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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 $\log_{10}$ cfu/mL and Tol1 lost 0.4 ± 0.2 and 1.4 ± 0.9 $\log_{10}$ 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 $\log_{10}$ cfu/mL at the same endpoints). In experimental endocarditis, penicillin treatment induced a significant reduction in vegetation bacterial densities between Tol1 (4.1 $\log_{10}$ cfu/g) and Tol1Δ*ccpA* (2.4 $\log_{10}$ 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,[7–9] meningitis,[10] pharyngitis[11] and osteomyelitis.[12] Moreover, they are likely to represent a reservoir of survivors potentially able to develop further resistance during prolonged exposure to antibiotics.[13,14]

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*).[15] 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.[15] 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),[16] which is a global regulatory mechanism allowing bacteria to utilize the most efficient carbon source for their growth.[17] 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.[18]

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 restitution restored penicillin tolerance in the test tube as well as *in vivo*.

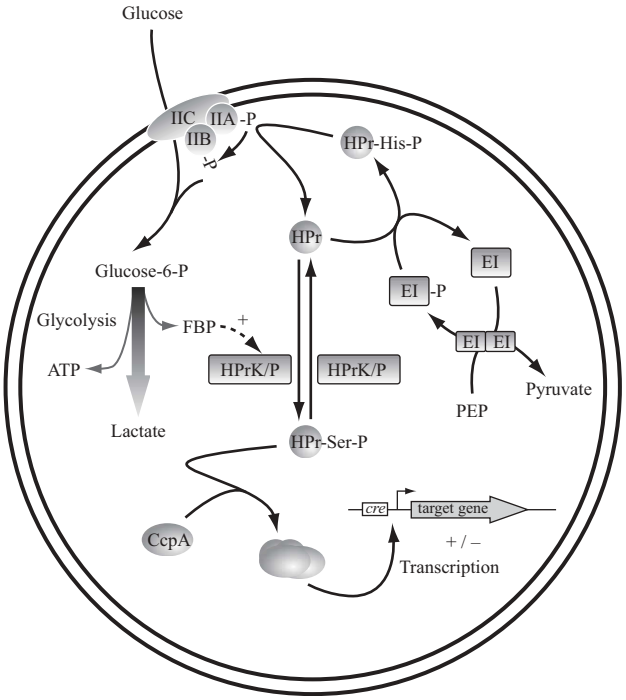

Figure 1. CcpA-dependent carbon catabolite repression in low-GC (<50 mol%) Gram-positive bacteria. The phosphotransferase protein (HPr) is phosphorylated on a histidine residue by enzyme I (EI) at the expense of phosphoenolpyruvate (PEP), leading to HPr-His-P. The phosphoryl group is then transferred 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 repressed or activated. Figure adapted from references 48–50.

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 10^5–10^6 cfu/mL of diluted exponential cultures as a final inoculum.[19] For time–kill experiments, penicillin G at a final concentration of 2 mg/L (equivalent to 500× the MIC) was added to streptococcal cultures in the exponential phase of growth (OD_{600} of 0.2, corresponding to $\sim 1 \times 10^8$ cfu/mL). Samples were removed just before antibiotic addition as well as 24 and 48 h later, serially diluted and plated for viable counts. Antibiotic carryover on the agar plates was avoided as described previously.[8] The numbers of survivors giving rise to colonies were determined after 48 h of incubation at 37°C.

DNA manipulations and transformation, plasmids and oligonucleotides

The preparation of *S. gordonii* genomic DNA was done according to a published method.[20] Conventional agarose gel electrophoresis, restriction endonuclease digests, DNA ligations and PCR amplifications were performed using standard techniques.[21] 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

Table 1. *S. gordonii* strains used in this study

Designation	Relevant genotype, phenotype or description	Source or reference
WT	spontaneous streptomycin-resistant strain of *S. gordonii* Challis DL1; susceptible to penicillin-induced killing; Sm^r	15
Tol1	a penicillin-tolerant mutant of the WT selected by penicillin cycling; Sm^r	15
WT Δ*ccpA*	*ccpA* deletion mutant of the WT; Em^r Sm^r	this work
Tol1 Δ*ccpA*	*ccpA* deletion mutant of the Tol1; Em^r Sm^r	this work
Tol1 Δ*ccpA*(+)	A *ccpA* restored Tol1 Δ*ccpA*; Sm^r	this work
CI	*S. gordonii* clinical isolate	patient with bacteraemia
3165	*S. gordonii* NCTC 3165	UK National Culture Collection
7865	*S. gordonii* NCTC 7865	UK National Culture Collection

Sm^r, streptomycin resistant; Em^r, erythromycin resistant; NCTC, National Collection of Type Cultures.

(NanoDrop Technologies, Wilmington, DE, USA). The preparation of *S. gordonii* competent cells and the transformation experiments were done as previously described.[20] The plasmids and primers used in the study are described in Table 2. Primer synthesis and sequencing reactions were done by Microsynth GmbH (Balgach, Switzerland).

RNA manipulations and real-time quantitative PCR

Total RNA from *S. gordonii* cultures was extracted at an OD_{600} of 0.2, just before and 10 min after penicillin addition, using the RNeasy Mini Kit (Qiagen) combined with the RNA protect bacterial reagent (Qiagen) according with the manufacturer's instructions. Potential DNA contamination was minimized by treating the isolated RNA with RNase-free DNase (Qiagen). The quantity and quality of the RNA were assessed using both a spectrophotometer and formaldehyde RNA gel electrophoresis.[21] Reverse transcription of 1 μg of total RNA was performed using the Omniscript RT kit (Qiagen) with random decamer primers (Microsynth) and RNasin (Promega). Relative gene expression analysis by real-time quantitative PCR was carried out on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), using a SYBR Green PCR master mix (Applied Biosystems). Primers for target amplification (Table 2) were designed using the Primer Express v2.0 software (Applied Biosystems). The 16S rRNA was used as a normalization factor. Results were interpreted using the Q-Gene software.[22]

Table 2. Plasmids and oligonucleotide primers used in this study

Name	Description or 5′-3′ sequence	Reference
Plasmids		
pJDC9	streptococcal suicide vector; Em^R	47
Oligonucleotide primers		
erm-K7-DAM104	CCGGGCCCAAAATTTGTTTGATTTGTATCTTAAAATTTTGTATAATAGG	24
erm-K7-DAM105	GGGGATCCAAAAACTGCCGACTGTAAAAAGTACAGTCGGCAGCGACTCATAGAATTATTTC	24
erm-PA	GGCGCGCCCCGGGCCCAAAATTTGTTTGAT	23
erm-PB	GGCCGGCCAGTCGGCAGCGACTCATAGAAT	23
*ccpA*_L5	AAGAAACGGTGCTAGAAGC	
*ccpA*_L3	GGCGCGCCAGGCGATCAATTACTTCC	
*ccpA*_R5	GGCCGGCCCTTTAGTTGATGATATCAATGG	
*ccpA*_R3	CTGTCTCACTCAATTTTATGAC	
Ins*ccpA* 5	GTTATTTCTCAACACTCGCCAAGG	
Ins*ccpA* 3	TTGCGCTTAGCAAGGAATGTG	
ccpA RT 5	CTTCTAAAGCAACGGCTGCTTT	
ccpA RT 3	CGCAATCAGCTAGTCCGTTTAA	
arcA RT 5	ACTATCCATTCGCTATCGATCCA	
arcA RT 3	GATACTGCGTTACCAATTGTAGCAA	
16S RT 5	GGAAACGATAGCTAATACCGCATAA	
16S RT 3	GAGCCGTTACCTCACCTACTAGCTAA	

Underlined portions of oligonucleotides represent engineered restriction sites: GG/CGCGCC, *Asc*I; GGCCGG/CC, *Fse*I.

Southern blot

Southern-blot experiments were performed with streptococcal chromosomal DNA digested overnight at 37°C with *Dra*I and *Xba*I according to standard methods.[21] 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.[23] 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.[24] 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′ *Asc*I and a 3′ *Fse*I 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 *Asc*I and an *Fse*I 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, Δ*ccpA* mutants produced slightly smaller and less shiny colonies, an observation which parallels recent results in *S. pneumoniae*.[25] On the other hand, growth rate in BHI broth assessed by OD_{600} and chain length formation observed by phase-contrast microscopy were identical between Δ*ccpA* 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 Δ*ccpA* mutants. Tol1 Δ*ccpA* 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.[26] 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).[8] 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.[8] 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% CO_2 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 retested *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[16,27] 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.[28] 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 Ins*ccpA* 5 and Ins*ccpA* 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).

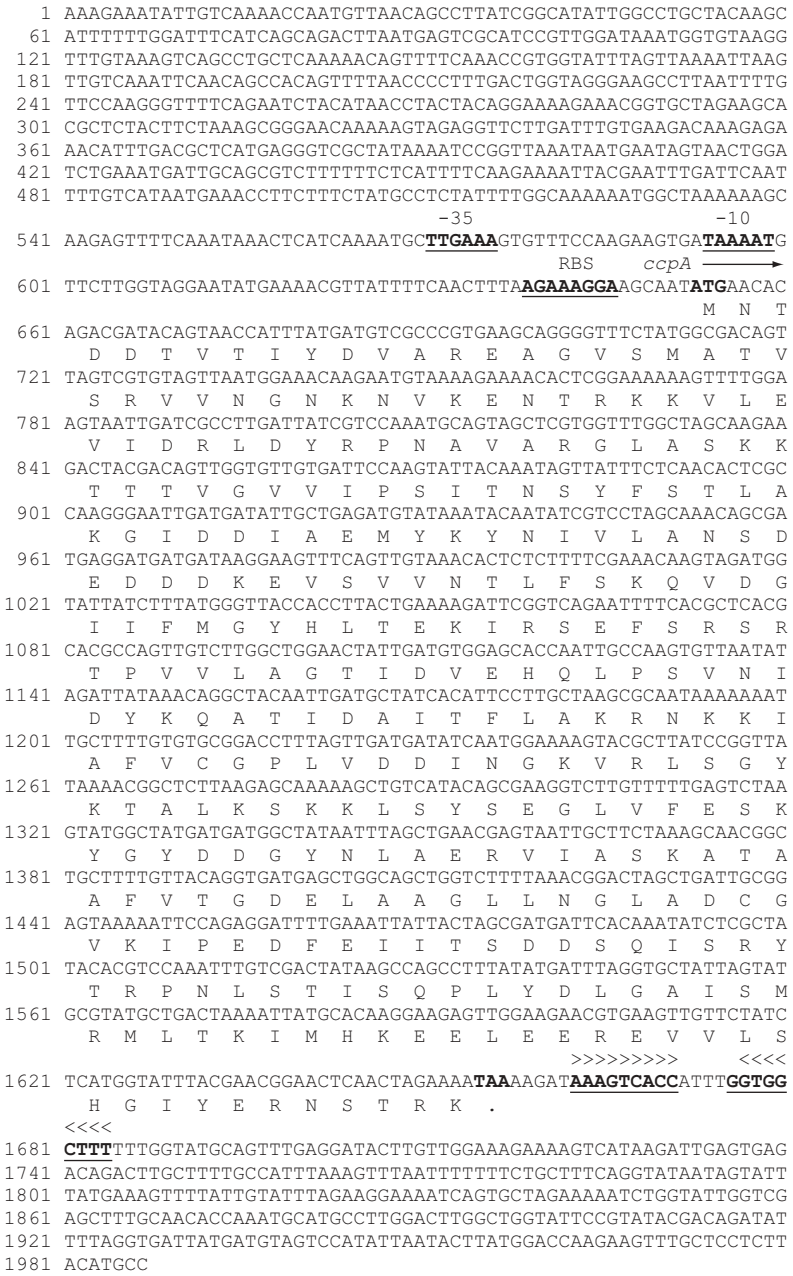

Figure 2. Sequence analysis of the *S. gordonii ccpA* locus. The deduced amino acid sequence is given for *ccpA*. Putative −35 and −10 promoter elements, as well as ribosome-binding site and transcription terminator, are underlined.

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.[28] Therefore, we chose to inactivate *ccpA* using the PCR ligation mutagenesis technique[23] 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 ± 0.6 and $5.3 \pm 0.6 \log_{10}$ cfu/mL in viable counts in time–kill experiments after 24 and 48 h of exposure, respectively, whereas the tolerant mutant lost 0.4 ± 0.2 and $1.4 \pm 0.9 \log_{10}$ cfu/mL at the same endpoints (Table 3). Accordingly, the MBC/MIC ratios of penicillin were ≤ 4 for the parent WT and >500 for the tolerant mutant Tol1 (Table 3). This value confirms the tolerant phenotype of the tolerant mutant, as defined by an MBC/MIC ratio >32 for tolerant bacteria.[5] Second, although the inactivation of *ccpA* did not affect the susceptibility to drug-induced killing

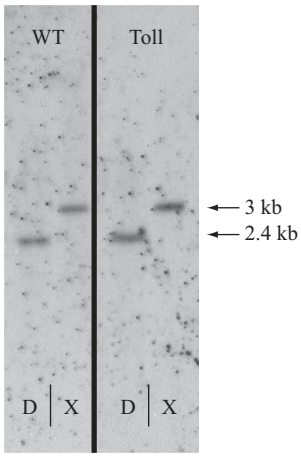

Figure 3. Southern-blot analysis using a digoxigenin-labelled probe targeting the inner part of the *ccpA* gene showing the presence of one single copy of the gene at the expected locus in both wild-type (WT) and Tol1. Predicted band sizes were 2.39 and 2.95 kb for *Dra*I and *Xba*I digestions, respectively. D, *Dra*I-digested chromosomal DNA; X, *Xba*I-digested chromosomal DNA.

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 Δ*ccpA* 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

Table 3. MICs, MBCs and time–kill determinations of penicillin for the test strains

	Penicillin susceptibility			Loss of viability[a] ($\log_{10}$ cfu/mL)	
Strain	MIC (mg/L)	MBC (mg/L)	MBC/MIC ratio	24 h	48 h
WT	0.008	0.016	2	3.5 ± 0.6	5.3 ± 0.6
WTΔ*ccpA*	0.004	0.016	4	3.3 ± 0.5	5.6 ± 0.5
Tol1	0.008	>4	>500	0.4 ± 0.2	1.4 ± 0.9
Tol1Δ *ccpA*	0.008	0.032	4	2.5 ± 0.7	4.9 ± 0.7
Tol1Δ *ccpA* (+)	0.004	>4	>1000	0.9 ± 0.2	1.8 ± 0.8

[a]Mean (±SD) of three independent experiments.

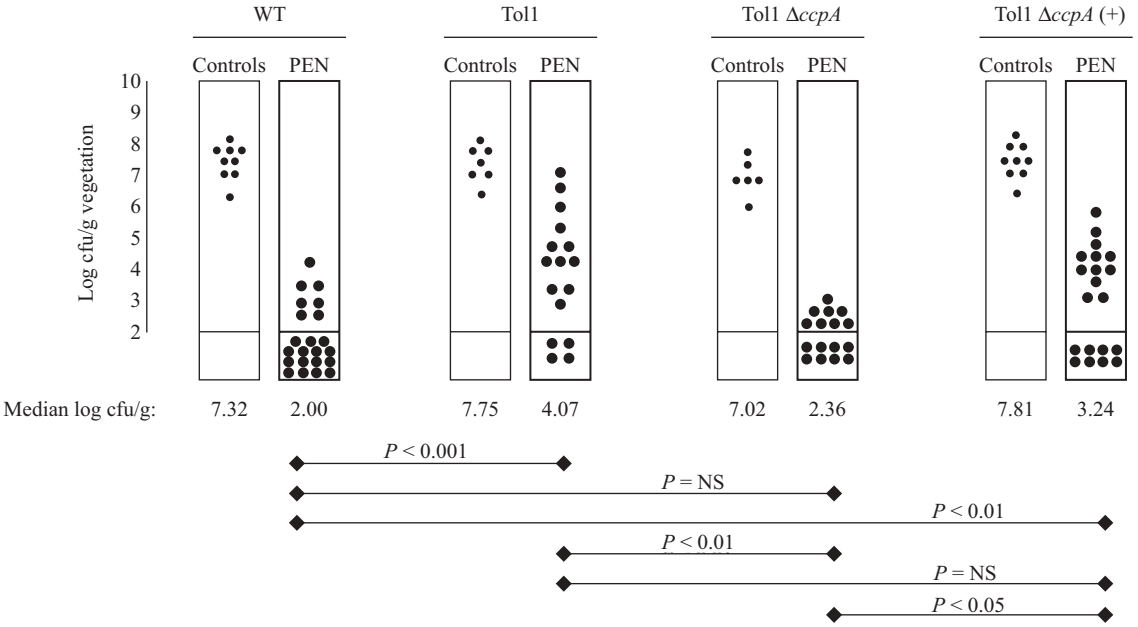

Figure 4. Outcome of 2 days of penicillin (PEN) therapy of experimental endocarditis due to either *S. gordonii* wild-type (WT), its penicillin-tolerant derivative (Tol1), the *ccpA*-deleted Tol1 (Tol1 Δ*ccpA*) or its restored derivative [Tol1 Δ*ccpA* (+)]. Each dot above the bars at 2 $\log_{10}$ cfu/g 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.

responsible for tolerance,[15] the deregulation of *arc* pointed towards possible alterations in upstream regulatory systems, particularly CCR.[16,18]

The present results indicate that inactivating *ccpA*—a central element of CCR (Figure 1)—in a representative tolerant mutant of *S. gordonii* (Tol1) almost completely restored its susceptibility to penicillin-induced killing both *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 *Bacillus subtilis*, CCR modulates the expression of up to 250 genes.[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,[30] PBP2b,[31] the cell wall branching proteins MurM and MurN,[32] the PsaA ABC transporter,[33] the ZmpB metalloprotease,[34] the heat-shock protein ClpC,[35] the ABC transporter, the signalling peptide and two-component system locus *vex123-pep27-vncRS*,[36] the autolysin LytB,[37] the lysozyme LytC,[38] the capsular polysaccharide[39] and the two-component system *lytSR* which regulates the antiholins LrgAB and the CidAB holins.[40,41] Some of them are still debated.[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 *S. pneumoniae*.[39] In *S. pneumoniae* D39, deletion of *ccpA* induced a down-regulation of the capsular locus,[28] whereas capsule production was unchanged in a *ccpA* deletion mutant of *S. pneumoniae* TIGR4.[25] Thus, the role of *ccpA* in capsule production in streptococci is not clear. In addition, *S. gordonii* appears to be an unencapsulated bacterium. Furthermore, a link between antibiotic tolerance and carbohydrate metabolism through the regulation of *S. aureus cidABC* and *lrgAB* genes has recently been described.[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 *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 *S. gordonii in vitro* and restore therapeutic efficacy of penicillin *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.[46] 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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