Loss of penicillin tolerance by inactivating the carbon catabolite repression determinant CcpA in *Streptococcus gordonii*

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**Objectives:** Antibiotic tolerance is a phenomenon allowing bacteria to withstand drug-induced killing. Here, we studied a penicillin-tolerant mutant of *Streptococcus gordonii* (Tol1), which was shown to be deregulated in the expression of the arginine deiminase operon (*arc*). *arc* was not directly responsible for tolerance, but is controlled by the global regulator CcpA. Therefore, we sought whether CcpA might be implicated in tolerance.

**Methods:** The *ccpA* gene was characterized and subsequently inactivated by PCR ligation mutagenesis in both the susceptible wild-type (WT) and Tol1. The minimal inhibitory concentration and time–kill curves for the strains were determined and the outcome of penicillin treatment in experimental endocarditis assessed.

**Results:** *ccpA* sequence and expression were similar between the WT and Tol1 strains. In killing assays, the WT lost 3.5 ± 0.6 and 5.3 ± 0.6 log10 cfu/mL and Tol1 lost 0.4 ± 0.2 and 1.4 ± 0.9 log10 cfu/mL after 24 and 48 h of penicillin exposure, respectively. Deletion of *ccpA* almost totally restored Tol1 kill susceptibility (loss of 2.5 ± 0.7 and 4.9 ± 0.7 log10 cfu/mL at the same endpoints). In experimental endocarditis, penicillin treatment induced a significant reduction in vegetation bacterial densities between Tol1 (4.1 log10 cfu/g) and Tol1Δ*ccpA* (2.4 log10 cfu/g). Restitution of *ccpA* re-established the tolerant phenotype both in vitro and in vivo.

**Conclusions:** CcpA, a global regulator of the carbon catabolite repression system, is implicated in penicillin tolerance both in vitro and in vivo. This links antibiotic survival to bacterial sugar metabolism. However, since *ccpA* sequence and expression were similar between the WT and Tol1 strains, other factors are probably involved in tolerance.

Keywords: experimental endocarditis, antibiotics, arginine deiminase

**Introduction**

Bacteria have evolved two principal mechanisms to evade the killing effect of antibiotics: resistance and tolerance. Bacteria resistant to antibiotics are characterized by their ability to grow in the presence of drug concentrations higher than the one inhibiting the growth of susceptible members of the same species. Hence, resistant bacteria have an increased minimal inhibitory concentration (MIC) of the drug. However, when exposed to antibiotic concentrations exceeding their new MIC, resistant bacteria remain sensitive to the antibiotic killing effect. Resistance is of utmost clinical importance and its mechanisms are widely studied.1

In contrast, antibiotic-tolerant bacteria have an unchanged MIC. However, they have a considerably increased ability to survive drug-induced killing, even at drug concentrations exceeding their MIC by several orders of magnitude.2 In other words, bactericidal drugs act as mere bacteriostatic agents towards tolerant bacteria.

The first laboratory tolerant mutant was reported in *Streptococcus pneumoniae* in 1970.3 In 1974 and 1977, the isolation of clinical specimens of *Staphylococcus aureus* showing tolerance to various β-lactams indicated that this phenomenon was not only a laboratory finding.4,5 Since then, retrospective screenings of bacterial collections have identified the existence of tolerant strains in samples dating from the 1950s. Thus, antibiotic tolerance is not a recent phenomenon, but rather a phenotype which has been overlooked in the microbiology laboratory because of the lack of proper detection techniques.6

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Tolerant bacteria have been associated with treatment failures in endocarditis, meningitis, pharyngitis, and osteomyelitis. Moreover, they are likely to represent a reservoir of survivors potentially able to develop further resistance during prolonged exposure to antibiotics.

In previous work, it was observed that independently generated tolerant mutants of Streptococcus gordonii were deregulated in the expression of the arginine deiminase operon (arc). Although arc was expressed at the end of the exponential phase of growth in the kill-susceptible parent strain, it was expressed constitutively in the tolerant derivatives. Yet, deregulation of arc was not responsible for tolerance by itself. Indeed, its inactivation did not alter the tolerance phenotype. Therefore, we hypothesized that arc deregulation might represent an indirect marker of an as yet unknown factor (or factors) responsible for tolerance.

Expression of the arc operon is under the control of carbon catabolite repression (CCR), which is a global regulatory mechanism allowing bacteria to utilize the most efficient carbon source for their growth. CCR acts upstream of arc and affects the expression of numerous other genes, one or several of which might control tolerance. In Gram-positive bacteria, one of the trans-acting factors involved in CCR is the carbon catabolite control protein A (CcpA) depicted in Figure 1. Here, we show that CcpA is central to tolerance. Its inactivation almost completely abolished the tolerance phenotype—i.e. restored susceptibility to antibiotic-induced killing—both in vitro and in rats with experimental endocarditis. In symmetry, its reactivation restored penicillin tolerance in the test tube as well as in vivo.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains used in this study are described in Table 1. Streptococci were grown at 37°C in brain heart infusion (BHI) broth (Difco, Detroit, MI, USA) without aeration or on Columbia agar (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) supplemented with 3% human blood. Growth of the cultures was followed by measurement of optical density at a wavelength of 600 nm (OD_{600}) using an Ultrospec 500 pro spectrophotometer (Amersham Biosciences, Piscataway, NJ, USA), as well as by viable counts on agar plates. When appropriate, antibiotics were added to the medium at the following concentrations: streptomycin 100 mg/L and erythromycin 5 mg/L. Bacterial stocks were stored at −80°C in BHI broth supplemented with 10% (v/v) glycerol.

Antibiotics, enzymes and chemicals

Penicillin G was purchased from Hoechst-Pharma (Zurich, Switzerland). Restriction enzymes (New England Biolabs Inc., Beverly, MA, USA), HotStar Taq DNA polymerase (Qiagen GmbH, Hilden, Germany) and T4 DNA ligase (Promega Corp., Madison, WI, USA) were used according to the manufacturer’s recommendations. All other chemicals were reagent-grade, commercially available products.

Susceptibility testing and time–kill curves

The MICs and minimal bactericidal concentrations (MBCs) were determined by a standard macrodilution method, with concentrations of dilutions being parts of the multidomain structure EIIABC. The uptake of glucose, by the transporter domain enzyme IIC (IIC), is associated with its phosphorylation by IIB, yielding glucose-6-phosphate. Glucose-6-phosphate going through the energy-producing glycolysis pathway produces glycolytic intermediates such as fructose 1,6-bisphosphate (FBP). This stimulates the kinase activity of HPr kinase/phosphatase (HPrK/P), generating a serine-phosphorylated form of HPr (HPr-Ser-P). A CcpA dimer binds to two HPr-Ser-P, and the resulting heterotetramer regulates the expression of target genes by binding to catabolite responsive element (cre) sequences in their promoters. Depending on the position of cre, transcription is either derepressed or activated. Figure adapted from references 48–50.

DNA manipulations and transformation, plasmids and oligonucleotides

The preparation of S. gordonii genomic DNA was done according to a published method. Conventional agarose gel electrophoresis, restriction endonuclease digests, DNA ligations and PCR amplifications were performed using standard techniques. DNA fragments were purified from gel or solution using a QIAquick DNA purification kit (Qiagen). Small-scale purification of plasmid DNA was performed using a QIAprep spin miniprep kit (Qiagen). DNA quantification was done on an ND-1000 spectrophotometer.

Figure 1. CcpA-dependent carbon catabolite repression in low-GC Gram-positive bacteria. The phosphotransferase protein (HPr) is phosphorylated on a histidine residue by enzyme I (EI) at the expense of phosphoenolpyruvate (PEP), leading to HPr-His-P. The phosphoryl group is then transferred to enzyme IIA (IIA) and further to enzyme IIB (IIB), both being parts of the multidomain structure EIIABC. The uptake of glucose, by the transporter domain enzyme IIC (IIC), is associated with its phosphorylation by IIB, yielding glucose-6-phosphate. Glucose-6-phosphate going through the energy-producing glycolysis pathway produces glycolytic intermediates such as fructose 1,6-bisphosphate (FBP). This stimulates the kinase activity of HPr kinase/phosphatase (HPrK/P), generating a serine-phosphorylated form of HPr (HPr-Ser-P). A CcpA dimer binds to two HPr-Ser-P, and the resulting heterotetramer regulates the expression of target genes by binding to catabolite responsive element (cre) sequences in their promoters. Depending on the position of cre, transcription is either derepressed or activated. Figure adapted from references 48–50.
Role of CcpA in penicillin tolerance

Table 1.  S. gordonii strains used in this study

<table>
<thead>
<tr>
<th>Designation</th>
<th>Relevant genotype, phenotype or description</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td>WT</td>
<td>spontaneous streptomycin-resistant strain of S. gordonii Challis DL1; susceptible to penicillin-induced killing; Sm&lt;sup&gt;r&lt;/sup&gt;</td>
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<tr>
<td>Tol1</td>
<td>a penicillin-tolerant mutant of the WT selected by penicillin cycling; Sm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>15</td>
</tr>
<tr>
<td>WT ΔccpA</td>
<td>ccpA deletion mutant of the WT; Em&lt;sup&gt;r&lt;/sup&gt; Sm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>this work</td>
</tr>
<tr>
<td>Tol1 ΔccpA</td>
<td>ccpA deletion mutant of the Tol1; Em&lt;sup&gt;r&lt;/sup&gt; Sm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>this work</td>
</tr>
<tr>
<td>Tol1 ΔccpA(+)</td>
<td>A ccpA restored Tol1 ΔccpA; Sm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>this work</td>
</tr>
<tr>
<td>CI</td>
<td>S. gordonii clinical isolate</td>
<td>patient with bacteraemia</td>
</tr>
<tr>
<td>3165</td>
<td>S. gordonii NCTC 3165</td>
<td>UK National Culture Collection</td>
</tr>
<tr>
<td>7865</td>
<td>S. gordonii NCTC 7865</td>
<td>UK National Culture Collection</td>
</tr>
</tbody>
</table>

Sm<sup>r</sup>, streptomycin resistant; Em<sup>r</sup>, erythromycin resistant; NCTC, National Collection of Type Cultures.

Table 2. Plasmids and oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Description or 5’-3’ sequence</th>
<th>Reference</th>
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<tr>
<td>Plasmids</td>
<td>pJDC9 streptococcal suicide vector; Em&lt;sup&gt;R&lt;/sup&gt;</td>
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<tr>
<td>16S RT 3</td>
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Underlined portions of oligonucleotides represent engineered restriction sites: GG/CAGG/AscI; GGGGCGG/C, FseI.
Southern blot

Southern-blot experiments were performed with streptococcal chromosomal DNA digested overnight at 37 °C with DraI and XhoI according to standard methods. Hybridizations with digoxigenin-labelled PCR DNA probes and chemiluminescent revelation were done with a DIG-High Prime DNA Labelling and Detection Starter Kit II (Roche Applied Science, Rotkreuz, Switzerland) according to the manufacturer’s instructions.

Construction of ccpA-deleted strains of the wild-type and tolerant S. gordonii

Deletion mutants were generated using the PCR ligation mutagenesis technique. First, an erythromycin resistance cassette was PCR-amplified from pJDC9 using primers erm-K7-DAM104 and erm-K7-DAM105. These two primers introduce both a strong promoter and a transcription terminator, respectively, before and after the coding sequence. Next, a second PCR was performed using this cassette as a template and primers erm-PA and erm-PB in order to introduce a 5' Ascl and a 3' FseI restriction site. This final cassette was double digested with these two enzymes.

In parallel, a sequence overlapping the 5' portion of the ccpA gene was amplified using primers ccpA_L5 and ccpA_L3. A second sequence overlapping the 3' portion of ccpA was amplified using primers ccpA_R5 and ccpA_R3. The 5' and 3' overlapping portions contained an Ascl and an FseI site, respectively. The two amplicons were digested with these enzymes and ligated separately to the resistance cassette. The ligation product was amplified using primers ccpA_L5 and ccpA_R3 in order to generate the whole chimera construct. Finally, the 2 kb construct was transformed into competent WT and Tol1. Recombinants were selected on erythromycin-containing agar plates and purified, and the correct inactivation of ccpA was assessed by PCR and Southern blot (data not shown).

Restitution of ccpA in the Tol1 ΔccpA strain

When grown on blood agar plates, ΔccpA mutants produced slightly smaller and less shiny colonies, an observation which parallels previously described recent results in S. pneumoniae. On the other hand, growth rate in BHI broth assessed by OD600 and chain length formation observed by phase-contrast microscopy were identical between ΔccpA mutants and their parent strains (data not shown). We took advantage of the agar-plate phenotype to detect restitution of the bona fide gene in ΔccpA mutants. Tol1 ΔccpA competent cells were transformed with a PCR product encompassing the full ccpA open reading frame amplified from Tol1 genomic DNA using primers ccpA_L5 and ccpA_R3. Reversion to the morphology of parental ccpA(+) colonies correlated with the restitution of the complete ccpA gene, as assessed by PCR and DNA sequencing (data not shown).

Rat model of experimental endocarditis

The permission for experimentation on living animals regarding the present work was granted by the State Veterinary Office of the ‘Canton de Vaud’ (permission 879.5). Catheter-induced aortic vegetations were produced in female Wistar rats (180–200 g) as previously described. Twenty-four hours later, groups of 5–10 animals were inoculated intravenously with 0.5 mL of saline containing 10^7 cfu of exponential-phase streptococci. This inoculum consistently infected 100% of vegetations in untreated animals (data not shown).

The experiments were repeated two or more times and the results were pooled. Intra-operative mortality was ~10%, mostly due to catheter-induced cardiac arrhythmia.

Penicillin treatment and evaluation of infection

Control rats were sacrificed at the time of treatment onset, i.e. 16 h after inoculation, in order to measure the severity of valve infection at the start of therapy. Treated animals received procaine penicillin (300 000 U/kg) given subcutaneously every 12 h for a total of 2 days. This regimen produced peak and trough antibiotic levels in the serum of rats, which approximated drug concentrations in the serum of humans during intravenous penicillin therapy. Treated rats were killed 12 h after the trough level of the last antibiotic dose, a time at which no antibiotic was detectable in the serum anymore. Euthanasia was performed in a 100% CO2 atmosphere. The cardiac vegetations were dissected, weighed, homogenized in 1 mL of saline, serially diluted and plated for viable colony counts. Colonies growing on the plates were enumerated after 48 h of incubation at 37 °C. The dilution technique permitted detection of >2 log_{10} cfu/g of tissue. Vegetations with negative cultures were given a value of 2 log_{10} cfu/g, the lower limit of detection, in subsequent calculations for statistical analysis. Plating was done on both antibiotic-containing and antibiotic-free agar to ascertain the stability of the markers. Bacteria recovered from infected valves were restested in vitro to assess the stability of both the phenotype and genotype of the strains.

Statistical analysis

Median bacterial titres in the vegetations of penicillin-treated groups were compared by the non-parametric Kruskal–Wallis test with Dunn’s multiple comparison test. Differences were considered significant when the P value was <0.05.

Results

Identification and analysis of a ccpA homologue in S. gordonii

A ccpA homologue was sought in the S. gordonii database available at The Institute for Genomic Research (http://www.tigr.org) by comparison with previously published sequences and PCR-amplified using primers ccpA_L5 and ccpA_R3. A 1005 bp open reading frame was identified, encoding a 334 amino acid protein showing 87% identity and 94% similarity to its S. pneumoniae homologue. Inspection of the region upstream of the putative ATG start codon revealed candidates for the −35 and −10 promoter elements, as well as for the ribosome-binding site (Figure 2). A region of dyad symmetry, representing a putative transcription terminator, is present five bases after the TAA stop codon. This indicates that the ccpA homologue is most probably transcribed as a single messenger RNA. A Southern-blot experiment using a 300 bp probe targeting the inner part of ccpA synthesized using primers InscPA 5 and InscPA 3 revealed that a single copy of the gene was present in both WT and Tol1 strains (Figure 3). Moreover, the exact same sequence could be amplified from three other strains of S. gordonii originating from different culture collections (CI, 3165, 7865), thus indicating that the gene is well conserved. The ccpA nucleotide sequence was submitted to the GenBank/EMBL/DDBJ databases (accession number DQ157896).
Role of CcpA in penicillin tolerance

Inactivation of ccpA leads to loss of antibiotic tolerance in vitro

A study describing an S. pneumoniae ccpA homologue—called regM—showed that its inactivation using insertion-duplication mutagenesis was unstable in vivo and resulted in a high rate of reversion due to excision of the insert. Therefore, we chose to inactivate ccpA using the PCR ligation mutagenesis technique based on omega recombination (see Materials and methods).

To assess the effect of the ccpA deletion on the in vitro susceptibility to the antibiotic, we determined the MICs and MBCs as well as the loss of viable counts after exposure of the test bacteria to 2 mg/L (i.e. 500 × the MIC) of penicillin G (Table 3). The MICs of penicillin were not affected by the ccpA inactivation or restitution. On the other hand, differential results were obtained regarding drug-induced killing. First, the parent strain lost 3.5 ± 0.2 log10 cfu/mL at the same endpoints. Second, although the inactivation of ccpA did not affect the susceptibility to drug-induced killing, the tolerant mutant showed a high rate of inactivation using insertion-duplication mutagenesis. Putative −35 and −10 promoter elements, as well as ribosome-binding site and transcription terminator, are underlined.

![Figure 2. Sequence analysis of the S. gordonii ccpA locus. The deduced amino acid sequence is given for ccpA. Putative −35 and −10 promoter elements, as well as ribosome-binding site and transcription terminator, are underlined.](image-url)
in the WT, it almost fully restored kill-susceptibility in the tolerant mutant, as indicated by MBC/MIC ratios and viable losses in time kill experiments (Table 3). Third, restitution of ccpA restored kill-resistance in the tolerant mutant. Thus, ccpA appeared a key element in the tolerant phenotype of Tol1 (Table 3).

Inactivation of ccpA leads to loss of penicillin tolerance during treatment of experimental endocarditis

The impact of ccpA inactivation was tested in rats with experimental endocarditis receiving penicillin therapy (Figure 4). The four test organisms were equally able to infect damaged valves as shown by similar bacterial densities in the vegetations at the start of therapy (median log_{10} cfu/g: 7.20–7.81; P > 0.05 between groups). However, as for time–kill experiments, they demonstrated different responses to therapy. First, the penicillin-susceptible parent strain was successfully eradicated by penicillin in most animals. Second, the tolerant mutant resulted in a very significant number of treatment failures, thus confirming the detrimental effect of tolerance on penicillin therapy.8 Third, deletion of ccpA in the tolerant mutant restored penicillin efficacy to the level of the susceptible strain, thus confirming the loss of tolerance observed in vitro.

Finally, restitution of the bona fide ccpA gene in the ΔccpA tolerant mutant restored treatment failure in animals, as predicted from test-tube experiments. Of note, in rats left untreated throughout the experiment, no spontaneous bacterial clearance was observed and densities were always higher than in untreated controls sacrificed at the onset of penicillin therapy (data not shown). Therefore, the critical impact of ccpA on tolerance was also relevant in vivo.

Nucleotide sequence and analysis of ccpA expression

The converging results of both in vitro and in vivo experiments point to an important role of CcpA in the survival mechanism of S. gordonii to antibiotic treatment. The next logical step was to determine whether a mutation in either the ccpA coding sequence or promoter could be responsible for the tolerance phenotype. The sequence of the ccpA open reading frame plus an additional 400 nucleotides upstream of it was identical in both the parent and the tolerant mutant (data not shown). Since no differences were found in the ccpA gene and its putative regulatory region, we assessed whether a difference could be found in the transcription of ccpA between the parent and the tolerant mutant, using quantitative determination of ccpA mRNA. Culture samples of the WT and Tol1 were taken just before penicillin addition and 10 min after antibiotic challenge. The results failed to show a significant difference of ccpA expression between WT and Tol1, either before or after penicillin exposure.

Taken together, these results indicate that CcpA itself is not the primary cause of kill-survival, but that it serves as a central hub for as yet undetermined players involved in tolerance.

Discussion

Previous work in our laboratory indicated that multiple exposures of S. gordonii to penicillin could select for spontaneous mutants that were tolerant to the drug.15 When such mutants were generated separately from individual cultures, 6 out of 10 had a deregulation in the arc operon, indicating that a majority of them shared some kind of common tolerance mechanism. Since arc or arc mutations were not directly

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<th>Strain</th>
<th>Penicillin susceptibility</th>
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<tr>
<td></td>
<td>MIC (mg/L)</td>
<td>MBC (mg/L)</td>
<td>MBC/MIC ratio</td>
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<tr>
<td>WT</td>
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<td>WTΔccpA</td>
<td>0.004</td>
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<tr>
<td>Tol1</td>
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<td>&gt;500</td>
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<tr>
<td>Tol1 Δ ccpA</td>
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<td>4</td>
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<tr>
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<td>&gt;4</td>
<td>&gt;1000</td>
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</table>

^aMean (+SD) of three independent experiments.
Role of CcpA in penicillin tolerance

![Graph showing the outcome of penicillin (PEN) therapy for experimental endocarditis due to either S. gordonii wild-type (WT), its penicillin-tolerant derivative (Tol1), the ccpA-deleted Tol1 (Tol1 ΔccpA) or its restored derivative (Tol1 ΔccpA (+)). Each dot above the bars at 2 log10 cfu/g represents the bacterial density in the vegetation of a single animal. Dots under the bars represent sterile vegetations. Statistical differences were evaluated by the non-parametric Kruskal–Wallis test with Dunn’s multiple comparison test. NS, not significant.]

Figure 4. Outcome of 2 days of penicillin (PEN) therapy of experimental endocarditis due to either S. gordonii wild-type (WT), its penicillin-tolerant derivative (Tol1), the ccpA-deleted Tol1 (Tol1 ΔccpA) or its restored derivative (Tol1 ΔccpA (+)). Each dot above the bars at 2 log10 cfu/g represents the bacterial density in the vegetation of a single animal. Dots under the bars represent sterile vegetations. Statistical differences were evaluated by the non-parametric Kruskal–Wallis test with Dunn’s multiple comparison test. NS, not significant.

### Role of CcpA in penicillin tolerance

responsible for tolerance,\textsuperscript{15} the deregulation of arc pointed towards possible alterations in upstream regulatory systems, particularly CCR.\textsuperscript{16,18}

The present results indicate that inactivating ccpA—a central element of CCR (Figure 1)—in a representative tolerant mutant of \textit{S. gordonii} (Tol1) almost completely restored its susceptibility to penicillin-induced killing both \textit{in vitro} and in rats with experimental endocarditis. Nevertheless, the experiments disclosed that ccpA was unlikely to be the primary effector of tolerance because it carried no mutations in its structural gene or promoter region, and it was expressed similarly in the kill-susceptible parent and the tolerant mutant during growth.

The CCR pathway regulates the expression of numerous genes—including the arc operon—in response to the availability of carbohydrates in the medium. In the reference Gram-positive organism \textit{Bacillus subtilis}, CCR modulates the expression of up to 250 genes.\textsuperscript{29} The number of genes affected in streptococci is unknown, but certainly involves numerous elements as well. Thus, one or several genes implicated in CCR or regulated by it could represent a final effector of tolerance in the Tol1 mutant.

Genes or gene products associated with tolerance in streptococci and staphylococci include the major autolysin LytA,\textsuperscript{30} PBP2b,\textsuperscript{31} the cell wall branching proteins MurM and MurN,\textsuperscript{32} the PsA ABC transporter,\textsuperscript{33} the ZmpB metalloprotease,\textsuperscript{34} the heat-shock protein ClpC,\textsuperscript{35} the ABC transporter, the signalling peptide and two-component system locus \textit{vex123-peg27-vncRS},\textsuperscript{36} the autolysin LytB,\textsuperscript{37} the lysozyme LytC,\textsuperscript{38} the capsular polysaccharide\textsuperscript{39} and the two-component system \textit{lytSR} which regulates the antiholins LrgAB and the CidAB holins.\textsuperscript{40,41} Some of them are still debated.\textsuperscript{42–44} Detailing the effect of each of these genes is beyond the scope of this discussion, but it is noteworthy that the capsular polysaccharide has been shown to contribute to tolerance in \textit{S. pneumoniae}.\textsuperscript{39} In \textit{S. pneumoniae}

D39, deletion of ccpA induced a down-regulation of the capsular locus,\textsuperscript{28} whereas capsule production was unchanged in a ccpA deletion mutant of \textit{S. pneumoniae} TIGR4.\textsuperscript{25} Thus, the role of ccpA in capsule production in streptococci is not clear. In addition, \textit{S. gordonii} appears to be an unencapsulated bacterium. Furthermore, a link between antibiotic tolerance and carbohydrate metabolism through the regulation of \textit{S. aureus} \textit{cidABC} and \textit{lrgAB} genes has recently been described.\textsuperscript{45} Thus, a normally functioning ccpA could allow the expression of one or multiple tolerance effector genes, whereas an altered ccpA could alter their expression and restore kill-susceptibility. This model complies with the observation described here.

It is the analysis of the upstream regulation of the arc operon that led to ccpA, the integrity of which is indispensable for the phenotypic expression of tolerance. Yet, in spite of an unaltered and normally expressed ccpA gene in the tolerant mutant Tol1, arc was indeed deregulated in this very same organism. This suggests the existence of one or several mutations that must affect directly or indirectly the expression of arc and maybe the function (but not the expression) of ccpA as well. Identifying such mutations is the object of ongoing experiments.

In summary, this study indicates that ccpA is important for the phenotypic expression of tolerance in certain tolerant mutants of \textit{S. gordonii} arising spontaneously during penicillin exposure. Our results indicate that the ccpA gene product is likely to act indirectly by allowing the functional expression of other effectors of tolerance. Importantly, down-modulation of ccpA could restore kill-susceptibility of tolerant \textit{S. gordonii} \textit{in vitro} and restore therapeutic efficacy of penicillin \textit{in vivo}. It is important to examine whether this is also true for tolerant mutants of other species. Interestingly, it has recently been shown that ccpA deletion induced a 4-fold reduction in oxacillin resistance levels in a highly methicillin-resistant strain of...
S. aureus. Thus, CcpA must be a hub in the pathway of drug-induced bacterial death and might represent a new target to promote drug-induced killing of tolerant bacteria.

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Transparency declarations

None to declare.

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