sensitizes *S. aureus* towards AMPs of the human innate immune system. Therefore, bacterial killing of the WT *S. aureus* JE2 and isogenic *atpA* (ATP synthase subunit alpha) transposon mutant was assessed following 2 h exposure to human cationic AMPs, comprising histatin-5, LL-37 and hBD1-4 at the concentrations highlighted in Fig. 1a. The *atpA* mutant was more susceptible to hBD4 and hBD2 compared with the WT (Fig. 1a). The *atpA* mutant displayed a 63-fold greater reduction in viable cells compared with WT upon treatment with hBD4. For hBD2, the *atpA* mutant displayed a fivefold greater reduction in viable cells compared with WT. A minor increase in bactericidal activity against the *atpA* mutant was detected for histatin-5 and LL-37, whereas no differences between WT and *atpA* survival were detected upon treatment with hBD1 nor hBD3.

We assessed hBD4-mediated killing of other ATP synthase mutants, namely *atpB* (ATP synthase subunit A) and *atpG* (ATP synthase subunit gamma) and both mutants displayed increased susceptibility to hBD4, similarly to the *atpA* mutant (Fig. 1b).

Since the *atpA* mutant has a hyperpolarized membrane²⁵, we also assessed hBD4 susceptibility of the *menD* transposon mutant, which has a depolarized membrane³⁷. The *menD* mutant was indeed more tolerant to hBD4 compared with WT, as no reduction in viable cell count was observed following 2 h exposure to hBD4 at 5 μM (Fig. 1b), suggesting that the magnitude of the membrane potential is an important determinant for hBD4 susceptibility.

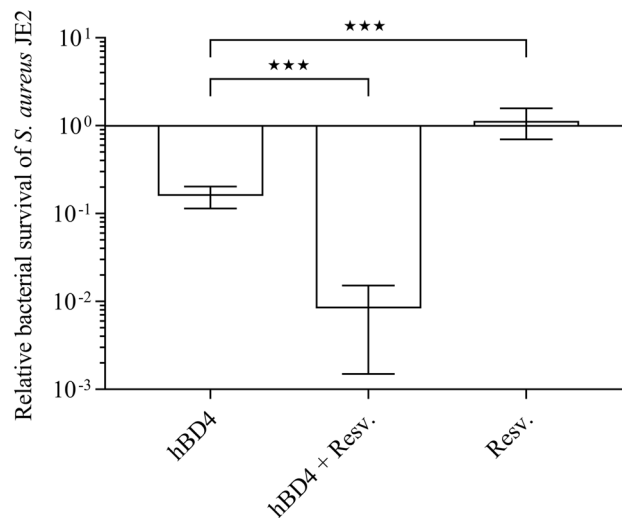


Figure 2. Resveratrol sensitizes *S. aureus* to hBD4. Survival of *S. aureus* JE2 was assessed for resveratrol (32 µg/ml) and hBD4 (5 µM), either alone or in combination. Each value provided is the mean ± SEM derived from at least three independent measurements. ★ $p < 0.05$, ★★ $p < 0.01$ and ★★★ $p < 0.001$.

Similarly we observed this correlation between magnitude of membrane potential and polymyxin B susceptibility, where the *menD* mutant was more resistant towards polymyxin B compared with the WT (Supplementary Table S1). The *menD* mutant is auxotrophic for menadione and supplementation with the compound re-sensitized the *menD* mutant to polymyxin B (Supplementary Table S1).

Taken together, inactivation of the ATP synthase sensitizes *S. aureus* to specific human AMPs and the magnitude of the membrane potential correlates with hBD4 susceptibility. This correlation also applies more broadly to include the non-human AMP, polymyxin B.

The ATP synthase inhibitor resveratrol sensitizes *S. aureus* towards hBD4. Resveratrol is a putative ATP synthase inhibitor in *S. aureus*³⁰ and therefore, we assessed if resveratrol could sensitize *S. aureus* JE2 towards hBD4. Resveratrol has growth-inhibitory properties with a MIC of 256 µg/ml, but at a sub-inhibitory concentration (0.125 × MIC) it had no impact on *S. aureus* viability (Fig. 2). Importantly, however, when supplemented in combination with hBD4, resveratrol increased killing of WT *S. aureus* by 20-fold compared with hBD4 alone.

Similarly, supplementation of resveratrol at sub-inhibitory concentrations (0.0625x–0.25 × MIC) sensitized *S. aureus* JE2 to polymyxin B (Supplementary Table S2).

These result suggests that ATP synthase inhibition with resveratrol may be an attractive approach to sensitize *S. aureus* towards hBD4.

The *atpA* mutant is more susceptible to killing by human neutrophils. Log-phase bacteria of the WT and *atpA* mutant were opsonized with pooled normal human serum, phagocytosed by neutrophils, and subsequently incubated for one hour before determination of surviving cells. The *atpA* mutant was more susceptible to neutrophil killing than the WT and after one hour of incubation with neutrophils, only 39.2% of the *atpA* cells survived compared with 49.9% for the WT ($P = 0.006$) (Fig. 3). The uptake of the strains into the neutrophils was similar (Data not shown), suggesting that the increased killing of the *atpA* is due to antimicrobial activities of the neutrophils and not due to alterations in phagocytosis rates.

As neutrophils normally use both, oxygen-dependent and non-oxygen-dependent killing mechanisms, including antimicrobial peptides³⁸, we compared survival of *atpA* and WT in neutrophils treated with the NADPH oxidase inhibitor diphenyleneiodonium (DPI), which suppresses the formation of reactive oxygen species¹⁸. Suppression of the oxidative burst limited killing of *S. aureus* (Fig. 3). After one hour incubation only 74.3% of the *atpA* cells survived in DPI-treated neutrophils, compared to 91.4% for the WT ($P = 0.0196$) (Fig. 3). These data suggest that the *atpA* mutant is more susceptible towards the oxygen-independent antimicrobial activities of neutrophils.

Discussion

Antimicrobial peptides are an important part of the innate immune system and the AMPs display activity against a wide range of bacterial-, fungal- and viral species⁶. Several human AMPs however display low inhibitory activity against *S. aureus*¹⁴. For example, the human β-defensins 1–4 are produced by keratinocytes and are key in protecting against skin infections⁶. hBD3 displays greater bactericidal activity against *S. aureus* than the other β-defensins^{14–16}, and hBD3 is important for keratinocytes in killing *S. aureus*¹⁷. Production of AMPs in the skin and in the nasal passages plays a major role in preventing *S. aureus* persistent colonization and people with defects in hBD3 production have enhanced nasal colonization of *S. aureus*³⁹. Our results point to a novel

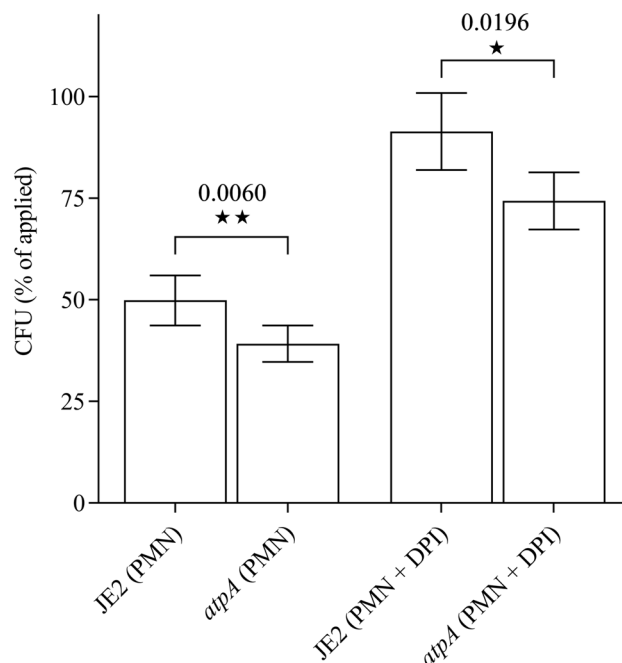


Figure 3. Neutrophil-mediated killing of *S. aureus*. The percentage of viable opsonized WT and *atpA* mutant cells following incubation with neutrophils (PMN) for 1 h. Surviving cells are expressed in percentage of the initial counts. Diphenyleneiodonium (DPI) is a NADPH oxidase inhibitor. Each value provided is the mean \pm SEM derived from at least seven independent measurements. $\star p < 0.05$, $\star\star p < 0.01$ and $\star\star\star p < 0.001$.

type of antimicrobial therapy, whereby the susceptibility of the pathogen is enhanced towards the natural human antimicrobial peptides. Here we demonstrate the potential for *S. aureus*, but it may be applicable to other human pathogens as well.

The energetic state of bacterial membranes can affect the susceptibility towards AMPs in different bacterial species⁸ and for some conventional classes of antibiotics as well, i.e. aminoglycosides⁴⁰. Recently, we demonstrated that ATP synthase mutants of *S. aureus* become more sensitive towards polymyxins²⁵. ATP synthase inactivation confers hyperpolarization of the membrane^{25,28} and larger membrane potentials can facilitate AMP insertion into membranes⁸. In this study, we demonstrate that the activities of certain human AMPs are affected by the magnitude of the membrane potential. ATP synthase mutants have a hyperpolarized membrane^{25,28} and become more sensitive towards hBD2 and hBD4 and to a minor degree towards LL-37 and histatin-5 (Fig. 1a). Contrarily, a *menD* mutant with a depolarized membrane is less sensitive towards hBD4 (Fig. 1b).

Our finding that membrane depolarization protects *S. aureus* from hBD4-mediated killing corroborate previous studies demonstrating that *S. aureus* SCVs are less susceptible to different AMPs. For *S. aureus*, electron-transport chain deficient mutants are less susceptible to killing by thrombin-induced PMP-1 (tPMP-1)⁴¹, nisin²⁴, lactoferrin B⁴² and human AMPs, including hBD2, hBD3 and LL-37²³. Another study, with genetically defined *menD* and *hemB* mutants in different *S. aureus* genetic backgrounds did however not observe changes in susceptibility to LL-37⁴³. Killing by the human neutrophil defensin 1 (hNP-1) is similar in WT and mutants with impaired electron transport chains^{24,41,44}. These observations indicate that membrane potential changes only affect the activity of specific AMPs.

It has been suggested that membrane depolarization and subsequently increased tolerance towards AMPs of the innate immune system is a survival strategy that enable intracellular persistence of *S. aureus* in eukaryotic cells⁴⁵. Here we demonstrate that inactivation of the ATP synthase contrarily sensitizes *S. aureus* to neutrophil-killing (Fig. 3). The increased susceptibility to neutrophil-killing is also evident when the oxidative burst is suppressed (Fig. 3), suggesting that this effect is mediated by increased susceptibility to AMPs produced by neutrophils³⁸.

It is not only in *S. aureus* that AMP sensitivity is modulated by the magnitude of the membrane potential. In *E. coli*, inactivation of the ATP synthase also leads to hyperpolarization of the membrane⁴⁶ and ATP synthase mutants are more sensitive to colistin⁴⁷ and aminoglycosides^{40,47}. Deletion of the gene *phoP* in *E. coli* conferred hyperpolarization of the membrane and a concomitant increase in activity of polymyxin B, while collapsing the proton gradient with the protonophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) abrogated this effect⁴⁸. Dissipation of membrane potential with CCCP also impaired killing of *E. coli* with the AMP indolicidin⁴⁹. For *Salmonella enterica* Typhimurium, impairment of the electron transport chain reduces AMP activity, e.g. a *hemB* mutant displays a fourfold increase in MIC for colistin⁵⁰. Even respiration-deficient mutants of the fungus *Candida albicans* experience reduced sensitivity to histatin-5 and chemical inhibition of the electron transport chain with sodium azide or CCCP treatment also protects *C. albicans* against histatin-5 killing^{51,52}.

The ATP synthase may potentially be targeted to facilitate killing by AMPs of the innate immune system and hence be essential under in vivo conditions. By employing the Tn-seq methodology, the ATP synthase has been identified in several studies as essential during in vivo conditions, such as in abscess formation or osteomyelitis, while the ATP synthase is dispensable during growth in laboratory medium^{53–55}. Recently, Grosser and colleagues demonstrated that an ATP synthase mutant indeed is severely attenuated in a murine skin abscess model²⁸. ATP synthase inactivation confers pleiotropic effects, including attenuated growth under anaerobic conditions, increased sensitivity towards peroxide and nitric oxide stresses²⁸. Whether virulence attenuation of the ATP synthase mutant in the murine skin abscess model is mediated by a single phenotypic trait or a combination thereof remain unexplored in the study²⁸ and here we provide an additional phenotype that may contribute to the attenuated virulence, namely increased sensitivity of *S. aureus* to different AMPs of the innate immune system.

Inhibition of the ATP synthase may potentially have therapeutic value either as a monotherapy or in combination with AMPs or aminoglycosides. Many ATP synthase inhibitors have been identified²⁹, however several of these, e.g. oligomycin A, are not selective for bacterial ATP synthases and also blocks human mitochondrial ATP synthases³¹. The ATP synthase has been clinically validated as a druggable target in recent years with the antibiotic bedaquiline that selectively inhibits ATP synthases of Mycobacteria⁵⁶. We demonstrated that the ATP synthase inhibitor resveratrol, a commonly used nutraceutical⁵⁷, sensitizes *S. aureus* towards hBD4 (Fig. 2). Resveratrol has previously been assessed for clearance of acne skin lesions⁵⁸ and has recently been shown to reduce abscess formation by *S. aureus* when used as a monotherapy⁵⁹. It will be important in future animal experiments to elucidate, whether resveratrol in combination with human defensins are superior in treating topical *S. aureus* infections compared with the respective monotherapies.

It is encouraging that bacterial ATP synthases are sufficiently different from human ATP synthases, which enables identification of selective bacterial ATP synthase inhibitors that are not toxic to human cells³². Selective staphylococcal ATP synthase inhibitors may provide a novel class of antibacterial therapies that is based on sensitizing *S. aureus* towards the AMPs of the innate immune system. Additionally, such inhibitors can be adjuvants that potentiate the activity of conventional antibiotics, such as aminoglycosides and polymyxins^{25,30}. Several AMPs are in clinical development³, whose activity potentially also can be enhanced by co-administration of ATP synthase inhibitors.

In summary, we have investigated the possibility of sensitizing *S. aureus* towards human AMPs by targeting the ATP synthase and our results suggest that it may be a novel strategy for development of new antimicrobial therapeutics.

Received: 18 February 2020; Accepted: 8 June 2020

Published online: 09 July 2020

References

- Lewis, K. Platforms for antibiotic discovery. *Nat. Rev. Drug Discov.* **12**, 371–387 (2013).
- Butler, M. S., Blaskovich, M. A. & Cooper, M. A. Antibiotics in the clinical pipeline at the end of 2015. *J. Antibiot.* **70**, 3–24 (2017).
- Mahlapuu, M., Häkansson, J., Ringstad, L. & Björn, C. Antimicrobial peptides: an emerging category of therapeutic agents. *Front. Cell. Infect. Microbiol.* **6**, 194 (2016).
- Hancock, R. E., Haney, E. F. & Gill, E. E. The immunology of host defence peptides: beyond antimicrobial activity. *Nat. Rev. Immunol.* **16**, 321 (2016).
- De Smet, K. & Contreras, R. Human antimicrobial peptides: defensins, cathelicidins and histatins. *Biotech. Lett.* **27**, 1337–1347 (2005).
- Otto, M. *Staphylococcus* colonization of the skin and antimicrobial peptides. *Expert Rev. Dermatol.* **5**, 183–195 (2010).
- Xhindoli, D. *et al.* The human cathelicidin LL-37—pore-forming antibacterial peptide and host-cell modulator. *Biochimica et Biophysica Acta (BBA)-Biomembranes* **1858**, 546–566 (2016).
- Yeaman, M. R. & Yount, N. Y. Mechanisms of antimicrobial peptide action and resistance. *Pharmacol. Rev.* **55**, 27–55 (2003).
- Omardeen, S., Brul, S. & Zaat, S. A. Antimicrobial activity of cationic antimicrobial peptides against gram-positives: current progress made in understanding the mode of action and the response of bacteria. *Front. Cell Dev. Biol.* **4**, 111 (2016).
- Sass, V. *et al.* Human β -defensin 3 inhibits cell wall biosynthesis in Staphylococci. *Infect. Immun.* **78**, 2793–2800 (2010).
- Andersson, D. I., Hughes, D. & Kubicek-Sutherland, J. Z. Mechanisms and consequences of bacterial resistance to antimicrobial peptides. *Drug Resist. Updates* **26**, 43–57 (2016).
- Lowy, F. D. *Staphylococcus aureus* infections. *N. Engl. J. Med.* **339**, 520–532 (1998).
- Gordon, R. J. & Lowy, F. D. Pathogenesis of methicillin-resistant *Staphylococcus aureus* infection. *Clin. Infect. Dis.* **46**, S350–S359 (2008).
- Midorikawa, K. *et al.* *Staphylococcus aureus* susceptibility to innate antimicrobial peptides, β -defensins and CAP18, expressed by human keratinocytes. *Infect. Immun.* **71**, 3730–3739 (2003).
- Chen, X. *et al.* Synergistic effect of antibacterial agents human β -defensins, cathelicidin LL-37 and lysozyme against *Staphylococcus aureus* and *Escherichia coli*. *J. Dermatol. Sci.* **40**, 123–132 (2005).
- Kubicek-Sutherland, J. Z. *et al.* Antimicrobial peptide exposure selects for *Staphylococcus aureus* resistance to human defence peptides. *J. Antimicrob. Chemother.* **72**, 115–127 (2017).
- Kisich, K. O. *et al.* The constitutive capacity of human keratinocytes to kill *Staphylococcus aureus* is dependent on β -defensin 3. *J. Investig. Dermatol.* **127**, 2368–2380 (2007).
- Peschel, A. *et al.* *Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with l-lysine. *J. Exp. Med.* **193**, 1067–1076 (2001).
- Peschel, A. *et al.* Inactivation of the *dlt* operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides. *J. Biol. Chem.* **274**, 8405–8410 (1999).
- Koprivnjak, T., Weidenmaier, C., Peschel, A. & Weiss, J. P. Wall teichoic acid deficiency in *Staphylococcus aureus* confers selective resistance to mammalian group IIA phospholipase A2 and human β -defensin 3. *Infect. Immun.* **76**, 2169–2176 (2008).
- Kagan, B. L., Selsted, M. E., Ganz, T. & Lehrer, R. I. Antimicrobial defensin peptides form voltage-dependent ion-permeable channels in planar lipid bilayer membranes. *Proc. Natl. Acad. Sci.* **87**, 210–214 (1990).
- Proctor, R. A. *et al.* Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nat. Rev. Microbiol.* **4**, 295–305 (2006).

23. Gläser, R., Becker, K., von Eiff, C., Meyer-Hoffert, U. & Harder, J. Decreased susceptibility of *Staphylococcus aureus* small-colony variants toward human antimicrobial peptides. *J. Investig. Dermatol.* **134**, 2347–2350 (2014).
24. Koo, S.-P., Bayer, A. S., Sahl, H.-G., Proctor, R. A. & Yeaman, M. R. Staphylocidal action of thrombin-induced platelet microbicidal protein is not solely dependent on transmembrane potential. *Infect. Immun.* **64**, 1070–1074 (1996).
25. Vestergaard, M. *et al.* Inhibition of the ATP Synthase Eliminates the Intrinsic Resistance of *Staphylococcus aureus* towards Polymyxins. *mBio* **8**, e01114–e01117 (2017).
26. Zavascki, A. P., Goldani, L. Z., Li, J. & Nation, R. L. Polymyxin B for the treatment of multidrug-resistant pathogens: a critical review. *J. Antimicrob. Chemother.* **60**, 1206–1215 (2007).
27. Deckers-Hebestreit, G. & Altendorf, K. The F₀F₁-type ATP synthases of bacteria: structure and function of the F₀ complex. *Annu. Rev. Microbiol.* **50**, 791–824 (1996).
28. Grosser, M. R. *et al.* Genetic requirements for *Staphylococcus aureus* nitric oxide resistance and virulence. *PLoS Pathog.* **14**, e1006907 (2018).
29. Hong, S. & Pedersen, P. L. ATP synthase and the actions of inhibitors utilized to study its roles in human health, disease, and other scientific areas. *Microbiol. Mol. Biol. Rev.* **72**, 590–641 (2008).
30. Nøhr-Meldgaard, K., Ovsepian, A., Ingmer, H. & Vestergaard, M. Resveratrol enhances the efficacy of aminoglycosides against *Staphylococcus aureus*. *Int. J. Antimicrob. Agents* **52**, 390–396 (2018).
31. Balemans, W. *et al.* Novel antibiotics targeting respiratory ATP synthesis in Gram-positive pathogenic bacteria. *Antimicrob. Agents Chemother.* **56**, 4131–4139 (2012).
32. Haagsma, A. C. *et al.* Selectivity of TMC207 towards mycobacterial ATP synthase compared with that towards the eukaryotic homologue. *Antimicrob. Agents Chemother.* **53**, 1290–1292 (2009).
33. Gledhill, J. R., Montgomery, M. G., Leslie, A. G. & Walker, J. E. Mechanism of inhibition of bovine F₁-ATPase by resveratrol and related polyphenols. *Proc. Natl. Acad. Sci.* **104**, 13632–13637 (2007).
34. Dadi, P. K., Ahmad, M. & Ahmad, Z. Inhibition of ATPase activity of *Escherichia coli* ATP synthase by polyphenols. *Int. J. Biol. Macromol.* **45**, 72–79 (2009).
35. Vestergaard, M. & Ingmer, H. Antibacterial and antifungal properties of resveratrol. *Int. J. Antimicrob. Agents* **53**, 716–723 (2019).
36. Troelstra, A. *et al.* Dual effects of soluble CD14 on LPS priming of neutrophils. *J. Leukoc. Biol.* **61**, 173–178 (1997).
37. Vestergaard, M. *et al.* Novel pathways for ameliorating the fitness cost of gentamicin resistant small colony variants. *Front. Microbiol.* **7**, 1866 (2016).
38. Amulic, B., Cazalet, C., Hayes, G. L., Metzler, K. D. & Zychlinsky, A. Neutrophil function: from mechanisms to disease. *Annu. Rev. Immunol.* **30**, 459–489 (2012).
39. Zanger, P., Nurjadi, D., Vath, B. & Kreamsner, P. G. Persistent nasal carriage of *Staphylococcus aureus* is associated with deficient induction of human β -defensin 3 after sterile wounding of healthy skin *in vivo*. *Infect. Immun.* **79**, 2658–2662 (2011).
40. Lobritz, M. A. *et al.* Antibiotic efficacy is linked to bacterial cellular respiration. *Proc. Natl. Acad. Sci.* **112**, 8173–8180 (2015).
41. Yeaman, M. R., Bayer, A. S., Koo, S.-P., Foss, W. & Sullam, P. M. Platelet microbicidal proteins and neutrophil defensin disrupt the *Staphylococcus aureus* cytoplasmic membrane by distinct mechanisms of action. *J. Clin. Invest.* **101**, 178–187 (1998).
42. Samuelsen, Ø *et al.* *Staphylococcus aureus* small colony variants are resistant to the antimicrobial peptide lactoferricin B. *J. Antimicrob. Chemother.* **56**, 1126–1129 (2005).
43. Zhang, P., Wright, J. A., Tymon, A. & Nair, S. P. Bicarbonate induces high-level resistance to the human antimicrobial peptide LL-37 in *Staphylococcus aureus* small colony variants. *J. Antimicrob. Chemother.* **73**, 615–619 (2017).
44. Sadowska, B. *et al.* Characteristics of *Staphylococcus aureus* isolated from airways of cystic fibrosis patients, and their small colony variants. *FEMS Immunol. Med. Microbiol.* **32**, 191–197 (2002).
45. Kahl, B. C. Small colony variants (SCVs) of *Staphylococcus aureus*—a bacterial survival strategy. *Infect. Genet. Evolut.* **21**, 515–522 (2014).
46. Jensen, P. R. & Michelsen, O. Carbon and energy metabolism of *atp* mutants of *Escherichia coli*. *J. Bacteriol.* **174**, 7635–7641 (1992).
47. Liu, A. *et al.* Antibiotic sensitivity profiles determined with an *Escherichia coli* gene knockout collection: generating an antibiotic bar code. *Antimicrob. Agents Chemother.* **54**, 1393–1403 (2010).
48. Alteri, C. J., Lindner, J. R., Reiss, D. J., Smith, S. N. & Mobley, H. L. The broadly conserved regulator PhoP links pathogen virulence and membrane potential in *Escherichia coli*. *Mol. Microbiol.* **82**, 145–163 (2011).
49. Falla, T. J., Karunaratne, D. N. & Hancock, R. E. Mode of action of the antimicrobial peptide indolicidin. *J. Biol. Chem.* **271**, 19298–19303 (1996).
50. Pránting, M. & Andersson, D. I. Mechanisms and physiological effects of protamine resistance in *Salmonella enterica* serovar Typhimurium LT2. *J. Antimicrob. Chemother.* **65**, 876–887 (2010).
51. Gyurko, C., Lendenmann, U., Troxler, R. F. & Oppenheim, F. G. *Candida albicans* mutants deficient in respiration are resistant to the small cationic salivary antimicrobial peptide histatin 5. *Antimicrob. Agents Chemother.* **44**, 348–354 (2000).
52. Koshlukova, S. E., Lloyd, T. L., Araujo, M. W. & Edgerton, M. Salivary histatin 5 induces non-lytic release of ATP from *Candida albicans* leading to cell death. *J. Biol. Chem.* **274**, 18872–18879 (1999).
53. Ibberson, C. B. *et al.* Co-infecting microorganisms dramatically alter pathogen gene essentiality during polymicrobial infection. *Nat. Microbiol.* **2**, 17079 (2017).
54. Wilde, A. D. *et al.* Bacterial hypoxic responses revealed as critical determinants of the host-pathogen outcome by TnSeq analysis of *Staphylococcus aureus* invasive infection. *PLoS Pathog.* **11**, e1005341 (2015).
55. Valentino, M. D. *et al.* Genes contributing to *Staphylococcus aureus* fitness in abscess-and infection-related ecologies. *MBio* **5**, e01729–e01714 (2014).
56. Mahajan, R. Bedaquiline: first FDA-approved tuberculosis drug in 40 years. *Int. J. Appl. Basic Med. Res.* **3**, 1 (2013).
57. Aschemann-Witzel, J. & Grunert, K. G. Resveratrol food supplements: a survey on the role of individual consumer characteristics in predicting the attitudes and adoption intentions of US American and Danish respondents. *BMC Public Health* **15**, 110 (2015).
58. Fabbrocini, G. *et al.* Resveratrol-containing gel for the treatment of acne vulgaris. *Am. J. Clin. Dermatol.* **12**, 133–141 (2011).
59. Duan, J. *et al.* Subinhibitory concentrations of resveratrol reduce alpha-hemolysin production in *Staphylococcus aureus* isolates by downregulating *saerS*. *Emerg. Microbes Infect.* **7**, 1–10 (2018).
60. Fey, P. D. *et al.* A genetic resource for rapid and comprehensive phenotype screening of nonessential *Staphylococcus aureus* genes. *MBio* **4**, e00537–e00512 (2013).

Acknowledgements

The work is funded by the Danish Research Council for Independent Research, Technology and Production nr. 7017-00079B for HI and by grants from the German Research Foundation (PE 805/5-2, TRR156, and SFB766 [to A. P.] and TRR34 [to D. K. and A. P.]) and the German Center for Infection Research (DZIF) to A.P. and D.K. The authors acknowledge infrastructural support by the Cluster of Excellence EXC2124 Controlling Microbes to Fight Infections.

Author contributions

MV, AP, DK and HI conceived and designed the study. Experiments were performed by MV, LL, KNM and CB. All authors contributed in analysis of data and writing of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at <https://doi.org/10.1038/s41598-020-68146-4>.

Correspondence and requests for materials should be addressed to H.I.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2020