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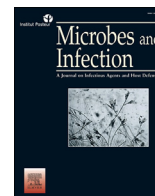
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Short communication

Staphylococcus aureus NAD kinase is required for envelop and antibiotic stress responses

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ABSTRACT

Global burden of infectious diseases and antimicrobial resistance are major public health issues calling for innovative control measures. Bacterial NAD kinase (NADK) is a crucial enzyme for production of NADP(H) and growth. In *Staphylococcus aureus*, NADK promotes pathogenesis by supporting production of key virulence determinants. Here, we find that knockdown of NADK by CRISPR interference sensitizes *S. aureus* to osmotic stress and to stresses induced by antibiotics targeting the envelop as well as replication, transcription and translation. Thus, NADK represents a promising target for the development of inhibitors which could be used in combination with current antibiotics.

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1. Introduction

Antimicrobial resistance (AMR) is a major societal and public health issue with an estimated US\$100 trillion cost and a global fatality foreseen to reach 10 million people annually by 2050 [1]. A first comprehensive study reported 4.95 million deaths associated with bacterial AMR and 1.27 million deaths directly imputable to resistance worldwide in 2019 [2]. For the same year, the Global Burden Disease Study revealed an estimated 13.7 million deaths related to infections, including 7.7 million deaths associated with 33 pathogenic bacteria [3]. The study, which excluded *Mycobacterium tuberculosis*, highlighted that *Staphylococcus aureus* was the leading cause of death in 135 countries. Current burden of infectious diseases, emergence of pathogenic microorganisms and worrisome spread of AMR call for urgent identification of novel drug targets and development of innovative antimicrobial therapies. We have previously discovered a new class of antibacterials that inhibit *S. aureus* NAD kinase (NADK), an original drug target [4]. The NAD kinase is the only enzyme that can phosphorylate NAD⁺/NADH to NADP⁺/NADPH, which are essential cofactors contributing to important metabolic pathways in all living organisms [5–7]. Recently, we reported that *S. aureus* NADK promotes pathogenesis by protecting bacteria from host antimicrobial defense mechanisms and by supporting production of major virulence factors [8]. The purpose of this study is to determine the role of NADK in response to stresses

mimicking those encountered during the infectious process, with a particular focus on stresses induced by antibiotic treatment. CRISPR interference was used to knockdown NADK expression to evaluate the contribution of the enzyme to bacterial survival when *S. aureus* was exposed to these stresses.

2. Materials and methods

2.1. Plasmids, bacterial strains and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1. All plasmids were maintained in *Escherichia coli* TOP10. The restriction deficient *S. aureus* strain RN4220 was transformed with plasmids isolated from *E. coli*. The *S. aureus* Xen36 strain was transformed with plasmids isolated from the RN4220 strain as previously described [4]. *E. coli* was grown in lysogeny broth (LB) medium (Difco) with shaking at 200 rpm or on LB agar plates at 37 °C. *S. aureus* was grown in brain heart infusion (BHI) broth (BD) or in tryptic soy broth (TSB) (Difco) with shaking at 200 rpm or on BHI agar plates at 37 °C. When required, culture medium was supplemented with antibiotics (carbenicillin 100 µg/mL for *E. coli*, chloramphenicol 15 µg/mL or anhydrotetracycline 100 ng/mL for *S. aureus*).

2.2. Bacterial growth measurement

Overnight *S. aureus* cultures in BHI at 37 °C were diluted 1:100 into BHI broth. For NADK inhibition experiments, culture medium was supplemented with 100 ng/mL anhydrotetracycline (Sigma–Aldrich)

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Table 1
Strains and plasmids used in this study.

Strains and plasmids	Characteristics	References
Strains		
<i>E. coli</i> strain TOP10	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74</i> <i>recA1</i> <i>araD139</i> Δ (<i>ara-leu</i>) 7697 <i>galU</i> <i>galK</i> <i>rpsL</i> (Str ^R) <i>endA1</i> <i>nupG</i>	Invitrogen
<i>S. aureus</i> strains		
Xen36	Strain derived from a clinical isolate from a bacteremic patient	PerkinElmer [9]
Xen36/pSD1	Xen36 strain carrying plasmid pSD1	[4]
Xen36/NADK sgRNA	Xen36 strain carrying plasmid pSD1 <i>ppnK</i>	[4]
Plasmids		
pSD1	dCas9 ATc-inducible and sgRNA constitutive expression plasmid	[10]
pSD1 <i>ppnK</i>	pSD1 plasmid carrying sgRNA targeting <i>S. aureus</i> NADK gene <i>ppnK</i>	[4]

and 25 μ g/mL chloramphenicol when required. For stress experiments, culture medium was supplemented at indicated concentrations with polymyxin B, kanamycin, vancomycin, rifampicin (Sigma–Aldrich), triclosan, sodium chloride (Merck) or levofloxacin (USP), with or without 10 μ g/mL catalase (Sigma). Bacterial suspensions were incubated in 96-well plates or in Erlenmeyer, with shaking at 200 rpm at 37 °C. Growth was monitored with a microplate reader (Glomax Discover, Promega). Experiments were performed at least three times.

2.3. Statistical analysis

Results are expressed as means \pm SEM of 3 replicates. Statistical analysis was performed using GraphPad Prism (GraphPad Prism

9.1.2 software). One-way ANOVA was used to compare data. Differences between groups were considered significant when the *p* value was lower than 0.05.

3. Results

3.1. NADK inhibition increases *S. aureus* susceptibility to envelop stresses

At each step of the infectious process, *S. aureus* has to cope with stressful host microenvironments. Among them envelop stress derives from multiple sources such as antimicrobial peptides, bile acids and osmotic stress [11,12]. Thus, we investigated the effects of NADK deficiency on *S. aureus* sensitivity to envelop stresses. Since NADK has been shown to be essential for *S. aureus* growth [13], we used CRISPR interference-based NADK inhibition to address specifically the role of NADK. We first studied the role of NADK in response to osmotic stress upon addition of NaCl to culture broth. The NADK knockdown strain (*S. aureus*/NADK sgRNA) showed increased sensitivity to NaCl treatment at concentrations of 4% and above, while the empty vector control strain (*S. aureus*/pSD1) was not significantly affected up to 8% NaCl (Fig. 1A). Next, we treated *S. aureus* containing the empty vector or the NADK knockdown strain with triclosan, a membranotropic antibacterial agent and an inhibitor of FabI, an enoyl-ACP reductase implicated in the last step of bacterial fatty acid elongation [14,15]. The NADK knockdown strain displayed a higher susceptibility to triclosan treatment at 0.5 nM and higher concentrations compared to 0.25 nM treatment or untreated conditions (Fig. 1B). In contrast, treatment of the *S. aureus*/pSD1 strain with at least 1 nM triclosan was necessary to inhibit growth. We then wondered if NADK knockdown might impact bacterial survival under stress induced by envelop-targeting antibiotics, a condition bacteria face during infection treatment. To

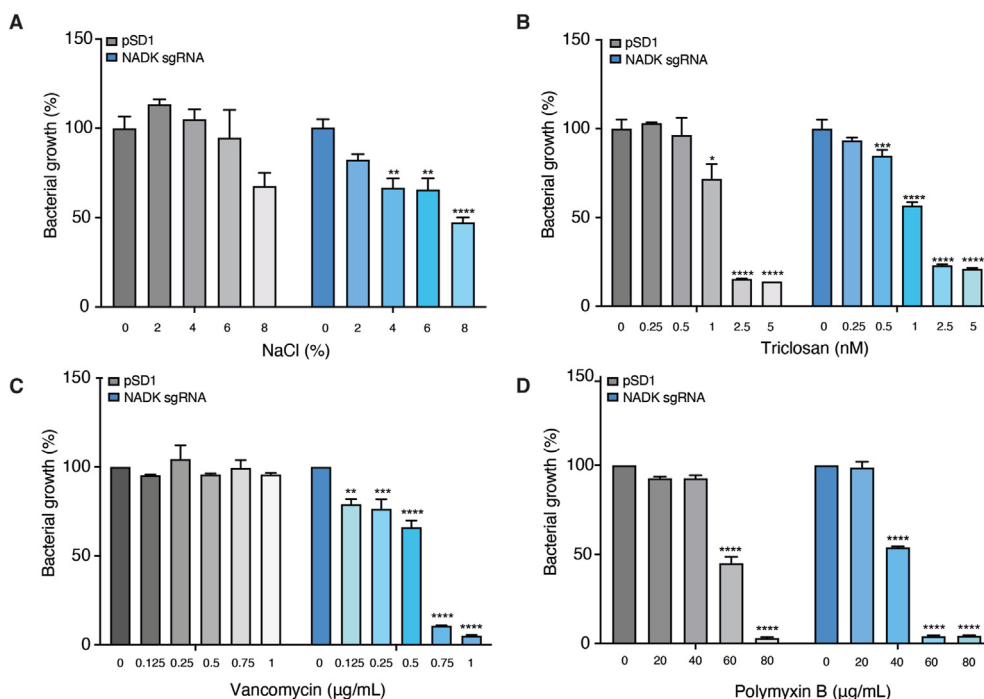


Fig. 1. NADK inhibition synergizes with envelop stress to kill *S. aureus*. (A–E) Bacterial growth was monitored at OD_{600nm} in BHI broth at 37 °C. Percentage of growth of *S. aureus* containing the empty vector (pSD1) or the NADK knockdown strain (NADK sgRNA) exposed for 6 h to increasing concentrations of NaCl (A), triclosan (B), vancomycin (C), or polymyxin B (D) relative to untreated condition. Data shown are representative of three independent experiments. Bars indicate the standard error of the means of biological replicates. Comparison of data from each treated condition to data from the corresponding untreated condition was performed using one-way analysis of variance (***p* < 0.01, ****p* < 0.001, *****p* < 0.0001).

test this hypothesis, we treated *S. aureus*/pSD1 or *S. aureus*/NADK sgRNA strains with subinhibitory concentrations of antibiotics targeting the bacterial envelop and compared their growth after 6 h of treatment. We first used vancomycin, a clinically used antibiotic which binds the D-ala-D-ala moiety of murein monomers, thereby inhibiting their incorporation to peptidoglycan chains and subsequent cell wall synthesis [16]. Strikingly, the NADK knockdown strain showed increased susceptibility to vancomycin treatment at 0.125 µg/mL and higher concentrations (Fig. 1C), while growth of the *S. aureus*/pSD1 strain remained unaffected irrespective of vancomycin treatments. We then treated both strains with polymyxin B, an amphipathic cyclic lipopeptide which disrupts bacterial membranes. Polymyxin B is generally used to treat infection caused by some Gram-negative bacteria but it has been shown to kill some strains of *S. aureus in vitro* at high concentration [17]. As expected, a concentration of polymyxin B as high as 40 µg/mL did not inhibit growth of the *S. aureus*/pSD1 strain (Fig. 1D). In contrast, the NADK knockdown strain was susceptible to treatment with 40 µg/mL polymyxin B (Fig. 1D). Together, these results emphasize the role of NADK in protecting *S. aureus* from envelop stresses, and fit our previous study showing that inhibition of NADK sensitizes *S. aureus* to combined activities of lysozyme and lysostaphin, two enzymes cleaving peptidoglycan [8]. Most importantly, they reveal the synergistic antibacterial effect of NADK inhibition and envelop stresses.

3.2. NADK inhibition increases *S. aureus* susceptibility to stresses induced by several classes of antibiotics

We next investigated whether NADK inhibition could synergize with antibiotic-induced stresses other than envelop stress to mediate antibacterial activity. We used levofloxacin which inhibits DNA synthesis and induces DNA damage, rifampicin which inhibits RNA synthesis and kanamycin which inhibits protein synthesis. Treatment with a subinhibitory concentration of levofloxacin, i.e., 0.0625 µg/mL, led to decreased bacterial growth upon NADK knockdown (Fig. 2A). NADK inhibition also sensitized *S. aureus* to rifampicin, at a concentration of 2 ng/mL (Fig. 2B). The NADK knockdown strain showed a more modest but significant increased susceptibility to kanamycin treatment at 46 µg/mL and higher concentrations (Fig. 2C), while growth of the *S. aureus*/pSD1 strain was not affected by any of the kanamycin treatments. Together, these results indicate that NADK protect *S. aureus* from multiple antibiotic-induced stresses and show the synergistic antibacterial effect of NADK inhibition and inhibition of other essential bacterial functions by clinically used antibiotics.

3.3. NADK inhibition increases *S. aureus* susceptibility to antibiotic stresses in a process mediated by reactive oxygen species and alternative pathways

We have reported that NADK contributes to *S. aureus* resistance to reactive oxygen species (ROS) [8]. To address how NADK protects *S. aureus* from antibiotic stresses, we thus investigated the effects of NADK knock-down on antibiotic sensitivity in presence of the antioxidant enzyme catalase, which catalyzes hydrogen peroxide dismutation into water and oxygen. First, we treated the *S. aureus*/pSD1 strain with subinhibitory concentrations of vancomycin, polymyxin B, levofloxacin, rifampicin or kanamycin and measured bacterial growth with or without catalase (Fig. 3A–E). Supplementation of the culture medium with catalase did not have any significant effect on growth in presence or absence of each antibiotic compound (Fig. 3A–E). Similarly, addition of catalase had no significant effect on growth of the NADK knockdown strain in absence of antibiotic treatment (Fig. 3A–E). In contrast, catalase had a positive effect on growth of the *S. aureus*/NADK sgRNA strain treated

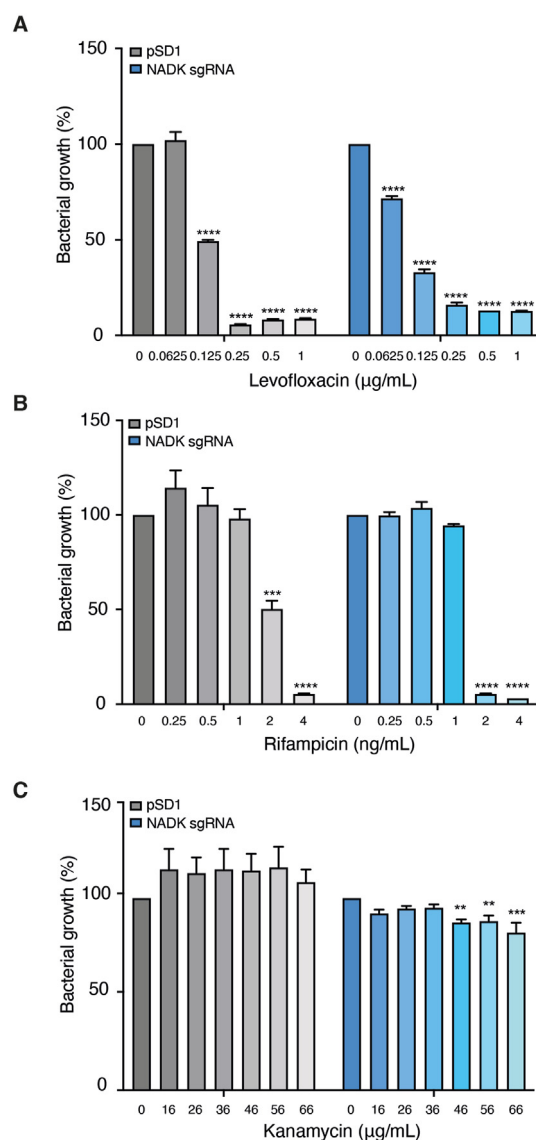


Fig. 2. NADK inhibition increases bacterial susceptibility to diverse classes of antibiotics. (A–C) Bacterial growth was monitored at OD_{600nm} in BHI broth at 37 °C. Percentage of growth of *S. aureus* containing the empty vector (pSD1) or the NADK knockdown strain (NADK sgRNA) exposed for 6 h to increasing concentrations of levofloxacin (A), rifampicin (B) or kanamycin (C), relative to the untreated condition. Data shown are representative of three independent experiments. Bars indicate the standard error of the means of biological replicates. Comparison of data from each treated condition to data from the corresponding untreated condition was performed using one-way analysis of variance (**p < 0.01, ***p < 0.001, ****p < 0.0001).

with antibiotics (Fig. 3A–E). Indeed, catalase treatment rescued the growth defect of the NADK knockdown strain upon polymyxin B, levofloxacin, rifampicin and kanamycin treatments (Fig. 3B, C, 3D, 3E). On the other hand, catalase did not protect bacteria from growth inhibition by vancomycin, as it was unable to restore growth to the levels of non-treated bacteria (Fig. 3A). Taken together, our results show that NADK protects *S. aureus* from antibiotic stresses through antioxidant mechanisms and suggest the contribution of additional pathways in response to vancomycin treatment.

4. Discussion

In this study, we investigated the role of *S. aureus* NADK in response to envelop stress and antibiotic treatment. Salt tolerance

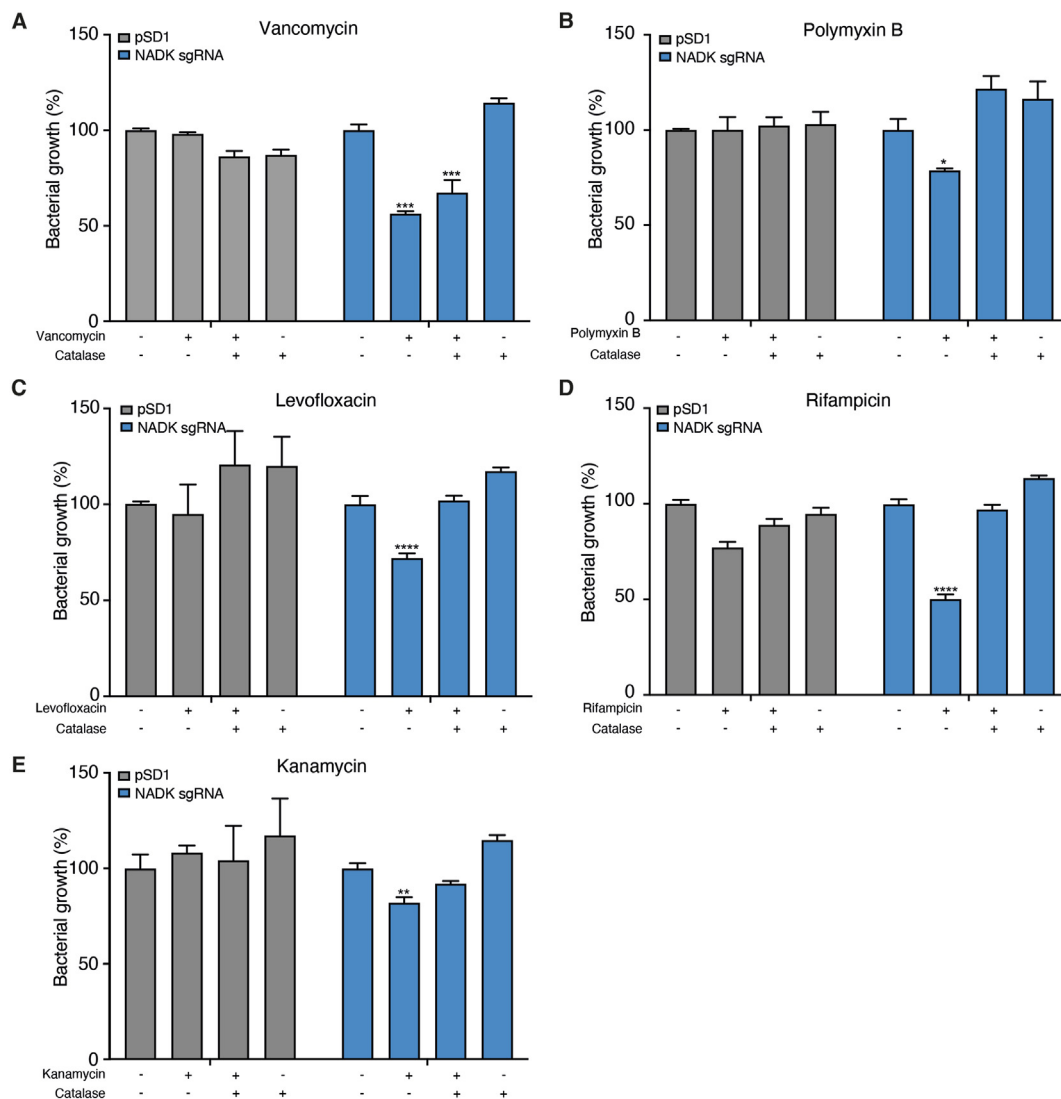


Fig. 3. NADK inhibition increases antibiotic susceptibility in oxidative and non-oxidative manners. Bacterial growth was monitored at OD_{600nm} in BHI broth at 37 °C. Percentage of growth of *S. aureus* containing the empty vector (pSD1) or the NADK knockdown strain (NADK sgRNA) exposed for 6 h to 0.5 µg/mL vancomycin (A), 40 µg/mL polymyxin B (B), 0.125 µg/mL levofloxacin (C), 1.5 ng/mL rifampicin (D) or 60 µg/mL kanamycin (E), with or without 10 µg/mL catalase, relative to the untreated condition. Data shown are representative of three independent experiments. Bars indicate the standard error of the means of biological replicates. Comparison of data from each treated condition to data from the corresponding untreated condition was performed using one-way analysis of variance (**p < 0.01, ***p < 0.001, ****p < 0.0001).

contributes to *S. aureus* capacity to colonize the skin and grow in some food products. Here, we showed that knockdown of NADK expression by CRISPR interference sensitizes *S. aureus* to sodium chloride. It has been shown that upon NaCl exposure *S. aureus* increases expression of proline/betaine transporter ProP, glycine betaine transporter OpuD1, oxygen-dependent choline dehydrogenase BetA and betaine aldehyde dehydrogenase BetB, which converts betaine aldehyde to osmoprotectant glycine betaine [18]. We have recently reported that knockdown of *S. aureus* NADK leads to decreased levels of betaine aldehyde dehydrogenase [8]. Thus, NADK possibly contributes to regulate intracellular osmotic pressure by supporting osmolyte production. Decreasing NADK expression also led to increased sensitivity to triclosan, a broad-spectrum antibacterial agent that intercalates into bacterial membranes and inhibits bacterial fatty acid biosynthesis by targeting the NADH-dependent enoyl reductase FabI [14,15]. While reduction of NADK expression does not significantly change the level of FabI [8], it could contribute to triclosan sensitization by modulating

NAD(H)/NADP(H) homeostasis and subsequently triclosan-mediated inhibition of FabI enzymatic activity. Indeed, NADP(H) is required in the two reduction steps of fatty acid synthesis catalyzed by FabI and the beta keto-ACP reductase FabG in *E. coli* [19]. Additionally, lowering NADK expression increased *S. aureus* susceptibility to vancomycin, which inhibits peptidoglycan synthesis [16]. Along these lines, we recently reported that inhibition of NADK lowers the levels of enzymes that regulates peptidoglycan biosynthesis such as the amidase LytH, which controls the density of peptidoglycan assembly sites and whose inactivation sensitizes *S. aureus* to oxacillin [20]. NADK may thus contribute to *S. aureus* resistance to different types of envelop stresses by promoting functional fatty acid biosynthesis, maintenance of bacterial membrane integrity and proper peptidoglycan biosynthesis. Interestingly, we found that inhibition of NADK could potentiate the antibacterial activity of antibiotics targeting the cell envelop but also that of antibiotics targeting replication, transcription and translation such as levofloxacin, rifampicin and kanamycin,

respectively. Although controversial, ROS production has been proposed as a common antibiotic-mediated killing mechanism [21–23]. ROS have been shown to contribute to bactericidal activity of quinolone antibiotics [24]. Both quinolones and aminoglycosides promote ROS formation mediated by TCA cycle and NADH depletion [25–27]. Polymyxins have also been suggested to induce ROS production through TCA cycle activation and NAD⁺/NADH ratio increase [28]. NADK is the only source of NADP(H) and as such it constitutes a protective enzyme against oxidative stress [29]. We have recently demonstrated that NADK inhibition leads to growth deficiency of *S. aureus* exposed to hydrogen peroxide [8]. Furthermore, we have documented a drop in the levels of *S. aureus* antioxidant enzymes such as catalase, superoxide dismutase, thioredoxin, peroxiredoxin and methionine sulfoxide reductase upon NADK knockdown [8]. Accordingly, we found that catalase could protect *S. aureus* expressing reduced levels of NADK from growth inhibition by various antibiotics. In our experimental setting, catalase alleviated toxicity of antibiotics that belong to polypeptides, fluoroquinolones, ansamycins and aminoglycosides. Therefore, toxic effects of ROS upon NADK inhibition contribute to sensitization to different classes of antibiotics. In addition, non-oxidative mechanisms presumably contribute to sensitization, as catalase treatment did not mitigate growth defect of the NADK knockdown strain in presence of vancomycin. NADK deficiency could lead to aforementioned impaired regulation of peptidoglycan synthesis and mediate inhibition of important pathways of central metabolism which might synergize with inhibition of cell wall synthesis by vancomycin, ultimately leading to growth defects [30].

The World Health Organization included *S. aureus* in the list of high-priority bacteria for which new antibiotics are urgently needed (<https://www.who.int/news/item/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed>). Our study paves the way for development of NADK inhibitors that could be used in combination with current antibiotics to fight multidrug resistant bacteria.

Author contributions

Conceptualization, O.D. and C.L.; methodology, C.L.; investigation and validation, C.L. and L.M.; visualization, C.L.; writing-original draft, O.D. and C.L.; writing-review and editing, O.D., C.L., L.M. and J.P.C.; supervision, O.D.; project administration, O.D.; funding acquisition, O.D. and J.P.C. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no conflict of interests.

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References

[1] Piddock LJV. Reflecting on the final report of the O'Neill review on antimicrobial resistance. *Lancet Infect Dis* 2016;16:767–8. [https://doi.org/10.1016/S1473-3099\(16\)30127-X](https://doi.org/10.1016/S1473-3099(16)30127-X).

[2] Murray CJ, Ikuta KS, Sharara F, Swetschinski L, Robles Aguilar G, Gray A, et al. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *Lancet* 2022;S0140673621027240. [https://doi.org/10.1016/S0140-6736\(21\)02724-0](https://doi.org/10.1016/S0140-6736(21)02724-0).

[3] Ikuta KS, Swetschinski LR, Robles Aguilar G, Sharara F, Mestrovic T, Gray AP, et al. Global mortality associated with 33 bacterial pathogens in 2019: a systematic analysis for the Global Burden of Disease Study 2019. *Lancet* 2022;400:2221–48. [https://doi.org/10.1016/S0140-6736\(22\)02185-7](https://doi.org/10.1016/S0140-6736(22)02185-7).

[4] Gelin M, Paoletti J, Huteau V, Nahori M, Leseigneur C, Dugue L, et al. From substrate to fragments to inhibitor active in vivo against *Staphylococcus aureus*. *ACS Infect Dis* 2020;6:422–35. <https://doi.org/10.1021/acscinf.9b00368>.

[5] Chandel NS. NADPH—the forgotten reducing equivalent. *Cold Spring Harbor Perspect Biol* 2021;13:a040550. <https://doi.org/10.1101/cshperspect.a040550>.

[6] Chini CCS, Zeidler JD, Kashyap S, Warner G, Chini EN. Evolving concepts in NAD⁺ metabolism. *Cell Metabol* 2021;S1550413121001698. <https://doi.org/10.1016/j.cmet.2021.04.003>.

[7] Sharma R, Hartman TE, Beites T, Kim J-H, Eoh H, Engelhart CA, et al. Metabolically distinct roles of NAD synthetase and NAD kinase define the essentiality of NAD and NADP in *Mycobacterium tuberculosis*. *mBio* 2023:e00340. <https://doi.org/10.1128/mbio.00340-23>.

[8] Leseigneur C, Boucontet L, Duchateau M, Pizarro-Cerdá J, Matondo M, Colucci-Guyon E, et al. NAD kinase promotes *Staphylococcus aureus* pathogenesis by supporting production of virulence factors and protective enzymes. *Elife* 2022;11:e79941. <https://doi.org/10.7554/eLife.79941>.

[9] Blaise O, Leseigneur C, Cokelaer T, Capuzzo E, Frescaline N, Dussurget O. Complete genome sequences of bioluminescent *Staphylococcus aureus* strains Xen31 and Xen36, derived from two clinical isolates. *Microbiol Resour Annot* 2023;12:e00024. <https://doi.org/10.1128/mra.00024-23>.

[10] Zhao C, Shu X, Sun B. Construction of a gene knockdown system based on catalytically inactive (“Dead”) Cas9 (dCas9) in *Staphylococcus aureus*. *Appl Environ Microbiol* 2017;83. <https://doi.org/10.1128/AEM.00291-17>.

[11] Fang FC, Frawley ER, Tapscott T, Vázquez-Torres A. Bacterial stress responses during host infection. *Cell Host Microbe* 2016;20:133–43. <https://doi.org/10.1016/j.chom.2016.07.009>.

[12] Avican K, Aldahdooh J, Togninalli M, Mahmud AKMF, Tang J, Borgwardt KM, et al. rNA atlas of human bacterial pathogens uncovers stress dynamics linked to infection. *Nat Commun* 2021;12:3282. <https://doi.org/10.1038/s41467-021-23588-w>.

[13] Chaudhuri RR, Allen AG, Owen PJ, Shalom G, Stone K, Harrison M, et al. Comprehensive identification of essential *Staphylococcus aureus* genes using Transposon-Mediated Differential Hybridisation (TMDH). *BMC Genom* 2009;10:291–318. <https://doi.org/10.1186/1471-2164-10-291>.

[14] Lu H, Tonge PJ. Inhibitors of FabI, an enzyme drug target in the bacterial fatty acid biosynthesis pathway. *Acc Chem Res* 2008;41:11–20. <https://doi.org/10.1021/ar700156e>.

[15] Guillen J, Bernabeu A, Shapiro S, Villalain J. Location and orientation of Triclosan in phospholipid model membranes. *Eur Biophys J* 2004;33. <https://doi.org/10.1007/s00249-003-0378-8>.

[16] Rubinstein E, Keynan Y. Vancomycin revisited – 60 Years later. *Front Public Health* 2014;2. <https://doi.org/10.3389/fpubh.2014.00217>.

[17] Yoshida T, Hiramatsu K. Potent *in vitro* bactericidal activity of polymyxin B against methicillin-resistant *Staphylococcus aureus* (MRSA). *Microbiol Immunol* 1993;37:853–9. <https://doi.org/10.1111/j.1348-0421.1993.tb01716.x>.

[18] Ming T, Geng L, Feng Y, Lu C, Zhou J, Li Y, et al. iTRAQ-based quantitative proteomic profiling of *Staphylococcus aureus* under different osmotic stress conditions. *Front Microbiol* 2019;10:1082. <https://doi.org/10.3389/fmicb.2019.01082>.

[19] Li W, Wu H, Li M, San K. Effect of NADPH availability on free fatty acid production in *Escherichia coli*. *Biotechnol Bioeng* 2018;115:444–52. <https://doi.org/10.1002/bit.26464>.

[20] Do T, Schaefer K, Santiago AG, Coe KA, Fernandes PB, Kahne D, et al. *Staphylococcus aureus* cell growth and division are regulated by an amidase that trims peptides from uncrosslinked peptidoglycan. *Nat Microbiol* 2020;5:291–303. <https://doi.org/10.1038/s41564-019-0632-1>.

[21] Van Acker H, Coenye T. The role of reactive oxygen species in antibiotic-mediated killing of bacteria. *Trends Microbiol* 2017;25:456–66. <https://doi.org/10.1016/j.tim.2016.12.008>.

[22] Dwyer DJ, Collins JJ, Walker GC. Unraveling the physiological complexities of antibiotic lethality. *Annu Rev Pharmacol Toxicol* 2015;55:313–32. <https://doi.org/10.1146/annurev-pharmtox-010814-124712>.

[23] Baquero F, Levin BR. Proximate and ultimate causes of the bactericidal action of antibiotics. *Nat Rev Microbiol* 2021;19:123–32. <https://doi.org/10.1038/s41579-020-00443-1>.

[24] Kottur J, Nair DT. Reactive oxygen species play an important role in the bactericidal activity of quinolone antibiotics. *Angew Chem* 2016;128:2443–6. <https://doi.org/10.1002/ange.201509340>.

[25] Kohanski MA, Dwyer DJ, Collins JJ. How antibiotics kill bacteria: from targets to networks. *Nat Rev Microbiol* 2010;8:423–35. <https://doi.org/10.1038/nrmicro2333>.

[26] Belenky P, Ye JD, Porter CBM, Cohen NR, Lobritz MA, Ferrante T, et al. Bactericidal antibiotics induce toxic metabolic perturbations that lead to cellular damage. *Cell Rep* 2015;13:968–80. <https://doi.org/10.1016/j.celrep.2015.09.059>.

- [27] Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA, Collins JJ. A common mechanism of cellular death induced by bactericidal antibiotics. *Cell* 2007;130:797–810. <https://doi.org/10.1016/j.cell.2007.06.049>.
- [28] Yin J, Meng Q, Cheng D, Fu J, Luo Q, Liu Y, et al. Mechanisms of bactericidal action and resistance of polymyxins for Gram-positive bacteria. *Appl Microbiol Biotechnol* 2020;104:3771–80. <https://doi.org/10.1007/s00253-020-10525-y>.
- [29] Grose JH, Joss L, Velick SF, Roth JR. Evidence that feedback inhibition of NAD kinase controls responses to oxidative stress. *Proc Natl Acad Sci U S A* 2006;103:7601–6. <https://doi.org/10.1073/pnas.0602494103>.
- [30] Yang JH, Bening SC, Collins JJ. Antibiotic efficacy — context matters. *Curr Opin Microbiol* 2017;39:73–80. <https://doi.org/10.1016/j.mib.2017.09.002>.