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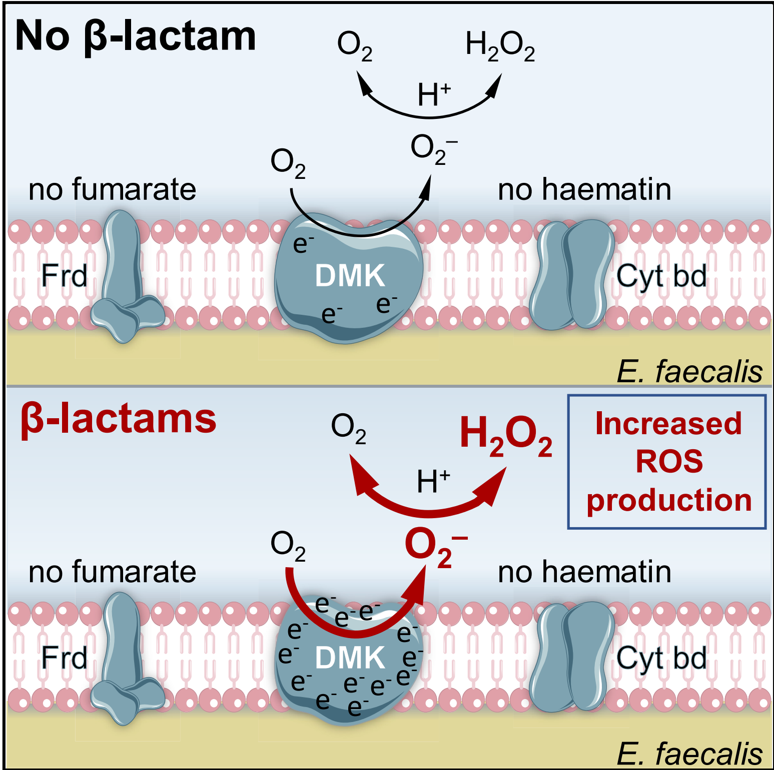
β-Lactam Exposure Triggers Reactive Oxygen Species Formation in *Enterococcus faecalis* via the Respiratory Chain Component DMK

Graphical Abstract



Highlights

- β-lactams trigger a strong increase in ROS production in *E. faecalis*
- ROS production is mainly dependent on the oxidation of membrane-associated DMK
- ROS production results from increased electron flux through DMK in absence of respiration

Authors

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In Brief

Léger et al. show that β-lactams treatment enhances superoxide and hydrogen peroxide production in *Enterococcus faecalis* in a mainly demethylmenaquinone (DMK)-dependent process. In the absence of respiration, the antibiotics trigger the accumulation of the reduced form of DMK, a respiratory chain component, which increases the adventitious reactivity with oxygen.

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β-Lactam Exposure Triggers Reactive Oxygen Species Formation in *Enterococcus faecalis* via the Respiratory Chain Component DMK

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SUMMARY

Whereas the primary actions of β-lactams are well characterized, their downstream effects are less well understood. Although their targets are extracellular, β-lactams stimulate respiration in *Escherichia coli* leading to increased intracellular accumulation of reactive oxygen species (ROS). Here, we show that β-lactams over a large concentration range trigger a strong increase in ROS production in *Enterococcus faecalis* under aerobic, but not anaerobic, conditions. Both amoxicillin, to which the bacterium is susceptible, and cefotaxime, to which *E. faecalis* is resistant, triggers this response. This stimulation of ROS formation depends mainly on demethylmenaquinone (DMK), a component of the *E. faecalis* respiratory chain, but in contrast to *E. coli* is observed only in the absence of respiration. Our results suggest that in *E. faecalis,* β-lactams increase electron flux through the respiratory chain, thereby stimulating the auto-oxidation of reduced DMK in the absence of respiration, which triggers increased extracellular ROS production.

INTRODUCTION

Beta-lactam antibiotics are bactericidal drugs that block the activity of extracellular enzymes, known as penicillin-binding proteins (PBPs) that catalyze cross-linking of peptidoglycan peptide side chains thereby inhibiting cell wall synthesis (Tipper and Strominger, 1965; Wise and Park, 1965). It is generally believed that killing by β-lactams is due to their bacteriolytic activity. However, bacteriolysis cannot fully explain the lethality of these antibiotics. For example, penicillin has been shown to kill pathogens like *Streptococcus pyogenes* and *Enterococcus hirae* efficiently without inducing a significant lysis of the cells (Daneo-Moore et al., 1988; Gutmann and Tomasz, 1982; McDowell and Lemanski, 1988). A more recent study concluded that the killing of *Enterococcus faecalis* induced by amoxicillin was due to two distinct mechanisms, one involving autolysins and a second action of the drug independent of lysis (Dubée et al., 2011).

In several works, the secondary actions of β-lactams were analyzed. A recent study showed that β-lactams induce a deleterious futile cycle of cell wall synthesis and degradation thereby depleting cellular resources contributing to their killing activity in *Escherichia coli* (Cho et al., 2014). Other studies showed that β-lactams and other bactericidal antibiotics induce complex toxic metabolic perturbations leading to cell damage (Kohanski et al., 2007; Belenky et al., 2015; Dwyer et al., 2009). The authors proposed that these drugs induce an intracellular oxidative stress by increasing respiration and with it the release of reactive oxygen species (ROS) from the respiratory chain overwhelming the cellular antioxidant defenses, leading to DNA damage and finally to cell death (Kohanski et al., 2007; Dwyer et al., 2009). These studies were mainly conducted with *E. coli* but numerous follow up studies on different gram-negative and gram-positive bacteria were in accordance with the proposed ROS-based model (for recent review, see Dwyer et al., 2015). Concerning β-lactams, major results supporting the induction of oxidative stress by these drugs are: (1) amoxicillin strongly induced the expression of the *recA* gene encoding the major recombinase involved in DNA repair, caused DNA damage, and in the presence of ferric iron, induced the expression of anti-oxidant activities in *Pseudomonas* (Yeom et al., 2010); (2) compared to the wild-type parent strain, *Acinetobacter baumannii* and *E. coli recA* mutants were more susceptible to β-lactams (Kohanski et al., 2007; Aranda et al., 2011); (3) a combination of an iron chelator and a radical scavenger as well as the natural antioxidant glutathione provided protection against oxacillin-mediated killing of *Staphylococcus aureus* (Liu et al., 2012); and (4) reduction of the Fenton reaction by iron chelation by the ferritin-like protein Fri is suggested to be at the basis of β-lactam tolerance of *Listeria monocytogenes* (Krawczyk-Balska et al., 2012). Despite these substantial data, the ROS-based killing model was later challenged by other studies persuasively demonstrating that killing by the bactericidal antibiotics used in the former studies was independent from oxidative stress (Ezraty et al., 2013; Keren et al., 2013; Liu and Imlay, 2013).

These controversial viewpoints reflect that our mechanistic understanding of the action of bactericidal antibiotics on bacteria remains incomplete. In this report, we use the health-care-associated pathogen *E. faecalis* as a model bacterium to test the proposal that bactericidal antibiotics induce ROS formation. This gram-positive facultative aerobic/anaerobic bacterium is a usual member of the intestinal microbiome of humans and has

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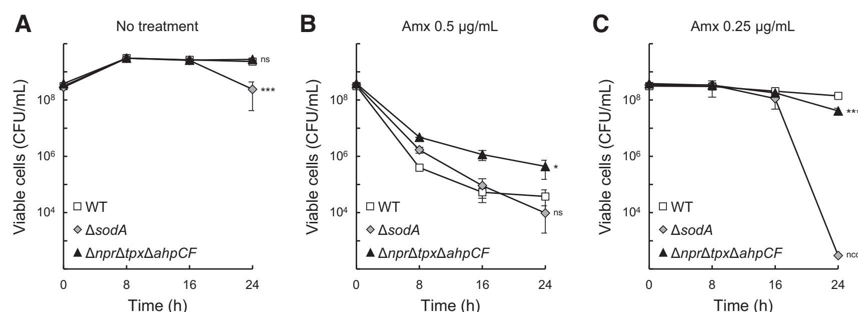

Figure 1. The Δ*sodA* Mutant Is Sensitive to Amoxicillin at 0.25 μg/mL

Survival of *E. faecalis* JH2-2 wild-type strain (squares), Δ*sodA* (diamonds), and Δ*npr*Δ*tpx*Δ*ahpCF* (triangles) mutants growing under aerobic conditions in the absence of antibiotic (A), in the presence of 0.5 μg/mL (B) or 0.25 μg/mL amoxicillin (C). Data are represented as means of 2 to 5 experiments, and error bars depict SDs. Asterisks indicate a statistically significant difference between mutants and the wild-type strain at 24 h. *$p < 0.05$, ***$p < 0.001$ (Student's t test). ncd, no colonies detected. See also Figures S1 and S3.

interesting properties to address this important question of downstream actions of antibiotics. Compared to other gram-positive cocci, enterococci exhibit decreased susceptibility to penicillins and high-level resistance to cephalosporins. We showed previously that this β-lactam tolerance as well as tolerance to vancomycin is linked to the superoxide dismutase SodA in *E. faecalis* and *E. faecium* (Bizzini et al., 2009; Ladjouzi et al., 2013). Unlike *E. coli*, enterococci have no tricarboxylic acid cycle, a pathway central in the Kohanski et al. (2007) model. Furthermore, respiration, responsible for ROS formation in this model, is facultative and only functional in the presence of hematin or fumarate, although respiration by extracellular electron transfer in iron-enriched biofilms has been reported recently for this species (Keogh et al., 2018). *E. faecalis* can produce extracellular superoxide (O_2^-) through autoxidation of membrane-associated demethylmenaquinone (DMK) (Huycke et al., 2001). It has been demonstrated that this radical is also produced in the mammalian intestinal tract (Huycke and Moore, 2002; Moore et al., 2004) and is responsible for chromosomal instability (Wang and Huycke, 2007; Wang et al., 2008), inflammation, and colorectal cancer (CRC) (Balish and Warner, 2002; Kim et al., 2005). The objective of the present work was to analyze if β-lactam antibiotics increase ROS formation in *E. faecalis* wild-type cells using drug concentrations potentially encountered by these pathogens during antimicrobial treatment. We show that this is indeed the case, and provide a mechanistic explanation for this secondary action of these cell-wall targeting drugs.

RESULTS

Superoxide Dismutase-Deficient Mutant Is Sensitive to Amoxicillin

Amoxicillin is still one of the treatments of choice for enterococcal infections that lack mechanisms for high-level resistance (Mercuro et al., 2018). Therefore, we first analyzed whether this bactericidal drug induces an oxidative stress in *E. faecalis*. Because enterococci exhibit a high resistance to oxidants (Flahaut et al., 1998), we reasoned that the most direct way to explore the proposed role of ROS in amoxicillin mortality would be to use mutants defective either in O_2^- or in peroxide defenses as indicators of oxidative stress. *E. faecalis* harbors a manganese-cofactored superoxide dismutase (MnSod or SodA) and three peroxidases, the NADH peroxidase (Npr), the alkyl hydroperoxide reductase (AhpCF), and a thiol peroxidase (Tpx). In this work, the Δ*sodA* and Δ*npr*Δ*tpx*Δ*ahpCF* mutants known to be hypersensitive to ROS were used (La Carbona et al., 2007; Verneuil et al., 2006). Because under our experimental conditions the minimal inhibition concentration (MIC) of amoxicillin was 0.5 μg/mL for all strains, we tested two different concentrations (0.25 and 0.5 μg/mL). As shown in Figure 1A, in the absence of amoxicillin, growth of the triple peroxidase mutant was comparable to that of the parental strain whereas survival of the Δ*sodA* mutant decreased slightly between 16 h and 24 h of incubation under the used aeration condition. This is likely due to the generation and accumulation of ROS, because no decrease in survival of the Δ*sodA* mutant was observed with cultures grown in anaerobic conditions (Figure S1A). Rapid killing of all strains was observed with 0.5 μg/mL of amoxicillin (Figure 1B). No significant differences in survival between the wild-type and the Δ*sodA* mutant strain were observed under this condition, whereas the triple peroxidase mutant was somewhat more resistant to killing by the antibiotic than the two former strains (Figure 1B). Rapid killing also occurred under anaerobic conditions at this amoxicillin concentration (Figure S1B). We concluded that at 0.5 μg/mL of the antibiotic, lethality of the bacteria was independent of an oxidative stress. At 0.25 μg/mL of amoxicillin (half MIC), the survival of all strains was comparable during the first 16 h of incubation (Figure 1C). During this period, killing of the strains was minimal and increased slightly in the case of the wild-type and triple peroxidase mutant after 24 h of exposition to the antibiotic. In contrast, the killing of the Δ*sodA* mutant decreased by ∼5.7 $\log_{10}$ between 16 and 24 h (Figure 1C). This impressive mortality of the Δ*sodA* mutant was not observed under anaerobic conditions (Figure S1C). These results are consistent with a ROS-based killing mode of the MnSod-deficient *E. faecalis* mutant at half MIC amoxicillin. Decrease of survival starts after 16 h of aerobic amoxicillin treatment suggesting that ROS accumulates until creating some sort of damage that became lethal for the Δ*sodA* mutant.

Amoxicillin Promotes H_2O_2 Generation

O_2^- is readily dismuted into H_2O_2 ($2\ O_2^- \leftrightarrow H_2O_2 + O_2$) (Bielski and Richter, 1977), and therefore we wondered if catalase that catalyzes the dismutation of H_2O_2 to molecular oxygen and water would decrease killing by amoxicillin. As shown in Figure 2A, the survival of the Δ*sodA* mutant was nearly restored to the wild-type level in the presence of active catalase (but not with heat-inactivated catalase) strongly suggesting that besides O_2^-, H_2O_2 is also generated during amoxicillin treatment and causatively contributes to antibiotic-mediated killing of the Δ*sodA* mutant. In the

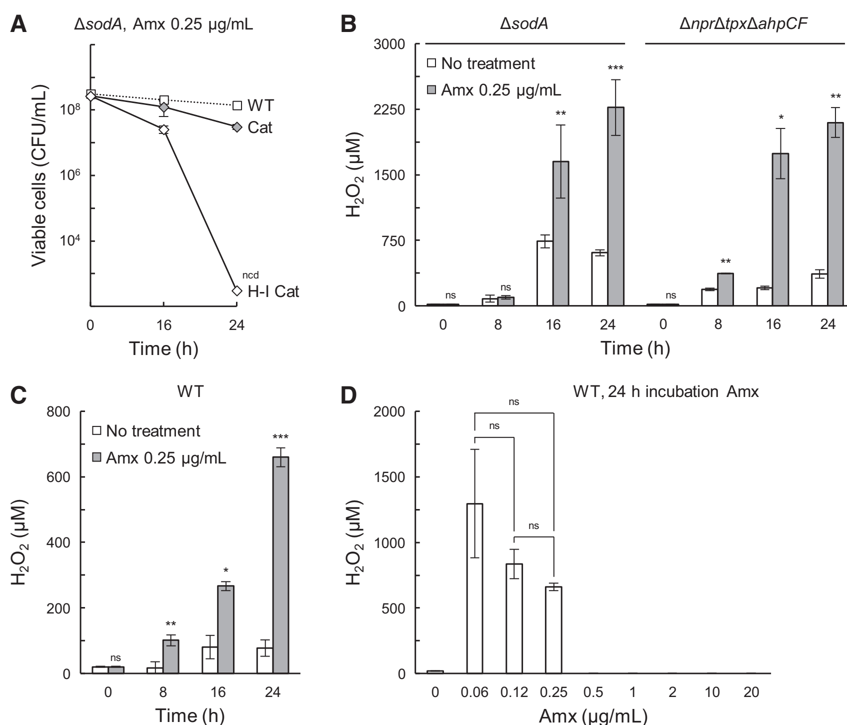

Figure 2. Amoxicillin Promotes H_2O_2 Generation

(A) Survival of the Δ*sodA* mutant exposed to 0.25 μg/mL amoxicillin under aerobic conditions in the presence of ~500 U/mL bovine catalase (Cat) or heat-inactivated catalase (H-I Cat). For comparisons, survival curve of the wild-type strain exposed to 0.25 μg/mL amoxicillin under aerobic conditions is also shown.

(B and C) H_2O_2 concentration of supernatants of Δ*sodA* and Δ*npr*Δ*tpx*Δ*ahpCF* mutants (B) and wild-type strain (C) cultures in the absence of antibiotic (open bars) or after treatment with 0.25 μg/mL amoxicillin (closed bars) under aerobic conditions.

(D) H_2O_2 in culture supernatants of wild-type strain cultivated under various concentrations of amoxicillin for 24 h under aerobic conditions.

Data are represented as means of 2 to 4 experiments, and error bars depict SDs. For H_2O_2 assays, asterisks indicate a statistically significant difference between treated and untreated cells. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Student's t test). ncd, no colonies detected. See also Figure S3.

next step, we verified this hypothesis by directly quantifying H_2O_2 accumulation by *E. faecalis* cultivated with and without amoxicillin. In the absence of the antibiotic, the Δ*sodA* mutant generated H_2O_2 reaching a maximum concentration of around 700 μM after 16 h of incubation and was slightly lower at 24 h (Figure 2B). No H_2O_2 was detected in cultures treated with 0.5 μg/mL of amoxicillin, which is likely due to the rapid ROS-independent killing of the cells at this concentration. However, in the presence of half MIC amoxicillin, cells produced H_2O_2 continuously over the whole incubation time to reach >2,000 μM after 24 h; that is around 4 times more than in the absence of the drug (Figure 2B). We wondered then if amoxicillin treatments also induce an increased H_2O_2 production in wild-type cells that are not killed by 0.25 μg/mL of amoxicillin. Relative to the Δ*sodA* mutant, the H_2O_2 concentrations measured were globally lower, but we found that even in the parental strain, the amoxicillin treatment triggered an increased continuous accumulation of H_2O_2 over the 24 h incubation period (Figure 2C). At the end, the H_2O_2 concentration accumulated was >600 μM in the presence of 0.25 μg/mL of the drug that correspond to 8.4-fold more of this ROS than in the absence of the antibiotic (Figure 2C). We then extended these analyses by testing H_2O_2 production in the presence of a wider range of amoxicillin concentrations. As expected, no H_2O_2 accumulation was observed with lethal concentrations (≥0.5 μg/mL) of the drug (Figure 2D). However, concentrations of amoxicillin below 0.25 μg/mL seems to trigger more accumulation of H_2O_2 although the differences were not statistically significant when compared to 0.25 μg/mL (Figure 2D). Of note, H_2O_2 levels accumulated by the Δ*npr*Δ*tpx*Δ*ahpCF* strain were of the same order of magnitude as those determined with the Δ*sodA* mutant (Figure 2B). We concluded from the combined results that amoxicillin stimulates H_2O_2 formation in *E. faecalis*.

A *recA* Mutant Is as Resistant as the Wild-Type Strain to Amoxicillin

We wondered if the amoxicillin treatment would also increase intracellular ROS production. In *E. coli*, ampicillin induces the SOS response and DNA damage, which has been attributed to the increased formation of hydroxyl radicals by the drug (Kohanski et al., 2007; Miller et al., 2004). *E. coli* and *A. baumanii* strains deficient in RecA, a protein promoting the central steps in recombination and recombinational DNA repair, are more sensitive to ampicillin, supporting the increased intracellular ROS production and DNA damage in cells exposed to this antibiotic (Aranda et al., 2011; Kohanski et al., 2007). We used a well-defined Δ*recA* mutant (Boumghar-Bourtchaï et al., 2009) to analyze if this scenario also holds true in *E. faecalis* cells treated with amoxicillin. This mutant was highly more susceptible to UV radiation (Figure S2A) but was as resistant as wild-type strain to a treatment with 0.25 μg/mL of amoxicillin (Figure S2B). We concluded that intracellular concentration of oxidants should be low, because no RecA-dependent DNA damages are caused by the drug in *E. faecalis*.

Amoxicillin-Induced H_2O_2 Accumulation Appears to Depend Mainly on DMK

Next, we addressed the question about the molecular mechanism(s) of drug-induced ROS formation. The cell death pathway model described in *E. coli* proposed the involvement of the respiratory chain as the primary source of increased intracellular ROS formation by antibiotics (Kohanski et al., 2007). *E. faecalis*

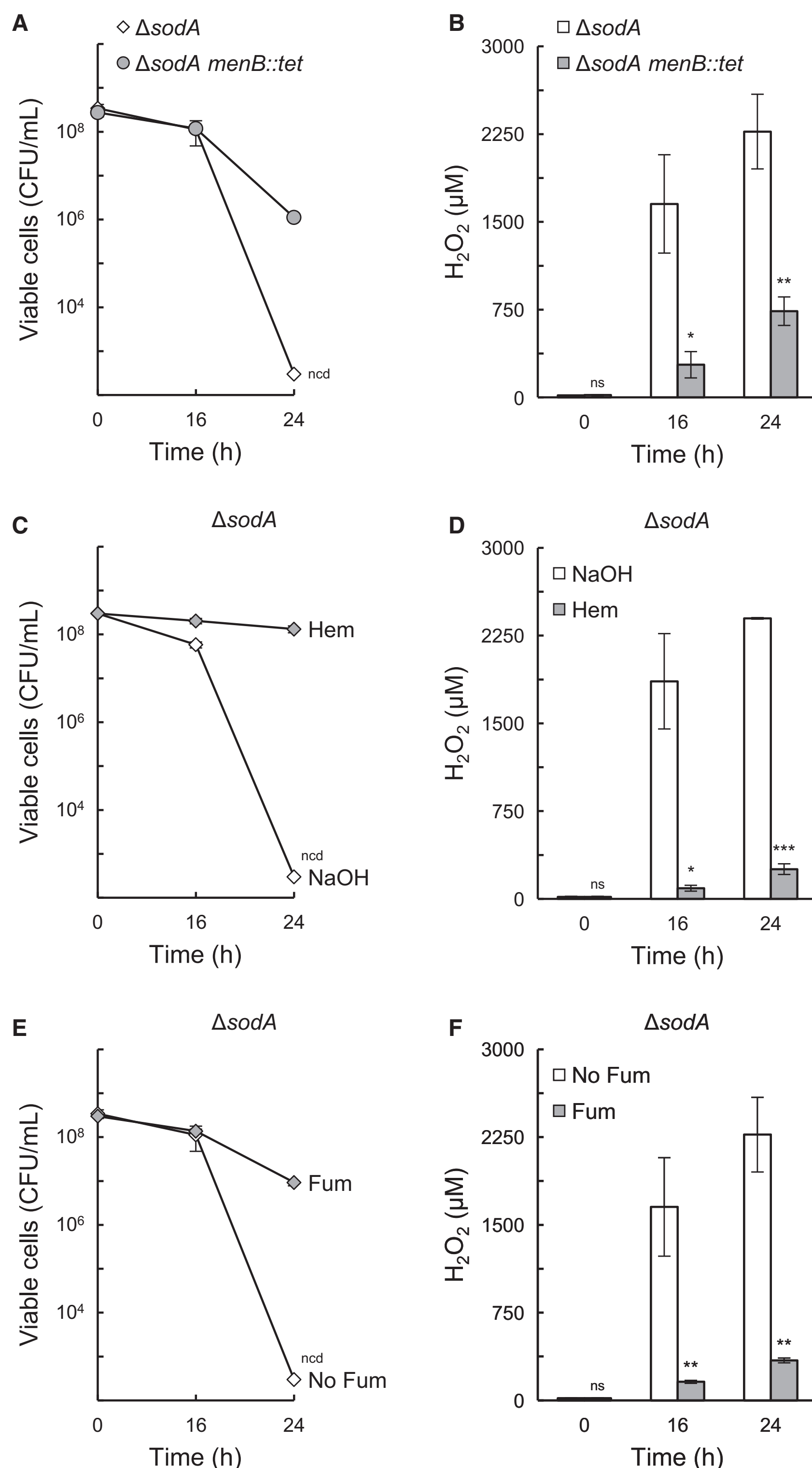

Figure 3. Amoxicillin-Induced H_2O_2 Accumulation Appears to Depend Mainly on DMK

(A–F) Cultures are realized with amoxicillin at 0.25 μg/mL under aerobic conditions.

(A) Survival of Δ*sodA* (diamonds) and Δ*sodA menB::tet* (circles) mutants.

(B) H_2O_2 concentration of supernatants of Δ*sodA* (open squares) and Δ*sodA menB::tet* (closed squares) cultures.

(C) Survival of the Δ*sodA* mutant in the presence of 8 μM hematin/0.56 μM NaOH (closed diamonds) or 0.56 μM NaOH (open diamonds).

(D) H_2O_2 concentration of supernatants of Δ*sodA* mutant cultures supplemented with 8 μM hematin/0.56 μM NaOH (closed bars) or 0.56 μM NaOH (open bars).

(E) Survival of the Δ*sodA* mutant in the presence (closed diamonds) or absence (open diamonds) of 8 mM fumarate.

(F) H_2O_2 concentration of supernatants of Δ*sodA* mutant cultures supplemented with (closed bars) or without (open bars) 8 mM fumarate.

Data are represented as means of 2 to 4 experiments, and error bars depict SDs. For H_2O_2 assays, asterisks indicate a statistically significant difference between 2 datasets. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Student's t test). ncd, no colonies detected. See also Figure S2.

is capable of aerobic respiration, and the presence of cytochromes and DMK has been identified in its membrane (Huycke et al., 2001). However, because enterococci lack the ability to synthesize heme, their cytochromes are only functional in the presence of a source for this prosthetic group (Pritchard and Wimpenny, 1978; Ritchey and Seeley, 1974). In the absence of heme, aerobic respiration is blocked, and it has been demonstrated that this leads to an increase in extracellular O_2^- generation through adventitious auto-oxidation of membrane-associated reduced DMK ($DMKH_2$) (Huycke et al., 2001). Because a known H_2O_2-producing pathway in *E. faecalis* is the spontaneous disproportionation of extracellular O_2^- (Huycke et al., 2002), we wondered whether the amoxicillin-induced ROS formation could be a result of the stimulation of auto-oxidation of DMK. To test this possibility, we measured survival of a Δ*sodA menB::tet* double mutant in the presence of amoxicillin. The *menB* gene encodes 1,4-dihydroxy-2-naphthoic acid synthase catalyzing an essential step in the DMK biosynthesis pathway (Huycke et al., 2001). Relative to the Δ*sodA* single mutant, survival of the Δ*sodA menB::tet* double mutant increased by >4 log_{10} (Figure 3A), likely due to reduced ROS production. This was confirmed by measuring H_2O_2 concentrations in the culture medium of the *menB::tet* single and the Δ*sodA menB::tet* double mutants. H_2O_2 concentration in the former strain was under the detection limit and the latter strain accumulated 6 times and 3 times less H_2O_2 than the

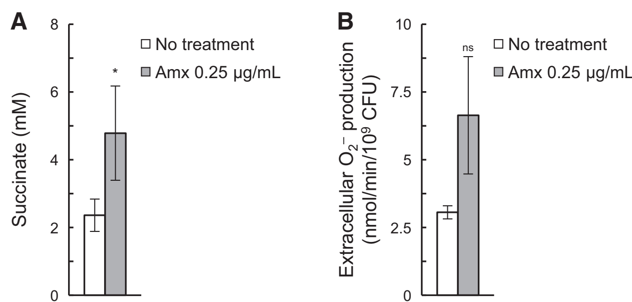

Figure 4. Amoxicillin Appears to Cause an Increased Electron Flow through DMK

(A) Succinate production in the wild-type strain after 4 h of growth in the presence of 20 mM fumarate in the absence of antibiotic (open bars) or after treatment with 0.25 μg/mL amoxicillin (closed bars) under aerobic conditions.

(B) Extracellular O_2^- production of wild-type strain after 24 h of growth in the absence of antibiotic (open bar) or after treatment with 0.25 μg/mL amoxicillin (closed bar) under aerobic conditions.

Data are represented as means of 2 to 4 experiments, and error bars depict SDs. Asterisks indicate a statistically significant difference between mutants and the wild-type strain at 24 h. *$p < 0.05$ (Student's t test). See also Figure S3.

Δ*sodA* single mutant at 16 h and 24 h, respectively (Figure 3B). This strongly suggested DMK as the major source of H_2O_2 accumulation.

If right, depletion of electrons from the $DMKH_2$ pool should also decrease killing by amoxicillin. This was tested by the addition of hematin, which induces synthesis of cytochrome *bd* oxidase allowing aerobic respiration as well as by addition of fumarate that allows anaerobic respiration via a fumarate reductase (Huycke et al., 2001). In the presence of hematin, killing of the Δ*sodA* mutant (Figure 3C) and accumulation of H_2O_2 were significantly reduced (Figure 3D). However, it was recently reported that in addition to its respiratory role, cytochrome *bd* from *E. coli* displays catalase activity (Borisov et al., 2013). If true for the cytochrome *bd* of *E. faecalis*, it may reduce amoxicillin-induced H_2O_2 accumulation. Moreover, *E. faecalis* also harbors a gene encoding a heme-dependent catalase (Frankenberg et al., 2002) and it could not be excluded that it may also contribute to H_2O_2 degradation in the presence of hematin. Therefore, we performed experiments in the presence of amoxicillin and fumarate. *E. faecalis* expresses fumarate reductase, a membrane-associated enzyme that catalyzes the reduction of fumarate to succinate. Electrons are provided by $DMKH_2$, and this process allows non-oxidative respiration in the absence of hematin (Huycke, 2002). The addition of fumarate improved the survival (Figure 3E) and reduced H_2O_2 accumulation (Figure 3F) of the Δ*sodA* mutant. Collectively, these results strongly suggest that amoxicillin-induced H_2O_2 accumulation depends mainly on auto-oxidation reactions involving DMK.

Amoxicillin Appears to Cause an Increased Electron Flow through DMK

Stimulation of the auto-oxidation reactions involving DMK by amoxicillin suggests an increase in electron flow through the respiratory chain of *E. faecalis*. We used fumarate respiration to verify this hypothesis. Because *E. faecalis* does not express succinate dehydrogenase activity (Aue and Deiel, 1967), an increase in succinate formation in the presence of exogenous fumarate and amoxicillin would reflect an increased electron flux through the respiratory chain. We therefore analyzed succinate levels in the supernatants of wild-type cultures challenged with the antibiotic and found that, relative to untreated control, amoxicillin induced a statistically significant 2-fold increase in succinate production rate (Figure 4A), supporting the hypothesis that amoxicillin causes an increase in electron flow through DMK and fumarate reductase.

In the absence of fumarate and hematin, an increased electron flux through DMK cycle would similarly elevate the rate of O_2^- formation, by increasing auto-oxidation reactions. Thus, we evaluated the extracellular O_2^- production rate of amoxicillin-treated and untreated cells and found that the antibiotic induced a >2-fold increase in the formation of O_2^- per minute for 10^9 colony-forming unit (CFU) (Figure 4B). However, the difference between untreated and treated cells seems not statistically significant. Furthermore, it could not be excluded that part of the cells in the culture have been damaged by the antibiotic treatment and consequently are not able to form colonies. These cells might be still metabolically active and produce O_2^- that might overestimate the production rate of this radical. Nevertheless, as we observed a similar statistically significant increase in succinate production rate (Figure 4A), we concluded that these results support the hypothesis that amoxicillin causes an increased electron flow through DMK that, in absence of fumarate and hematin, promotes the formation of O_2^-.

Cefotaxime Also Increases ROS Production in *E. faecalis*

E. faecalis is intrinsically highly resistant to cephalosporins. Therefore, we wondered whether this β-lactam would also stimulate the generation of ROS. As shown in Figure S3, this is indeed the case. The Δ*sodA* mutant was ∼6.1 $\log_{10}$ more susceptible than the parental strain to treatment with 1 μg/mL cefotaxime, a third-generation cephalosporin (Figure S3A). The antibiotic promoted H_2O_2 production in both strains (Figures S3B and S3C), and this stimulation was observed over a wide concentration rate of the antibiotic in the wild-type strain (Figure S3D). Cefotaxime treatment also increased, relative to untreated cells, both extracellular O_2^- production by ∼2-fold (Figure S3E) as well as fumarate respiration (Figure S3F).

DISCUSSION

The present study contributes to an ongoing discussion concerning the secondary mode of actions of antibiotics (Liu and Imlay, 2013). A better understanding of these less well understood downstream effects might pave the way for the development of new treatment strategies (Brynildsen et al., 2013). Here, we clearly demonstrate that β-lactams increase the production of ROS in *E. faecalis*, whether in the wild-type strain or the Δ*sodA* and Δ*npr*Δ*tpx*Δ*ahpCF* mutants. Unexpectedly and unrelated to antibiotic treatment, the Δ*sodA* mutant, for hitherto unknown reasons, accumulated more H_2O_2 than the wild-type strain (Figures 2B and 2C). Because the mutant deficient of all peroxidases of *E. faecalis* accumulated comparable high concentrations of H_2O_2 (Figure 2B), it might be that peroxidases are inactive in the Δ*sodA* background.

In *E. coli*, the respiratory chain is suspected to be at the basis of ROS generation under antibiotic treatment (Kohanski et al., 2007). Our results show that the ROS production appears to depend mainly on DMK, which is consistent with this proposal. However, in contrast to the Kohanski et al. (2007) study, significant ROS production is only observed in our study if electrons are stalled in the DMK pool, which is the case in environments lacking hematin or fumarate. In the presence of β-lactams, the pool of $DMKH_2$ might increase which, consequently, will stimulate O_2^- formation. In the presence of hematin or fumarate, electrons will not accumulate in the DMK pool strongly attenuating the adventitious reaction of molecular oxygen with $DMKH_2$. However, further experimentations are now needed to decipher the detailed molecular changes induced by β-lactam antibiotics leading to the increased generation of ROS in *E. faecalis*.

It is worth mentioning that in a previous study we analyzed tolerance of *E. faecalis* to high antibiotic concentration (20× MIC) (Bizzini et al., 2009). We focused in this former study mainly on vancomycin, a glycopeptide inhibiting cell wall synthesis by targeting the pentapeptide precursors. Whereas the wild-type strain was tolerant to 20× MIC of this drug, survival of the Δ*sodA* mutant decreased by $4\log_{10}$. In contrast to the present study, this decrease in survival was independent of DMK, because the Δ*sodA menB::tet* double mutant was as sensitive to vancomycin as the Δ*sodA* single mutant. This difference to the present work is likely due to the different antibiotics and/or concentrations used in the two studies.

Albesa et al. (2004) showed that *S. aureus*, *E. coli*, and *E. faecalis* sensitive to ciprofloxacin exhibited oxidative stress when they were incubated with the antibiotic, whereas resistant strains did not. Surprisingly, our results demonstrate that cefotaxime also increase ROS generation in *E. faecalis* although these bacteria are highly resistant to the drug (MIC $\geq$ 256 μg/mL). Cephalosporin resistance of enterococci is not fully understood but one well-characterized component is a specialized low-affinity PBP (Pbp5) that remains active in the presence of these drugs. Pbp5 permits cross-linking of peptidoglycan and thereby allows growth of the bacteria in the presence of the antibiotic (Arbeloa et al., 2004; Signoretto et al., 1994). Another key component necessary for intrinsic cephalosporin resistance in *E. faecalis* is the CroR-CroS two-component signal transduction system (Djorić and Kristich, 2015). The loss of intrinsic resistance to cephalosporins of CroRS mutants remains obscure, but it has been reported that oxidative stress enhances intrinsic cephalosporin resistance in *E. faecalis* and in a CroR-CroS-dependent manner (Djorić and Kristich, 2015). The authors showed that mutants accumulating more H_2O_2 than the parental strain or wild-type cultures incubated with extracellular added H_2O_2 demonstrated increased resistance to ceftriaxone. This increased resistance is specific for cephalosporins, because no increase in MICs has been observed with other cell wall targeting drugs including the β-lactam ampicillin. In view of these results, it might be suggested that the increased synthesis of O_2^- and H_2O_2 during β-lactam treatment evidenced in our study corresponds to an active adaptive stress response with the aim to better survive the antibiotic challenge.

E. faecalis is suspected to play a role in colorectal carcinogenesis. This pro-carcinogenic potential seems to be linked with its capacity to generate O_2^- by the auto-oxidation of DMK, because tumor formation was observed with wild-type bacteria but not with *menB* mutants (Wang et al., 2012). The results presented in this communication might therefore reveal an important concern on the careful use of β-lactam antibiotics, because amoxicillin increases significantly the generation of ROS in *E. faecalis* that could increase the risk of CRC development. Interestingly, several recent nested case-control studies analyzing large population-based databases demonstrated indeed a higher risk for CRC with increased number of antibiotic courses and exposure intensities, especially with the most frequently prescribed penicillins (Cao et al., 2018; Dik et al., 2016). Our results might indicate that *E. faecalis* could be a prime candidate to provide a mechanistic explanation for the increased incidence of CRC after therapy with β-lactams. This risk might be even amplified in patients treated with broad-spectrum cephalosporins, because enterococci exhibit intrinsic resistance to these antibiotics, enabling them to proliferate and achieve abnormally high densities in the gastrointestinal tract under these conditions (Donskey et al., 2000).

In conclusion, our study demonstrates that β-lactam antibiotics, besides inhibiting their extracellular targets, have clearly off-target intracellular effects in *E. faecalis*. They seem to modify metabolism leading to an increased production of ROS mainly via DMK auto-oxidation. In the long run, this could be harmful to patients frequently treated with these drugs. Mechanisms by which β-lactams elicit these metabolic alterations are still poorly understood on the molecular level and their understanding might allow the development of new drugs devoid of these undesired downstream effects. Next steps will be to identify the signaling cascade activated by the drug's action outside the cell and the rearrangements of intracellular metabolism triggered by these signals.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.10.080.

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AUTHOR CONTRIBUTIONS

Conceptualization, L.L., A.B.-V., A.B., A.H., and N.V.; Methodology, L.L., A.B.-V., and M.C.; Validation, L.L., A.B.-V., A.B., A.H., and N.V.; Formal Analysis, L.L. and A.B.-V.; Investigation, L.L., A.B.-V., and M.C.; Resources, L.L., A.B.-V., A.B., A.H., and N.V.; Writing – Original Draft Preparation, L.L., A.B.-V., A.B., A.H., and N.V.; Writing – Review & Editing, L.L., A.B.-V., A.B., A.H., and N.V.; Visualization, L.L., A.B.-V., A.B., A.H., and N.V.; Supervision, A.B., A.H., and N.V.; Project Administration, A.B.-V. and N.V.; Funding Acquisition, A.B., A.H., and N.V.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and Virus Strains		
Enterococcus faecalis JH2-2	Jacob and Hobbs, 1974	N/A
Enterococcus faecalis JH2-2 Δ*npr*Δ*tpx*Δ*ahpCF*	La Carbona et al., 2007	N/A
Enterococcus faecalis JH2-2 Δ*recA*	Boumghar-Bourtchaï et al., 2009	N/A
Enterococcus faecalis JH2-2 Δ*sodA*	Verneuil et al., 2006	N/A
Enterococcus faecalis JH2-2 Δ*sodA menB::tet*	Bizzini et al., 2009	N/A
Enterococcus faecalis JH2-2 *menB::tet*	This paper	N/A
Escherichia coli VE14188	Rigottier-Gois et al., 2011	N/A
Chemicals, Peptides, and Recombinant Proteins		
Amoxicillin	Panpharma	Cat#3400956035885
Bacteriological agar type E	BIOKAR Diagnostics	Cat#A1012GC
Catalase from bovine liver	Sigma-Aldrich	Cat#C30
Cefotaxime	Panpharma	Cat#3400956333943
Cytochrome *c* from equine heart	Sigma-Aldrich	Cat#C2506
D(+)-Glucose monohydrate for microbiology	Merck	Cat#1.08342
Fumaric acid	Sigma-Aldrich	Cat#47910
Haematin	Sigma-Aldrich	Cat#H5533
L-Ascorbic acid	Fisher Bioreagents	Cat#BP351
Magnesium sulfate	Prolabo	Cat#25164.265
Meat extract	BIOKAR Diagnostics	Cat#A1710
Pepton USP	BIOKAR Diagnostics	Cat#A1401
Sodium glycerophosphate hydrate	VWR	Cat#27874.295
Sodium hydroxide	Carlo Erba Reagents	Cat#480507
Soytone	BIOKAR Diagnostics	Cat#A1601
Succinic acid	Fluka	Cat#14079
Sulfuric acid	Carlo Erba Reagents	Cat#410301
Superoxide Dismutase from *Escherichia coli*	Sigma-Aldrich	Cat#S5639
Tetracycline·HCl	SERVA	Cat#35866
Yeast extract	BIOKAR Diagnostics	Cat#A1202
Critical Commercial Assays		
Aminex® HPX-87H column 300x7.8 mm	Biorad	Cat#1250140
Amplex® Red Hydrogen Peroxide/peroxidase Assay Kit	Invitrogen	Cat#A22188
Oligonucleotides		
menB::pVE14218 forward: GGCGATCGGACTAAACAATTG	N/A	N/A
menB::pVE14218 reverse: TGATGAAACGGCACGGATAG	N/A	N/A
menB forward: CTCTAAAGTGCCGATTCTTGTG	This paper	N/A
menB reverse: GATCGAAATCTGGTGTCCGT	This paper	N/A
Recombinant DNA		
pGhost3	Maguin et al., 1992	N/A
menB::pVE14218 (Tet)	Bizzini et al., 2009	N/A
Software and Algorithms		
Borwin chromatography	JMBS Developments	N/A
GraphPad Prism 5	GraphPad	https://www.graphpad.com

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
SoftMax® Pro 5.4.4	Molecular Devices	https://www.moleculardevices.com/products/microplate-readers/acquisition-and-analysis-software/softmax-pro-software

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Aurélie Budin-Verneuil (aurelie.verneuil@unicaen.fr).

All unique/stable reagents generated in this study are available from the Lead Contact without restriction.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

The bacterial strains used in this study are listed in Table S1. Strains of *E. faecalis* were grown in M17 medium (Terzaghi and Sandine, 1975) supplemented with 0.3% glucose (GM17) at 37°C (Table S2). *E. coli* VE14188 was cultivated at 37°C in Luria-Bertani (LB) broth (Miller, 1972) with agitation or on LB agar. When appropriate, tetracycline (10 μg/mL for *E. faecalis*, 12.5 μg/mL for *E. coli*) was added to the medium.

METHOD DETAILS

Survival

For overnight cultures, a single colony was grown at 37°C in 10 mL of GM17, with tetracycline if necessary, for about 16 hours. For survival in aerobic conditions, the culture was diluted in 100 mL of GM17 in a 1000 mL Erlenmeyer flask to obtain a final OD_{600nm} of 0.01. The bacterial suspension was incubated at 37°C with moderate shaking (60 rpm) for about 3 hours until reaching OD_{600nm} of 0.5 (exponential growth phase). Cells were then collected by centrifugation (10 minutes at 3,000 g) and resuspended in fresh GM17 pre-warmed at 37°C. Eight milliliters of culture were then distributed into 100 mL Erlenmeyer flasks and 2 mL of antibiotics [diluted in GM17 to obtain the defined final concentration of amoxicillin or cefotaxime (Panpharma, France), respectively] were added. In the case of the controls without antibiotic, 2 mL of GM17 were added to these flasks. Samples were collected immediately (T_0) and after incubation for 8 h, 16 h and 24 h at 37°C with shaking at 60 rpm. All samples were then serially diluted in 0.9% saline, spread onto GM17 plates, and incubated at 37°C for about 48 hours. Survival was determined by counting colonies on plates harboring between 30 and 300 colonies.

For survival in anaerobic conditions, an overnight culture (~16 hours) was diluted in 100 mL of GM17 in a 100 mL sealed bottle to obtain a final OD_{600nm} of 0.01 and incubated at 37°C without agitation for about 3 hours until OD_{600nm} of 0.5. The survival experiments were carried out as previously described except that it was performed in 10 mL sealed tubes containing 10 mL of the above culture and incubated without agitation.

When used, catalase, fumarate, or haematin (Sigma-Aldrich, MO) were diluted to a final concentration of 500 U/mL, 8 mM and 8 μM, respectively. Haematin was prepared as a 20 mM stock solution in 1.4 mM NaOH. Catalase was inactivated by boiling for 5 min.

H_2O_2 Assay

The concentrations of H_2O_2 were measured in the supernatants using Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, OR) as described previously (Bizzini et al., 2009). Briefly, supernatants of cultures treated with antibiotics as described above were collected at the indicated time points by centrifugation (10 min at 3,000 g) and diluted 100-fold in 50 mM sodium phosphate buffer, pH 7.4. The H_2O_2 concentrations were determined using 50 μL of these dilutions and 50 μL of a working solution of 100 μM Amplex Red reagent and 0.2 U/mL HRP. In parallel, H_2O_2 standard curves (0 to 5 μM) were prepared using GM17 diluted 100-fold in 1X Reaction Buffer. Reactions were incubated at room temperature for 30 minutes. Fluorescence was then measured with a FlexStation 3 reader (Molecular Devices, CA) using excitation at 530 nm and fluorescence detection at 590 nm. Background fluorescence, determined from no-H_2O_2 control reactions, has been subtracted for each value.

Construction of the menB::tet Mutant

To construct the *menB::tet* mutant, we used the recombinant pVE14218 (a pORI derivative plasmid) harboring an internal fragment of *menB* gene (*menB::pVE14218*) previously used to construct the Δ*sodA menB::tet* double mutant (Bizzini et al., 2009). The *menB::tet* mutant was obtained following a single crossing over insertion mutagenesis based on a two-vector system using the plasmids pGhost3 (Maguin et al., 1992), which provides functional $RepA^{Ts}$, and the conditionally replicating pVE14218 (Tet^R) (Rigottier-Gois et al., 2011).

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HPLC Analysis of Succinate Production

Succinate concentrations were determined by HPLC (Waters 600 Controller). As described in "Survival" section, cells were treated with antibiotics in presence of fumarate (20 mM) for 4 hours and collected by centrifugation. The filtrates of supernatants as well as internal standards were separated on an Aminex HPX-87H column (Biorad) maintained at 65°C and eluted for 30 min using a 5 mM H_2SO_4 mobile phase with a flow rate of 0.6 μl/min. Peak areas of substrates and derivative products were determined with the Borwin chromatography software (JMBS Developments).

Extracellular O_2^- Assay

Cells treated with antibiotics as described above were collected after 24 hours by centrifugation (10 min at 3,000 g), washed twice with Washing Buffer (7.5 mM ammonium sulfate, 6 mM sodium chloride, 33 mM potassium dihydrogenphosphate, 60 mM potassium hydrogenphosphate, and 1 mM magnesium chloride, pH 7.3), and resuspended in Washing Buffer supplemented with 0.33% glucose to an approximate OD_{550nm} of 0.2. The extracellular O_2^- production was measured as described by Huycke et al. (1996) with slight modifications. Briefly, after being warmed to room temperature, ferricytochrome *c* was added to cell suspension in 1 cm-cuvettes to obtain a final concentration of 20 μM, and the reduction of ferricytochrome *c* was monitored over 10 min at 550 nm in the presence or absence of SOD (25 μg/mL; Sigma-Aldrich, MO) in a SmartSpecTMPlus spectrophotometer (Biorad, CA). The rate of ferricytochrome *c* reduction was calculated using linear regression. The rate of reduction in the absence of SOD was corrected from the rate measured in its absence. O_2^- production was calculated as described by Huycke et al. (1996), using an extinction coefficient of 21.5 mM^{-1} cm^{-1} for reduced cytochrome *c*. In contrast to Huycke et al. (1996), viable counts were determined to normalize the rate of O_2^- production (nmol/min) to 10^9 CFU.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical parameters and significances are reported in the figure legends and figures. Means are considered significantly different when $p < 0.05$. In figures, asterisks denote statistical significances as calculated by Student's t test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Statistical analyses were performed in GraphPad Prism 5. No statistical calculations could be done for 24 hour survivals lacking CFU counts (refer to Figures 1, 2, 3 and S3). These results are highlighted in the corresponding figures by "ncd" meaning "no colonies detected."

DATA AND CODE AVAILABILITY

The published article includes all datasets generated or analyzed during this study.