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Opposite Effects of Mn\(^{2+}\) and Zn\(^{2+}\) on PsaR-Mediated Expression of the Virulence Genes \(pcpA\), \(prtA\), and \(psaBCA\) of \textit{Streptococcus pneumoniae}\(^{\dagger}\)

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Homeostasis of Zn\(^{2+}\) and Mn\(^{2+}\) is important for the physiology and virulence of the human pathogen \textit{Streptococcus pneumoniae}. Here, transcriptome analysis was used to determine the response of \textit{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 \(pca\) 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 \textit{S. pneumoniae}, suggesting that the ratio of these metal ions exerts an important influence on pneumococcal pathogenesis.

The human pathogen \textit{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 \textit{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 \textit{S. pneumoniae} could face is Zn\(^{2+}\), which is present in the human body in concentrations ranging from a few \(\mu\)M to over 100 \(\mu\)M (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 \textit{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 (\(paaB/C/D\), \(pitB/C/D\), and \(pim\)) (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 \(pca\) genes is repressed by the DtxR family regulator PsaR in response to high Mn\(^{2+}\) concentrations (31). \textit{S. pneumoniae} also contains systems involved in cation efflux (58), such as \(czzD\), which has recently been shown to be responsible for Zn\(^{2+}\) resistance (39). The presence in \textit{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 \textit{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, \(\beta\)-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. \(\beta\)-Galactosidase assays were performed as described previously (38).
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 CaCl2, 50 μM MgCl2, 5 μM FeCl2, 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 ZnSO4, MgCl2, CaCl2, NiCl2, CuSO4, and FeSO4. 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 (spr1945-1 and PpcpA Rev_4). All fragments were cloned as EcoRI/BamHI fragments were run in 0.44 M Tris-borate buffer (pH 8.3) at 100 V for 90 min. Determination of the Mn 2+ -induced gene found in the microarray 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/cybertstatistical 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
| Strain or plasmid | Description | 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 |
| D39nisRK | D39 ΔgutA::nisRK; Trmp<sup>a</sup> | 37 |
| MP102 | D39 ΔexcD | This work |
| RW100 | D39 ΔpsaR | This work |
| RW101 | D39nisRK ΔpsaR | This work |
| RW102 | D39 ΔgutA::Ppcp4-ΔlacZ; Tet<sup>b</sup> | This work |
| RW103 | D39 ΔgutA::PnrD-ΔlacZ; Tet<sup>b</sup> | This work |
| RW104 | D39nisRK pRTA-ΔlacZ; Erm<sup>c</sup> | This work |
| RW105 | D39 ΔgutA::Psr0576-ΔlacZ; Tet<sup>b</sup> | This work |
| RW106 | D39 ΔgutA::PpsaR-ΔlacZ; Tet<sup>b</sup> | This work |
| RW107 | RW100 ΔgutA::Ppcp4-ΔlacZ; Tet<sup>b</sup> | This work |
| RW108 | RW100 ΔgutA::PnrD-ΔlacZ; Tet<sup>b</sup> | This work |
| RW109 | RW101 pRTA-ΔlacZ; Erm<sup>c</sup> | This work |
| RW110 | RW100 ΔgutA::Psr0576-ΔlacZ; Tet<sup>b</sup> | This work |
| RW111 | RW100 ΔgutA::PpsaR-ΔlacZ; Tet<sup>b</sup> | This work |
| RW112 | D39 ΔgutA::PpsaB-ΔlacZ; Tet<sup>b</sup> | This work |
| RW113 | RW100 ΔgutA::PpsaB-ΔlacZ; Tet<sup>b</sup> | This work |
| RW114 | RW121 ΔgutA::Ppcp4-ΔlacZ; Tet<sup>b</sup> | This work |
| RW115 | RW120 with mariner insertion in psaR; Spec<sup>c</sup> | This work |
| RW116 | D39 ΔpsaCA::ermR; Erm<sup>c</sup> | This work |
| RW117 | MP102 ΔgutA::Ppcp4-ΔlacZ; Tet<sup>b</sup> | This work |
| RW118 | D39 ΔgutA::Ppcp4-3-ΔlacZ; Tet<sup>b</sup> | This work |
| RW119 | D39 ΔgutA::Ppcp4-3a-ΔlacZ; Tet<sup>b</sup> | This work |
| RW120 | D39 ΔgutA::Ppcp4-4-ΔlacZ; Tet<sup>b</sup> | This work |
| RW121 | D39 ΔgutA::Ppcp4-5-ΔlacZ; Tet<sup>b</sup> | This work |
| RW122 | D39 ΔgutA::Ppcp4-Δrev3-ΔlacZ; Tet<sup>b</sup> | This work |
| RW123 | D39 ΔgutA::Ppcp4-Δrev4-ΔlacZ; Tet<sup>b</sup> | This work |
| RW124 | RW100 ΔgutA::Ppcp4-3-ΔlacZ; Tet<sup>b</sup> | This work |
| RW125 | RW100 ΔgutA::Ppcp4-3a-ΔlacZ; Tet<sup>b</sup> | This work |
| RW126 | RW100 ΔgutA::Ppcp4-4-ΔlacZ; Tet<sup>b</sup> | This work |
| RW127 | RW100 ΔgutA::Ppcp4-5-ΔlacZ; Tet<sup>b</sup> | This work |
| RW128 | RW100 ΔgutA::Ppcp4-Δrev3-ΔlacZ; Tet<sup>b</sup> | This work |
| RW129 | RW100 ΔgutA::Ppcp4-Δrev4-ΔlacZ; Tet<sup>b</sup> | This work |
| **E. coli** EC1000 | Km<sup>c</sup>; MC1000 derivative carrying a single copy of the pWV01 repA gene in glnB | 45 |
| **L. lactis** NZ9000 | MG1363 ΔrepN::nisRK | 41 |
| **Plasmids** | | |
| pR412T7 | Spec<sup>c</sup>; derivative of pR412 (49) | 8 |
| pORI13 | Erm<sup>d</sup>; orf<sup>e</sup> repA<sup>f</sup>; promoterless lacZ, for single-copy chromosomal lacZ fusions. | 70 |
| pORI280 | Erm<sup>d</sup>; orf<sup>e</sup> repA<sup>f</sup>; deletion derivative of pWV01; constitutive lacZ expression from P32 promoter | 45 |
| pPP2 | Amp<sup>c</sup> Tet<sup>b</sup>; promoterless lacZ; for replacement of bgaA (spr0565) with promoter- lacZ fusions; derivative of pPP1 | 22 |
| pNZ8048 | Cm<sup>c</sup>; nisin-inducible PnisA | 16 |
| pNG8048E | Cm<sup>c</sup> Erm<sup>c</sup>; nisin-inducible PnisA, pNZ8048 derivative containing Erm<sup>c</sup> gene to facilitate cloning | Laboratory collection |
| pRW1 | pORI13::prsA-ΔlacZ | This work |
| pRW2 | pPP2 Pspr0276 | This work |
| pRW3 | pPP2 PrsdD | This work |
| pRW4 | pPP2 PpsaR | This work |
| pRW5 | pPP2 Ppcp4 | This work |
| pRW6 | pPP2 PpsaB | This work |
| pRW7 | pPP2 Ppcp4-3 | This work |
| pRW12 | pPP2 Ppcp4-3a | This work |
| pRW13 | pPP2 Ppcp4-4 | This work |
| pRW14 | pPP2 Ppcp4-5 | This work |
| pRW15 | pPP2 Ppcp4-Δrev3 | This work |
| pRW16 | pPP2 Ppcp4-Δrev4 | This work |
| pRW20 | pORI280 ΔpsaR | 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<sup>b</sup>) in the PsaR binding box | This work |
| pRW23 | pRW12 containing a point mutation (A→G, bp −184<sup>b</sup>) in the PsaR binding box | This work |
| pRW24 | pRW12 containing two point mutations (A→G, bp −184<sup>b</sup>; A→C, bp −185<sup>b</sup>) in the PsaR binding box | This work |
| pRW25 | pNG8048E carrying psaR-strep downstream of PnisA | This work |

<sup>a</sup> Erm<sup>c</sup>, erythromycin resistance; Tet<sup>b</sup>, tetracycline resistance; Cm<sup>c</sup>, chloramphenicol resistance; Spec<sup>c</sup>, spectinomycin resistance.

<sup>b</sup> Where the first base of the ppcp4 start codon (ATG) is +1.

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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 PpcpA-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 PpcpA-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 PpcpA-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, Pspr0183 were constructed and introduced in both the wild type and the psaR mutant. As expected, in the wild type expression of prtA and PpsaB increased upon addition of Zn^{2+} to the GM17 growth medium (Tables 6 and 7). In the psaR

### TABLE 2. Oligonucleotide primers used in this study

| Primer          | Nucleotide sequence (5' to 3') | Restriction site |
|-----------------|--------------------------------|-----------------|
| PBMrIRPi       | AGACCGGGGACTTATCAGCC           | EcoRI           |
| PBMrTn1        | CTAGCCGACCCCTATCTATG           | BamHI           |
| TMr_1          | TGCAATTAACTAGCGACCCATCTATG     | EcoRI           |
| TMr_4          | GGAATCCATTCGCGTCATTACGGG      | BamHI           |
| PpsaB-1-lacZ   | CGGAAATCTCCTTTTTTTTTACACT    | EcoRI           |
| PpsaB-2-lacZ   | CGGAGATCCATTTTTTTTTTTATG     | EcoRI           |
| pspr1485-1     | CGGAATCCGTTAGTTTTTTTTTTTTAGT | EcoRI           |
| PpcpA_f_2      | CGGAATCCATTTTTTTTTTTTTTTTTCT | EcoRI           |
| PpcpA_f_3      | CGGAATCCATTTTTTTTTTTTTTTTTCT | EcoRI           |
| PpcpA_rev_1    | CGGAATCCATTTTTTTTTTTTTTTTTTTCT | EcoRI           |
| PpcpA_rev_2    | CGGAATCCATTTTTTTTTTTTTTTTTTTCT | EcoRI           |
| marR-lacZ1     | CGGAATCCATTTTTTTTTTTTTTTTTTTCT | EcoRI           |
| marR-lacZ2     | CGGAATCCATTTTTTTTTTTTTTTTTTTCT | EcoRI           |
| marR-del1      | CGGAATCCATTTTTTTTTTTTTTTTTTTCT | EcoRI           |
| marR-del2      | CGGAATCCATTTTTTTTTTTTTTTTTTTCT | EcoRI           |
| marR-del3      | CGGAATCCATTTTTTTTTTTTTTTTTTTCT | EcoRI           |
| marR-del4      | CGGAATCCATTTTTTTTTTTTTTTTTTTCT | EcoRI           |
| PrptA-1        | CGGAATCCATTTTTTTTTTTTTTTTTTTCT | EcoRI           |
| PrptA-2        | CGGAATCCATTTTTTTTTTTTTTTTTTTCT | EcoRI           |
| spr0561-1      | CGGAATCCATTTTTTTTTTTTTTTTTTTCT | EcoRI           |
| spr0561-2      | CGGAATCCATTTTTTTTTTTTTTTTTTTCT | EcoRI           |
| PSpr183-1      | CGGAATCCATTTTTTTTTTTTTTTTTTTCT | EcoRI           |
| PSpr183-2      | CGGAATCCATTTTTTTTTTTTTTTTTTTCT | EcoRI           |
| Ppspr1945-1    | CGGAATCCATTTTTTTTTTTTTTTTTTTCT | EcoRI           |
| Ppspr1945-2    | CGGAATCCATTTTTTTTTTTTTTTTTTTCT | EcoRI           |
| PpcpA-box1     | CGGAATCCATTTTTTTTTTTTTTTTTTTCT | EcoRI           |
| PpcpA-box2     | CGGAATCCATTTTTTTTTTTTTTTTTTTCT | EcoRI           |
| PpcpA-mut1.1   | CGGAATCCATTTTTTTTTTTTTTTTTTTCT | EcoRI           |
| PpcpA-mut1.2   | CGGAATCCATTTTTTTTTTTTTTTTTTTCT | EcoRI           |
| PpcpA-mut2.1   | CGGAATCCATTTTTTTTTTTTTTTTTTTCT | EcoRI           |
| PpcpA-mut2.2   | CGGAATCCATTTTTTTTTTTTTTTTTTTCT | EcoRI           |
| PpcpA-mut3.1   | CGGAATCCATTTTTTTTTTTTTTTTTTTCT | EcoRI           |
| PpcpA-mut3.2   | CGGAATCCATTTTTTTTTTTTTTTTTTTCT | EcoRI           |
| Spr1480OX-2    | CGGAATCCATTTTTTTTTTTTTTTTTTTCT | EcoRI           |
| Spr1480OX-new  | CGGAATCCATTTTTTTTTTTTTTTTTTTCT | EcoRI           |
| Spr1480-Ctermstrep_OX | CGGAATCCATTTTTTTTTTTTTTTTTTTCT | EcoRI           |
| Spr14800X-1    | CGGAATCCATTTTTTTTTTTTTTTTTTTCT | EcoRI           |

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To construct a markerless deletion mutant of psaR and found that in this mutant expression of PpcpA-lacZ is highly derepressed independent of Zn^{2+} (Table 5), suggesting that PsaR is responsible for Zn^{2+}-dependent derepression of pcpA expression.
TABLE 3. Summary of transcriptome comparison of \textit{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-\(\beta\)-glucosidase BglA | -7.6     |
| SP0305          | Phosphotransferase system cellubiose-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 cellubiose-specific component IIA | -3.0     |
| SP0309          | Hypothetical protein | -7.6     |
| SP0310          | Phosphotransferase system cellubiose-specific component IIC | -6.0     |
| SP0338          | Putative ATP-dependent ctp protease, ATP binding subunit CtpL | -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$^c$  |
| 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$^c$  |
| 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.

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

A transcriptional fusion in the presence of various metal cations

| Addition to GM17 (mM) | Mean (SD) \(\beta\)-galactosidase activity (Miller units) |
|-----------------------|-----------------------------------------------------|
| None                  | 4 (1)                                               |
| Zn$^{2+}$ (0.2)       | 54 (12)                                             |
| Cu$^{2+}$ (0.4)       | 388 (45)                                            |
| 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$^{3+}$ (0.1)       | 21 (6)                                              |
| Mg$^{2+}$ (1.0)       | 4 (1)                                               |
| Mg$^{2+}$ (10)        | 2 (1)                                               |

$^a$ \(\beta\)-Galactosidase of a \textit{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.
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 PpcpA-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 PpcpA-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 PpcpA-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 PpcpA-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 (PprtA and PpcpA) or overlapping with (PpsaB) the predicted core promoter regions, that might serve as the PsaR operator was uncovered.

To dissect the promoter of pcpA experimentally, we per-
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 $\beta$-galactosidase activity of $P_{pcpA}-4$ was 82% identity with $P_{prtA}$.

**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.

**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 $\beta$-galactosidase activities (Miller units) of the promoter truncations in wild-type D39 (wt, strains RW131 to RW134) and the 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.
in wild-type D39 was 1.197 ± 78 Miller units, which is similar to the values for \( \text{PcpA}^{-1} \) in GM17 and GM17 with \( \text{Zn}^{2+} \) (Fig. 2), indicating that the effects of \( \text{Zn}^{2+} \) and \( \text{Mn}^{2+} \) 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 (\( \text{PcpA}^{-1}\text{a} \)) 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 \( \text{PcpA} \) promoter. For subclone \( \text{PpcpcA}^{-1} \), expression was close to zero under all conditions. Deletion of the same region as in \( \text{PpcpcA}^{-1} \) but now from the 3′ side (\( \text{PpcpcA}^{-1}\text{rev} \)) versus \( \text{PpcpcA}^{-1}\text{rev} \); 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 \( \text{PpcpcA}^{-1} \) and \( \text{PpcpcA}^{-1} \) (Fig. 2 and 1B).

To further show that the predicted PsAR operator is functional, a 64-bp DNA fragment of the \( \text{pcpcA} \) promoter comprising the PsAR operator was put into plasmid pNG8048E, which replicates in \( \text{S. pneumoniae} \) (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 \( \text{PpcpcA-lacZ} \) transcriptional fusion, transcription from \( \text{PpcpcA} \) was strongly derepressed, showing that this 64-bp stretch of DNA titrates away the repressive effect of PsAR on the expression of \( \text{pcpcA} \) (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 \( \text{pcpcA} \). The entire \( \text{S. pneumoniae} \) 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 regions of \( \text{pcpcA}, \text{psaB}, \) and \( \text{prtA} \) (Fig. 3A to C, lanes 3). PsAR-Strep did not bind under any condition to a truncated \( \text{pcpcA} \) promoter lacking the PsAR binding box (Fig. 3D). Besides \( \text{Mn}^{2+}, 0.05 \text{mM} \text{Co}^{2+} \) was also able to stimulate the binding of PsAR-Strep to the promoter fragments, whereas 0.05 mM \( \text{Fe}^{2+}, \text{Cu}^{2+}, \text{Ni}^{2+} \), and \( \text{Zn}^{2+} \) did not (data not shown). These data show that the PsAR-Strep–DNA interaction was specific and indicates that PsAR directly functions as a \( \text{Mn}^{2+} \)-dependent repressor of its target genes. Based on the lacZ expression studies, we hypothesized that \( \text{Zn}^{2+} \) should somehow impair PsAR binding to fulfill its function as an \( \text{Mn}^{2+} \)-dependent repressor. Therefore, experiments addressing the influence of \( \text{Zn}^{2+} \) on the in vitro PsAR-Strep–DNA interaction in the presence of \( \text{Mn}^{2+} \) were performed (Fig. 3A, B, and C, lanes 4 to 8). These demonstrated that the stimulatory effect of \( \text{Mn}^{2+} \) on the binding of PsAR-Strep to all three promoter fragments was counteracted by the addition of \( \text{Zn}^{2+} \). There was also a weaker counteracting effect of \( \text{Cu}^{2+} \) for the \( \text{pcpcA} \) and \( \text{prtA} \) promoters (Fig. 3A and C, lanes 8). Thus, \( \text{Mn}^{2+} \) stimulates PsAR binding to the operators in the \( \text{pcpcA}, \text{psaB}, \) and \( \text{prtA} \) promoters, whereas in the presence of \( \text{Zn}^{2+} \), PsAR binding is abolished, indicating that \( \text{Zn}^{2+} \) and \( \text{Zn}^{2+} \) exert their regulatory effects on \( \text{pcpcA}, \text{prtA}, \) and \( \text{psaBCA} \) expression directly through PsAR.

**DISCUSSION**

In this study, we analyzed the transcriptional change of the human pathogen \( \text{S. pneumoniae} \) in response to a high level of \( \text{Zn}^{2+} \). Expression of several genes and operons with diverse functions was affected by \( \text{Zn}^{2+} \), including \( \text{pcpcA}, \text{prtA}, \) and \( \text{psaBCA} \). The observed \( \text{Zn}^{2+} \)-dependent expression of these virulence genes was shown to be directly mediated by the \( \text{Mn}^{2+} \)-responsive repressor PsAR (31). We further demonstrate that this is caused by \( \text{Mn}^{2+} \)-dependent binding of PsAR to and \( \text{Zn}^{2+} \)-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 \( \text{Mn}^{2+} \) and \( \text{Zn}^{2+} \) may vary greatly between different sites in the human body. For example, in lung tissue the total concentration of \( \text{Mn}^{2+} \) is approximately 0.2 μg/g (wet weight) (3.6 μM) and that of \( \text{Zn}^{2+} \) is 15 μg/g (wet weight) (229 μM), whereas in the blood serum concentrations of \( \text{Mn}^{2+} \) and \( \text{Zn}^{2+} \) are 0.5 ng/ml (9 nM) and 1.0

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**TABLE 8. Mutational analysis of the PsAR operator**

| PsAR box | Sequence (5′→3′)* | Mean (SD) β-galactosidase activity (Miller units) for the following strain and medium: |
|----------|------------------|---------------------------------------------------------------------------------|
| Wild type | AAATTAAAGTGTTTTAAATTT | Wild type<br>GM17 | 733 (123) | 835 (122) | 1,288 (170) | 1,331 (167) |
| mut 1    | AAATTAAAGTGTTTTAAATTT | GM17 + Zn2+ (0.4 mM) | 74 (15) | 376 (78) | 1,160 (135) | 1,271 (122) |
| mut 2    | AAATTAAAGTGTTTTAAATTT | GM17 + Zn2+ (0.4 mM) | 121 (25) | 416 (75) | 1,300 (101) | 1,278 (69) |
| mut 3    | AAATTAAAGTGTTTTAAATTT | GM17 + Zn2+ (0.4 mM) | 58 (12) | 464 (16) | 1,389 (111) | 1,378 (178) |

* Sequences of wild-type and mutant PsAR boxes of the \( \text{pcpcA} \) promoter; point mutations are in bold.
FIG. 3. In vitro interaction of PsaR-Strep with the pcpA (A), psaB (B), and prtA (C) promoter regions and with a truncated PpcpA fragment lacking the PsaR operator (D). Purified PsaR-Strep was added at concentrations of 5 nM (PcpA), 11 nM (PprtA), and 25 nM (PpsaB). 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, pcp4 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, MsrR 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 CczA in *B. subtilis* (24). CczA normally is activated for DNA release in the presence of Zn$^{2+}$, but Cu$^{2+}$ inhibits the Zn$^{2+}$-induced allosteric, 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. gordoni* 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 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, 5′-AAATTAAAACAATTCTC-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, *rma*, *rbg*, *rbg*, *rc*, and *rfl* (25), weak repression of *rlrA* by PsaR likely explains the up-regulation 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*.

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