Opposite effects of Mn2+ and Zn2+ on PsaR-Mediated expression of the virulence genes pcpA, prtA, and psaBCA of Streptococcus pneumoniae

Kloosterman, Tomas G.; Witwicki, Robert M.; van der Kooi-Pol, Magdalena M.; Bijlsma, Jetta J. E.; Kuipers, Oscar

Published in:
Journal of Bacteriology

DOI:
10.1128/JB.00307-08

Publication date:
2008

JOURNAL OF BACTERIOLOGY, Aug. 2008, p. 5382–5393 Vol. 190, No. 15
0021-9193/08/$08.00+0 doi:10.1128/JB.00307-08

Opposite Effects of Mn^{2+} and Zn^{2+} on PsaR-Mediated Expression of the Virulence Genes *pcpA*, *prtA*, and *psaBCA* of *Streptococcus pneumoniae*[▽]

Tomas G. Kloosterman, Robert M. Witwicki, Magdalena M. van der Kooi-Pol, Jetta J. E. Bijlsma,† and Oscar P. Kuipers*

Department of Molecular Genetics, University of Groningen, Groningen Biomolecular Sciences and Biotechnology Institute, P.O. Box 14, 9750 AA Haren, The Netherlands

Received 28 February 2008/Accepted 22 May 2008

Homeostasis of Zn^{2+} and Mn^{2+} is important for the physiology and virulence of the human pathogen *Streptococcus pneumoniae*. Here, transcriptome analysis was used to determine the response of *S. pneumoniae* D39 to a high concentration of Zn^{2+}. Interestingly, virulence genes encoding the choline binding protein PcpA, the extracellular serine protease PrtA, and the Mn^{2+} uptake system PsaBC(A) were strongly upregulated in the presence of Zn^{2+}. Using random mutagenesis, a previously described Mn^{2+}-responsive transcriptional repressor, PsaR, was found to mediate the observed Zn^{2+}-dependent derepression. In addition, PsaR is also responsible for the Mn^{2+}-dependent repression of these genes. Subsequently, we investigated how these opposite effects are mediated by the same regulator. In vitro binding of purified PsaR to the *prtA*, *pcpA*, and *psaB* promoters was stimulated by Mn^{2+}, whereas Zn^{2+} destroyed the interaction of PsaR with its target promoters. Mutational analysis of the *pcpA* promoter demonstrated the presence of a PsaR operator that mediates the transcriptional effects. In conclusion, PsaR is responsible for the counteracting effects of Mn^{2+} and Zn^{2+} on the expression of several virulence genes in *S. pneumoniae*, suggesting that the ratio of these metal ions exerts an important influence on pneumococcal pathogenesis.

The human pathogen *Streptococcus pneumoniae* is a commensal of the nasopharynx, but it can become virulent and infect the lungs, middle ear, brain, and bloodstream, causing severe diseases such as pneumonia, otitis media, meningitis, or sepsis (see references 10 and 61 for reviews). Little is known about the specific environmental changes that *S. pneumoniae* encounters during infection of the human body and the way in which these affect virulence. One important environmental factor is probably the concentration of metal ions, which form a class of nutrients that are important for bacteria in small amounts but are often toxic in larger amounts (9, 20, 57, 58).

An important metal ion that *S. pneumoniae* could face is Zn^{2+}, which is present in the human body in concentrations ranging from a few μM to over 100 μM (81). In the host, Zn^{2+} is of great importance for immunity, as it is necessary for proper functioning of immune cells (28), and mild Zn^{2+} deficiency severely affects immune function (72). Zn^{2+} levels are elevated during inflammation (53), and Zn^{2+} administration reduces airway infections in children in developing countries (71). Moreover, Zn^{2+} deficiency results in an increased risk of pneumococcal infection and death in mice (75) and in a lower immune response to the pneumococcal antigen PspA (76). Interestingly, several studies suggest that the use of and response to metal ions, such as Zn^{2+}, Mn^{2+}, and Fe^{2+}, is important for the virulence and physiology of pathogenic streptococci (3, 11, 12, 18, 30, 32, 39, 40, 46, 50, 54, 55, 63, 64, 66, 67).

In *S. pneumoniae* several systems dedicated to the acquisition of specific metal ions have been studied. These are the PsaBCA Mn^{2+} transporter (18, 44, 50), the AdcCBA Zn^{2+} uptake system (18, 19), and three iron uptake loci (*piaABCD*, *piuBCDA*, and *pit*) (11, 12). The PsaBCA complex is involved in virulence, oxidative stress, penicillin stress, competence, and adhesion via interaction with human E-cadherin (2, 6, 18, 32, 48, 50, 59, 60, 79). Expression of the *psaBCA* genes is repressed by the DtxR family regulator PsaR in response to high Mn^{2+} concentrations (31). *S. pneumoniae* also contains systems involved in cation efflux (58), such as *czcD*, which has recently been shown to be responsible for Zn^{2+} resistance (39). The presence in *S. pneumoniae* of this Zn^{2+} efflux system together with the AdcCBA Zn^{2+} uptake system indicates that this pathogen has to deal with fluctuating Zn^{2+} levels in the human body.

Therefore, we explored the response of *S. pneumoniae* to a high Zn^{2+}concentration by means of transcriptome analysis. Interestingly, Zn^{2+} was found to increase expression of several genes involved in virulence, namely, *prtA*, *pcpA*, and *psaBC*, which could be counteracted by Mn^{2+}. Subsequent research showed that the transcriptional regulator PsaR is directly responsible for the Mn^{2+}-dependent repression and the Zn^{2+}-dependent derepression of these genes.

MATERIALS AND METHODS

DNA techniques, β-galactosidase assays, bacterial strains, and growth conditions. All DNA manipulation techniques, growth conditions, and media were the same as described previously (37, 38) unless indicated otherwise. β-Galactosidase assays were performed as described previously (38).

* Corresponding author. Mailing address: Department of Molecular Genetics, University of Groningen, Groningen Biomolecular Sciences and Biotechnology Institute, P.O. Box 14, 9750 AA Haren, The Netherlands. Phone: 31-50-3632093. Fax: 31-50-3632348. E-mail: o.p.kuipers@rug.nl.

† Present address: Molecular Bacteriology, Department of Medical Microbiology, University Medical Centre Groningen, Hanzeplein 1, 9713 GZ Groningen, The Netherlands.

▽ Published ahead of print on 30 May 2008.

Chelex-treated M17 was prepared by autoclaving 2% Chelex 100 resin (Bio-Rad) with M17, followed by 2 h of stirring. After removal of the resin, 50 μM $CaCl_2$, 50 μM $MgCl_2$, 5 μM $FeCl_3$, and 0.25% glucose were added, and the resulting medium, designated GM17chel, was used as growth medium as specified in Results. Metal ions were added as the salts $ZnSO_4$, $MnCl_2$, $MgCl_2$, $CaCl_2$, $NiCl_2$, $CuSO_4$, and $FeSO_4$. The strains and plasmids used or constructed in this study are listed in Table 1. Primers are listed in Table 2. Since most work presented in this paper was carried out before publication of the D39 genome sequence (43), primer sequences are based on the R6 genome sequence (27).

Construction of transcriptional *lacZ* fusions. Ectopic *lacZ* fusions to the *pcpA* (spr1945), *psaR* (spr1480), *psaB* (spr1492), *nrdD* (spr0183), and spr0276 promoters were made in pPP2 with primer pairs pspr1945-1/pspr1945-2, marR-lacZ1/marR-lacZ2, PpsaB-1-lacZ/PpsaB-2-lacZ, Pspr183-1.2/Pspr183-2.2, and Pspr0267-1/Pspr0276-2, yielding plasmids pRW5, pRW4, pRW6, pRW3, and pRW2. A chromosomal *lacZ* fusion to the 3′ end of *prtA* (spr0561) was made with primer pair spr0561-1/spr0561-2 in plasmid pORI13, leading to plasmid pRW1. In all cases *Escherichia coli* EC1000 was used as the cloning host. The *lacZ* fusion constructs were introduced into wild-type D39 and D39 Δ*psaR* (RW100) in the case of pPP2 (integration via double crossover in the *bgaA* gene) and into D39*nisRK* and its isogenic *psaR* mutant (RW101) in the case of pORI13 (integration by single crossover), giving strains RW102 to RW113. The P*pcpA-lacZ* fusion was also introduced into the *psaCA* (RW121) and *czcD* (MP102) deletion strains, giving strains RW114 and RW122. All plasmid constructs were checked by sequencing, and new loci created with these plasmids were verified by PCR.

Random mutagenesis screen. Random mutagenesis using the *Himar1* MarC9 mariner transposon was performed essentially as described previously (42, 49). pR412-T7 (8), a derivative of pR412, was used as the source of the *spec* mariner transposon. Mutated R6 chromosomal DNA was transformed into strain R6, yielding a mutant library of approximately 20,000 CFU. Chromosomal DNA of this library was used to perform random mutagenesis in strain D39 Δ*bgaA*::P*pcpA-lacZ*. Mutants were selected on GM17 with 1% sheep blood, 0.006% X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 1.5 μg/ml tetracycline, and 130 μg/ml spectinomycin. Transposon insertion sites in mutants showing derepression of expression of the P*pcpA-lacZ* transcriptional fusion were identified by the direct sequencing method as described previously (34), using primer PBMrTn1 with PBMrIRPi (nested).

Construction of deletion strains. An in-frame marker-free deletion of *psaR* (spr1480) was constructed with plasmid pORI280 essentially as described previously (37), using primer pairs marR-del1/marR-del2 and marR-del3/marR-del4 and *E. coli* EC1000 as the cloning host. The mutant was checked by PCR and DNA sequencing.

A deletion strain of *psaCA* was made with primer pairs psaCA-KO-1/psaCA-KO-2 and psaCA-KO-1/psaCA-KO-4 by overlap extension PCR (73) and allelic replacement with an erythromycin resistance cassette, yielding strain RW121.

Construction of *pcpA* promoter subclones in pPP2. The following promoter subclones of the *pcpA* promoter were made in pPP2 (primer pairs are in parentheses): P*pcpA*-3 (PpcpA_f_2 and PpcpA_rev_1), P*pcpA*-3a (PpcpA_f_3a and PpcpA_rev_1), P*pcpA*-4 (PpcpA_f_3 and PpcpA_rev_1), P*pcpA*-5 (PpcpA_f_4 and PpcpA_rev_1), P*pcpA*-rev3 (spr1945-1and PpcpA_rev_3), and P*pcpA*-rev4 (spr1945-1 and PpcpA_rev_4). All fragments were cloned as EcoRI/BamHI fragments in the same sites of pPP2, yielding plasmids pRW11 to pRW16. The constructs were sequenced and introduced into strains D39 and D39Δ*psaR* (RW100), giving strains RW131 to RW136 and RW141 to RW146.

Construction of point mutations in the PsaR binding site in P*pcpA*. A pNG8048E derivative containing a 64-bp fragment of the *pcpA* promoter comprising the PsaR binding site was constructed by annealing oligonucleotides P-pcpA-box1/P-pcpA-box2 and cloning them into NcoI/XbaI-digested pNG8048E, giving plasmid pRW21. This plasmid was used as a template for a PCR with Phusion DNA polymerase using the primer pairs PcpA_mut1.1/PcpA_mut1.2, PpcpA_mut2.1/PpcpA_mut2.2, and PpcpA_mut3.1/PpcpA_mut3.2, giving rise to plasmids pRW22 and pRW23, each with one point mutation in the predicted PsaR binding site, and pRW24, with two point mutations in the predicted PsaR binding site, respectively. The constructs were checked by DNA sequencing.

Microarray analyses. For microarray analysis, the transcriptome of wild-type D39 grown in four biological replicates in GM17 was compared to the transcriptome of the same strain grown in four biological replicates in GM17 plus 0.25 mM $ZnSO_4$. Growth, RNA isolation, labeling, hybridization, and analysis of slides were done essentially as described previously (38). Since the analysis was performed by interslide comparisons (i.e., the wild-type transcriptome analyzed with four slides was compared to the wild-type transcriptome in GM17 plus Zn^{2+} analyzed with four different slides), scaled signals from PostPrep of the slides hybridized to the GM17 samples were pairwise compared with the scaled signals of the slides hybridized to the GM17-Zn^{2+} samples. The resultant table with the scaled (and normalized) signals was used as input for the CyberT variant for statistical analysis of control versus experimental data, using a local running copy of the CyberT algorithm for paired data (http://molgen.biol.rug.nl/cgi-bin/cybert/CyberT-8.0.form.pl?DATATYPE=CE). Genes were considered differentially expressed when the Bayesian *P* value was <0.001 and the false discovery rate was <0.01, unless otherwise stated (80).

Overexpression and purification of Strep-tagged PsaR. For the overexpression of a C-terminally Strep-tagged variant of PsaR, *psaR* was amplified from D39 chromosomal DNA using primers Spr1480OX-1 and Spr1480-Ctermstrep_OX. The resulting PCR product was digested with RcaI/XbaI and cloned into the NcoI/XbaI sites of pNG8048E, yielding plasmid pRW25. Overexpression in *Lactococcus lactis* NZ9000 was done essentially as described previously (41). Purification of PsaR-Strep from *L. lactis* was performed using the Streptactin column from IBA according to the supplier's instructions. Buffers without EDTA were used, and the purified protein was stored at a concentration of 0.15 mg/ml in the elution buffer (100 mM Tris-HCl [pH 8], 150 mM NaCl, 2.5 mM desthiobiotin, 1 mM β-mercaptoethanol) with 10% glycerol at −80°C.

EMSAs. Electrophoretic mobility shift assays (EMSAs) were performed with [γ-^{33}P]ATP-labeled probes in buffer containing 20 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 0.1 mM dithiothreitol, 8.7% (wt/vol) glycerol, 62.5 mM KCl, 25 μg/ml bovine serum albumin, 25 μg/ml poly(dI-dC), and 3000 cpm of [γ-^{33}P]ATP-labeled PCR product. Various metal ions were added in concentrations as specified in Results. As probes, PCR products comprising the promoter regions of *psaB* (primers PpsaB-1-lacZ/PpsaB-2-lacZ), *pcpA* (PpcpA_f_2/PpcpA_rev_1), and *prtA* (PprtA-1/PprtA-2) were used. As a control, a PCR fragment of the *pcpA* promoter without the intact PsaR operator was used (PpcpA_f_4/PpcpA_rev_1). Reaction mixtures were incubated at 37°C for 10 min before loading on gels. Gels were run in 0.44 M Tris-borate buffer (pH 8.3) at 100 V for 90 min.

Measurement of concentrations of Mn^{2+} and Zn^{2+} in growth media. Using atomic absorption spectroscopy on a Vista AX-CCD simultaneous ICP-AES spectrometer, the concentrations of Mn^{2+} and Zn^{2+} in GM17 and GM17chel were determined at wavelengths (nm) of 257.610/259.372 (Mn) and 202.548/213.857 (Zn).

Microarray accession number. Microarray data have been deposited to the Gene Expression Omnibus (GEO) and can be accessed via http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE11438. In addition, slide images and raw and processed data are available at http://molgen.biol.rug.nl/publication/zinc_data/.

RESULTS

Identification of Zn^{2+}-regulated genes in *S. pneumoniae*. To identify genes that are regulated by a high level of Zn^{2+} in *S. pneumoniae*, we compared the transcriptome of wild-type D39 grown in GM17 with that of the same strain grown in GM17 with 0.25 mM $ZnSO_4$ (Table 3). With this concentration of Zn^{2+}, growth is not affected (39). The most upregulated genes were *pcpA*, encoding a choline binding protein (69) involved in virulence (31), and Zn^{2+} resistance gene *czcD* (39). Also upregulated were the serine protease gene *prtA* and the Mn^{2+} ABC transporter genes *psaBC*, which are both involved in virulence (6, 7, 32, 50), and an operon (SP0202 to SP0207) encoding, among other proteins, the NrdDG ribonucleoside triphosphate reductase, which is involved in synthesis of deoxyribonucleoside triphosphates (33, 78). An operon consisting of genes involved in cellobiose metabolism (SP0303 to SP0310) (52) was strongly downregulated. Thus, expression of genes with a variety of functions is affected by growth of *S. pneumoniae* in medium with an elevated Zn^{2+} concentration.

Zn^{2+}-dependent expression of *pcpA* is mediated by PsaR. To investigate in more detail the transcriptional regulation of *pcpA*, the most Zn^{2+}-induced gene found in the microarray experiment, a transcriptional *lacZ* fusion to the *pcpA* promoter was constructed by use of plasmid pPP2, which integrates into the *bgaA* locus (22). Using strain D39 containing this P*pcpA-lacZ* transcriptional fusion, it was demonstrated that only ele-

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference or source
S. pneumoniae		
D39	Serotype 2 strain, *cps2*	4, 43; laboratory of P. Hermans
R6	D39 (cps2 2538–9862) with increased transformation efficiency	27
D39*nisRK*	D39 Δ*bgaA*::*nisRK*; Trmp^r	37
MP102	D39 Δ*czcD*	This work
RW100	D39 Δ*psaR*	This work
RW101	D39*nisRK* Δ*psaR*	This work
RW102	D39 Δ*bgaA*::P*pcpA-lacZ*; Tet^r	This work
RW103	D39 Δ*bgaA*::P*nrdD-lacZ*; Tet^r	This work
RW104	D39*nisRK prtA-lacZ*; Erm^r	This work
RW105	D39 Δ*bgaA*::Pspr0276-*lacZ*; Tet^r	This work
RW106	D39 Δ*bgaA*::P*psaR-lacZ*; Tet^r	This work
RW107	RW100 Δ*bgaA*::P*pcpA-lacZ*; Tet^r	This work
RW108	RW100 Δ*bgaA*::P*nrdD-lacZ*; Tet^r	This work
RW109	RW101 *prtA-lacZ*; Erm^r	This work
RW110	RW100 Δ*bgaA*::Pspr0276-*lacZ*; Tet^r	This work
RW111	RW100 Δ*bgaA*::P*psaR-lacZ*; Tet^r	This work
RW112	D39 Δ*bgaA*::P*psaB-lacZ*; Tet^r	This work
RW113	RW100 Δ*bgaA*::P*psaB-lacZ*; Tet^r	This work
RW114	RW121 Δ*bgaA*::P*pcpA-lacZ*; Tet^r	This work
RW120	RW103 with mariner insertion in *psaR*; Spec^r	This work
RW121	D39 Δ*psaCA*::*ermR*; Erm^r	This work
RW122	MP102 Δ*bgaA*::P*pcpA-lacZ*; Tet^r	This work
RW131	D39 Δ*bgaA*::P*pcpA*-3-*lacZ*; Tet^r	This work
RW132	D39 Δ*bgaA*::P*pcpA*-3a-*lacZ*; Tet^r	This work
RW133	D39 Δ*bgaA*::P*pcpA*-4-*lacZ*; Tet^r	This work
RW134	D39 Δ*bgaA*::P*pcpA*-5-*lacZ*; Tet^r	This work
RW135	D39 Δ*bgaA*::P*pcpA*-rev3-*lacZ*; Tet^r	This work
RW136	D39 Δ*bgaA*::P*pcpA*-rev4-*lacZ*; Tet^r	This work
RW141	RW100 Δ*bgaA*::P*pcpA*-3-*lacZ*; Tet^r	This work
RW142	RW100 Δ*bgaA*::P*pcpA*-3a-*lacZ*; Tet^r	This work
RW143	RW100 Δ*bgaA*::P*pcpA*-4-*lacZ*; Tet^r	This work
RW144	RW100 Δ*bgaA*::P*pcpA*-5-*lacZ*; Tet^r	This work
RW145	RW100 Δ*bgaA*::P*pcpA*-rev3-*lacZ*; Tet^r	This work
RW146	RW100 Δ*bgaA*::P*pcpA*-rev4-*lacZ*; Tet^r	This work
E. coli EC1000	Km^r; MC1000 derivative carrying a single copy of the pWV01 *repA* gene in *glgB*	45
L. lactis NZ9000	MG1363 Δ*pepN*::*nisRK*	41
Plasmids		
pR412T7	Spec^r; derivative of pR412 (49)	8
pORI13	Erm^r; *ori*$^+$ *repA*$^-$; promoterless *lacZ*, for single-copy chromosomal *lacZ* fusions.	70
pORI280	Erm^r; *ori*$^+$ *repA*$^-$; deletion derivative of pWV01; constitutive *lacZ* expression from P32 promoter	45
pPP2	Amp^r Tet^r; promoterless *lacZ*; for replacement of *bgaA* (spr0565) with promoter-*lacZ* fusions; derivative of pPP1	22
pNZ8048	Cm^r; nisin-inducible P*nisA*	16
PNG8048E	Cm^r Erm^r; nisin-inducible P*nisA*, pNZ8048 derivative containing Erm^r gene to facilitate cloning	Laboratory collection
pRW1	pORI13::*prtA-lacZ*	This work
pRW2	pPP2 Pspr0276	This work
pRW3	pPP2 P*nrdD*	This work
pRW4	pPP2 P*psaR*	This work
pRW5	pPP2 P*pcpA*	This work
pRW6	pPP2 P*psaB*	This work
pRW11	pPP2 P*pcpA*-3	This work
pRW12	pPP2 P*pcpA*-3a	This work
pRW13	pPP2 P*pcpA*-4	This work
pRW14	pPP2 P*pcpA*-5	This work
pRW15	pPP2 P*pcpA*-rev3	This work
pRW16	pPP2 P*pcpA*-rev4	This work
pRW20	pORI280 Δ*psaR*	This work
pRW21	pNG8048E containing a 64-bp fragment comprising the PsaR binding site	This work
pRW22	pRW12 containing a point mutation (T→G, bp −186[b]) in the PsaR binding box	This work
pRW23	pRW12 containing a point mutation (A→G, bp −184[b]) in the PsaR binding box	This work
pRW24	pRW12 containing two point mutations (A→G, bp −184[b]; A→C, bp −185[b]) in the PsaR binding box	This work
pRW25	pNG8048E carrying *psaR-strep* downstream of P*nisA*	This work

[a] Erm^r, erythromycin resistance; Tet^r, tetracycline resistance; Cm^r, chloramphenicol resistance; Spec^r, spectimomycin resistance.
[b] Where the first base of the *pcpA* start codon (ATG) is +1.

TABLE 2. Oligonucleotide primers used in this study

Primer	Nucleotide sequence (5′ to 3′)	Restriction site
PBMrIRPi	AGACCGGGGACTTATCAGCC	
PBMrTn1	CTAGCGACGCCATCTATGTG	
TMr_1	TGCATTTAATACTAGCGACGCCATCTATGTGTC	
TMr_4	GGATCCATTCGCGTCAATTCGAGGGG	
PpsaB-1-lacZ	CGGAATTCTTCCAAGTTTTTTACACTTG	EcoRI
PpsaB-2-lacZ	CGGGATCCATTGTTGGTCCATGGAGCAC	BamHI
pspr1945-1	CGGAATTCCCTTCAAATTTTAAGTCC	EcoRI
pspr1945-2	CGGGATCCGTTAATGATAATATTGTAG	BamHI
PpcpA_f_2	CGGAATTCTAATTTCTTTTTTAACCCAC	EcoRI
PpcpA_f_3	CGGAATTCGTGGGTTAATTTTCCTTGAC	EcoRI
PpcpA_f_3a	CGGAATTCTAAAAAAGAAATTAAAGTGG	EcoRI
PpcpA_f_4	CGGAATTCAATGTACTACTCTATTCTAC	EcoRI
PpcpA_rev_1	CGGGATCCAGGATTGGTTCATTAGGGAC	BamHI
PpcpA_rev_3	CGGGATCCGTCAAGGAAAATTAACCCAC	BamHI
PpcpA_rev_4	CGGGATCCCTAGTAGAATAGAGTAGTAC	BamHI
marR-lacZ1	CGGAATTCGCTATTTTCGTCATATCC	EcoRI
marR-lacZ2	CGGGATCCCATTTTAGATAGTCTTCTTTG	BamHI
marR-del1	TGCTCTAGACAATTGCCCACCAGTCCCG	XbaI
marR-del2	TTCTTTGTTTGGGGTCATTC	
marR-del3	GACCCCAAACAAAGAAGTCGAGAAAATCAACTAAT	
marR-del4	GAAGATCTCTTTTGTCAGCTGAACGA	BglII
PprtA-1	CGGAATTCTAGCGCTGATATTTCATC	
PprtA-2	CGGGATCCGTTAGTGACAATACTGTG	
spr0561-1	CGGAATTCGATACGGGAGAGGTAAGTG	EcoRI
spr0561-2	CGGGATCCGAAATTGTCTCATCACCTC	BamHI
PSpr183-1.2	CGGAATTCCAACCTAAGGTGATTGTGG	EcoRI
PSpr183-2.2	CGGGATCCGAATTTCTGTAATAATTCGC	BamHI
Pspr0276-1	CGGAATTCGAATTAGAATTATTGTGAG	BamHI
Pspr0276-2	CGGGATCCGAGCAGCAACAGCACCACC	XbaI
Ery-for	TAACGATTATGCCGATAACT	
Ery-rev	GCATGCATCGATTAGATCTC	
psaCA-KO-1	AAATCCTTACACCGAATTGC	
psaCA-KO-2	GAGATCTAATCGATGCATGCTTCTGCAATCATAGGTCACCTCC	
psaCA-KO-3	AGTTATCGGCATAATCGTTAGACAAGATTGCTGAAGGATTGG	
psaCA-KO-4	TGCGCGTGCTAATAGGTGCC	
P-pcpA-box1	CATGTCTTTTTTAACACGGGTTAAAAAAGAAATTAAAGTGGGTTAATTTTCCTTGACTT AAAATTTAA	
P-pcpA-box2	CTAGTTAAATTTTAAGTCAAGGAAAATTAACCCACTTTAATTTCTTTTTTAACCCGTGT TAAAAAAGA	
PcpA_mut1.1	GATTTCTTTTTTAACCCGTG	
PcpA_mut1.2	AAAGTGGGTTAATTTTCCTTG	
PpcpA_mut2.1	GTAATTTCTTTTTTAACCCGTG	
PpcpA_mut2.2	AGTGGGTTAATTTTCCTTGAC	
PpcpA_mut3.1	GCAATTTCTTTTTTAACCCGTG	
PpcpA_mut3.2	AGTGGGTTAATTTTCCTTGAC	
Spr1480OX-2	TGCTCTAGATTAGTTGATTTTCTCGACATAG	XbaI
spr1480-OX-new	CGAGCCATCATGAAACATCATCATCATCATCATACCCCAAACAAAGAAGACTATC	RcaI
Spr1480-Ctermstrep_OX	TGCTCTAGATTATTTTTCAAATTGTGGATGGCTCCAAGCGCTGTTGATTTTCTCGACAT AGAGTTG	XbaI
Spr1480OX-1	CGAGCCATCATGACCCCAAACAAAGAAGAC	RcaI

vated Zn^{2+} concentrations lead to high expression from the *pcpA* promoter, although also some weak induction could be seen for Co^{2+} and Fe^{2+} (Table 4).

To identify the factor repressing transcription of *pcpA* under normal growth conditions, strain D39 P*pcpA-lacZ* (RW102) was randomly mutagenized with the mariner transposon (42, 49) and blue colonies were screened for on GM17 agar plates with X-Gal but without Zn^{2+} supplementation. Among 7,200 CFU, one blue clone was found, containing a transposon insertion in the gene encoding the MarR family transcriptional regulator PsaR (spr1480, SPD_1450, SP1638). The insertion in *psaR* gave rise to high expression of the P*pcpA-lacZ* fusion, which was independent of Zn^{2+} (data not shown). We constructed a markerless deletion mutant of *psaR* and found that in this mutant expression of P*pcpA-lacZ* is highly derepressed independent of Zn^{2+} (Table 5), suggesting that PsaR is responsible for Zn^{2+}-dependent derepression of *pcpA* expression.

PsaR regulates *prtA* and *psaBCA* in a Zn^{2+}-dependent way. To test whether PsaR is also responsible for the Zn^{2+}-dependent expression of the other genes identified in the microarray analysis, transcriptional *lacZ* fusions to *prtA*, P*psaB*, and P*nrdD* (Pspr0183) were constructed and introduced in both the wild type and the *psaR* mutant. As expected, in the wild type expression of *prtA* and P*psaB* increased upon addition of Zn^{2+} to the GM17 growth medium (Tables 6 and 7). In the *psaR*

TABLE 3. Summary of transcriptome comparison of *S. pneumoniae* strain D39 grown in GM17 and in GM17 with addition of 0.25 mM Zn^{2+}

TIGR4 locus tag	Function (TIGR annotation)	Ratio[a]
SP0202	Anaerobic ribonucleoside triphosphate reductase NrdD	2.5
SP0204	Predicted acetyltransferase, GNAT family	2.4
SP0205	Anaerobic ribonucleoside triphosphate reductase activating protein NrdG	1.8[b]
SP0206	Hypothetical protein; predicted uridine kinase	1.8[b]
SP0207	Hypothetical protein; predicted uridine kinase	2.2
SP0303	6-Phospho-β-glucosidase BglA	−7.6
SP0305	Phosphotransferase system cellobiose-specific component IIB	−4.1
SP0306	Putative transcriptional regulator; possible antiterminator BglG	−14.8
SP0307	Phosphotransferase system, IIA component	−6.3[b]
SP0308	Phosphotransferase system cellobiose-specific component IIA	−3.0
SP0309	Hypothetical protein	−7.6
SP0310	Phosphotransferase system cellobiose-specific component IIC	−6.0
SP0338	Putative ATP-dependent *clp* protease, ATP binding subunit ClpL	−2.0
SP0515	Heat-inducible transcription repressor HrcA	−2.0
SP0516	Heat shock protein GrpE	−1.6
SP0517	Chaperone protein DnaK (heat shock protein 70)	−1.4
SP0518	Hypothetical protein	−2.1
SP0519	Chaperone protein DnaJ	−1.4
SP0640	Hypothetical protein	1.5[b]
SP0641	Cell wall-associated serine proteinase precursor PrtA	2.7
SP0645	Putative phosphotransferase system IIA component	2.8
SP0646	Phosphotransferase system, IIB component, putative	2.4
SP0879	Hypothetical protein	−2.1
SP1648	Manganese (and/or zinc) ABC transporter, ATP binding protein PsaB	2.3
SP1649[c]	Manganese (and/or zinc) ABC transporter, permease protein PsaC	2.6
SP1762	Hypothetical protein	2.7
SP1855	Alcohol dehydrogenase, zinc-containing AdhB	1.7
SP1856	Transcriptional regulator, MerR family	2.0[b]
SP1857	Cation efflux system protein CzcD	7.2
SP1935	Hypothetical protein	2.8
SP2136	Choline binding protein; surface protein PcpA	8.5

[a] Ratios of >2.0 or <−2.0 (wild-type D39 compared to wild-type D39 plus 0.25 mM Zn^{2+}) are shown; in some cases neighboring genes with lower fold changes are also indicated.
[b] Ratio with a false discovery rate of >0.01 but <0.1.
[c] For SP1650 (*psaA*), the number of observations (replicates) was too low and hence no significance was obtained.

mutant expression was derepressed in GM17, showing that PsaR mediates the Zn^{2+}-dependent expression of *prtA* and *psaBCA* as well. Transcription from P*nrdD* was two- to three-fold higher in cells grown in GM17 with Zn^{2+} compared to GM17, but this was not affected by the *psaR* mutation (data not shown). We also tested the effect of metal ions on the expression of *psaR* itself. A P*psaR-lacZ* transcriptional fusion was highly expressed in GM17 but was not influenced by Zn^{2+} and Mn^{2+} or by the *psaR* mutation (data not shown).

TABLE 4. Expression of P*pcpA* specifically in the presence of various metal cations[a]

Addition to GM17 (mM)	Mean (SD) β-galactosidase activity (Miller units)
None	4 (1)
Zn^{2+} (0.2)	54 (12)
Zn^{2+} (0.4)	388 (45)
Cu^{2+} (0.05)	3 (1)
Cu^{2+} (0.1)	3 (1)
Co^{2+} (0.05)	8 (3)
Co^{2+} (0.1)	16 (3)
Ni^{2+} (0.1)	4 (1)
Ni^{2+} (0.4)	5 (2)
Fe^{2+} (0.1)	9 (2)
Fe^{2+} (0.4)	21 (6)
Mg^{2+} (1.0)	4 (1)
Mg^{2+} (10)	2 (1)

[a] β-Galactosidase of a P*pcpA-lacZ* transcriptional fusion was measured in the wild-type strain D39 (strain RW102) grown in GM17 with the indicated metal ions. For Zn^{2+}, Ni^{2+}, Co^{2+}, and Cu^{2+} the concentrations used have similar effects on growth (see also reference 39). Values are from three experiments.

The expression of the cellobiose utilization operon SP303 to SP310 (spr0276 to spr0282, R6 annotation) was further analyzed using a Pspr0276-*lacZ* transcriptional fusion. Surprisingly, in GM17 the expression was hardly measurable, and no effect of Zn^{2+} was observed (data not shown). However, in the absence of glucose as well as in the presence of cellobiose, Pspr0276 was highly expressed (data not shown). It is not immediately clear what the reason is for the downregulation of this operon in the transcriptome analysis.

Regulation of P*psaB*, P*pcpA*, and *prtA* depends on the balance between Mn^{2+} and Zn^{2+}. In an earlier study, expression of *pcpA* and *psaBCA* was demonstrated to be repressed by PsaR in the presence of Mn^{2+} (31). To specify how Mn^{2+} and Zn^{2+} influence PsaR activity, we analyzed the expression of transcriptional *lacZ* fusions to P*pcpA*, P*psaB*, and *prtA* in the presence of various concentrations of both Mn^{2+} and Zn^{2+} in GM17 medium and in GM17 medium treated with the metal ion chelator Chelex 100 resin (GM17chel). GM17 contains 2.0 μM Mn^{2+} and 8.1 μM Zn^{2+} and GM17chel contains 0.02 μM Mn^{2+} and 0.00 μM Zn^{2+}, showing that the Chelex treatment effectively removed these cations. In GM17, upregulation of the expression of all three *lacZ* fusions in the presence of a high concentration of Zn^{2+} was nullified by the addition of

TABLE 5. β-Galactosidase activities of the wild-type D39 and the Δ*psaR*, Δ*psaCA*, and Δ*czcD* strains, all containing the P*pcpA-lacZ* transcriptional fusion, grown in GM17 and GM17chel supplemented with metal ions[a]

Medium and addition(s) (mM)	Mean (SD) β-galactosidase activity (Miller units)[b] in:			
	Wild type	Δ*psaR* mutant	Δ*psaCA* mutant	Δ*czcD* mutant
GM17				
None	6 (3)	1,204 (67)	594 (55)	18 (5)
Zn^{2+} (0.1)	17 (4)	ND	ND	69 (12)
Zn^{2+} (0.2)	66 (21)	1,102 (277)	ND	ND
Zn^{2+} (0.4)	417 (113)	1,189 (115)	446 (37)	ND
Zn^{2+} (0.4) + Mn^{2+} (0.01)	33 (12)	1,119 (113)	237 (22)	ND
Zn^{2+} (0.4) + Mn^{2+} (0.05)	11 (4)	1,219 (203)	7 (4)	ND
Zn^{2+} (0.4) + Fe^{2+} (0.05)	269 (22)	ND	ND	ND
GM17chel				
None	315 (47)	1,217 (222)	ND	ND
Zn^{2+} (0.2)	424 (35)	1,260 (102)	ND	ND
Mn^{2+} (0.05)	6 (2)	1,315 (78)	ND	ND

[a] The wild-type D39 and the Δ*psaR*, Δ*psaCA*, and Δ*czcD* strains are strains RW102, RW107, RW114, and RW122, respectively.
[b] Values are from three experiments. ND, not determined.

Mn^{2+} when added in a concentration of 0.01 to 0.05 mM, which is about 10 to 40 times lower than the concentration of Zn^{2+} (Tables 5, 6, and 7). This repressive effect was specific for Mn^{2+}, since 0.05 mM Ni^{2+}, Cu^{2+}, and Co^{2+} had no effect on the Zn^{2+}-dependent expression of the P*pcpA-lacZ* transcriptional fusion (data not shown); for Fe^{2+}, there was only a weak repressive effect (Table 5). In GM17chel, expression of all three *lacZ* fusions was derepressed compared to that in GM17 (Tables 5, 6, and 7), which was expected because of the much lower concentration of Mn^{2+} after Chelex treatment. Addition of Zn^{2+} increased the derepression even more, while Mn^{2+} led to repression of expression again, which is in agreement with the observations made in GM17 (Tables 5, 6, and 7).

To investigate whether the opposite effects of Zn^{2+} and Mn^{2+} are the result of the competition for uptake of these cations, expression of the P*pcpA-lacZ* fusion in *czcD* and *psaCA* deletion mutants was measured. In a *czcD* deletion mutant, which is, as a consequence of impaired Zn^{2+} efflux, likely to accumulate higher intracellular levels of this metal ion (39), expression of P*pcpA-lacZ* in both GM17 and GM17 with 0.1 mM Zn^{2+} (the highest possible concentration for the *czcD* deletion mutant) was higher than that in the wild type (Table 5). In a *psaCA* deletion mutant, which is impaired in uptake of Mn^{2+} into the cell (50) the expression of P*pcpA-lacZ* was also highly derepressed, However, addition of Mn^{2+}, albeit at a higher concentration than with the wild type, still led to repression (Table 5). These results suggest that the observed regulatory effects on expression of *pcpA*, *prtA*, and *psaBCA* that are induced by Mn^{2+} and Zn^{2+} converge at the level of transcriptional regulation by PsaR.

Identification of a PsaR operator in the promoters of *pcpA*, *prtA*, and *psaB*. Using Gibbs Motif Sampler (77), a palindromic sequence (Fig. 1A and B), located just upstream of (P*prtA* and P*pcpA*) or overlapping with (P*psaB*) the predicted core promoter regions, that might serve as the PsaR operator was uncovered.

To dissect the promoter of *pcpA* experimentally, we per-

TABLE 6. β-Galactosidase activities of the wild-type D39 and the Δ*psaR* strain, containing the *prtA-lacZ* transcriptional fusion, grown in GM17 and GM17chel supplemented with metal ions[a]

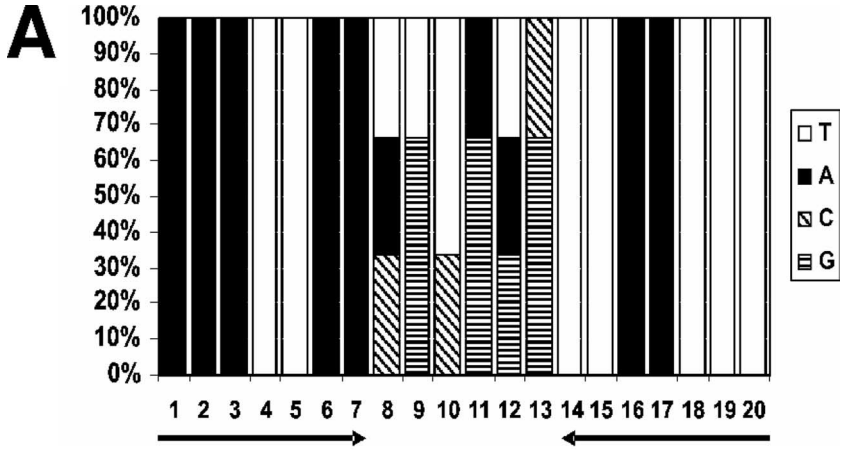
Medium and addition(s) (mM)	Mean (SD) β-galactosidase activity (Miller units)[b] in:	
	Wild type	Δ*psaR* mutant
GM17		
None	0.2 (0.06)	2.2 (0.3)
Zn^{2+} (0.2)	0.6 (0.17)	2.3 (0.5)
Zn^{2+} (0.4)	1.2 (0.05)	2.1 (0.4)
Zn^{2+} (0.4) + Mn^{2+} (0.01)	0.5 (0.09)	2.1 (0.2)
Zn^{2+} (0.4) + Mn^{2+} (0.05)	0.2 (0.08)	2.1 (0.2)
GM17chel		
None	0.5 (0.03)	2.2 (0.1)
Zn^{2+} (0.2)	0.9 (0.05)	2.0 (0.4)
Mn^{2+} (0.05)	0.2 (0.04)	2.1 (0.4)

[a] The wild-type D39 and the Δ*psaR* strain are strains RW104 and RW109, respectively. *lacZ* was fused to the 3′ end of *prtA* on the native chromosomal location, using plasmid pORI13. This might explain the much lower β-galactosidase activity compared to the values for the *lacZ* fusions with P*pcpA* and P*psaB* (Tables 5 and 7).
[b] Values are from three experiments.

TABLE 7. β-Galactosidase activities of the wild-type D39 and the Δ*psaR* strain, containing the P*psaB-lacZ* transcriptional fusion, grown in GM17 and GM17chel supplemented with metal ions[a]

Medium and addition(s) (mM)	Mean (SD) β-galactosidase activity (Miller units)[b] in:	
	Wild type	Δ*psaR* mutant
GM17		
None	134 (34)	1,167 (121)
Zn^{2+} (0.2)	448 (110)	970 (78)
Zn^{2+} (0.4)	760 (124)	1,089 (98)
Zn^{2+} (0.4) + Mn^{2+} (0.01)	651 (18)	1,205 (144)
Zn^{2+} (0.4) + Mn^{2+} (0.05)	142 (11)	1,072 (262)
GM17chel		
None	583 (41)	1,107 (220)
Zn^{2+} (0.2)	901 (33)	1,180 (102)
Mn^{2+} (0.05)	111 (27)	1,023 (45)

[a] The wild-type D39 and the Δ*psaR* strain are strains RW112 and RW113, respectively.
[b] Values are from three experiments.



B

>P*pcpA*

CACGGGTTAAAAAAGAAATTAAAGTGGGTTAATTTTCC**TTGACT**TAAAATTTAAAAA**TG**

ATATACTGTGTTTATGGAGAGATGACAATTTATAAAATGTACTACTCTATTCTACTAGA

TAATGATTGAATAAAACTTTTTATAACAACGGCTAGCAAAGTTATAATTTTATATCTAT

TAGAAAATAAATATGTA**AGGA**AATTTAAA*ATG*

>P*prtA*

CGTGTGCTAAAAAAATAAATTAATGCATGTTAATTTTT**TTGACT**TAAATTTTTTTAAA**TG**

ATATACTGTTTTTATAGAGAGATAACAATTATATATAAATGTACTACTCTATTTCCTAG

ATAATACTTAGTAAAACTTTTTATAACAAAGGCTAGCAAAGTTAAAGTTTATTATCTAT

TGGAAGTTAAATAAATATGTA**AGGA**ATTAAAA*ATG*

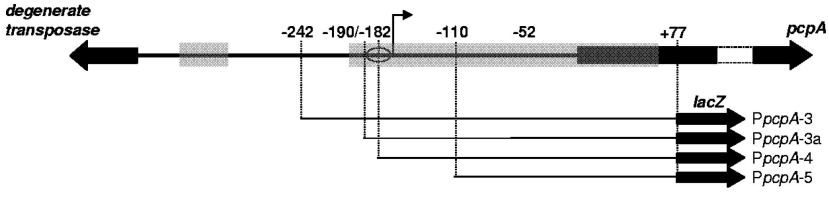
>P*psaB*

TTTCAGTTATTATTTTAATTTTTTCAAAAATTAAC**TTGACT**TAATTTTTTTTTTAATGT

ATATTAAGAGACAGG**AGGA**ATACAAGTTT*ATG*

FIG. 1. Identification of a putative PsaR operator. (A) Weight matrix of the identified PsaR operator as present in the promoter regions of *pcpA*, *prtA*, and *psaB*. (B) Positions of the PsaR operator (shaded) in the promoter regions of *pcpA*, *prtA*, and *psaB*. Putative core promoter sequences are in bold. The ribosome binding sites are in bold and underlined. Start codons are in italic.

formed a promoter subcloning experiment where the *pcpA* promoter was truncated from the 5′ end and fused to *lacZ* in the reporter plasmid pPP2 (Fig. 2). Expression of a promoter fragment truncated upstream of the predicted operator (P*pcpA*-3) was Zn^{2+} dependent, but as expected, deletion of half of the identified operator (P*pcpA*-4) led to fully derepressed, Zn^{2+}-independent expression (Fig. 2). In the presence of 0.05 mM Mn^{2+}, the β-galactosidase activity of P*pcpA*-4



	GM17		GM17 + Zn^{2+}	
	wt	*psaR*	wt	*psaR*
P*pcpA*-3	9 (3)	1107 (108)	756 (56)	902 (115)
P*pcpA*-3a	1135 (216)	1180 (205)	1199 (152)	957 (165)
P*pcpA*-4	1212 (136)	892 (156)	1223 (112)	1127 (215)
P*pcpA*-5	6 (2)	5 (1)	4 (2)	4 (2)

FIG. 2. Subcloning of P*pcpA*. A schematic overview of the P*pcpA* truncations is shown. Numbers indicate the positions of the truncations, which were fused to *lacZ*, relative to the putative *pcpA* start site (+1). The flag indicates the position of the core promoter, and the oval indicates the putative PsaR operator. Gray-shaded areas indicate regions of similarity with P*prtA* (89% identity for the short stretch and 82% identity for the long stretch). The table on the right gives β-galactosidase activities (Miller units) of the promoter truncations in wild-type D39 (wt, strains RW131 to RW134) and the Δ*psaR* mutant (*psaR*, strains RW141 to RW144) grown in GM17 and in GM17 plus 0.5 mM Zn^{2+}. Standard deviations of three measurements are given in parentheses.

TABLE 8. Mutational analysis of the PsaR operator

PsaR box	Sequence (5′→3′)[a]	Mean (SD) β-galactosidase activity (Miller units) for the following strain and medium[b]:			
		Wild type		Δ*psaR* mutant	
		GM17	GM17 + Zn^{2+} (0.4 mM)	GM17	GM17 + Zn^{2+} (0.4 mM)
Wild type	AAATTAAAGTGGGTTAATTT	733 (123)	853 (122)	1,288 (170)	1,331 (167)
mut 1	AAAT**G**AAAGTGGGTTAATTT	74 (15)	376 (78)	1,160 (135)	1,271 (122)
mut 2	AAATTA**G**AGTGGGTTAATTT	121 (25)	416 (75)	1,300 (101)	1,278 (69)
mut 3	AAATT**CG**AGTGGGTTAATTT	58 (12)	464 (16)	1,389 (111)	1,378 (178)

[a] Sequences of wild-type and mutant PsaR boxes of the *pcpA* promoter; point mutations are in bold.
[b] β-Galactosidase activities of wild-type D39 and the Δ*psaR* mutant harboring the P*pcpA-lacZ* transcriptional fusion (strains RW102 and RW107) containing plasmid pRW21 (wild-type PsaR box), pRW22 (PsaR box mut1), pRW23 (PsaR box mut2), or pRW24 (PsaR box mut3).

in wild-type D39 was 1,197 ± 78 Miller units, which is similar to the values for P*pcpA*-4 in GM17 and GM17 with Zn^{2+} (Fig. 2), indicating that the effects of Zn^{2+} and Mn^{2+} are mediated by the same PsaR operator site. To determine if the operator sequence was fully identified, a truncation of the promoter region 8 bp upstream of the operator sequence (P*pcpA*-3a) was constructed, which gave rise to full derepression, suggesting that additional bases 5′ to the operator are also important for PsaR-mediated repression of the *pcpA* promoter. For subclone P*pcpA*-5, expression was close to zero under all conditions. Deletion of the same region as in P*pcpA*-5 but now from the 3′ side (P*pcpA*-rev3 versus P*pcpA*-rev4; strains RW135, RW136, and RW145, RW146) confirmed that promoter activity locates exclusively to this area (data not shown). This demonstrates that the core promoter sequence is located in the region between the 5′ base pair positions of subclones P*pcpA*-4 and P*pcpA*-5 (Fig. 2 and 1B).

To further show that the predicted PsaR operator is functional, a 64-bp DNA fragment of the *pcpA* promoter comprising the PsaR operator was put into plasmid pNG8048E, which replicates in *S. pneumoniae* (37). Subsequently, several point mutations in the first half of the motif were introduced (Table 8). By putting the wild-type construct into D39 containing the P*pcpA-lacZ* transcriptional fusion, transcription from P*pcpA* was strongly derepressed, showing that this 64-bp stretch of DNA titrates away the repressive effect of PsaR on the expression of *pcpA* (Table 8). However, with the constructs containing the mutated PsaR boxes, this derepressive effect was not present (Table 8). This shows clearly that the bases in the predicted PsaR binding box are required for PsaR-dependent repression of *pcpA*. The entire *S. pneumoniae* R6, D39, and TIGR4 sequences were searched with a weight matrix of the PsaR operator sequence (Fig. 1A) using Genome2D (5), but the motif was not found in additional promoter regions (data not shown). In conclusion, a PsaR regulatory element in the promoters of *pcpA*, *psaB*, and *prtA* was identified.

Binding of PsaR-Strep to the *pcpA*, *psaB*, and *prtA* promoters in the presence of Mn^{2+} is counteracted by Zn^{2+}. To find out whether the observed Zn^{2+}- and Mn^{2+}-dependent effects on expression of the PsaR targets are caused by direct modulation of the DNA binding activity of the PsaR protein, EMSAs were performed with purified Strep-tagged PsaR (PsaR-Strep). PsaR-Strep alone did not shift the promoter regions of *pcpA*, *psaB*, and *prtA* (Fig. 3A to C, lanes 2). However, in the presence of Mn^{2+}, PsaR-Strep did bind to the promoter regions of *pcpA*, *psaB*, and *prtA* (Fig. 3A to C, lanes 3). PsaR-Strep did not bind under any condition to a truncated *pcpA* promoter lacking the PsaR binding box (Fig. 3D). Besides Mn^{2+}, 0.05 mM Co^{2+} was also able to stimulate the binding of PsaR-Strep to the promoter fragments, whereas 0.05 mM Fe^{2+}, Cu^{2+}, Ni^{2+}, and Zn^{2+} did not (data not shown). These data show that the PsaR-Strep–DNA interaction was specific and indicates that PsaR directly functions as a Mn^{2+}-dependent repressor of its target genes. Based on the *lacZ* expression studies, we hypothesized that Zn^{2+} should somehow impair PsaR binding to fulfill its function as an Mn^{2+}-dependent repressor. Therefore, experiments addressing the influence of Zn^{2+} on the in vitro PsaR-Strep–DNA interaction in the presence of Mn^{2+} were performed (Fig. 3A, B, and C, lanes 4 to 8). These demonstrated that the stimulatory effect of Mn^{2+} on the binding of PsaR-Strep to all three promoter fragments was counteracted by the addition of Zn^{2+}. There was also a weaker counteracting effect of Cu^{2+} for the *pcpA* and *prtA* promoters (Fig. 3A and C, lanes 8). Thus, Mn^{2+} stimulates PsaR binding to the operators in the *pcpA*, *psaB*, and *prtA* promoters, whereas in the presence of Zn^{2+}, PsaR binding is abolished, indicating that Mn^{2+} and Zn^{2+} exert their regulatory effects on *pcpA*, *prtA*, and *psaBCA* expression directly through PsaR.

DISCUSSION

In this study, we analyzed the transcriptome change of the human pathogen *S. pneumoniae* in response to a high level of Zn^{2+}. Expression of several genes and operons with diverse functions was affected by Zn^{2+}, including *pcpA*, *prtA*, and *psaBCA*. The observed Zn^{2+}-dependent expression of these virulence genes was shown to be directly mediated by the Mn^{2+}-responsive repressor PsaR (31). We further demonstrate that this is caused by Mn^{2+}-dependent binding of PsaR to and Zn^{2+}-dependent release from the promoters of these genes. Thus, these data represent an intriguing insight in the opposite regulatory effects of two metal cations on the expression of a set of virulence genes, mediated by a single transcriptional repressor.

The concentrations as well as the ratio of Mn^{2+} and Zn^{2+} may vary greatly between different sites in the human body. For example, in lung tissue the total concentration of Mn^{2+} is approximately 0.2 μg/g (wet weight) (3.6 μM) and that of Zn^{2+} is 15 μg/g (wet weight) (229 μM), whereas in the blood serum concentrations of Mn^{2+} and Zn^{2+} are 0.5 ng/ml (9 nM) and 1.0

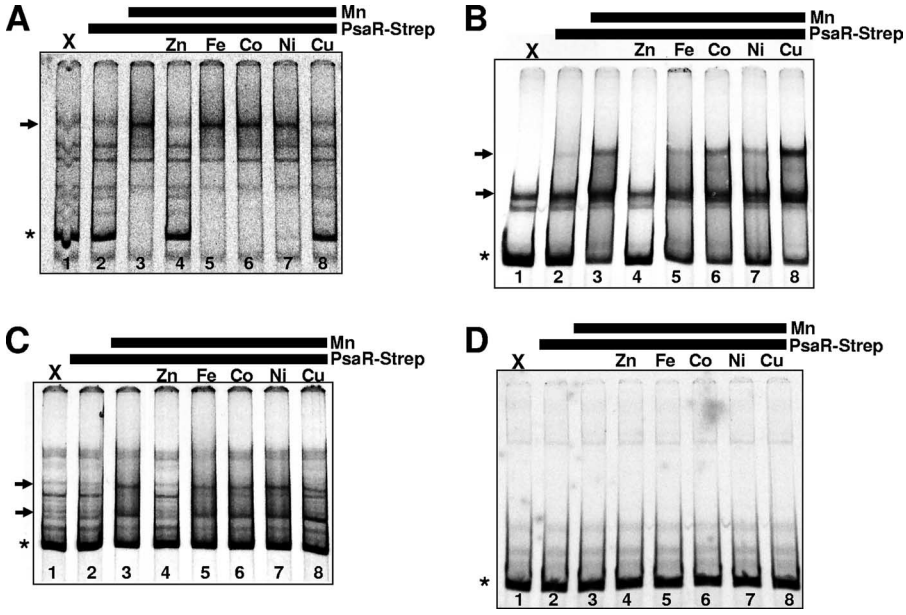


FIG. 3. In vitro interaction of PsaR-Strep with the *pcpA* (A), *psaB* (B), and *prtA* (C) promoter regions and with a truncated P*pcpA* fragment lacking the PsaR operator (D). Purified PsaR-Strep was added at concentrations of 5 nM (P*pcpA*), 11 nM (P*prtA*), and 25 nM (P*psaB*). Metal ions were added as indicated above the lanes at a concentration of 50 μM. X, free probe. The horizontal bar above lanes 3 to 8 indicates the presence of Mn^{2+}, and the horizontal bar above lanes 2 to 8 indicates the presence of PsaR-Strep. Arrows indicate the positions of the shifted probes, and asterisks indicate the position of the free probe. The presence of weaker bands which run higher than the free probe in the gels is a phenomenon that has also been seen by others in similar experiments. These bands may represent unspecific PCR products or single-stranded DNA (1, 15).

μg/ml (15.3 μM), respectively (81). Control of availability of both ions is of importance to the host, as a recent study showed that the human immune system employs chelation of Mn^{2+} and Zn^{2+} by calprotectin as a way to inhibit bacterial growth in tissue abscesses (13). On the other hand, sufficiently high levels of Zn^{2+} are required for proper functioning of the immune system (72). Thus, it is likely that the concentrations of Mn^{2+} and Zn^{2+} fluctuate greatly in the environment, which will lead to varying concentrations of these metal ions in the cytoplasm of *S. pneumoniae*. However, virulence studies that have been carried out so far with respect to *pcpA*, *prtA*, and *psaBCA* do not point to a specific site where the proteins encoded by these genes are needed (7, 26, 31, 47, 50, 51, 62). Interestingly, *pcpA* is also regulated by the nutritional regulator CodY (26), meaning that the concentrations of both metal ions as well as amino acids affect *pcpA* expression.

Homologs of PsaR in other organisms seem to have slightly different functions. In *Streptococcus gordonii*, ScaR is an Mn^{2+}-dependent repressor of the Sca (Mn^{2+}) permease (29). In *Streptococcus pyogenes*, MtsR regulates the Mn^{2+}-specific ABC transporter MtsABC in response to Mn^{2+}, while the heme-specific HtsABC transporter is repressed by MtsR in response to Fe^{3+} (23). The *Streptococcus mutans* SloR regulates several genes involved in biofilm formation, genetic competence, oxidative stress tolerance, and adherence in response to Mn^{2+} and, to a lesser extent, Fe^{3+} (35, 63, 67, 74).

The EMSAs performed in this study are in line with the transcriptional data and suggest that a direct effect of Zn^{2+} and Mn^{2+} on the PsaR-promoter interaction causes the observed transcriptional effects. However, in the EMSAs Mn^{2+} does not overcome the Zn^{2+} effect at equimolar concentration, whereas in vivo, only low (but repressive) concentrations of Mn^{2+} are counteracted by Zn^{2+}. This might be because of different intracellular concentrations/availabilities of these metal ions compared to the extracellular concentrations and indicates that in vivo Mn^{2+} is the principal effector. It will be interesting to know why Zn^{2+} and Mn^{2+} have these opposite effects on the DNA binding properties and activity of PsaR.

Clues about this could come from recent structural studies on DtxR from *Corynebacterium diphtheriae* (14) and MntR, a DtxR family protein from *Bacillus subtilis* that responds to Mn^{2+} (65). Both DtxR and MntR contain two metal binding sites per monomer: a low-affinity site and a high-affinity site (14, 21). MntR binds metal ions with affinities that roughly follow the Irving-Williams series, where Mn^{2+} displays the lowest affinity for MntR and Zn^{2+} the highest (21). As MntR has a much higher affinity for Zn^{2+} than Mn^{2+} but only very poorly activates DNA binding of MntR, the specificity of MntR is not correlated with the metal binding affinity (21). The conformation of Mn^{2+}-bound MntR differs from the Zn^{2+}-bound conformation with respect to the occupancy of the metal binding sites: Mn^{2+} binds to two sites, whereas only one Zn^{2+} ion binds to MntR, which does not allow binding of a second one (36). Metal binding at the second site is proposed to be required for DNA binding, as it promotes a disorder-to-order transition of MntR structure (17). PsaR shares 25% and 15% sequence identity with DtxR and MntR, respectively. Moreover, sequence alignment shows that most residues that constitute the metal ion binding sites in DtxR and MntR are conserved in PsaR (data not shown). Therefore, it might be that Zn^{2+} prevents Mn^{2+} binding to PsaR, rendering PsaR in a monomeric or destabilized state, and in this way counteracts Mn^{2+}-induced DNA binding and transcriptional repression.

Interference with the effect of one metal ion on a metal-

sensory protein by another metal ion has been reported recently for CzrA in *B. subtilis* (24). CzrA normally is activated for DNA release in the presence of Zn^{2+}, but Cu^{2+} inhibits the Zn^{2+}-induced allostery, since in vitro the protein preferentially binds Cu^{2+}. However, these effects are not seen in vivo. High levels of Cu^{2+} in the growth medium induces the Fur regulon in *B. subtilis* (56). Thus, opposite effects of metals on regulation of gene expression seems to occur with other classes of metalloregulatory proteins as well.

The identified PsaR binding site is similar to operator sequences of PsaR homologs in other streptococcal species. *S. gordonii* ScaR binds to a similar region in the *scaA* promoter but also to a second inverted repeat (29). This second repeat is present in the promoter region of *psaB* in *S. pneumoniae* but not in the promoters of *pcpA* and *prtA*. SloR in *S. mutans* also likely exerts its repressive effect on *sloABC* through a larger palindromic sequence that includes the conserved region that we identify (35). Apart from the promoters of *prtA*, *pcpA*, and *psaBCA*, no others that contain the identified PsaR operator in their promoter regions could be found in the R6 and TIGR4 genomes. This suggests that the PsaR regulon consists of only these genes in *S. pneumoniae*, in contrast to the case for *S. mutans*, where SloR directly regulates a large number of genes (67). It is very likely that also in TIGR4 the activity of PsaR is dependent on both Zn^{2+} and Mn^{2+}, since, apart from one amino acid difference (Asn161→Ser), TIGR4 PsaR is identical to the R6 and D39 PsaR sequences (data not shown). Johnston et al. (31) also found a repressive effect of Mn^{2+} and PsaR on the expression of the *rlrA* pathogenicity islet (SP0461 to SP0465). The *rlrA* locus is not present in the genomes of D39 and R6, making the effect of PsaR on *rlrA* and the downstream genes a strain-dependent phenomenon. There is a possible PsaR operator in the *rlrA* promoter region with a perfectly conserved first half-site, but a very degenerate second half-site, 5′-**AAATTAA**AACAAC**TT**CCAT**C**-3′ (consensus bases are in bold). Point mutations in the conserved bases of the first half of the operator destroyed PsaR-dependent regulation for the *pcpA* promoter (Table 8). However, we did not test the effect of mutations in the second half-site. Therefore, it cannot be excluded that an operator consisting of an intact first repeat and a degenerate inverted repeat, as is the case for the putative operator in the *rlrA* promoter, is still able to confer some weak PsaR-dependent regulation. As RlrA activates expression of the genes downstream of *rlrA*, namely, *rrgA*, *rrgB*, *rrgC*, and *srtB* (25), weak repression of *rlrA* by PsaR likely explains the upregulation of the *rlrA* locus in the *psaR* mutant (31, 68).

In conclusion, this study indicates that the relative availabilities of Zn^{2+} and Mn^{2+} in the human body could modulate the expression of several virulence genes and in this way affect the outcome of infection by *S. pneumoniae*.

ACKNOWLEDGMENTS

We thank D. Morrison for the generous gift of competence-stimulating peptide. We thank Anne de Jong for help with the DNA microarray production and analysis.

T.G.K. and J.J.E.B. are supported by IOP Genomics grant IGE03002 from the Dutch Ministry of Economic Affairs.

REFERENCES

1. **Albano, M., W. K. Smits, L. T. Ho, B. Kraigher, I. Mandic-Mulec, O. P. Kuipers, and D. Dubnau.** 2005. The Rok protein of *Bacillus subtilis* represses genes for cell surface and extracellular functions. J. Bacteriol. **187:**2010–2019.
2. **Anderton, J. M., G. Rajam, S. Romero-Steiner, S. Summer, A. P. Kowalczyk, G. M. Carlone, J. S. Sampson, and E. W. Ades.** 2007. E-cadherin is a receptor for the common protein pneumococcal surface adhesin A (PsaA) of Streptococcus pneumoniae. Microb. Pathog. **42:**225–236.
3. **Ando, M., Y. C. Manabe, P. J. Converse, E. Miyazaki, R. Harrison, J. R. Murphy, and W. R. Bishai.** 2003. Characterization of the role of the divalent metal ion-dependent transcriptional repressor MntR in the virulence of *Staphylococcus aureus*. Infect. Immun. **71:**2584–2590.
4. **Avery, O. T., C. M. Macleod, and M. McCarty.** 1944. Studies on the chemical nature of the substance inducing transformation of pneumococcal types. Induction of transformation by a desoxyribonucleic acid fraction isolated from Pneumococcus type III. Mol. Med. **1:**344–365.
5. **Baerends, R. J. S., W. K. Smits, A. de Jong, L. W. Hamoen, J. Kok, and O. P. Kuipers.** 2004. Genome2D: a visualization tool for the rapid analysis of bacterial transcriptome data. Genome Biol. **5:**R37.
6. **Berry, A. M., and J. C. Paton.** 1996. Sequence heterogeneity of PsaA, a 37-kilodalton putative adhesin essential for virulence of *Streptococcus pneumoniae*. Infect. Immun. **64:**5255–5262.
7. **Bethe, G., R. Nau, A. Wellmer, R. Hakenbeck, R. R. Reinert, H. P. Heinz, and G. Zysk.** 2001. The cell wall-associated serine protease PrtA: a highly conserved virulence factor of Streptococcus pneumoniae. FEMS Microbiol. Lett. **205:**99–104.
8. **Bijlsma, J. J., P. Burghout, T. G. Kloosterman, H. J. Bootsma, A. de Jong, P. W. Hermans, and O. P. Kuipers.** 2007. Development of genomic array footprinting for identification of conditionally essential genes in *Streptococcus pneumoniae*. Appl. Environ. Microbiol. **73:**1514–1524.
9. **Blencowe, D. K., and A. P. Morby.** 2003. Zn(II) metabolism in prokaryotes. FEMS Microbiol. Rev. **27:**291–311.
10. **Bogaert, D., R. de Groot, and P. W. Hermans.** 2004. Streptococcus pneumoniae colonisation: the key to pneumococcal disease. Lancet Infect. Dis. **4:**144–154.
11. **Brown, J. S., S. M. Gilliland, and D. W. Holden.** 2001. A Streptococcus pneumoniae pathogenicity island encoding an ABC transporter involved in iron uptake and virulence. Mol. Microbiol. **40:**572–585.
12. **Brown, J. S., S. M. Gilliland, J. Ruiz-Albert, and D. W. Holden.** 2002. Characterization of pit, a *Streptococcus pneumoniae* iron uptake ABC transporter. Infect. Immun. **70:**4389–4398.
13. **Corbin, B. D., E. H. Seeley, A. Raab, J. Feldmann, M. R. Miller, V. J. Torres, K. L. Anderson, B. M. Dattilo, P. M. Dunman, R. Gerads, R. M. Caprioli, W. Nacken, W. J. Chazin, and E. P. Skaar.** 2008. Metal chelation and inhibition of bacterial growth in tissue abscesses. Science **319:**962–965.
14. **D'Aquino, J. A., J. Tetenbaum-Novatt, A. White, F. Berkovitch, and D. Ringe.** 2005. Mechanism of metal ion activation of the diphtheria toxin repressor DtxR. Proc. Natl. Acad. Sci. USA **102:**18408–18413.
15. **den Hengst, C. D., S. A. F. T. van Hijum, J. M. Geurts, A. Nauta, J. Kok, and O. P. Kuipers.** 2005. The Lactococcus lactis CodY regulon: identification of a conserved cis-regulatory element. J. Biol. Chem. **280:**34332–34342.
16. **de Ruyter, P. G., O. P. Kuipers, and W. M. de Vos.** 1996. Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. Appl. Environ. Microbiol. **62:**3662–3667.
17. **DeWitt, M. A., J. I. Kliegman, J. D. Helmann, R. G. Brennan, D. L. Farrens, and A. Glasfeld.** 2007. The conformations of the manganese transport regulator of Bacillus subtilis in its metal-free state. J. Mol. Biol. **365:**1257–1265.
18. **Dintilhac, A., G. Alloing, C. Granadel, and J. P. Claverys.** 1997. Competence and virulence of Streptococcus pneumoniae: Adc and PsaA mutants exhibit a requirement for Zn and Mn resulting from inactivation of putative ABC metal permeases. Mol. Microbiol. **25:**727–739.
19. **Dintilhac, A., and J. P. Claverys.** 1997. The adc locus, which affects competence for genetic transformation in Streptococcus pneumoniae, encodes an ABC transporter with a putative lipoprotein homologous to a family of streptococcal adhesins. Res. Microbiol. **148:**119–131.
20. **Finney, L. A., and T. V. O'Halloran.** 2003. Transition metal speciation in the cell: insights from the chemistry of metal ion receptors. Science **300:**931–936.
21. **Golynskiy, M. V., W. A. Gunderson, M. P. Hendrich, and S. M. Cohen.** 2006. Metal binding studies and EPR spectroscopy of the manganese transport regulator MntR. Biochemistry **45:**15359–15372.
22. **Halfmann, A., R. Hakenbeck, and R. Bruckner.** 2007. A new integrative reporter plasmid for Streptococcus pneumoniae. FEMS Microbiol. Lett. **268:**217–224.
23. **Hanks, T. S., M. Liu, M. J. McClure, M. Fukumura, A. Duffy, and B. Lei.** 2006. Differential regulation of iron- and manganese-specific MtsABC and heme-specific HtsABC transporters by the metalloregulator MtsR of group A *Streptococcus*. Infect. Immun. **74:**5132–5139.
24. **Harvie, D. R., C. Andreini, G. Cavallaro, W. Meng, B. A. Connolly, K. Yoshida, Y. Fujita, C. R. Harwood, D. S. Radford, S. Tottey, J. S. Cavet, and N. J. Robinson.** 2006. Predicting metals sensed by ArsR-SmtB repressors: allosteric interference by a non-effector metal. Mol. Microbiol. **59:**1341–1356.
25. **Hava, D. L., C. J. Hemsley, and A. Camilli.** 2003. Transcriptional regulation

in the *Streptococcus pneumoniae rlrA* pathogenicity islet by RlrA. J. Bacteriol. **185:**413–421.
26. **Hendriksen, W. T., H. J. Bootsma, S. Estevao, T. Hoogenboezem, A. de Jong, R. de Groot, O. P. Kuipers, and P. W. Hermans.** 2008. CodY of *Streptococcus pneumoniae*: link between nutritional gene regulation and colonization. J. Bacteriol. **190:**590–601
27. **Hoskins, J., W. E. Alborn, Jr., J. Arnold, L. C. Blaszczak, S. Burgett, B. S. DeHoff, S. T. Estrem, L. Fritz, D. J. Fu, W. Fuller, C. Geringer, R. Gilmour, J. S. Glass, H. Khoja, A. R. Kraft, R. E. Lagace, D. J. LeBlanc, L. N. Lee, E. J. Lefkowitz, J. Lu, P. Matsushima, S. M. McAhren, M. McHenney, K. McLeaster, C. W. Mundy, T. I. Nicas, F. H. Norris, M. O'Gara, R. B. Peery, G. T. Robertson, P. Rockey, P. M. Sun, M. E. Winkler, Y. Yang, M. Young-Bellido, G. Zhao, C. A. Zook, R. H. Baltz, S. R. Jaskunas, P. R. Rosteck, Jr., P. L. Skatrud, and J. I. Glass.** 2001. Genome of the bacterium *Streptococcus pneumoniae* strain R6. J. Bacteriol. **183:**5709–5717.
28. **Ibs, K. H., and L. Rink.** 2003. Zinc-altered immune function. J. Nutr. **133:** 1452S–1456S.
29. **Jakubovics, N. S., A. W. Smith, and H. F. Jenkinson.** 2000. Expression of the virulence-related Sca (Mn2+) permease in Streptococcus gordonii is regulated by a diphtheria toxin metallorepressor-like protein ScaR. Mol. Microbiol. **38:**140–153.
30. **Janulczyk, R., S. Ricci, and L. Bjorck.** 2003. MtsABC is important for manganese and iron transport, oxidative stress resistance, and virulence of *Streptococcus pyogenes*. Infect. Immun. **71:**2656–2664.
31. **Johnston, J. W., D. E. Briles, L. E. Myers, and S. K. Hollingshead.** 2006. $Mn2^{+}$-dependent regulation of multiple genes in *Streptococcus pneumoniae* through PsaR and the resultant impact on virulence. Infect. Immun. **74:** 1171–1180.
32. **Johnston, J. W., L. E. Myers, M. M. Ochs, W. H. Benjamin, Jr., D. E. Briles, and S. K. Hollingshead.** 2004. Lipoprotein PsaA in virulence of *Streptococcus pneumoniae*: surface accessibility and role in protection from superoxide. Infect. Immun. **72:**5858–5867.
33. **Jordan, A., and P. Reichard.** 1998. Ribonucleotide reductases. Annu. Rev. Biochem. **67:**71–98.
34. **Karlyshev, A. V., M. J. Pallen, and B. W. Wren.** 2000. Single-primer PCR procedure for rapid identification of transposon insertion sites. BioTechniques **28:**1078–1082.
35. **Kitten, T., C. L. Munro, S. M. Michalek, and F. L. Macrina.** 2000. Genetic characterization of a *Streptococcus mutans* LraI family operon and role in virulence. Infect. Immun. **68:**4441–4451.
36. **Kliegman, J. I., S. L. Griner, J. D. Helmann, R. G. Brennan, and A. Glasfeld.** 2006. Structural basis for the metal-selective activation of the manganese transport regulator of Bacillus subtilis. Biochemistry **45:**3493–3505.
37. **Kloosterman, T. G., J. J. E. Bijlsma, J. Kok, and O. P. Kuipers.** 2006. To have neighbour's fare: extending the molecular toolbox for Streptococcus pneumoniae. Microbiology **152:**351–359.
38. **Kloosterman, T. G., W. T. Hendriksen, J. J. Bijlsma, H. J. Bootsma, S. A. van Hijum, J. Kok, P. W. Hermans, and O. P. Kuipers.** 2006. Regulation of glutamine and glutamate metabolism by GlnR and GlnA in Streptococcus pneumoniae. J. Biol. Chem. **281:**25097–25109.
39. **Kloosterman, T. G., M. M. van der Kooi-Pol, J. J. Bijlsma, and O. P. Kuipers.** 2007. The novel transcriptional regulator SczA mediates protection against Zn(2+) stress by activation of the Zn-resistance gene czcD in Streptococcus pneumoniae. Mol. Microbiol. **65:**1049–1063.
40. **Kolenbrander, P. E., R. N. Andersen, R. A. Baker, and H. F. Jenkinson.** 1998. The adhesion-associated *sca* operon in *Streptococcus gordonii* encodes an inducible high-affinity ABC transporter for $Mn2^{+}$ uptake. J. Bacteriol. **180:**290–295.
41. **Kuipers, O. P., P. G. Ruyter, M. Kleerebezem, and W. M. Vos.** 1998. Quorum sensing controlled gene expression in lactic acid bacteria. J. Biotechnol. **64:**15–21.
42. **Lampe, D. J., M. E. Churchill, and H. M. Robertson.** 1996. A purified mariner transposase is sufficient to mediate transposition in vitro. EMBO J. **15:**5470–5479.
43. **Lanie, J. A., W. L. Ng, K. M. Kazmierczak, T. M. Andrzejewski, T. M. Davidsen, K. J. Wayne, H. Tettelin, J. I. Glass, and M. E. Winkler.** 2007. Genome sequence of Avery's virulent serotype 2 strain D39 of *Streptococcus pneumoniae* and comparison with that of unencapsulated laboratory strain R6. J. Bacteriol. **189:**38–51.
44. **Lawrence, M. C., P. A. Pilling, V. C. Epa, A. M. Berry, A. D. Ogunniyi, and J. C. Paton.** 1998. The crystal structure of pneumococcal surface antigen PsaA reveals a metal-binding site and a novel structure for a putative ABC-type binding protein. Structure **6:**1553–1561.
45. **Leenhouts, K., G. Buist, A. Bolhuis, A. ten Berge, J. Kiel, I. Mierau, M. Dabrowska, G. Venema, and J. Kok.** 1996. A general system for generating unlabelled gene replacements in bacterial chromosomes. Mol. Gen. Genet. **253:**217–224.
46. **Loo, C. Y., K. Mitrakul, I. B. Voss, C. V. Hughes, and N. Ganeshkumar.** 2003. Involvement of the *adc* operon and manganese homeostasis in *Streptococcus gordonii* biofilm formation. J. Bacteriol. **185:**2887–2900.
47. **Marra, A., J. Asundi, M. Bartilson, S. Lawson, F. Fang, J. Christine, C. Wiesner, D. Brigham, W. P. Schneider, and A. E. Hromockyj.** 2002. Differential fluorescence induction analysis of *Streptococcus pneumoniae* identifies genes involved in pathogenesis. Infect. Immun. **70:**1422–1433.
48. **Marra, A., S. Lawson, J. S. Asundi, D. Brigham, and A. E. Hromockyj.** 2002. In vivo characterization of the psa genes from Streptococcus pneumoniae in multiple models of infection. Microbiology **148:**1483–1491.
49. **Martin, B., M. Prudhomme, G. Alloing, C. Granadel, and J. P. Claverys.** 2000. Cross-regulation of competence pheromone production and export in the early control of transformation in Streptococcus pneumoniae. Mol. Microbiol. **38:**867–878.
50. **McAllister, L. J., H. J. Tseng, A. D. Ogunniyi, M. P. Jennings, A. G. McEwan, and J. C. Paton.** 2004. Molecular analysis of the psa permease complex of Streptococcus pneumoniae. Mol. Microbiol. **53:**889–901.
51. **McCluskey, J., J. Hinds, S. Husain, A. Witney, and T. J. Mitchell.** 2004. A two-component system that controls the expression of pneumococcal surface antigen A (PsaA) and regulates virulence and resistance to oxidative stress in Streptococcus pneumoniae. Mol. Microbiol. **51:**1661–1675.
52. **McKessar, S. J., and R. Hakenbeck.** 2007. The two-component regulatory system TCS08 is involved in cellobiose metabolism of *Streptococcus pneumoniae* R6. J. Bacteriol. **189:**1342–1350.
53. **Milanino, R., M. Marrella, R. Gasperini, M. Pasqualicchio, and G. Velo.** 1993. Copper and zinc body levels in inflammation: an overview of the data obtained from animal and human studies. Agents Actions **39:**195–209.
54. **Mitrakul, K., C. Y. Loo, C. Gyurko, C. V. Hughes, and N. Ganeshkumar.** 2005. Mutational analysis of the adcCBA genes in Streptococcus gordonii biofilm formation. Oral Microbiol. Immunol. **20:**122–127.
55. **Montanez, G. E., M. N. Neely, and Z. Eichenbaum.** 2005. The streptococcal iron uptake (Siu) transporter is required for iron uptake and virulence in a zebrafish infection model. Microbiology **151:**3749–3757.
56. **Moore, C. M., A. Gaballa, M. Hui, R. W. Ye, and J. D. Helmann.** 2005. Genetic and physiological responses of Bacillus subtilis to metal ion stress. Mol. Microbiol. **57:**27–40.
57. **Moore, C. M., and J. D. Helmann.** 2005. Metal ion homeostasis in Bacillus subtilis. Curr. Opin. Microbiol. **8:**188–195.
58. **Nies, D. H.** 2003. Efflux-mediated heavy metal resistance in prokaryotes. FEMS Microbiol. Rev. **27:**313–339.
59. **Novak, R., J. S. Braun, E. Charpentier, and E. Tuomanen.** 1998. Penicillin tolerance genes of Streptococcus pneumoniae: the ABC-type manganese permease complex Psa. Mol. Microbiol. **29:**1285–1296.
60. **Novak, R., E. Tuomanen, and E. Charpentier.** 2000. The mystery of psaA and penicillin tolerance in Streptococcus pneumoniae. Mol. Microbiol. **36:** 1504–1505.
61. **Obaro, S., and R. Adegbola.** 2002. The pneumococcus: carriage, disease and conjugate vaccines. J. Med. Microbiol. **51:**98–104.
62. **Orihuela, C. J., J. N. Radin, J. E. Sublett, G. Gao, D. Kaushal, and E. I. Tuomanen.** 2004. Microarray analysis of pneumococcal gene expression during invasive disease. Infect. Immun. **72:**5582–5596.
63. **Paik, S., A. Brown, C. L. Munro, C. N. Cornelissen, and T. Kitten.** 2003. The *sloABCR* operon of *Streptococcus mutans* encodes an Mn and Fe transport system required for endocarditis virulence and its Mn-dependent repressor. J. Bacteriol. **185:**5967–5975.
64. **Panina, E. M., A. A. Mironov, and M. S. Gelfand.** 2003. Comparative genomics of bacterial zinc regulons: enhanced ion transport, pathogenesis, and rearrangement of ribosomal proteins. Proc. Natl. Acad. Sci. USA **100:**9912–9917.
65. **Que, Q., and J. D. Helmann.** 2000. Manganese homeostasis in Bacillus subtilis is regulated by MntR, a bifunctional regulator related to the diphtheria toxin repressor family of proteins. Mol. Microbiol. **35:**1454–1468.
66. **Ricci, S., R. Janulczyk, and L. Bjorck.** 2002. The regulator PerR is involved in oxidative stress response and iron homeostasis and is necessary for full virulence of *Streptococcus pyogenes*. Infect. Immun. **70:**4968–4976.
67. **Rolerson, E., A. Swick, L. Newlon, C. Palmer, Y. Pan, B. Keeshan, and G. Spatafora.** 2006. The SloR/Dlg metalloregulator modulates *Streptococcus mutans* virulence gene expression. J. Bacteriol. **188:**5033–5044.
68. **Rosch, J. W., B. Mann, J. Thornton, J. Sublett, and E. Tuomanen.** 2008. Convergence of regulatory networks on the pilus locus of *Streptococcus pneumoniae*. Infect. Immun. **76:**3187–3196.
69. **Sanchez-Beato, A. R., R. Lopez, and J. L. Garcia.** 1998. Molecular characterization of PcpA: a novel choline-binding protein of Streptococcus pneumoniae. FEMS Microbiol. Lett. **164:**207–214.
70. **Sanders, J. W., G. Venema, J. Kok, and K. Leenhouts.** 1998. Identification of a sodium chloride-regulated promoter in Lactococcus lactis by single-copy chromosomal fusion with a reporter gene. Mol. Gen. Genet. **257:**681–685.
71. **Sazawal, S., R. E. Black, S. Jalla, S. Mazumdar, A. Sinha, and M. K. Bhan.** 1998. Zinc supplementation reduces the incidence of acute lower respiratory infections in infants and preschool children: a double-blind, controlled trial. Pediatrics **102:**1–5.
72. **Shankar, A. H., and A. S. Prasad.** 1998. Zinc and immune function: the biological basis of altered resistance to infection. Am. J. Clin. Nutr. **68:**447S–463S.
73. **Song, J. H., K. S. Ko, J. Y. Lee, J. Y. Baek, W. S. Oh, H. S. Yoon, J. Y. Jeong, and J. Chun.** 2005. Identification of essential genes in Streptococcus pneumoniae by allelic replacement mutagenesis. Mol. Cells **19:**365–374.

74. **Spatafora, G., M. Moore, S. Landgren, E. Stonehouse, and S. Michalek.** 2001. Expression of Streptococcus mutans fimA is iron-responsive and regulated by a DtxR homologue. Microbiology **147:**1599–1610.
75. **Strand, T. A., D. E. Briles, H. K. Gjessing, A. Maage, M. K. Bhan, and H. Sommerfelt.** 2001. Pneumococcal pulmonary infection, septicaemia and survival in young zinc-depleted mice. Br. J. Nutr. **86:**301–306.
76. **Strand, T. A., S. K. Hollingshead, K. Julshamn, D. E. Briles, B. Blomberg, and H. Sommerfelt.** 2003. Effects of zinc deficiency and pneumococcal surface protein a immunization on zinc status and the risk of severe infection in mice. Infect. Immun. **71:**2009–2013.
77. **Thijs, G., K. Marchal, M. Lescot, S. Rombauts, M. B. De, P. Rouze, and Y. Moreau.** 2002. A Gibbs sampling method to detect overrepresented motifs in the upstream regions of coexpressed genes. J. Comput. Biol. **9:**447–464.
78. **Torrents, E., G. Buist, A. Liu, R. Eliasson, J. Kok, I. Gibert, A. Graslund, and P. Reichard.** 2000. The anaerobic (class III) ribonucleotide reductase from Lactococcus lactis. Catalytic properties and allosteric regulation of the pure enzyme system. J. Biol. Chem. **275:**2463–2471.
79. **Tseng, H. J., A. G. McEwan, J. C. Paton, and M. P. Jennings.** 2002. Virulence of *Streptococcus pneumoniae*: PsaA mutants are hypersensitive to oxidative stress. Infect. Immun. **70:**1635–1639.
80. **van Hijum, S. A. F. T., A. de Jong, R. J. S. Baerends, H. A. Karsens, N. E. Kramer, R. Larsen, C. D. den Hengst, C. J. Albers, J. Kok, and O. P. Kuipers.** 2005. A generally applicable validation scheme for the assessment of factors involved in reproducibility and quality of DNA-microarray data. BMC Genomics **6:**77.
81. **Versieck, J.** 1985. Trace elements in human body fluids and tissues. Crit. Rev. Clin. Lab. Sci. **22:**97–184.