Membrane depolarization kills dormant *Bacillus subtilis* cells by generating a lethal dose of ROS

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Article

Keywords: reactive oxygen species, *Bacillus subtilis*, membrane depolarization

Posted Date: August 24th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-806248/v1>

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Abstract

The bactericidal activity of several commonly used antibiotics have been shown to partially rely on the production of reactive oxygen species (ROS). Bacterial persister cells, an important cause of recurring infections, are tolerant to these antibiotics because they are in a dormant state. However, even dormant cells must maintain a membrane potential. Here we used *Bacillus subtilis* as model system to study the effect of membrane depolarization on dormant cells. Surprisingly, we found that membrane depolarization also leads to ROS production. In contrast to previous studies, this does not require the Fenton reaction and results primarily in superoxide radicals. Genetic analysis revealed that Rieske factor QcrA, the iron-sulfur subunit of complex III, is a primary source of superoxide radicals. Interestingly, the membrane distribution of QcrA changed upon membrane depolarization, suggesting a dissociation of complex III. Our data reveal an alternative mechanism by which antibiotics can cause lethal ROS levels, and may partially explain why membrane-targeting antibiotics are effective in eliminating persisters.

Introduction

Many commonly used antibiotics that interfere with protein synthesis, such as kanamycin and gentamicin, or cell wall synthesis, such as vancomycin and ampicillin, trigger the production of lethal levels of reactive oxygen species (ROS), which contributes to the bactericidal activity of these antibiotics¹⁻³. The produced ROS constitute primarily of hydroxyl radicals generated by the Fenton reaction. The free ferrous iron required for this reaction is assumed to originate from iron-sulfur proteins by a yet not fully understood mechanism¹⁻³. Despite this double mode of action, bacterial cells can tolerate these antibiotics by shutting down biosynthetic processes and entering a physiologically dormant state^{4,5}. This so called 'persister' state can be caused by activation of toxin-antitoxin systems^{5,6}, the stringent response⁷, SOS response^{8,9} or simple nutrient starvation¹⁰. Persister cells can resume growth when the antibiotic treatment ceases⁵, and are therefore believed to be an important source of recurrent infections^{11,12}. Therapeutic strategies for eradicating persister cells are in dire need.

We previously showed that the membrane potential is essential for the cellular localization of different bacterial peripheral membrane protein, such as the conserved cell division proteins FtsA and MinD¹³. Persister cells do not grow, but it is likely that they maintain a membrane potential. Presumably, dissipation of the membrane potential will also result in the delocalization of membrane-associated proteins in persister cells, which could affect their viability. We have investigated this possibility by using dormant, antibiotic-tolerant, *Bacillus subtilis* cells as a simple model system for persisters. Surprisingly, we found that dissipation of the membrane potential generates lethal levels of reactive oxygen species (ROS). This finding was counterintuitive since ROS production is generally linked to enhanced respiration¹⁴, and in fact membrane depolarization has been shown to reduce electron transfer in the respiratory chain of *B. subtilis*¹⁵. Moreover, we found that the Fenton reaction was not involved and that especially superoxide radicals were formed. By means of genetic analysis, we could pinpoint one of the sources of ROS to the conserved iron-sulphur cluster protein QcrA of respiratory complex III, also known as Rieske

factor¹⁶. Interestingly, microscopic analysis showed that QcrA delocalizes after membrane depolarization, possibly indicating that detachment of QcrA from complex III leads to superoxide radical formation. This novel killing mechanism may explain why membrane targeting compounds are successful in eradicating antibiotic tolerant persister cells^{17–21}

Results

Antibiotic tolerant *B. subtilis* cells

The antibiotic tolerance of persisters ensues from the fact that these cells are in a dormant state^{5,6}. It has been shown that *Escherichia coli* cells in the stationary growth phase display an antibiotic tolerance that is reminiscent of persister cells²². To examine whether the same is true for *B. subtilis*, we used a sporulation deficient mutant $\Delta spoII E$, since *B. subtilis* spores are insensitive to antibiotics and, thus, would confound the measurements. The $\Delta spoII E$ deletion mutant was grown overnight (18 h) in LB medium to stationary phase, and subsequently treated with 10 x minimal inhibitory concentrations of either vancomycin, kanamycin or ciprofloxacin for 10 h (see Table S4 for MIC). Samples for CFU measurements were taken every 2 h, and an exponentially growing culture was used as non-dormant comparison. As shown in Fig. 1, the latter culture was sensitive for all three antibiotics whereas the overnight culture was not, indicating that non-sporulating stationary phase *B. subtilis* cells can be used as a simple model system for antibiotic tolerant persister cells.

These stationary cells maintained membrane potential levels comparable to actively growing cells (Fig. S1). Many peripheral membrane proteins use an amphipathic alpha helix domain as a reversible membrane anchor and we have shown that the binding of such an anchor domain is strongly stimulated by the membrane potential¹³. Depolarization of the membrane potential might therefore disturb the normal localization of different peripheral membrane proteins, which, in turn, could affect the viability of persister cells. To test whether dormant *B. subtilis* cells are sensitive for membrane depolarization, we exposed them to the potassium ionophore valinomycin, which specifically dissipates the transmembrane potential^{23,24} (Fig. S1). To assure full dissipation over a 10 h incubation period, we used 100 μ M valinomycin which is 10 x the MIC for exponentially growing cells (Table S4). The addition of valinomycin reduced the viable count of exponentially growing cells by 90 % after 2 h incubation (Fig. 1c). The dormant culture showed some degree of resilience compared to its actively growing counterparts, but after some time the viability decreased and after 6 h approximately 90 % of the cells were killed (Fig. 1d), suggesting that membrane depolarizing compounds might indeed be useful to combat persisters.

Cause of killing

The killing kinetic of valinomycin is quite different between exponentially growing and dormant cells, and in the latter case showed a clear acceleration over time (Fig. 1c & 1d). This made us wonder what the

actual cause is why dormant cells eventually succumb when their membrane is depolarized. It has been shown that depolarization of the *B. subtilis* cell membrane results in an uncontrolled autolysin activity and cell lysis²⁵. To determine whether this could be responsible for the killing of dormant cells, we exposed a mutant lacking all major autolysins ($\Delta lytA$, $\Delta lytB$, $\Delta lytC$, $\Delta lytD$, $\Delta lytE$ and $\Delta lytF$) to valinomycin. However, this deletion mutant did not show an increase in viability (Fig. 2a). *E. coli* persisters can be killed by the induction of cryptic prophages²⁶. However, a *B. subtilis* strain cured of all prophages exhibited a similar sensitivity for valinomycin as the wild type strain (Fig. 2b), indicating that the reduction in viable count is also not related to prophage activation.

During the viable count measurements we noticed that after prolonged valinomycin treatment the colony size started to vary and smaller colonies emerged (Fig. 2c). Such variation in colony size is also observed when bacterial cells are treated with DNA damaging agents²⁷, suggesting that membrane depolarization might cause DNA damage. If this is the case, then a DNA repair mutant, such as a $\Delta recA$ mutant, should be more sensitive to valinomycin. This was indeed the case (Fig. 2d).

Generating ROS

A common cause of DNA damage is the accumulation of reactive oxygen species (ROS) in the cell. However, it seems unlikely that dormant *B. subtilis* cells would produce ROS upon membrane depolarization, since the accumulation of ROS is normally associated with hyper respiration, which can be prevented by membrane potential dissipating agents^{15,28,29}. Moreover, antibiotics that have been shown to generate ROS, including norfloxacin, vancomycin and kanamycin¹⁻³, are not active in dormant cells. ROS is primarily a by-product of aerobic respiration and under anaerobic conditions ROS levels are generally much lower³⁰. When dormant *B. subtilis* cells were placed into an anaerobic chamber for 10 h only a fraction of cells survived (Fig. 3a). Although *B. subtilis* can grow anaerobically, it requires certain medium conditions and sufficient time to adapt³¹, which likely explains this reduction in viability. Nevertheless, the fraction of surviving cells was still smaller when valinomycin was added (Fig. 3a).

Since the viability of *B. subtilis* is greatly affected by anaerobic conditions, even in the absence of valinomycin, this experiment did not reveal whether the DNA damage caused by valinomycin treatment was due to the accumulation of ROS. Therefore, we took another approach and tested the sensitivity of a *spx* deletion mutant. *Spx* is the key regulator of the oxidative stress response in *B. subtilis* and is required for survival in the presence of strong ROS-inducing compounds such as paraquat³². Interestingly, a Δspx deletion mutant was even more sensitive to valinomycin than the $\Delta recA$ mutant (Fig. 3b), supporting the idea that ROS is a key contributor to the killing of depolarized cells. Finally, to directly show that ROS was generated, we used the specific fluorescent ROS probe 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA)³. Dormant *B. subtilis* cells were incubated with H2DCFDA and exposed to either valinomycin or the well-known ROS inducer paraquat for 2 and 4 h³³. The increase in fluorescence in cells was measured using fluorescence light microscopy. As shown in Fig. 3c, incubation with valinomycin for 2 h

resulted in a strong increase in ROS that remained high over a 4 h period and even exceeded the effect of 1 mM paraquat. Thus, membrane depolarization of dormant *B. subtilis* cells does lead to the accumulation of lethal levels of ROS.

Determining ROS type

Hydroxyl radicals ($\cdot\text{OH}$) and superoxide radicals ($\text{O}_2^{\cdot-}$) are the main reactive oxygen species formed during aerobic growth³⁴. Incubation with antibiotics like norfloxacin, ampicillin and kanamycin results primarily in the formation of hydroxyl radicals made from hydrogen peroxide by the Fenton reaction¹⁻³. This conclusion was based on the fact that Fe^{2+} -specific chelators like ferrozine or bipyridyl and the hydroxyl radical scavenger thiourea reduced the formation of radicals and diminished the killing efficiency of these antibiotics¹⁻³. Moreover, increased levels of catalase, which removes hydrogen peroxide³⁵ also suppressed the killing by either ampicillin, gentamicin or norfloxacin³. Interestingly, the addition of 0.5 mM ferrozine had no effect on the viability of dormant cells when incubated with valinomycin, and 0.5 mM bipyridyl actually reduced the viability (Fig. 4a). Moreover, the presence of 150 mM thiourea also did not mitigate the effect of valinomycin and in fact enhanced its activity (Fig. 4b). In contrast to this, the presence of 10 mM tiron, a superoxide scavenger, almost completely inhibited the killing by valinomycin (Fig. 4b). Moreover, inactivation of KatA, the main catalase of *B. subtilis*³⁶, had no effect on the killing efficiency of valinomycin (Fig. 4c), whereas deleting *sodA*, encoding the main superoxide dismutase, strongly reduces the viable count upon valinomycin treatment (Fig. 4c). Indeed, ΔsodA cells show almost a doubling in fluorescence of the ROS probe H2DCFDA compared to wild type cells (Fig. 4d). Apparently, depolarization of dormant *B. subtilis* cells triggers the production of lethal levels of superoxide radicals, without involvement of the Fenton reaction, which is different from the ROS generation caused by antibiotics like norfloxacin, vancomycin and kanamycin.

Possible source of superoxide production

The accumulation of lethal levels of ROS upon exposure to bactericidal antibiotics is believed to be triggered by a surge in NADH consumption that induces a burst in superoxide generation via the respiratory chain. These superoxide radicals then destabilize iron-sulfur clusters, leading to free ferrous iron which enables the Fenton reaction, thus creating deadly levels of hydroxyl radicals¹⁻³. The TCA cycle is the main source of NADH (Fig. 5a), and it was shown that inactivation of either isocitrate dehydrogenase or aconitase reduces the killing activity of norfloxacin, vancomycin and kanamycin¹. When we inactivated *B. subtilis* pyruvate dehydrogenase, which fuels the TCA cycle, cells became not less but more sensitive to valinomycin (Fig. 5b, ΔpdhB), suggesting that there is no surge in NADH levels upon membrane depolarization. The other well-known source of ROS is the electron transport chain³⁷⁻⁴⁰. *B. subtilis* contains a classic electron transport chain composed of NADH dehydrogenase, succinate

dehydrogenase, cytochrome bc complex, cytochrome c oxidase, also known as complex I, II, III and IV, respectively (Fig. 5a). Complex I and II feed electrons to the menaquinone pool. Inactivating one of them, by either deleting *ndh*, *ndhF* or *sdhC*, did not mitigate the killing by valinomycin but made it worse (Fig. 5b). Inactivation of glycerol-3-phosphate dehydrogenase, which reduces glycerol-3-phosphate to dihydroxyacetone phosphate using menaquinone ⁴¹, had no effect on the viability count (Fig. 5b, $\Delta glpD$).

The absence of either the cytochrome b subunit ($\Delta qcrB$) or the cytochrome b/c subunit ($\Delta qcrC$) from complex III also strongly reduced the survival chance of valinomycin treated cells (Fig. 5c). Interestingly, when we deleted *qcrA*, encoding the Rieske-type iron-sulfur subunit of complex III, cells became more resilient to valinomycin, and after 10 h the viable count was more than 20-fold higher compared to wild type cells (Fig. 5c).

Impairing the expression of one of the cytochrome-c oxidase subunits of complex IV, by deleting either *ctaC*, *ctaD*, *ctaE* or *ctaF*, made dormant cells slightly more sensitive for membrane depolarization (Fig. 5d). *B. subtilis* contains two cytochrome-c electron carriers, cytochrome c550, which contains a transmembrane anchor, and cytochrome c551 that is anchored to the cell membrane via a diacyl glycerol tail (Fig. 5a) ⁴². Deleting one of them did not mitigate the killing by valinomycin (Fig. 5e, $\Delta cccA$ or $\Delta cccB$). Inactivating both, by removing enzymes involved in their biogenesis ($\Delta ccdA$, $\Delta resA$), strongly increased the sensitivity to valinomycin (Fig. 5e). *B. subtilis* possesses two alternative cytochrome oxidases that use quinol for their oxidation reaction, cytochrome bd oxidase and cytochrome aa3 oxidase (Fig. 5a). Inactivation one of them, by deleting either *qoxB* or *cydA*, considerably reduced the viability of dormant cells when incubated with valinomycin (Fig. 5f).

These surprising results suggest that not so much an active but rather an intact electron transport chain is important for the survival of dormant *B. subtilis* cells upon membrane depolarization, and that QcrA is a source of superoxide radicals. This Rieske protein contains a unique 2Fe-2S cluster in which one of the two iron atoms is held in place by two histidines rather than two cysteines. This cluster accepts an electron from the quinol anion and transfers it to the cytochrome heme iron ^{16,43,44}, and in fact this step is a well-known source of superoxide radicals in mitochondria ⁴⁵. To provide further support that QcrA is a likely source of the observed ROS when the membrane potential is dissipated, we deleted *qcrA* in the $\Delta recA$ background strain, which has an impaired DNA repair system and has been shown to be especially sensitive for valinomycin (Fig. 2d). As shown in Fig. 5g, the absence of QcrA clearly attenuated the valinomycin susceptibility of dormant $\Delta recA$ cells. Finally, we directly measured ROS production in the $\Delta qcrA$ mutant using the fluorescent probe H2DCFDA (Fig. 5h). Indeed, in the absence of the Rieske protein the average fluorescence signal after 4 h valinomycin treatment was considerably lower.

Cellular distribution of QcrA

It is difficult to understand how QcrA can produce lethal levels of superoxide radicals upon membrane depolarization. As mentioned in the introduction, depolarization of the cell membrane leads to the

delocalization of different membrane proteins, including some transmembrane proteins^{13,46}. Possibly, the localization of electron transport chain components is also affected. To examine this, we constructed GFP fusions with the transmembrane proteins QcrA, QcrB and QcrC of complex III, CtaC and CtaD of complex IV, and the main cytochrome C (CccA), and expressed these fusions from an ectopic locus in the genome. Of note, we have not checked whether the GFP fusions influenced their enzymatic activities, since we were only interested in their localization. As shown in Fig. 6a, all dormant cells showed a more or less uniform fluorescent membrane stain. Interestingly, incubation with valinomycin caused a strong clustering of GFP-QcrA over time, whereas the localization of the other fusions were unaffected. When we repeated the experiment with exponentially growing cells, the clustering of GFP-QcrA was already visible within 10 min after the addition of valinomycin, whereas the other fusions showed no delocalization, also not after 30 min (Fig. 6b). Possibly, the distinct clustering of QcrA upon membrane depolarization is somehow responsible for the production of superoxide radicals.

Discussion

In this study we provide evidence that QcrA, the Rieske protein subunit of complex III, produces lethal levels of superoxide radicals upon depolarization of the membrane of dormant *B. subtilis* cells. Depolarization changes the distribution of QcrA in the cell membrane from smooth to spotty, which is not the case for the other two subunits QcrB and QcrC. This seems to indicate a detachment of QcrA from the other subunits of complex III. We speculate that this hampers electron transfer from the iron-sulfur cluster of QcrA to the heme of QcrB, and/or exposes the iron-sulfur cluster to molecular oxygen, thereby facilitating superoxide radical formation. This might also be a reason why the $\Delta qcrB$ and $\Delta qcrC$ mutants are extra sensitive for valinomycin (Fig. 5).

How depolarization of the membrane would lead to the disintegration of complex III is unclear. However, in previous work we have shown that the membrane potential is important for the regular distribution of different membrane proteins in *B. subtilis*, likely related to the distribution of lipids with short, unsaturated or branched fatty acids that stimulate a more liquid disordered phase in the membrane⁴⁶. A comparable phenomenon has been observed in yeast where the distribution of specific transmembrane proton symporters is disturbed when the membrane potential is neutralized⁴⁷, and also in mammalian cells, where membrane depolarization affects the clustering of the GTPase Ras-K⁴⁸. These effects have been attributed to membrane potential dependent clustering of specific lipids⁴⁷⁻⁴⁹. Since a lipid membrane behaves like a capacitor, a voltage difference over the membrane will apply a compression force (Maxwell pressure), which is also referred to as electrostriction. It is assumed that this compression affects the packing of fatty acid chains⁵⁰⁻⁵². Possibly, the change in bilayer packing upon depolarization triggers the dissociation of QcrA from QcrBC. This dissociation might also explain why menaquinone-dependent electron transfer in the *B. subtilis* respiratory chain is facilitated by membrane energization¹⁵.

Mitochondrial Rieske protein

The mitochondrial Rieske iron-sulfur protein is one of the key sources of ROS in eukaryotic cells. High respiration levels increases ROS production to dangerous levels. On the other hand hypoxic/anoxic conditions can also trigger superoxide production by this protein ⁵³. The mechanism by which superoxide production is produced by hypoxia/anoxia is not clear ⁵⁴, but these low oxygen conditions can lead to a reduction of the mitochondrial membrane potential ^{55,56}. Interestingly, the bacterium *Paracoccus denitrificans*, which contains a respiratory chain similar to that of eukaryotic mitochondria, shows an increase in ROS production in response to hypoxic conditions, as well ⁵⁷. Based on these and our data, it might be interesting to examine whether the mitochondrial complex III also dissociates upon membrane depolarization, thereby exposing the Rieske subunit.

Role of the electron transport chain

It is assumed that the production of superoxide by the Rieske subunit occurs when the oxidized iron-sulfur cluster withdraws one electron from reduced menaquinone (menaquinol) ⁵⁸. Menaquinol is provided by either NADH dehydrogenase (complex I), succinate dehydrogenase (complex II) or glycerol-3-phosphate dehydrogenase. Inactivation of one of these protein complexes did not mitigate the effect of valinomycin, possibly because of their redundant activities. However, inactivation of pyruvate dehydrogenase, which will lower the production of NADH by the TCA cycle, also did not mitigate the effect of membrane depolarization. On the other hand, mutations that reduce the oxidation of the menaquinol pool, such as the inactivation of the two cytochrome Cs, and the alternative quinol oxidases cytochrome aa3 and bd oxidases, strongly diminishes the viability of cells exposed to valinomycin. Possibly, this raises the menaquinol levels and increases the production of superoxide radicals by the Rieske subunit.

ROS and antibiotics

Whether the production of ROS by bactericidal antibiotics contributes to their effectivity has been hotly debated when this phenomenon was first proposed in 2007 ^{1,3,59-61}. Our data supports the finding that antibiotics can induce lethal levels of ROS. However, the mechanism of ROS production that we found differs from what has been previously described. Bactericidal antibiotics produce hydroxyl radicals from endogenous hydrogen peroxide using the Fenton reaction. The free ferrous iron required for this reaction is assumed to originate from iron-sulfur proteins, whom iron-sulfur clusters have been compromised by superoxide radicals generated by the electron transport chain that has become highly active due to a surge in NADH ⁶². How this surge happens is still not entirely clear. It has been shown that in *E. coli* the two-component regulatory systems CpxRA and ArcAB are required for the induction of ROS by antibiotics. CpxRA senses and responds to aggregated and misfolded proteins in the bacterial cell envelope, and the ArcAB system is involved in sensing oxygen availability and the concomitant transcriptional regulation of oxidative and fermentative catabolism ⁶³. Based on work with the ribosome inhibitor gentamycin it was proposed that mistranslation and misfolded membrane or periplasmic proteins activate the sensor CpxA, which, by an unknown mechanism, activates ArcA, as a result of which the expression of a large number of metabolic and respiratory genes are activated, leading to increased respiration rates and ultimately

ROS production¹. Interestingly, accumulation of misfolded cell envelope proteins by gentamycin also affects the *E. coli* membrane potential¹. Whether a reduction of the membrane potential contributes to ROS formation in *E. coli* remains to be investigated. If this turns out to be the case, there is at least no Rieske protein containing complex III involved, since *E. coli* lacks this complex. Nevertheless, dormant *B. subtilis* mutants that lack QcrA still die upon membrane depolarization, albeit slower (Fig. 5c), and this might still involve ROS as the inactivation of the RecA-based DNA repair system renders $\Delta qcrA$ cells more sensitive towards valinomycin (compare Fig. 5c and 5g). Possibly, a change in lipid bilayer packing due to membrane potential dissipation also affects the assembly and/or proper membrane embedding of other electron transport chain components. The resulting inefficient electron transfer between components, possibly combined with an increased exposure of their Fe-containing prosthetic groups to oxygen, might then stimulate the production of ROS.

Relevance for persisters

B. subtilis is not a human pathogen, so the question arises how relevant our findings are for the fight against clinically relevant antibiotic tolerant persisters. Many pathogens, including *E. coli*, contain a non-canonical electron transport chain and lack complex III. However, depolarization of the cell membrane has multiple effects, including reducing ATP levels, delocalization of peripheral as well as transmembrane proteins, and possible ROS produced by the non-canonical electron transport chain component as described above. All these factors will affect the fitness of persister cells. A pathogen and well known persister that lacks complex III is *Staphylococcus aureus*. Dormant *S. aureus* cells are considerably more resistant to valinomycin than *B. subtilis* (Fig. S4), however when these *S. aureus* cells lacked one or both of its main superoxide dismutases they became substantially more sensitive for incubation with valinomycin (Fig. S4). These data support the notion that membrane depolarization can also result in the accumulation of lethal levels of superoxide radicals when no Rieske protein-containing complex III is present.

A notorious example of a pathogenic persister that does contain the Rieske protein subunit is *Mycobacterium tuberculosis*. It has been shown that the membrane potential is essential to maintain viability of non-growing mycobacterial cells²⁰. Interestingly, it was found that resistance of *M. tuberculosis* against membrane targeting lipophilic quinazoline derivatives can arise by mutations in QcrA⁶⁴. Both the lethal effect of membrane depolarization as well as the exposure to these quinazoline derivatives were attributed to ATP depletion. However, our results suggest that induction of superoxide radicals might be a more important factor contributing to the lethality of these interventions. In conclusion, membrane depolarization seems to be a promising method to kill bacterial persister cells since it leads to the production of lethal levels of ROS.

Experimental Procedure

Maintenance and growth of strains

Nutrient agar (NA) (Oxoid) was used for routine selection and maintenance of both *B. subtilis* and *E. coli* strains. Supplements were added as required: chloramphenicol (5 µg/ml), erythromycin (1 µg/ml), kanamycin (2 µg/ml), spectinomycin (100 µg/ml), tetracycline (10 µg/ml), ampicillin (100 µg/ml) 0.2 % xylose and 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). For liquid cultures cells were cultured in either a Spizizen minimum medium (SMM)⁶⁵, modified potassium rich Lysogeny Broth or Penassay broth (PAB). SMM contained (NH₄)₂SO₄ 15 mM, K₂HPO₄ 80 mM, KH₂PO₄ 44 mM, Na₃C₆H₅O₇ 3 mM, 0.5 % glucose, MgSO₄ 6 mM, 0.2 mg/ml tryptophan, 0.02 % casamino acids and 0.00011 % Fe-NH₄-citrate. Membrane depolarization by the potassium ionophore valinomycin requires the presence of sufficient potassium ions in the medium. Therefore, cells were grown in a modified LB medium composed of 10 g/l tryptone, 5 g/l yeast extract, 50 mM Hepes pH 7.5, and 300 mM KCl, here referred to as 'KCl-LB' medium²⁴.

Strain construction

Strains used in this study are listed in Table S1, and the plasmids and oligonucleotides used in this study are listed in tables Table S2 and S3, respectively. *B. subtilis* strain construction was performed according to established methods⁶⁶. For the labelling of ETC components with GFP, both N- as well as a C- terminal monomeric superfolder GFP (msfGFP) fusions were constructed, since it is possible that the GFP moiety interferes with membrane insertion. After microscopic inspection it appeared that only the N-terminal msfGFP fusions of QcrA, QcrB, QcrC, CccA, CtaC and CtaD showed a clear membrane signal, indicating proper membrane insertion. These N-terminal msfGFP fusions were used for the fluorescence microscopy experiments. The xylose-inducible msfGFP fusions were constructed as follows. Target genes were PCR amplified using forward and reverse primer pairs with genomic DNA of strain BSB1 as the template. The *amyE*-integration vector part with the xylose-inducible promoter and msfGFP sequences was amplified from pHJS105 using primer pairs EKP30 & EPK22 and MW226 & EKP36, for the N- and for C-terminal fusion, respectively. Target genes and vector amplicons were purified and assembled using two-fragment Gibson assembly. All plasmids were sequenced to confirm the constructs. Subsequently, the plasmids were transformed into PG344 strain resulting in double-crossover integrations, positioning the fusions in the *amyE* locus. Mutants were verified by PCR with primers TerS350 and TerS351 and by sequencing.

Minimum inhibitory concentration (MIC) measurement

The MIC values of *B. subtilis* strain PG344 was determined according to a standard protocol⁶⁷. Overnight *B. subtilis* PG344 cells were diluted in fresh medium and grown to exponential phase. Cells were then diluted to 1 × 10⁵ cells/ml in standard LB or KCl-LB supplemented with increasing concentrations of vancomycin, ciprofloxacin, kanamycin or valinomycin. The lowest concentration inhibiting visible growth after 18 h incubation at 37°C was taken as the MIC value.

Antibiotic susceptibility tests

For antibiotic susceptibility test of vancomycin, ciprofloxacin and kanamycin, cells were cultured in LB medium at 37°C with aeration whereas for valinomycin cells were cultured in KCl-LB medium. The

exponential phase or overnight dormant cells were incubated with 20 µg/ml vancomycin, 2 µg/ml ciprofloxacin, 10 µg/ml kanamycin or 100 µM valinomycin. The antibiotic concentration we used is 10-fold in excess of the MIC to ensure stability over the 10 h treatment period. 1 % DMSO was used as a negative control. Supplements were added as required: 10 mM tiron, 150 mM thiourea, 500 µM ferrozine and 500 µM 2,2'-bipyridyl. Cultures were incubated for 10 h at 37°C with aeration, and samples were taken every 2 h in order to determine the CFU through serial dilutions and plating on LB agar. Plates were incubated overnight and colonies were counted the following morning.

Membrane potential determination

The membrane potential levels of *B. subtilis* cells were determined using the voltage-sensitive dye 3,3'-Dipropylthiadicarbocyanine iodide [DiSC₃(5)] (Sigma-Aldrich) in a fluorescent microplate reader (Biotek Synergy) ²⁴. Briefly, early-mid exponential growth phase cells (OD₆₀₀ = ~ 0.4) or stationary growth phase cells (overnight culture) were diluted in medium supplemented with 50 µg/ml BSA to an OD₆₀₀ of 0.2 and then incubated with 1 µM DiSC₃(5) with shaking. The fluorescent base line, while the dye reached a stable accumulation in cells, was recorded every 42 sec with 651 nm excitation and 675 nm emission filters. After 3 min, valinomycin was added to the cells to a final concentration of 10 µM, and 1 % DMSO was added as negative control. Of note, we used a ten-times lower valinomycin concentration in this assay compared with the concentration used in the experiments because at high concentrations valinomycin affects the fluorescence of DiSC₃(5). After addition of antibiotics, the fluorescent signal was recorded for another 30 min.

Generation of anaerobic conditions

Strains were cultured in KCl-LB overnight at 37°C with aeration. Following incubation, cells were mixed with either 100 µM valinomycin or 1 % DMSO. Cultures were then placed into an anaerobic chamber and oxygen was removed using AnaeroGen 2.5L sachets. Cultures were incubated for 10 h under anaerobic conditions. The CFU was determined prior to incubation and after the 10 h period through serial dilutions and plating on nutrient agar plates.

Microscopy

Fluorescence microscopy experiments were performed using a Nikon Eclipse Ti equipped with a CFI Plan Intensilight HG 130-W lamp, and images were acquired by a C11440-22CU Hamamatsu ORCA camera with NIS-Elements software version 4.20.01. Samples consisting of 0.3 µl of cells were spotted onto a microscope slide coated with 1 % agarose film and sealed with a glass coverslip on top ⁶⁸.

The level of cellular ROS production was measured by using a cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) probe (ThermoFisher) ³. The H2DCFDA probe was dissolved in DMSO to make 10 mM stock aliquots and stored at -20°C under dry argon. Overnight cultures grown in KCl-LB were 10-fold diluted in the same medium and grown at 37°C for 2 h (OD₆₀₀ around 2.5). The probe was added to a final concentration of 10 µM, and the samples were protected from light and incubated for another 2 h (OD₆₀₀ around 4.0). Cells were then split and treated with either 100 µM

valinomycin, 1 % DMSO as negative or 1 mM paraquat (Sigma) as positive control. For visualization of ROS-fluorescence, cells of different treated time-points were quickly washed once, resuspended in PBS and imaged immediately. Images were analysed using Coli-Inspector which is a plugin for ImageJ (National Institutes of Health) ^{69,70}.

For visualization of electron transport chain proteins, overnight cultures of GFP fusion expressing cells were grown to mid-exponential phase in KCl-LB with 0.2 % xylose at 30°C. Samples were treated with either 100 µM valinomycin or 1 % DMSO and analysed using fluorescence microscopy at different time points. Images were analysed using ImageJ ⁶⁹.

Declarations

ACKNOWLEDGEMENTS

We would like to thank Dr. Yoshikazu Kawai and Dr. Kevin Waldron for strains and Dr. Michaela Wenzel for advices and comments. Funding for DG was provided by a Biotechnology and Biological Sciences Research Council (BBSRC) DTP Studentship BB/J014516/1. BW was funded by the China Scholarship Council (CSC). PG and HS were funded by BBSRC grants BB/I004238/1 and BB/I01327X/1, respectively, and LWH was partially funded by STW-Vici grant 12128 from the Netherlands Organization for Scientific Research (NWO).

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Figures

Figure 1

Antibiotic killing of non-growing *B. subtilis* cells. Survival curves of exponentially growing (a and c), and stationary (dormant) phase (b and d) *B. subtilis* cells incubated with different antibiotics. Cells were treated with either 20 $\mu\text{g/ml}$ vancomycin (van), 2 $\mu\text{g/ml}$ ciprofloxacin (cipro), 10 $\mu\text{g/ml}$ kanamycin (kan) or 100 μM valinomycin (val). 1 % DMSO was used as control. Percent survival was determined through

plating serial dilutions and counting viable cells every 2 h. Data shown reflect mean $\pm$ SD (standard deviation) of three biological replicates.

Figure 2

Possible cause of killing upon membrane potential dissipation. Survival curves of different stationary phase cells incubated with valinomycin (val). a Survival of a strain lacking the main autolysin encoding genes lytA, -B, -C, -D, -E and -F (Δ lyt). b Survival of a strain lacking all 6 prophages: skin, ICEBs1,

prophage1, prophage3, SP β , PBSX and pks (Δ prophages). c Colonies of stationary phase cells treated with valinomycin for 0 h and 10 h. Small colonies are indicated by red arrows. d Survival curves of a *recA* deletion strain (Δ *recA*). 100 μ M valinomycin was used and 1 % DMSO as negative control. wt indicate wild type cells. Data shown reflect mean $\pm$ SD of three biological replicates.

Figure 3

Generation of ROS. a Survival of stationary phase cells under anaerobic conditions in the absence or presence of 100 μ M valinomycin. The addition of 1 % DMSO was used as a control. Viable counts were determined after 0 and 10 h incubation. b Survival curves of stationary phase wild type (wt), Δ recA and Δ spx cells in the presence of 100 μ M valinomycin (val) or 1 % DMSO. Data shown reflect mean $\pm$ SD of three biological replicates. The viable counts of Δ recA and Δ spx cells in the presence of 1 % DMSO was similar to that of wild type cells and are not indicated here. c ROS production in stationary phase cells measured by the fluorescent ROS probe H₂DCFDA. Cells were treated with either 100 μ M valinomycin, 1 mM paraquat or 1 % DMSO for 2 and 4 h. The fluorescence intensities of 150 cells were measured microscopically and plotted.

Figure 4

Determining whether hydroxyl and/or superoxide radicals are formed. a Survival curves of stationary phase cells incubated with 100 μ M valinomycin (val) in the presence of either the iron chelators ferrozine (Fer, 500 μ M) or 2,2'-Bipyridyl (Bip, 500 μ M). b Survival curves of stationary phase cells incubated with 100 μ M valinomycin (val) in the presence of either the hydroxyl radical scavenger thiourea (TU, 150 mM) or the superoxide scavenger tiron (Tiron, 10 mM). c Survival curves of stationary phase Δ katA and Δ sodA

cells incubated with 100 μ M valinomycin (val). *kata* and *sodA* code for the main catalase and superoxide dismutase, respectively. The viable counts of cells in the presence of 1 % DMSO were similar to that of wild type cells and are not indicated. Data shown reflect mean $\pm$ SD of three biological replicates. d ROS production in stationary phase wt and Δ *sodA* cells after 4 h incubation with 100 μ M valinomycin (val) measured by the fluorescent ROS probe H2DCFDA. Control cells were treated with either 1 mM paraquat or 1 % DMSO for 4 h. The fluorescence intensities of 150 cells were measured microscopically and plotted.

Figure 5

A *qcrA* mutant increases valinomycin tolerance by generating less ROS. a Schematic depiction of the key enzymes in the *B. subtilis* TCA cycle and electron transport chain. Deleted genes encoding the different components are shown above the related subunits. The different components shown are pyruvate dehydrogenase (Pdh), glycerol-3-phosphate (G3P) dehydrogenase (Gdh), succinate dehydrogenase (Complex I, Sdh), NADH dehydrogenase (Complex II, Ndh), menaquinol pool (Q pool), cytochrome bc₁ complex (Complex III, cyt bc), cytochrome c₅₅₀ and c₅₅₁, cytochrome-c oxidase (Complex IV, c oxid), cytochrome aa₃ quinol oxidase (aa₃ oxid) and cytochrome bd ubiquinol oxidase (bd oxid). QcrA is the Rieske factor, menaquinol:cytochrome c oxidoreductase (iron-sulfur subunit), component of the cytochrome bc₁ complex. b-g Survival curves of the different deletion mutant grown to stationary phase and subsequently incubated with 100 μ M valinomycin (val). The affected redox step in the mutants is indicated in a. See main text for details. The viable counts of the mutants treated with 1 % DMSO were similar to wild type cells and are not shown here. Data shown reflect mean $\pm$ SD of three biological replicates. h ROS production in stationary phase wt and Δ *qcrA* cells after 4 h incubation with 100 μ M valinomycin (val) measured by the fluorescent ROS probe H₂DCFDA. Control cells were treated with 1 % DMSO for 4 h. The fluorescence intensities of 150 cells were measured microscopically and plotted.

Figure 6

Localization of cytochrome bc₁ and cytochrome-c oxidase subunits and cytochrome c550 fused to GFP. a Cellular localization of GFP tagged cytochrome bc₁ subunits QcrA (Rieske protein), QcrB and QcrC, cytochrome-c oxidase subunits CtaC and CtaD, and cytochrome c550 in stationary phase cells incubated with 100 μ M valinomycin for 1, 2 and 4 h. b Same as a except that the cells were sampled from the exponential phase and treated with 100 μ M valinomycin for only 10 to 30 min. Arrows indicate clustering

of the GFP-QcrA signal. Scale bars represent 3 μm . Large field images with more cells are shown in Fig. S2 and S3.

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