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The novel transcriptional regulator SczA mediates protection against Zn^{2+} stress by activation of the Zn^{2+}-resistance gene *czcD* in *Streptococcus pneumoniae*

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Summary

Maintenance of the intracellular homeostasis of metal ions is important for the virulence of many bacterial pathogens. Here, we demonstrate that the *czcD* gene of the human pathogen *Streptococcus pneumoniae* is involved in resistance against Zn^{2+}, and that its transcription is induced by the transition-metal ions Zn^{2+}, Co^{2+} and Ni^{2+}. Upstream of *czcD* a gene was identified, encoding a novel TetR family regulator, SczA, that is responsible for the metal ion-dependent activation of *czcD* expression. Transcriptome analyses revealed that in a *sczA* mutant expression of *czcD*, a gene encoding a MerR-family transcriptional regulator and a gene encoding a zinc-containing alcohol dehydrogenase (*adhB*) were downregulated. Activation of the *czcD* promoter by SczA is shown to proceed by Zn^{2+}-dependent binding of SczA to a conserved DNA motif. In the absence of Zn^{2+}, SczA binds to a second site in the *czcD* promoter, thereby fully blocking *czcD* expression. This is the first example of a metalloregulatory protein belonging to the TetR family that has been described. The presence in *S. pneumoniae* of the Zn^{2+}-resistance system characterized in this study might reflect the need for adjustment to a fluctuating Zn^{2+} pool encountered by this pathogen during infection of the human body.

Introduction

Metal ions are essential for the structure and function of many enzymes and regulatory proteins in bacteria. However, an excess of metal ions can be deleterious to the cell (Finney and O'Halloran, 2003). To maintain the homeostasis of metal ions, bacteria contain systems for metal ion uptake and efflux, of which the expression is tightly controlled by metal-responsive regulatory proteins (Hantke, 2001; Nies, 2003; Moore and Helmann, 2005; Pennella and Giedroc, 2005). Various systems involved in the uptake of metal ions have been studied in the human pathogen *Streptococcus pneumoniae*. Mn^{2+}, and possibly also Zn^{2+}, are taken up by the PsaBCA permease (Dintilhac *et al*., 1997; Lawrence *et al*., 1998; McAllister *et al*., 2004), whereas the *adcCBA* gene cluster likely encodes a Zn^{2+} ABC uptake system (Dintilhac *et al*., 1997; Dintilhac and Claverys, 1997). In addition, three iron uptake loci, *piaABCD, piuBCDA* and *pit*, have been described in *S. pneumoniae* (Brown *et al*., 2001; 2002). Both PsaBCA and AdcCBA, as well as their presumed substrates Mn^{2+} and Zn^{2+}, are important for competence of *S. pneumoniae* (Dintilhac *et al*., 1997; Dintilhac and Claverys, 1997). Furthermore, PsaA (Berry and Paton, 1996; Johnston *et al*., 2004), PiaA and PiuA (Brown *et al*., 2001) have been shown to contribute to virulence, and moreover, PsaA contributes to oxidative stress resistance (Tseng *et al*., 2002; Johnston *et al*., 2004).

So far, three regulators have been described in *S. pneumoniae*, that regulate metal ion uptake and contribute to virulence. First, the DtxR-family regulator PsaR represses *psaA* and other genes in the presence of a high Mn^{2+} concentration (Johnston *et al*., 2006). Second, the orphan response regulator RitR (Throup *et al*., 2000) functions as a repressor of iron uptake via the *piuABCD* operon (Ulijasz *et al*., 2004). Third, TCS04 activates expression of *psaBCA* (McCluskey *et al*., 2004).

Next to metal ion uptake systems, bacterial genomes encode genes that enable the cell to cope with high concentrations of metal ions (Nies, 2003). Prominent protein families include: (i) the resistance nodulation family (RND), which are proton-driven antiporters found in all kingdoms of life; (ii) the cation diffusion facilitators (CDF family) driven by a chemiosmotic gradient or a potassium gradient (Haney *et al*., 2005); and (iii) P-type ATPases that are driven by ATP hydrolysis (Nies, 2003). In several bacteria, the CDF-family protein CzcD is known to be an important heavy-metal ion-resistance determinant. In the

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Gram-negative bacterium *Ralstonia metallidurans* CH34 (= *Alcaligenes eutrophus*), the *czcRS* two-component system is involved in regulation of the *czcD* and *czcCBA* genes, that mediate resistance against Co^{2+}, Zn^{2+} and Cd^{2+} (Nies and Silver, 1989; van der Lelie *et al*., 1997). In *Staphylococcus aureus* CzrA, a member of the ArsR/SmtB family of DNA binding proteins, functions as a repressor of the *czr* operon, that consists of *czrA* and the gene encoding the CzcD homologue CzrB (Xiong and Jayaswal, 1998; Kuroda *et al*., 1999; Singh *et al*., 1999). CzrA-mediated repression is alleviated in the presence of Zn^{2+} and Co^{2+} (Xiong and Jayaswal, 1998; Kuroda *et al*., 1999; Singh *et al*., 1999). The *Bacillus subtilis* cation efflux pump *czcD*, which mediates resistance against Zn^{2+}, Co^{2+}, Ni^{2+} and Cu^{2+}, is regulated by an ArsR-type repressor ($CzrA_{BS}$) as well (Moore *et al*., 2005).

As metal ions like Zn^{2+}, Fe^{2+} and Cu^{2+} are also necessary for the proper functioning of the human immune system (Percival, 1998; Shankar and Prasad, 1998; Rink and Gabriel, 2000; Schaible and Kaufmann, 2004), they might have a significant influence on the interaction between *S. pneumoniae* and its host. Noteworthy, the concentration of Zn^{2+} may vary greatly between different sites in the human body (15 μM in serum *versus* 229 μM in the lungs) (Versieck, 1985). In the light of the pivotal role of CzcD homologues in metal ion homeostasis in other organisms, the function of *czcD* in *S. pneumoniae* was studied. In this pathogen we demonstrate that *czcD* is an important determinant for resistance against elevated levels of Zn^{2+}. Furthermore, a novel TetR-family regulator, SczA, is shown to function as a Zn^{2+}/Co^{2+}/Ni^{2+}-dependent transcriptional activator of *czcD* by binding to a regulatory *cis*-element in the *czcD* promoter, that is conserved in several related streptococci.

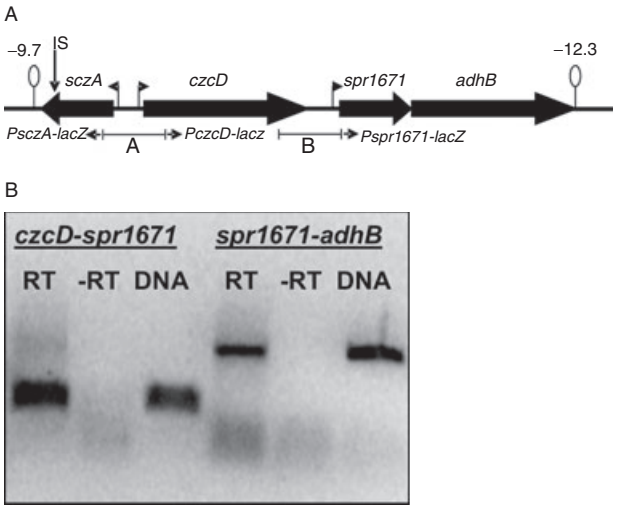

Fig. 1. Organization of the *czcD* operon in *S. pneumoniae*.
A. Schematic overview of the *czcD* locus in *S. pneumoniae* R6. Lollipops indicate terminator structures with the numbers indicating the predicted ΔG^0 in kcal mol^{-1}. Right- or left-pointing flags indicate promoters. Below the genes, fragments used for construction of the *lacZ* fusions to P*sczA* and P*czcD* (A) and P*spr1671* (B) in pPP2 are indicated. IS indicates the position of the mariner insertion in strain MP112.
B. RT-PCR using primers spr1671-2/czcDKO-3 (*czcD–spr1671* intergenic region) and spr1671-3/spr1671-4 (*spr1671–adhB* intergenic region). DNA, positive control PCR on D39 chromosomal DNA. 'RT' and '-RT' indicate PCR on total RNA isolated from D39 wild-type grown in GM17 + 0.05 mM Co^{2+}, with and without reverse transcription respectively.

Table 1. Transcriptional regulation of *czcD* in *S. pneumoniae* D39.

	β-Galactosidase activity		
Medium	wt	Δ*sczA*	Δ*czcD*
GM17	3 (2)	3 (2)	12 (3)
Zn^{2+} (0.1 mM)	66 (4)	3 (2)	85 (10)
Co^{2+} (0.05 mM)	78 (5)	5 (2)	25 (8)
Ni^{2+} (0.2 mM)	160 (16)	6 (2)	52 (9)

β-Galactosidase activity (Miller Units) is given for strains MP103 [D39 Δ*bgaA*::P*czcD–lacZ*, wt (wild-type)] and MP107 (D39 Δ*sczA* Δ*bgaA*::P*czcD–lacZ*, Δ*sczA*) and MP111 (D39 Δ*czcD* Δ*bgaA*::P*czcD–lacZ*, Δ*czcD*) grown in GM17 with the indicated metal ions. Standard deviation of three independent experiments is given between brackets.

Results

Regulation and function of czcD *in* S. pneumoniae

The *S. pneumoniae czcD* orthologue is located in a possible operon with the genes *spr1671*, encoding a MerR-family regulator, and *adhB*, encoding a zinc-containing alcohol dehydrogenase, followed by a putative terminator (Fig. 1A). To be able to study the expression of the *czcD* operon in detail, an ectopic transcriptional *lacZ* fusion was constructed to the predicted promoter of *czcD* in D39 (Fig. 1A). Of various metal ions tested, namely Mn^{2+}, Mg^{2+}, Fe^{2+}, Fe^{3+}, Zn^{2+}, Co^{2+}, Ni^{2+} and Cu^{2+}, only Zn^{2+}, Co^{2+} and Ni^{2+} caused induction of P*czcD–lacZ* expression (Table 1). To investigate the physiological function of CzcD in *S. pneumoniae*, growth of an in-frame *czcD* deletion strain was compared with that of the wild-type in GM17 (Terzaghi and Sandine, 1975; a complex medium used for growth of streptococci, based on casein, soy peptone, beef extract and yeast extract) supplemented with various metal ions. This showed that the *czcD* mutant displays a strongly decreased resistance against Zn^{2+} compared with the wild-type (Fig. 2A). In addition, resistance against Co^{2+} was also lower in the *czcD* mutant (Fig. 2B), while resistance to Ni^{2+} was slightly higher compared with the wild-type (Fig. 2C). These data indicate that CzcD protects *S. pneumoniae* primarily against Zn^{2+} toxicity, and to a lesser extent against Co^{2+}.

In *R. metallidurans*, deletion of *czcD* resulted in increased transcription of the *czcCBA* heavy-metal-resistance genes, likely due to an increase in the intrac-

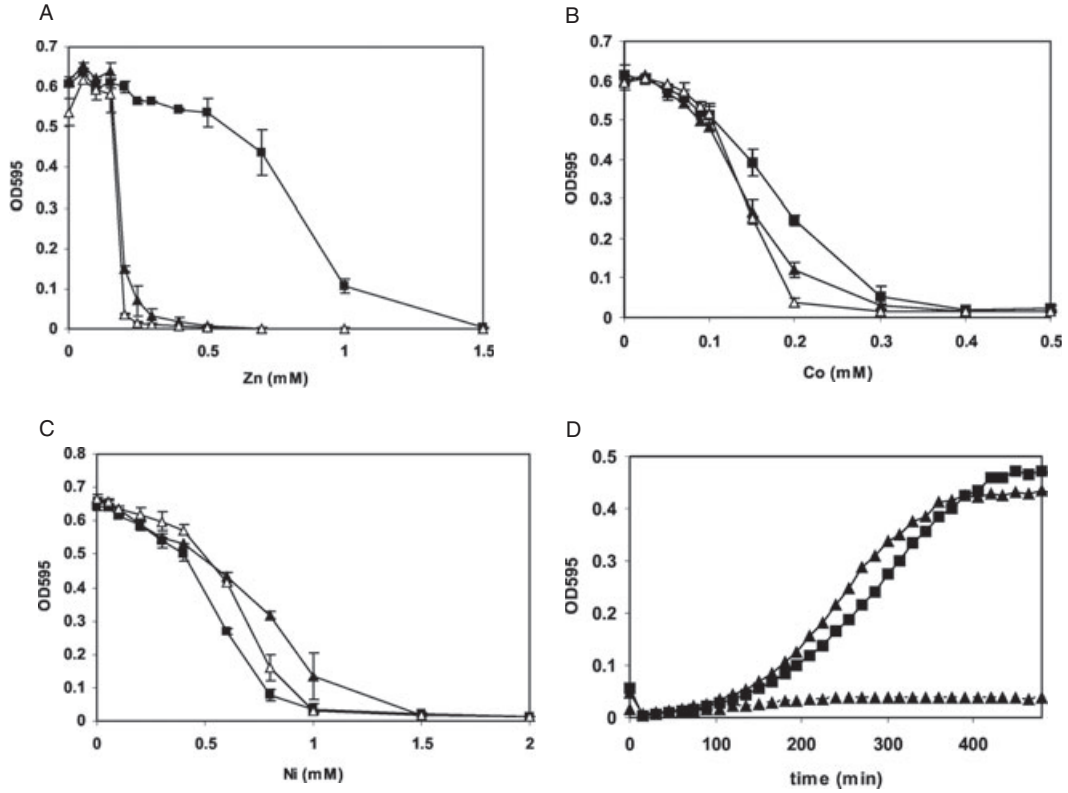

Fig. 2. Optical densities at 595 nm (OD_{595}) after 8 h of growth of D39 (black squares), MP102 (D39 Δ*czcD*, white triangles) and MP100 (D39 Δ*sczA*, black triangles) in GM17 with increasing concentrations of Zn^{2+} (A), Co^{2+} (B) or Ni^{2+} (C). Averages and standard deviations of three experiments are shown.
D. Growth of MP101 (D39*nisRK* Δ*sczA*) containing H_6-*sczA* under control of the nisin promoter (pMP6, triangles) or the empty vector (pNZ8048, triangles, discontinuous line) and D39*nisRK* containing pNZ8048 (squares) in GM17 with 0.3 mM Zn^{2+} and 2 ng ml^{-1} nisin.

ellular concentration of metal cations (Nies, 1992; Anton *et al*., 1999; Grosse *et al*., 2004). To investigate the possibility that in *S. pneumoniae* CzcD has a similar effect on expression of its own gene, we measured expression of the P*czcD–lacZ* transcriptional fusion in the *czcD*-deletion mutant (Table 1). In the absence of added metal ions, there was expression from P*czcD* in the mutant but not in the wild-type. The induction of expression by Zn^{2+} was slightly higher in the *czcD* mutant than in the wild-type. Surprisingly, however, expression from the *czcD* promoter (P*czcD*) in response to Co^{2+} and Ni^{2+} was threefold lower in the *czcD* mutant compared with the wild-type. Thus, deletion of *czcD* interferes with the responsiveness of P*czcD* to Co^{2+} and Ni^{2+}.

Identification of SczA, a novel TetR-family regulator involved in activation of czcD

BLAST searches revealed that the *S. pneumoniae* genome does not contain orthologues of the ArsR-type repressor *czrA* that regulates expression of *czcD* in both *B. subtilis* and *S. aureus* (Xiong and Jayaswal, 1998; Singh *et al*., 1999; Kuroda *et al*., 1999; Moore *et al*., 2005). Therefore, we used strain MP103 (D39 Δ*bgaA*::P*czcD–lacZ*) to perform random mutagenesis with the *Himar1* MarC9 transposon (Lampe *et al*., 1996), screening for mutants that are disturbed in the Zn^{2+}-dependent induction of P*czcD–lacZ* expression. Random mutants were selected in the presence of a subinhibitory concentration of 0.25 mM Zn^{2+} and X-gal, a condition that normally leads to expression of P*czcD–lacZ* and thus blue colonies. Five white colonies were found among 12 500 blue colony-forming units. The transposon insertion sites were determined to be all at the same position in *spr1673*, encoding a TetR-family regulator (Ramos *et al*., 2005), which lies upstream of *czcD* (Fig. 1A). To unambiguously prove that *spr1673* is involved in activation of *czcD*, we replaced the gene with a spectinomycin-resistance marker. In the resulting mutant, *czcD* expression was no longer induced by Co^{2+}, Zn^{2+} or Ni^{2+} (Table 1). We propose the name SczA for the newly identified regulator, which stands for streptococcal *czcD* activator. Thus, SczA activates transcription of the *czcD* gene in *S. pneumoniae*, in response to Co^{2+}, Zn^{2+} or Ni^{2+}.

As SczA activates expression of *czcD*, we hypothesized that the *sczA* mutant has the same phenotype as the *czcD*

Table 2. Summary of transcriptome comparison of *S. pneumoniae* strains MP100 (D39 Δ*sczA*) with D39 wild-type grown in GM17 with 0.05 mM Co^{2+}.

Gene name[a]	Function[b]	Ratio[c]
SP0202/spr0183	Anaerobic ribonucleoside-triphosphate reductase NrdD	2.7
SP0203	Hypothetical protein	2.5
SP0204/spr0184	Predicted acetyltransferase, GNAT family	2.6
SP0205/spr0185	Anaerobic ribonucleoside-triphosphate reductase activating protein NrdG	2.5
SP0206	Hypothetical protein; uridine kinase	2.6
SP0207/spr0186	Hypothetical protein; uridine kinase	2.6
SP1855/spr1670	Alcohol dehydrogenase, zinc-containing, AdhB	–3.9
SP1856/spr1671	Transcriptional regulator, MerR family	–3.8
SP1857/spr1672	Cation efflux system protein CzcD	–17.5
SP1858/spr1673	TetR-family transcriptional regulator protein SczA	–6.2

a. Gene numbers refer to TIGR4 and R6 locus tags.
b. TIGR annotation/R6 annotation (Hoskins *et al*., 2001; Tettelin *et al*., 2001).
c. Ratios > 2.0 or < –2.0 (signal intensity for MP100 divided by that for D39), which have a Bayesian *P*-value < 0.001 and a false discovery rate < 0.01.

mutant. This is indeed the case (Fig. 2A–C). *In trans* expression of a his-tagged SczA version complemented the Zn^{2+}-sensitive phenotype of the *sczA* mutant, excluding the possibility of polar effects of the *sczA* deletion on *czcD* (Fig. 2D).

Microarray analysis of the sczA *mutant*

To determine the influence of the *sczA* mutation on the transcriptome of *S. pneumoniae* D39, the wild-type was compared with the isogenic *sczA* mutant by use of DNA microarrays. To exclude effects due to the increased toxicity of Zn^{2+} to the *sczA* mutant compared with the wild-type, strains were grown in GM17 with 0.05 mM Co^{2+}. Under these conditions, expression from P*czcD* is activated by SczA, and growth of the mutant and the wild-type is comparable (Fig. 2). The transcriptome analysis showed that in addition to *czcD*, also two downstream genes, namely the MerR-family regulator *spr1671* and the zinc-containing alcohol dehydrogenase *adhB* (*spr1670*), were strongly downregulated in the mutant (Table 2).

There is a 243 bp intergenic region between *czcD* and *spr1671*, but no intergenic region between *spr1671* and *adhB* (Fig. 1A). Thus, *spr1671* and *adhB* are likely to be regulated by SczA either via P*czcD* or via a putative promoter just upstream of *spr1671*. To investigate this, a transcriptional *lacZ* fusion with the *czcD–spr1671* intergenic region was constructed (Fig. 1A). This *lacZ* fusion displayed 7 Miller Units promoter activity, which was neither influenced by the *sczA* mutation nor by the addition of metal ions to the medium (data not shown). In addition, reverse transcription polymerase chain reaction (RT-PCR) showed that *spr1671* and *adhB* are located on the same transcript as *czcD* (Fig. 1B). Thus, we conclude that *spr1671* and *adhB* are regulated by SczA via P*czcD* and are at least partially co-transcribed with *czcD*, i.e. form an operon with it.

A transcriptional unit (*spr0183–spr0186*) containing the class III nucleotide reductase encoding genes *nrdD* and *nrdG*, involved in ribonucleoside triphosphate synthesis, was upregulated in the *sczA* mutant, suggesting that SczA is directly or indirectly a repressor of these genes. Using a *lacZ* fusion to the promoter of *nrdD* (*spr0183*), the array data could be validated (Table 3). However, expression of P*nrdD* also seems to be affected by another factor, as an effect of SczA was only seen in the presence of Co^{2+}. In conclusion, under these experimental conditions, SczA influences the expression of only a limited number of genes.

The sczA–czcD *genomic organization is conserved among several streptococcal species*

Using BLAST searches, several organisms were found to contain an orthologue of SczA. Only in *S. mitis* (90% identity), *S. thermophilus* (73% identity), *S. pyogenes* (50% identity) and *S. agalactiae* (48% identity), which contain the closest SczA homologues, the putative *sczA* genes were located immediately next to a *czcD* orthologue (data not shown). This suggests that in these streptococci, SczA has a similar regulatory function of *czcD* expression as we here found in *S. pneumoniae*.

Table 3. Expression (Miller Units) of a P*spr0183–lacZ* transcriptional fusion in D39 wild-type (wt; strain MP106) and D39 Δ*sczA* (strain MP110) in GM17 and GM17 with 0.05 mM Co^{2+}.

	β-Galactosidase activity	
Medium	wt	Δ*sczA*
GM17	18 (3)	21 (3)
Co^{2+} (0.05 mM)	32 (2)	103 (5)

Standard deviation of three independent experiments is given between brackets.

Fig. 3. Identification of a SczA operator sequence in P*czcD*. Position of putative SczA operators (grey shading) in P*czcD* of *S. pneumoniae* R6 (Spneu), *S. pyogenes* MGAS5005 (Spyo), *S. agalactiae* A9 (Saga), *S. thermophilus* CNRZ1066 (Sther) and *S. mitis* NCTC 12261 (Smit). The palindrome is indicated with inverted arrows (→ ←). The consensus sequence is given below the alignment. W = A or T, Y = C or T. Putative core promoter sequences are underlined. Ribosome binding sites are in bold face. Start codons of *czcD* and *sczA* are in italic type. N, inserted base to align sequences.

As the *sczA–czcD* gene order is conserved in the above-mentioned streptococci, the *sczA–czcD* intergenic regions from these five organisms were subjected to the online tool Gibbs Motif Sampler (Thijs *et al*., 2002). This resulted in the identification of a conserved palindromic sequence, which is present in the *sczA–czcD* intergenic region of each of the five organisms, and of several conserved residues that are present just upstream of this palindrome (Fig. 3). Searching the entire *S. pneumoniae* R6 and D39 genomes for the presence of the putative SczA operator using Genome2D (Baerends *et al*., 2004) did not reveal additional promoter regions containing a similar sequence.

*Identification and verification of SczA operators in P*czcD

To find out whether the identified conserved DNA motif (hereafter designated as motif 1, Figs 4 and 8B) mediates the SczA-dependent transcriptional control of P*czcD*, several *lacZ* fusions were constructed, in which P*czcD* was truncated from the 5′ end (Fig. 4). This showed that removal of the few conserved bases upstream of the palindromic sequence of motif 1 (P*czcD*-2), or the whole (P*czcD*-4) or half (P*czcD*-3) of the palindromic sequence, led to abolishment of the SczA-dependent activation in the presence of Zn^{2+}, whereas a promoter fragment truncated just upstream of motif 1 (P*czcD*-2b) still showed SczA- and Zn^{2+}-dependent activation (Fig. 4). The same was seen when Co^{2+} was used to induce expression (data not shown).

To determine whether motif 1 functions as a binding site for SczA, the promoter truncations were used in direct binding assays with purified H_6-SczA (Fig. 5A), which was functional when expressed in *S. pneumoniae* (Fig. 2D). Surprisingly, H_6-SczA specifically bound to all truncations, even to P*czcD*-4, which entirely lacks motif 1 (Fig. 5A). However, only with motif 1 present in its full length, including the bases upstream of the palindrome (P*czcD*-2b), binding resulted in complexes of higher molecular weight (MW) (Fig. 5A).

Apparently, H_6-SczA also binds to another, more downstream site in P*czcD*. Closer examination of this 3′ region of P*czcD* revealed a perfect inverted repeat (motif 2, Figs 4 and 8B), of which the second half (5′-TGTTCA-3′) is identical to the first half of the palindromic part of the SczA operator (Fig. 8B), which therefore could function as an additional SczA binding site. This motif 2 is, however, not conserved in the other streptococci. To investigate the effect of motif 2 on the SczA-dependent activation of

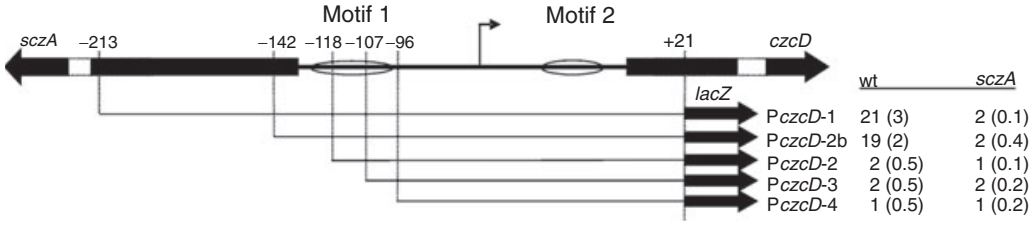

Fig. 4. Subcloning of P*czcD*. A schematic drawing of P*czcD* truncations is shown. Positions of the truncations relative to the ATG start codon of *czcD* are given. The promoter is indicated with a flag. Ovals indicate the position of the two motifs that might function as operator sequences for SczA (motif 1 and motif 2). The table on the right gives β-galactosidase activity (Miller Units) of the promoter truncations in D39 wild-type (wt, strains MP120–MP124) and the Δ*sczA* mutant (*sczA*, strains MP130–MP134) grown in GM17 + 0.1 mM Zn^{2+}. Standard deviations of three independent measurements are given between brackets.

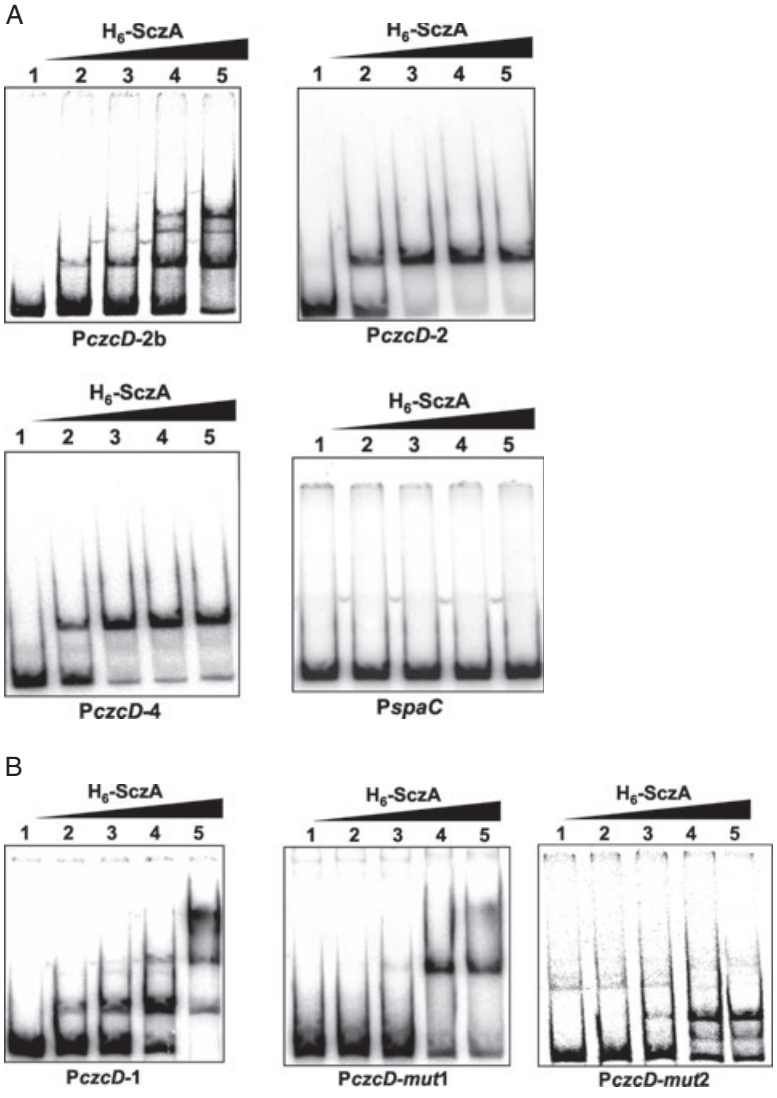

Fig. 5. *In vitro* interaction of H_6-SczA with P*czcD*.
A. EMSAs of H_6-SczA with P*czcD* truncations P*czcD*-2b, P*czcD*-2 and P*czcD*-4 (see Fig. 4). The *spaC* promoter was used as a negative control. Purified H_6-SczA was added in concentrations of 0, 1.15×10^{-8}, 2.3×10^{-8}, 4.6×10^{-8} and 7×10^{-8} M in lanes 1–5 respectively.
B. EMSAs of H_6-SczA binding to P*czcD*-1 (see Fig. 4) and its derivatives P*czcD-mut*1 and P*czcD-mut*2 (see Fig. 8B). Conditions were the same as given under 'A'.

P*czcD*, four subsequent point-mutations were introduced in promoter fragment P*czcD*-1 (Fig. 8B). Expression from this P*czcD* fragment (P*czcD-mut*1) in the presence of Zn^{2+} or Co^{2+} was four times higher than P*czcD*-1, and it was also slightly expressed in GM17 (Fig. 6A). Conversely, introduction of four subsequent point-mutations in motif 1 (Fig. 8B, P*czcD-mut*2) abolished expression in the presence of Zn^{2+} or Co^{2+} completely (data not shown). Binding of purified H_6-SczA to P*czcD-mut*1 resulted only in the band of higher MW (Fig. 5A), whereas binding to P*czcD-mut*2 resulted only in the low-MW bands (Fig. 5B). Thus, motif 2 functions as a binding site for SczA, giving rise to the complex of low MW. This interaction is not necessary for the activation of P*czcD* by SczA, but moderates activation of *czcD* expression. Motif 1, on the other hand, mediates the SczA-dependent activation of the *czcD* promoter.

As SczA activates P*czcD*, we wondered whether it also affects expression of its own promoter, which is located on the same intergenic region (Fig. 1A), and whether this is mediated by motif 2. Therefore, expression of the wild-type (P*sczA*-1) and a *sczA* promoter with the four point-mutations in motif 2 (P*sczA-mut*1) was analysed in the wild-type strain and the *sczA* mutant (Fig. 6B). This showed that SczA has a twofold repressive effect on the expression of its own gene, which was, however, independent of the addition of the metal ions Zn^{2+} and Co^{2+} (Fig. 6B). The autorepressive effect of SczA was fully relieved upon mutation of motif 2 (Fig. 6B). Taken together, our experiments demonstrate the functionality of a SczA operator sequence (motif 1) that mediates activation of P*czcD*, as well as a second sequence (motif 2) that mediates autorepression of *sczA* and furthermore weakens activation of P*czcD*.

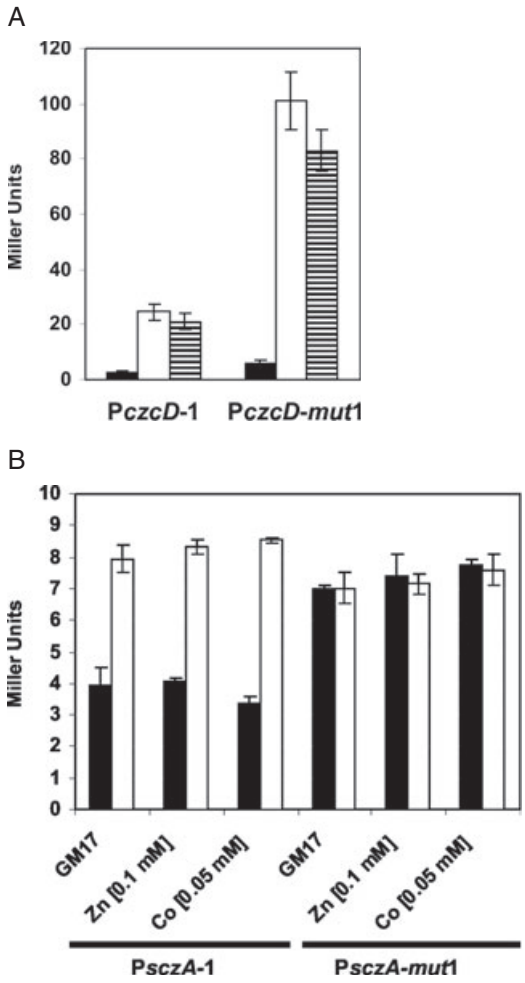

Fig. 6. Effect of motif 2 on transcriptional regulation of the *czcD* and *sczA* promoters.
A. Expression of P*czcD*-1–*lacZ* and P*czcD-mut*1–*lacZ* transcriptional fusions in D39 wild-type (strains MP120 and MP125) in GM17 (black bars), GM17 with 0.05 mM Co^{2+} (white bars) and GM17 with 0.1 mM Zn^{2+} (horizontally striped bars).
B. Expression of P*sczA*-1–*lacZ* and P*sczA-mut*1–*lacZ* transcriptional fusions in D39 wild-type (strains MP127 and MP128, black bars) and Δ*sczA* (strains MP137 and MP138, white bars) in GM17 with the indicated concentrations of metal ions.

*Zn^{2+}-dependent binding of SczA to P*czcD

To further elucidate the mechanism of transcriptional activation by SczA, the effect of metal ions on binding of H_6-SczA to P*czcD* was studied. To prevent interference with the binding of metal ions to H_6-SczA, running- and gel buffers without EDTA were used. Under these conditions, H_6-SczA binding to P*czcD*-1 resulted only in the higher-MW complex (binding to motif 1, Fig. 7A), as opposed to the formation of both high- and low-MW complexes with the standard TBE running buffer that contains 2 mM EDTA, which was used for the shifts in Fig. 5A. Binding of H_6-SczA to P*czcD*-1 in the absence of EDTA was stimulated by Zn^{2+}, Co^{2+} and Ni^{2+}, but not by Mn^{2+} and apparently Mg^{2+}, which is present in a 5 mM concentration in the binding buffer (Fig. 7A and B). Addition of EDTA to the reaction mixture, however, led to formation of the lower-MW complex (Fig. 7A and B). Titration of Zn^{2+} in the reactions with EDTA indeed led to formation of the higher-MW complex again, while Mn^{2+} did not (Fig. 7A). Also Co^{2+} and Ni^{2+} led to formation of the higher band (Fig. 7B). The fact that the low-MW band was only seen upon addition of EDTA, suggests that the purified H_6-SczA contains a low amount of metal ions, possibly Ni^{2+} as a consequence of the purification procedure, which favours formation of the higher-MW complex.

To analyse the effects observed above in more detail, DNase I footprint analyses were performed with H_6-SczA and P*czcD*, in the presence of EDTA and in the presence of both EDTA and Zn^{2+}(Fig. 8A and B). In the presence of EDTA, only protection was seen of motif 2, whereas the addition of Zn^{2+} led to disappearance of this protected

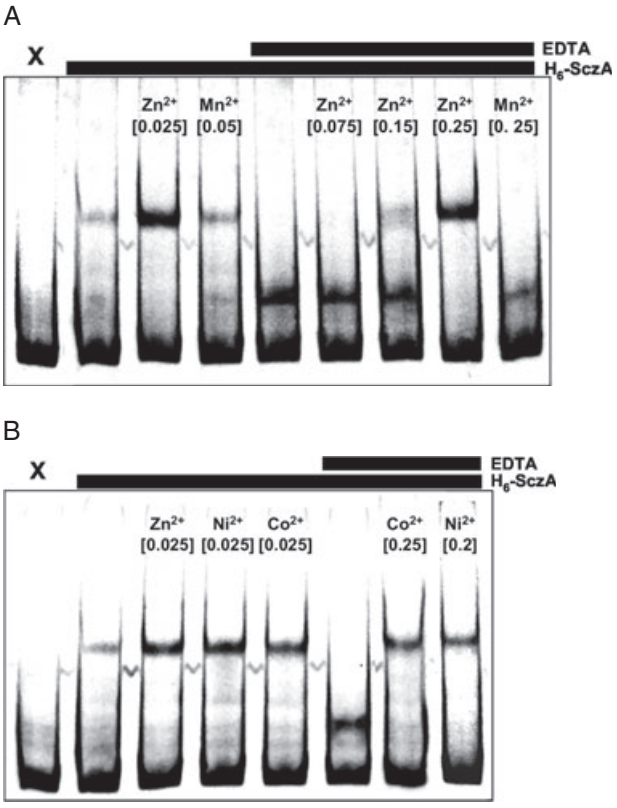

Fig. 7. Modulation of binding of H_6-SczA to P*czcD* by Zn^{2+}, Ni^{2+} and Co^{2+} analysed with EMSAs.
A. Effect of Zn^{2+} on binding of H_6-SczA to P*czcD*-1. H_6-SczA and EDTA were added as indicated above the gel in a concentration of 3.5×10^{-8} M and 0.3 mM respectively. Metal ions were added as indicated above the lanes, with concentrations in mM between brackets. The electrophoresis and gel buffer did not contain EDTA. X, free probe P*czcD*-1.
B. Effect of Co^{2+} and Ni^{2+} on binding of H_6-SczA to P*czcD*-1. Conditions were the same as described for A, except for the metal ions that are indicated in the figure.

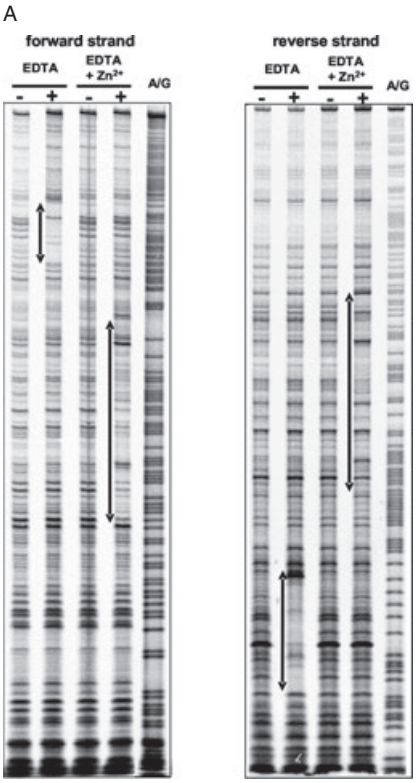

Fig. 8. A. DNase I footprinting analyses of the binding of H_6-SczA to P*czcD*. H_6-SczA was added in concentrations of 0 M (–) or 7×10^{-8} M (+). A/G indicates lane with Maxam–Gilbert sequence ladder. EDTA and Zn^{2+} were added as indicated in a concentration of 0.3 mM. Left panel, the P*czcD*-1 PCR fragment was labelled with the forward primer PczcD-for1. Right panel, the P*czcD*-1 PCR fragment was labelled with the reverse primer PczcD-rev. Regions of protection are indicated with two-pointed arrows on the left of the lanes.
B. Regions of protection in the P*czcD* sequence as found in the footprint analyses. The predicted SczA operator (motif 1) is boxed. The area protected in the footprint is in grey shading. Hypersensitive bases are in italics and underlined. The second SczA binding site (motif 2), a 15 bp inverted repeat, is indicated with arrows below the sequence. Putative –10 and –35 sequences are in bold. Base-pair mutations as introduced in P*czcD-mut*1 (and P*sczA-mut*1) and P*czcD-mut*2 are indicated in white above the sequence (positions –33 to –36). Numbers indicate base positions relative to the start codon (in italics) of *czcD*.

B

Forward strand

```
                          ---------------- motif 1 ----GACC--
-149 CGGTCAATGTTAGTCATATGGACACTTAAGGCAAATTGTTCAGAACTGAATAAAGCTGAC
                                                    -motif 2 CTCC--
-89  GTTTTGCTTCTATCCTTTCTTTGAGTTTTAGTGGATAATGATAATGAACAAGGTGTTCAT
-29  AAATCTATTATAACAAAGGAATGAGAAATATG
```

Reverse strand

```
                          ---------------- motif 1 ----GACC--
-149 CGGTCAATGTTAGTCATATGGACACTTAAGGCAAATTGTTCAGAACTGAATAAAGCTGAC
                                                    -motif 2 CTCC--
-89  GTTTTGCTTCTATCCTTTCTTTGAGTTTTAGTGGATAATGATAATGAACAAGGTGTTCAT
-29  AAATCTATTATAACAAAGGAATGAGAAATATG
```

region and the appearance of a protected area comprising motif 1 (Fig. 8A and B). Thus, the data argue for a model in which binding of SczA to motif 1 in P*czcD* is stimulated by Zn^{2+}, while SczA binding to motif 2 is relieved by Zn^{2+}, in this way accomplishing the Zn^{2+}-dependent activation of *czcD* expression (Fig. 9).

Discussion

In this study, we investigated the regulation of *czcD*, which we demonstrate to encode an important Zn^{2+}-resistance determinant in the human pathogen *S. pneumoniae*. A novel TetR-family regulator, SczA, was identified that activates expression of *czcD* in the presence of Zn^{2+}, Co^{2+} and Ni^{2+}. Moreover, we identified and demonstrated the functionality of an operator sequence for SczA. TetR-family regulators are known to be involved in a variety of functions, such as regulation of antibiotic biosynthesis, osmotic stress and multidrug resistance, and they usually function as repressors (Ramos *et al*., 2005). To our knowledge, this is the first metalloregulatory protein belonging to the TetR family identified so far. In addition, it is one of a few examples of regulators from the TetR family that function as a transcriptional activator (Lin *et al*., 2000; Croxatto *et al*., 2002; Ramos *et al*., 2005; Novakova *et al*., 2005; Christen *et al*., 2006).

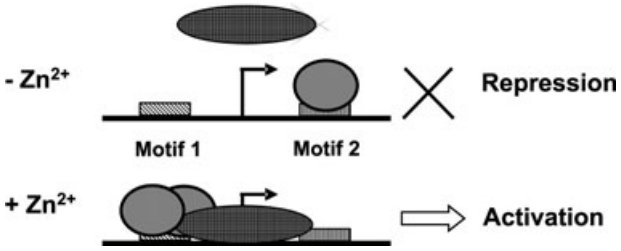

Fig. 9. Schematic model of the mechanism of activation of P*czcD* by SczA depending on Zn^{2+}. In the absence of Zn^{2+}, SczA binds to motif 2, thereby blocking transcription from P*czcD*. In the presence of Zn^{2+}, SczA binds to motif 1, which leads to activation of P*czcD*. Arrows indicate the *czcD* promoter. Big oval, RNA polymerase. Small oval, SczA. The binding of SczA to motif 1 in a higher oligomeric complex is indicated by the two overlapping SczA symbols, as opposed to the single (lower oligomeric state) SczA symbol bound to motif 2.

The CDF family, to which CzcD belongs, is an ubiquitous family of transporters present in prokaryotes and eukaryotes, which play a role in metal ion homeostasis, but also appear to fulfil other functions in the cell (Haney *et al*., 2005). We show that CzcD protects significantly against Zn^{2+} stress in *S. pneumoniae*, and to a lesser extent against Co^{2+} stress. Alignment shows that *S. pneumoniae* CczD contains the conserved aspartyl residues that are known to contribute to a metal ion binding site and metal ion transport in the CDF-family Zn^{2+} efflux pump YiiP of *Escherichia coli* (Wei and Fu, 2006). This suggests that CzcD also functions as a Zn^{2+} efflux pump in *S. pneumoniae*.

The observation that *czcD* in *S. pneumoniae* seems to be primarily involved in resistance towards Zn^{2+} raises the question as to the reason of the strong transcriptional response of P*czcD* to Co^{2+} and Ni^{2+}. Possibly, there are other efflux pumps in *S. pneumoniae* that are involved in resistance against Co^{2+} and Ni^{2+}. This would agree with the observed aberrant regulation of P*czcD* in the *czcD* mutant in response to Co^{2+} and Ni^{2+}, but not to Zn^{2+}. Other candidate heavy-metal-resistance genes encoded by the *S. pneumoniae* genome are *spr1466* (*cadD*), a putative cadmium-resistance transporter, and *spr1411*, encoding a CDF-family protein. A possible third metal ion-resistance gene might be *spr0641*, which shows homology to the PerR-regulated gene *pmtA* in *S. pyogenes* involved in resistance to Zn^{2+} (Brenot *et al*., 2007).

The transcriptional response of *czcD* in *S. pneumoniae* shows similarity to that found in *B. subtilis*, where *czcD* is induced in the presence of Zn^{2+}, Ni^{2+}, Co^{2+} and Cu^{2+}. However, the regulatory mechanism underlying this response is completely different, as in *B. subtilis*, an ArsR-type regulator, CzrA, functions as a repressor of *czcD* (Moore *et al*., 2005). Close homologues of CzrA (> 45% identity) are present in several *Staphylococcus*, *Bacillus* and *Listeriae* species, whereas SczA is only present (> 45% identity) in a small group of related streptococci, where the *sczA* gene is linked to *czcD*. Several other important groups of Gram-positive bacteria, such as lactococci and enterococci, do not seem to contain either regulator type, indicating a yet different regulatory factor governing expression of their *czcD* orthologues.

The microarray results show that SczA also regulates expression of *spr1671*, encoding a MerR-family regulator with homology (30% identity) to copper-responsive regulators, and *adhB*, which are located immediately downstream of *czcD*. The fact that *adhB* is regulated by SczA makes sense, as it is predicted to encode a Zn^{2+}-containing alcohol dehydrogenase. *AdhB* was found in an STM screen to be important for virulence in a lung infection model (Hava and Camilli, 2002). Also, s*pr1671* (*SP1856*) seems important for virulence based on STM screens (Hava and Camilli, 2002; Hava *et al*., 2003). Thus, SczA could indirectly influence *S. pneumoniae* virulence via *adhB* and *spr1671*.

The functionality of a putative SczA operator, predicted on the bases of sequence analysis of P*czcD* regions of different streptococci, was confirmed by promoter subcloning, EMSAs and DNase I footprinting. The operator appears to consist of a palindromic sequence, as well as a stretch of seven bases located just upstream of the palindrome, together making up motif 1. In addition, a second binding site for H_6-SczA was identified, that moderates expression of P*czcD* and is responsible for the autorepression by SczA. Binding of H_6-SczA to motif 1 was increased by Zn^{2+}, whereas binding to motif 2 was decreased in the presence of Zn^{2+}. This indicates that in the absence of Zn^{2+}, SczA binds to motif 2, thereby tightly shutting down P*czcD*. However, in the presence of Zn^{2+}, SczA binds to motif 1, resulting in activation of P*czcD*. As repression of transcription from the *czcD* and *sczA* promoters mediated by motif 2 was not relieved in the presence of Zn^{2+}, the relevance of SczA binding to motif 2 remains somewhat obscure. Maybe, this interaction is only destroyed under extreme metal ion intoxication, thereby giving an extra boost to *czcD* expression and, as a consequence, metal ion resistance. The observation that binding of H_6-SczA to motif 1 resulted in a band of higher MW than binding of H_6-SczA to motif 2, might indicate that monomeric SczA binds to motif 2, whereas an oligomeric conformation of SczA binds to motif 1 (Fig. 9).

Zn^{2+} has a profound influence on the immune function of the human body (Milanino *et al*., 1993; Shankar and Prasad, 1998; Schaible and Kaufmann, 2004; Thurnham *et al*., 2005). Generally, low Zn^{2+} levels lead to decreased functioning of the immune system, while physiologically normal concentrations [12–16 μM in plasma (Versieck,

1985)] secure normal functioning of the immune system (Shankar and Prasad, 1998; Rink and Gabriel, 2000; Ibs and Rink, 2003). High dosages of Zn^{2+} (0.1 mM) may even activate certain cells of the immune system (Ibs and Rink, 2003). Moreover, Zn^{2+} levels in the human body are increased during inflammation (Milanino *et al*., 1993; Thurnham *et al*., 2005). Thus, it is well possible that *S. pneumoniae* encounters fluctuating levels of Zn^{2+} during infection of the human body. One of the strategies *S. pneumoniae* could employ to cope with this is by activation of the *czcD* system in the presence of an elevated Zn^{2+} level. Thus, Zn^{2+} might be an important signal during the complex interplay between *S. pneumoniae* and humans.

Experimental procedures

Bacterial strains, growth conditions, materials and general DNA techniques

Bacterial strains are listed in Table 4. Growth of bacteria and DNA manipulation techniques were performed essentially as described previously (Kloosterman *et al*., 2006a,b). Metals were used as the following salts: $ZnSO_4$, $CoCl_2$, $NiSO_4$, $MgCl_2$, $MnCl_2$, $CuSO_4$, $FeCl_2$ and $FeCl_3$. D39 chromosomal DNA was used as a template for PCR reactions. Primers are listed in Table 5.

Random mutagenesis screen using the mariner transposon

Chromosomal DNA of *S. pneumoniae* R6 was mutagenized with the *Himar1* MarC9 transposase essentially as described (Lampe *et al*., 1996; Martin *et al*., 2000). pR412-T7 (Bijlsma *et al*., 2007), a derivative of pR412 (Martin *et al*., 2000), containing an outward-facing T7 promoter on each side of the mini-transposon, was used as the source of the 1146 bp long *spec mariner* mini-transposon. Mutated R6 chromosomal DNA was transformed to strain R6 to generate a mutant library of approximately 20 000 colony-forming units. Chromosomal DNA of this library was isolated and used to transform strain D39 Δ*bgaA*::P*czcD–lacZ* (MP103), and was plated on GM17 with 1% sheep blood, 0.006% Xgal, 1.5 μg ml^{-1} tetracycline and 130 μg ml^{-1} spectinomycin, and 0.25 mM $ZnSO_4$. Plates were incubated for 2 days at 37°C. Sites of transposon insertion were determined using an inverse PCR approach. In short, chromosomal DNA of mutants was digested with AvaII and self-ligated at a concentration of 0.02 μg DNA per μl. PCR was performed on this ligation mixture with primers TMr_1 and TMr_4, and the resulting PCR products were isolated from gel with the Qiagen gel-extraction kit and sequenced.

Construction of mutants

A mutant of *sczA* (*spr1673*) was made by allelic-replacement mutagenesis. In short, primers Spr1673KO-1/Spr1673KO-2 and Spr1673KO-3/Spr1673KO-4 were used to generate PCR products of the approximately 500 bp left and right flanking regions of *sczA*, which were, by means of overlap extension PCR (Song *et al*., 2005), fused to a spectinomycin-resistance gene amplified with primers Spec_Fp and Spec_Rp from plasmid pORI38. The resulting PCR product was transformed to D39 and D39*nisRK*, yielding strains MP100 and MP101, and the mutation was verified by PCR and sequencing.

An in-frame deletion of *czcD* (*spr1672*) was constructed using pORI280 (Leenhouts *et al*., 1996; Kloosterman *et al*., 2006a). In short, primer pairs czcD-KO-1/czcD-KO-2 and czcD-KO-3/czcD-KO-4 were used to generate approximately 600 bp PCR fragments of the left and right flanking regions of *czcD*, which were fused in a separate PCR reaction and cloned into the XbaI/BglII sites of pORI280 in *E. coli* EC1000, yielding pMP1. A *czcD* mutant in D39 (MP102) was obtained following the procedure described before (Kloosterman *et al*., 2006a), and the mutation was verified by PCR and sequencing.

Construction of lacZ *fusions*

Transcriptional fusions to *lacZ* were constructed in plasmid pPP2. Primer pairs PczcD-1/PczcD-2, spr1671-1/spr1671-2.1 and Pspr183-1.2/Pspr183-2.2 were used to generate PCR fragments spanning the *sczA–czcD*, *czcD–spr1671* intergenic regions and the *nrdD* promoter respectively, and cloned in the EcoRI/BamHI, XbaI/BamHI and EcoRI/BamHI sites of pPP2, using *E. coli* EC1000 as a host, yielding plasmids pMP2–pMP5. Correct constructs were transformed to D39 wild-type and MP100 to generate ectopic *lacZ* fusions in the *bgaA* locus, yielding strains MP103, MP105, MP106 and MP107, MP109 and MP110 respectively. The P*czcD–lacZ* fusion was introduced in the *czcD* mutant (MP102) as well, giving strain MP111.

Subcloning and mutation of the czcD *and* sczA *promoters*

Subclones (P*czcD*-1, P*czcD*-2, P*czcD*-2b, P*czcD*-3 and P*czcD*-4) of the *PczcD* were inserted in the EcoRI/BamHI sites of the *lacZ* reporter plasmid pPP2, using primer pairs PczcD_for1/PczcD_rev, PczcD_for2/PczcD_rev, PczcD_2b/PczcD_rev, PczcD_for3/PczcD_rev and PczcD_for4/PczcD_rev, giving plasmids pMP7 to pMP11. Four subsequent point-mutations were introduced into motif 2 in the *czcD* promoter as follows. PCR products were generated on D39 chromosomal DNA with primers PczcD-mut1.1/PczcD_for-1 and PczcD-mut1.2/PczcD_rev. These PCR products overlap for 17 bases and were fused in a separate PCR reaction with the outer primers PczcD-mut1.1 and PczcD_rev. The resulting PCR product was cloned as an EcoRI/BamHI fragment into pPP2, yielding plasmid pMP12. In the same way, four subsequent point-mutations were introduced in motif 2 in the *czcD* promoter, using primers PczcD-mut2.1/PczcD_for-1 and PczcD-mut2.2/PczcD_rev, yielding plasmid pMP15. The cloned sequences of these plasmids were verified by DNA sequencing, and they were subsequently transformed to D39 wild-type and its isogenic *sczA* mutant, giving strains MP120–MP126 and MP130–MP136.

The wild-type (P*sczA*-1) and mutated (P*sczA-mut*1) *sczA* promoters were amplified with primers PsczA-for1 and

Table 4. Strains and plasmids used in this study.

Strain/plasmid	Description	Reference or source
Strain		
S. pneumoniae		
D39	Serotype 2 strain, *cps2*	Avery *et al*. (1944), Lanie *et al*. (2007) lab. P. Hermans
D39*nisRK*	D39 Δ*bgaA*::*nisRK*; $Trmp^R$	Kloosterman *et al*. (2006a)
MP100	D39 Δ*sczA*; $Spec^R$	This work
MP101	D39*nisRK* Δ*sczA*; $Spec^R$	This work
MP102	D39 Δ*czcD*	This work
MP103	D39 Δ*bgaA*::P*czcD–lacZ*; Tet^R	This work
MP105	D39 Δ*bgaA*::P*spr1671–lacZ*; Tet^R	This work
MP106	D39 Δ*bgaA*::P*spr0183–lacZ*; Tet^R	This work
MP107	MP100 Δ*bgaA*::P*czcD–lacZ*; Tet^R $Spec^R$	This work
MP109	MP100 Δ*bgaA*::P*spr1671–lacZ*; Tet^R $Spec^R$	This work
MP110	MP100 Δ*bgaA*::P*spr0183–lacZ*; Tet^R $Spec^R$	This work
MP111	MP102 Δ*bgaA*::P*czcD–lacZ*; Tet^R	This work
MP112	MP103 with mariner insertion in *sczA* $Spec^R$	This work
MP120	D39 Δ*bgaA*::P*czcD*-1*–lacZ*; Tet^R	This work
MP121	D39 Δ*bgaA*::P*czcD*-2*–lacZ*; Tet^R	This work
MP122	D39 Δ*bgaA*::P*czcD*-2b*–lacZ*; Tet^R	This work
MP123	D39 Δ*bgaA*::P*czcD*-3*–lacZ*; Tet^R	This work
MP124	D39 Δ*bgaA*::P*czcD*-4*–lacZ*; Tet^R	This work
MP125	D39 Δ*bgaA*::P*czcD-mut*1*–lacZ*; Tet^R	This work
MP126	D39 Δ*bgaA*::P*czcD-mut*2*–lacZ*; Tet^R	This work
MP127	D39 Δ*bgaA*::P*sczA*-1*–lacZ*; Tet^R	This work
MP128	D39 Δ*bgaA*::P*sczA-mut*1*–lacZ*; Tet^R	This work
MP130	MP100 Δ*bgaA*::P*czcD*-1*–lacZ*; Tet^R $Spec^R$	This work
MP131	MP100 Δ*bgaA*::P*czcD*-2*–lacZ*; Tet^R $Spec^R$	This work
MP132	MP100 Δ*bgaA*::P*czcD*-2b*–lacZ*; Tet^R $Spec^R$	This work
MP133	MP100 Δ*bgaA*::P*czcD*-3*–lacZ*; Tet^R $Spec^R$	This work
MP134	MP100 Δ*bgaA*::P*czcD*-4*–lacZ*; Tet^R $Spec^R$	This work
MP135	MP100 Δ*bgaA*::P*czcD-mut*1*–lacZ*; Tet^R $Spec^R$	This work
MP126	MP100 Δ*bgaA*::P*czcD-mut*2*–lacZ*; Tet^R $Spec^R$	This work
MP137	MP100 Δ*bgaA*::P*sczA*-1*–lacZ*; Tet^R $Spec^R$	This work
MP138	MP100 Δ*bgaA*::P*sczA-mut*1*–lacZ*; Tet^R $Spec^R$	This work
L. lactis		
NZ9000	MG1363 Δ*pepN*::*nisRK*	Kuipers *et al*. (1998)
E. coli		
EC1000	Km^R; MC1000 derivative carrying a single copy of the pWV01 *repA* gene in *glgB*	Leenhouts *et al*. (1996)
Plasmid		
pR412-T7	SpecR; derivative of pR412 (Martin *et al*., 2000)	Bijlsma *et al*. (2007)
pORI13	Em^R; *ori*$^+$ *repA*$^-$; promoterless *lacZ*, for single-copy chromosomal *lacZ* fusions	Sanders *et al*. (1998)
pORI38	$Spec^R$; *ori*$^+$ *repA*$^-$; deletion derivative of pWV01	Leenhouts *et al*. (1996)
pNZ8048	Cm^R; nisin-inducible P*nisA*	de Ruyter *et al*. (1996)
pNG8048E	Cm^R Em^R; nisin-inducible P*nisA*, pNZ8048 derivative containing *em*^{R} gene to facilitate cloning	Laboratory collection
pORI280	Em^R; *ori*$^+$ *repA*$^-$; deletion derivative of pWV01; constitutive *lacZ* expression from P32 promoter	Leenhouts *et al*. (1996)
pPP2	Amp^R Tet^R; promoter-less *lacZ*. For replacement of *bgaA* (*spr0565*) with promoter–*lacZ* fusions. Derivative of pTP1	Halfmann *et al*. (2007)
pMP1	pORI280 Δ*czcD*	This work
pMP2	pPP2 P*czcD–lacZ*	This work
pMP3	pPP2 P*spr1671–lacZ*	This work
pMP5	pPP2 P*spr0183–lacZ*	This work
pMP6	pNG8048E carrying H_6-*sczA* downstream of P*nisA*	This work
pMP7	pPP2 P*czcD*-1*–lacZ*	This work
pMP8	pPP2 P*czcD*-2*–lacZ*	This work
pMP9	pPP2 P*czcD*-2b*–lacZ*	This work
pMP10	pPP2 P*czcD*-3*–lacZ*	This work
pMP11	pPP2 P*czcD*-4*–lacZ*	This work
pMP12	pPP2 P*czcD-mut*1*–lacZ*	This work
pMP13	pPP2 P*sczA*-1*–lacZ*	This work
pMP14	pPP2 P*sczA-mut*1*–lacZ*	This work
pMP15	pPP2 P*czcD-mut*2*-lacZ*	This work

$Trmp^R$, trimethoprim resistance; $Spec^R$, spectinomycin resistance; Em^R, erythromycin resistance; Tet^R, tetracycline resistance; Km^R, kanamycin resistance; Cm^R, chloramphenicol resistance.

Table 5. Oligonucleotide primers used in this study.

Name	Nucleotide sequence (5′ to 3′) (restriction enzyme sites underlined)	Restriction site
PczcD-1	CGGAATTCTAGATGGCTTTTTTGGTTTTGCTG	EcoRI
PczcD-2	CGGGATCCGCAGACTCAGAATAGACTCATTC	BamHI
PczcD_for1	CGGAATTCCTCGTAGCCCTTAGCATTCA	EcoRI
PczcD_for2	CGGAATTCCAAATTGTTCAGAACTGAAT	EcoRI
PczcD_2b	CGGAATTCTGTTAGTCATATGGACACTTAAGG	EcoRI
PczcD_for3	CGGAATTCGAACTGAATAAAGCTGACG	EcoRI
PczcD_for4	CGGAATTCAGCTGACGTTTTGCTTCTAT	EcoRI
PczcD_rev	CGGGATCCAACAGCATATTTTGCCTTCA	BamHI
PczcD-mut1.1	GGAGCCTTGTTCATTATCATTATCCAC	–
PczcD-mut1.2	ATAATGAACAAGGCTCCCATAAATCTATTATAACAAAGG	–
PczcD-mut2.1	**GACC**TAAAGCTGACGTTTTGCTTC	–
PczcD-mut2.2	CAAAACGTCAGCTTTA**GGTC**GTTCTGAACAATTTGCCTTAAGTG	–
czcD-KO-1	TGCTCTAGAAGGTCAATGTCTCGATAAAG	XbaI
czcD-KO-2	AGCATATTTTGCCTTCATATTTC	–
czcD-KO-3[a]	*ATATGAAGGCAAAATATGCT*AGTTATGAGCATCAACATTAG[a]	–
czcDKO-4	GAAGATCTCTGTAGCTGAGACAAGCGC	BglII
PsczA-for1	CGGGATCCCTCGTAGCCCTTAGCATTCA	BamHI
PsczA-rev	CGGAATTCAACAGCATATTTTGCCTTCA	EcoRI
Spr1673KO-1	CTAACAGATTGATAGTAATCG	–
Spr1673KO-2[b]	**TCCTCCTCACTATTTTGATTAG**ATACGGCGGTCAATGTTAGTC[b]	–
Spr1673KO-3[b]	**CGTTTTAGCGTTTATTTCGTTTAGT**TTATCTAGACCTTCTCATTCC[b]	–
Spr1673KO-4	CTGGACGGCAAGGGCTGGAC	–
spr1671-1	TGCTCTAGAAAGATTTTGCATCCGCAACC	XbaI
spr1671-2.1	CGGGATCCCGGCAGATTTAATATTCACAC	BamHI
spr1671-2	CGGGATCTCGGCAGATTTAATATTCACAC	–
spr1671-3	CATGCCATGGGAGCGCTTGTCTCAGCTACA	–
spr1671-4	GAAGATCTCAAAGCCCATACGCCCTCC	–
TMr_1	TGCATTTAATACTAGCGACGCCATCTATGTGTC	–
TMr_4	GGATCCATTCGCGTCAATTCGAGGGG	–
Spec_Fp	CTAATCAAAATAGTGAGGAGG	–
Spec_Rp	ACTAAACGAAATAAACGC	–
PspaC-1	CCAGTCCAGACTATTCGG	–
PspaC-2	CAGAGGTTGTTCTGG	–
Pspr183-1.2	CGGAATTCCAACCTAAGGTGATTGTGG	EcoRI
Pspr183-2.2	CGGGATCCGAATTTCTGTAATAATTCGC	BamHI
tetR-OX-1-H6	CGAGCCATCATGACTCATCATCATCATCATCATAACATTGACCG	RcaI
tetR-OX-2	CGGGATCCTCAATTTTTAGGAATGAGAAG	BamHI

a. Overlap with czcD-KO-2 in italics.
b. Overlap with *spec*R gene in bold.

PsczA-rev from plasmids pMP7 and pMP12 respectively, and cloned as EcoRI/BamHI fragments into pPP2. The resulting constructs were verified by DNA sequencing and introduced into strains D39 and MP100, yielding strains MP127, MP128, MP137 and MP138.

Construction of an SczA overexpression construct and purification of H_6-SczA

A his-tagged variant of *sczA* (*H_6-sczA*) was PCR amplified with primers tetR-OX-1-H6/tetR-OX-2, digested with RcaI and BamHI and cloned in the NcoI/BamHI sites of pNG8048E, using *Lactococcus lactis* NZ9000 as the cloning host, giving plasmid pMP6.

Overexpression in *L. lactis* and purification of H_6-SczA was performed essentially as described (Kloosterman *et al.*, 2006b). H_6-SczA was eluted from the Ni-NTA (Qiagen) beads with 250 mM imidazole. The protein was checked with SDS-PAGE and stored as frozen aliquots with 10% glycerol at –80°C.

Microarray analyses

For DNA microarray analysis, D39 wild-type and its isogenic *sczA* mutant (MP100) were grown as four biological replicates in GM17 with 0.05 mM $CoCl_2$ and harvested at an OD_{595} of approximately 0.3. All other procedures regarding microarray analyses were performed as described before (Kloosterman *et al.*, 2006b). Statistical analysis was performed as described (van Hijum *et al.*, 2005). A gene was considered differentially expressed when the Bayesian *P*-value < 0.001 and a false discovery rate < 0.01, and when at least five measurements were available. The raw and processed data are available at http://molgen.biol.rug.nl/publication/scza_data.

Growth experiments

Growth experiments were performed in microtiterplates in 220 μl volumes. As the inoculum, aliquots of cells frozen in the mid-exponential phase were used, washed once with the proper medium and diluted 1 to 40 for inoculation.

β-Galactosidase assays

β-Galactosidase assays were performed essentially as described (Kloosterman *et al*., 2006b). Strains were harvested at mid-logarithmic phase of growth. All experiments were performed at least *in triplo*.

SczA–DNA interaction studies

Electrophoretic mobility shift assays (EMSAs) were performed essentially as described previously (den Hengst *et al*., 2005). PCR products of P*czcD* (P*czcD*-1, P*czcD*-2, P*czcD*-2b, P*czcD*-4 and P*czcD-mut*1 and P*czcD-mut*2) were made with the primer pairs as given under 'subcloning of P*czcD*'. As a negative control, a PCR fragment of the P*spaC* promoter was amplified with primers Pspac-1/Pspac-2 from plasmid pDG148 (Stragier *et al*., 1988). The binding buffer was composed of 20 mM TrisHCl, pH 8.0, 5 mM $MgCl_2$, 0.1 mM dithiotreitol, 8.7% (w/v) glycerol, 62.5 mM KCl, 25 μg ml^{-1} bovine serum albumin, 50 μg ml^{-1} poly(dI–dC) and 3000 cpm of either [γ-^{32}P]ATP-labelled or [γ-^{33}P]ATP-labelled PCR product. Purified H_6-SczA, EDTA and metal ions were added as specified in the *Results* section. Reactions (20 μl) were incubated for 15 min at 37°C, after which they were run on a 6% poly-acrylamide gel for 80 min at 90 V. DNase I footprinting was performed essentially as described (den Hengst *et al*., 2005). In total, 150 000 cpm of [γ-^{33}P]ATP-labelled PCR product P*czcD*-1, amplified either with [γ-^{33}P]-labelled primer PczcD-for1 (forward strand) or with [γ-^{33}P]-labelled primer PczcD-rev (reverse strand), was used as probe in 40 μl of binding buffer containing H_6-SczA, EDTA and Zn^{2+} as specified in the *Results*. Buffer and reaction conditions were the same as for the EMSAs.

Reverse transcription (RT)-PCR

Reverse transcription reactions were performed as described under *Microarray analysis* on total RNA isolated from *S. pneumoniae* D39 grown in GM17 + 0.05 mM Co^{2+}, except that amino-allyl dUTP was replaced by dTTP. In parallel, reactions were performed in the same way, except that the reverse transcriptase enzyme Superscript III was omitted. These reactions were used as negative controls. PCRs were performed on 1/100 part of the RT reactions with primers as specified in the *Results*. As a positive control, PCRs were performed on 10 ng per reaction of chromosomal DNA of D39.

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