

RESEARCH ARTICLE

Ni²⁺-Dependent and PsaR-Mediated Regulation of the Virulence Genes *pcpA*, *psaBCA*, and *prtA* in *Streptococcus pneumoniae*

Irfan Manzoor^{1,2}, Sulman Shafeeq³, Oscar P. Kuipers^{1*}

1 Department of Molecular Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 7, 9747 AG, Groningen, The Netherlands, **2** Department of Bioinformatics and Biotechnology, Government College University, Faisalabad, Pakistan, **3** Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Nobels väg 16, 17177, Stockholm, Sweden

* o.p.kuipers@rug.nl



OPEN ACCESS

Citation: Manzoor I, Shafeeq S, Kuipers OP (2015) Ni²⁺-Dependent and PsaR-Mediated Regulation of the Virulence Genes *pcpA*, *psaBCA*, and *prtA* in *Streptococcus pneumoniae*. PLoS ONE 10(11): e0142839. doi:10.1371/journal.pone.0142839

Editor: Willem van Schaik, University Medical Center Utrecht, NETHERLANDS

Received: August 18, 2015

Accepted: October 27, 2015

Published: November 12, 2015

Copyright: © 2015 Manzoor et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: The DNA microarray data have been deposited to Gene Expression Omnibus (GEO) under the accession number GSE73818.

Funding: The authors received no specific funding for this work.

Competing Interests: The authors have declared that no competing interests exist.

Abstract

Previous studies have shown that the transcriptional regulator PsaR regulates the expression of the PsaR regulon consisting of genes encoding choline binding protein (PcpA), the extracellular serine protease (PrtA), and the Mn²⁺-uptake system (PsaBCA), in the presence of manganese (Mn²⁺), zinc (Zn²⁺), and cobalt (Co²⁺). In this study, we explore the Ni²⁺-dependent regulation of the PsaR regulon. We have demonstrated by qRT-PCR analysis, metal accumulation assays, β-galactosidase assays, and electrophoretic mobility shift assays that an elevated concentration of Ni²⁺ leads to strong induction of the PsaR regulon. Our ICP-MS data show that the Ni²⁺-dependent expression of the PsaR regulon is directly linked to high, cell-associated, concentration of Ni²⁺, which reduces the cell-associated concentration of Mn²⁺. *In vitro* studies with the purified PsaR protein showed that Ni²⁺ diminishes the Mn²⁺-dependent interaction of PsaR to the promoter regions of its target genes, confirming an opposite effect of Mn²⁺ and Ni²⁺ in the regulation of the PsaR regulon. Additionally, the Ni²⁺-dependent role of PsaR in the regulation of the PsaR regulon was studied by transcriptome analysis.

Introduction

Streptococcus pneumoniae, an encapsulated bacterium is a common cause of otitis media, bacterial meningitis, bacteremia, and pneumoniae, leading to millions of death every year, particularly in developing countries [1–3]. Although, *S. pneumoniae* has an asymptomatic association within the human nasopharyngeal cavity [4], it has also the ability to spread to other sites in the human body to cause severe infections [5–7]. The survival of *S. pneumoniae* in different niches inside the human body might depend on the availability of macro- and micro- nutrients on the respective infection sites. Metal ions are an integral part of nutrients, and play a vital role in the regulation of many cellular processes in *S. pneumoniae* [8–10]. The deprivation or excess of metal ions may result in impaired growth of bacterial cells [11]. Therefore, proper regulation of metal homeostasis is important for the survival of *S. pneumoniae*. For this

purpose, *S. pneumoniae* possesses metal uptake and -efflux systems that are specific to different metal ions, including manganese (Mn²⁺), zinc (Zn²⁺), copper (Cu²⁺), and iron (Fe²⁺) [12–16]. These systems are tightly regulated by different transcriptional regulators in the presence of specific metal ions [9,10,16–18]. For example, the expression of the *adc* operon encoding Zn²⁺ transporters is repressed by transcriptional regulator AdcR in the presence of Zn²⁺ [10,19]. The *cop* operon, encoding proteins for Cu²⁺ homeostasis is activated by transcriptional regulator CopY in the presence of Cu²⁺ [16]. The expression of Mn²⁺ uptake system *psaBCA* is regulated by transcriptional regulator PsaR and is dependent on the balance between Mn²⁺, Zn²⁺, and cobalt (Co²⁺) [20,21].

The interference or competition of metal ions for metal-sensory proteins has been reported for many bacteria, including *S. pneumoniae* [9,16,21–25]. The interplay of metal ions on specific protein depends on the concentration of metal ions, the nature of the coordinating ligands [26–28], and the effect of the Irving-William stability series (where the order is Mn²⁺ < Fe²⁺ < Co²⁺ < Ni²⁺ < Cu²⁺ > Zn²⁺) on protein metal ion affinity [29]. In *S. pneumoniae*, the CopY-mediated expression of the Cu²⁺-efflux system depends on the availability of Cu²⁺ and Zn²⁺, where the Cu²⁺ induced expression of Cu²⁺-efflux system is nullified by the addition of high Zn²⁺ concentrations [16]. Similarly, the expression of the PsaR regulon (*pcpA*, *psaBCA*, and *prtA*) is repressed by Mn²⁺ and derepressed by Zn²⁺ [9]. In our previous study, we have demonstrated the opposite effect of Mn²⁺ and Co²⁺ in the regulation of the PsaR regulon [21]. Moreover, metal ions can also compete to bind with extracellular proteins, which ultimately results in the impaired homeostasis of other metal ions. In *S. pneumoniae*, Zn²⁺ and Cd²⁺ have been shown to cause intracellular Mn²⁺ deficiency [30,31]. Several proteins with ligase activity have been reported to bind with nickel (Ni²⁺) [32]. However, the role of Ni²⁺ in pneumococcal metabolism and virulence has not been determined.

Here, we used qRT-PCR, β -galactosidase assays, EMSAs, and ICP-MS analyses to investigate the role of Ni²⁺ in the regulation of the PsaR regulon in *S. pneumoniae* D39. Our results demonstrate that the expression of the PsaR regulon is highly derepressed in the presence of Ni²⁺ and that a high concentration of Ni²⁺ causes cell-associated Mn²⁺ deficiency in *S. pneumoniae*. Furthermore, an opposite effect of Mn²⁺ and Ni²⁺ on the PsaR-mediated expression of the PsaR regulon is found.

Material and Methods

Bacterial strains, growth conditions and DNA manipulation

All the bacterial strains and plasmids used in this study are listed in Table 1. *S. pneumoniae* D39 was grown in 1% Chelex 100 resin (Bio-rad)-treated Chemically Defined Medium (CDMchelex). Salts of metal ions, *i.e.* MnSO₄ and NiSO₄ were added separately as specified in the Results section. *Escherichia coli* strain EC1000 was cultured at 37°C. The following concentrations of antibiotics were used in the media for the selection of strains where necessary: tetracycline: 2.5 $\mu\text{g ml}^{-1}$ for *S. pneumoniae*; chloramphenicol: 4 $\mu\text{g.ml}^{-1}$ for *Lactococcus lactis*; and ampicillin; 100 $\mu\text{g.ml}^{-1}$ for *E. coli*. Chromosomal DNA of *S. pneumoniae* D39 was used as a template for PCR amplification [33,34]. All bacterial strains used in this study were stored at -80°C in 10% (v/v) glycerol stock. Primers used in this study are based on the genome sequence of *S. pneumoniae* D39 and listed in Table 2.

β -galactosidase assays

The β -galactosidase assays were performed as described before [35] by using derivatives of *S. pneumoniae* D39 grown till mid-exponential phase of growth (OD₆₀₀ = 0.25) in triplicate in CDMchelex at 37°C supplemented with different metal ion concentrations (w/v) as mentioned

Table 1. List of strains and plasmids used in this study.

Strain/ plasmid	Description	Source
<i>S. pneumoniae</i>		
D39	Serotype 2 strain, <i>cps 2</i>	Laboratory of P. Hermans
RW100	D39 Δ <i>psaR</i>	[9]
RW104	D39 <i>nisRK</i> Δ <i>bgaA::PprtA-lacZ</i> ; Erm ^R	[9]
RW109	D39 <i>nisRK</i> Δ <i>psaR</i> Δ <i>bgaA::PprtA-lacZ</i> ; Erm ^R	[9]
IM402	D39 Δ <i>bgaA::PpsaB-lacZ</i> ; Tet ^R	[21]
IM403	D39 Δ <i>bgaA::PpcpA-lacZ</i> ; Tet ^R	[21]
IM451	RW100 Δ <i>bgaA::PpsaB-lacZ</i> ; Tet ^R	[21]
IM452	RW100 Δ <i>bgaA::PpcpA-lacZ</i> ; Tet ^R	[21]
<i>E. coli</i>		
EC1000	Km ^R ; MC1000 derivative carrying a single copy of the pWV1 <i>repA</i> gene in <i>glgB</i>	Laboratory collection
<i>L. lactis</i>		
NZ9000	MG1363 Δ <i>pepN::nisRK</i>	[39]

doi:10.1371/journal.pone.0142839.t001

in the Results section. Standard deviation was calculated from three independent replicates of each sample.

Quantitative real time (qRT)-PCR experiments

For qRT-PCR, *S. pneumoniae* D39 wild-type was grown in CDM with and without the addition of 0.3 mM Ni²⁺ and harvested at mid-exponential growth phase. RNA was isolated as described before [16]. Additionally, RNA was treated with DNase I (RNase-free) (Thermo Fisher Scientific, St. Leon-Rot, Germany) for 60 min at 37°C to remove any DNA contamination. qRT-PCR

Table 2. List of primers used in this study.

Name	Nucleotide Sequence (5'→3')
Primers for qRT-PCR	
prtA-F	GCAGCCTATGCCCTAATG
prtA-R	GTTTTAGTGTCTATTACAGG
pcpA-F	CCAATCCTAGCAGATACTCC
pcpA-R	GTAGGAATCGTGAATGG
psaB-F	CCTCAGTGTCTCCTACAAAG
psaB-R	GGCAATTCGGTGTAAAGG
psaC-F	CCATTTCTACAAAATGCCTT
psaC-R	TCCAAAGACAATGGCTCC
psaA-F	CTCGTTCTCTTTCTTTCTG
psaA-R	CTTAACGTCTTCAGGAA
gyrA-F	CGAGGCACGTATGAGCAAGA
gyrA-R	GACCAAGGGTCCCGTTTCAT

doi:10.1371/journal.pone.0142839.t002

was performed in triplicates as described before [16]. The transcription level of the target genes was normalized to *gyrA* transcription using the relative expression software tool [36].

Inductively coupled plasma-mass spectrometry (ICP-MS) analysis

To measure the intracellular concentrations of metal ions, *S. pneumoniae* D39 was grown till OD₆₀₀ = 0.2–0.25 in 20 ml CDMchelex supplemented with either 0.02 mM MnSO₄, 0.02 mM MnSO₄ + 0.1 mM NiSO₄, 0.02 mM MnSO₄ + 0.3 mM NiSO₄, or 0.02 mM MnSO₄ + 0.5 mM NiSO₄. Cell cultures were washed twice with CDMchelex medium and twice with overnight Chelex (Sigma) treated phosphate-buffered saline (PBS) with 1 mM nitrilotriacetic acid. The cell pellets were dried overnight in a Speedvac at room temperature and lysed in 2.5% nitric acid (Ultrapure, Sigma Aldrich) for 10 min at 95°C by vigorous vortexing. ICP-MS analysis on the lysed cell samples were performed as described before [31]. Amounts of metal ions are expressed in the Result section as μg g⁻¹ dry weight of cells.

DNA Microarray Analysis

To observe the impact of the *psaR* deletion on the transcriptome of *S. pneumoniae* in the presence of Ni²⁺, *S. pneumoniae* D39 wild-type and its isogenic *psaR* mutant (RW100) [9] were grown in two biological replicates in CDMchelex with 0.3 mM of NiSO₄·(H₂O)₆. Cells were harvested at the mid-exponential growth phase. Further experiments were performed essentially as described before [37]. DNA microarray data were analyzed by using the *MicroPrep* software package as described before [38]. To identify differentially expressed genes a Bayesian p-value <0.001 and a fold-change cut-off of ≥ 2 were applied. The DNA microarray data have been deposited to Gene Expression Omnibus (GEO) with accession number GSE73818.

Purification of Strep-tagged PsaR and Electrophoretic mobility shift assays

The overexpression and purification of C-terminally Strep-tagged PsaR was achieved in *L. lactis* NZ9000 essentially as described before [9,39]. Electrophoretic mobility shift assays (EMSAs) were performed essentially as described previously [10]. In short, PCR products of *PpcpA*, *PpsaB*, *PprtA*, and *PadcR* were labeled with [γ-³³P] ATP. EMSAs were carried out in buffer containing 20 mM Tris-HCL (pH 8.0), 5mM MgCl₂, 8.7% (w/v) glycerol, 62.5 mM KCL. 25 μg/ml bovine serum albumin, 25 μg/ml poly (dI-dC), and 5000 cpm of [γ-³³P] ATP-labeled PCR product. Reactions were incubated at 30°C for 30 min before loading on gels. Gels were run in 1 M Tris-borate buffer (PH 8.3) at 95 V for 90 min.

Results

Ni²⁺-dependent expression of the PsaR regulon in *S. pneumoniae*

In a previous study, we have shown that, like Zn²⁺, Co²⁺ also induces the expression of the PsaR regulon, while addition of Mn²⁺ causes repression of the PsaR regulon [21]. The PsaR regulon comprises the *psa* operon (*psaBCA*), encoding Mn²⁺-dependent ABC transporters, *pcpA*, encoding a choline binding protein and *prtA*, encoding a serine protease. In this study, we decided to explore the impact of Ni²⁺ on the expression of the PsaR regulon. To investigate the impact of Ni²⁺ on the expression of the PsaR regulon, cells were grown in CDM with either 0 or 0.3 mM Ni²⁺, and qRT-PCR was performed. qRT-PCR data revealed that the expression of *pcpA*, *psaBCA*, and *prtA* was highly upregulated in the presence of 0.3 mM Ni²⁺ compared to 0 mM Ni²⁺ (Table 3), suggesting the putative role of Ni²⁺ in the regulation of the PsaR regulon.

Table 3. The relative expression of *prrA*, *psaB*, *psaC*, *psaA*, and *pcpA* genes was normalized with the housekeeping gene *gyrA*. The log² fold increase is relative to the expression in the D39 wild-type grown in CDMchelex with 0.3 mM Ni²⁺ to that with 0 mM Ni²⁺. Standard deviation of three independent replications is given in parentheses.

Gene tag ^a	Function ^b	Fold Raito
<i>spd_0558</i>	Cell wall-associated serine protease PrtA	4.53 (1.22)
<i>spd_1461</i>	Manganese ABC transporter, ATP-binding protein, PsaB	2.83 (0.24)
<i>spd_1462</i>	Manganese ABC transporter, permease protein, PsaC	3.15 (0.30)
<i>spd_1463</i>	Manganese ABC transporter, ATP-binding protein, PsaA	5.88 (1.55)
<i>spd_1965</i>	Choline binding protein PcpA	10.53 (3.09)

^aGene numbers refer to D39 locus tags.

^bD39 annotation/TIGR4 annotation. [34,62]

doi:10.1371/journal.pone.0142839.t003

To further verify the role of Ni²⁺ in the regulation of the PsaR regulon in *S. pneumoniae*, the D39 wild-type strain containing either *PpcpA-lacZ*, *PpsaB-lacZ*, or *PprptA-lacZ* was grown in CDMchelex and CDMchelex-Mn²⁺ (CDMchelex without Mn²⁺) with the addition of 0, 0.1, 0.3 or 0.5 mM Ni²⁺, and β-galactosidase assays were performed. Our β-galactosidase data (Miller Units) revealed that the expression of the *PpcpA-lacZ*, *PpsaB-lacZ*, and *PprptA-lacZ* increased significantly with increasing concentrations of Ni²⁺ in CDMchelex and CDMchelex-Mn²⁺ (Table 4). However, the expression of these transcriptional *lacZ*-fusions was much higher in CDMchelex-Mn²⁺ compared to CDMchelex due to the unavailability of Mn²⁺ in CDMchelex-Mn²⁺. This data indicates that the expression of *pcpA*, *psaBCA*, and *prrA* is regulated by Ni²⁺ and in agreement with our qRT-PCR analysis data mentioned above.

PsaR mediates expression of the PsaR regulon in the presence of Ni²⁺

To check, whether the observed Ni²⁺-dependent high expression of the PsaR regulon is mediated by the Mn²⁺/Zn²⁺/Co²⁺-responsive transcriptional regulator PsaR, the *psaR* mutant strain (RW100) containing *PpcpA-lacZ*, *PpsaB-lacZ*, and *PprptA-lacZ* were grown in

Table 4. β-galactosidase activity (miller units) of *PpcpA-lacZ*, *PpsaB-lacZ*, and *PprptA-lacZ* in *S. pneumoniae* D39 wild-type and Δ*psaR* (RW100) grown in CDMchelex and CDMchelex-Mn²⁺ supplemented with various concentrations of Ni²⁺ (mM). Standard deviation of three independent replications is given in parentheses, whereas ND stands for not determined. Noteworthy, *lacZ* was fused to the 3' end of *prrA** on the native chromosomal location, using plasmid pOR113. This might explain the lower Miller Units of *PprptA* compared to *PpcpA* and *PpsaB*.

Medium	β-galactosidase Activity (Miller Units)					
	D39 (wt)			D39 Δ <i>psaR</i>		
	<i>PpcpA</i>	<i>PpsaB</i>	<i>PprptA</i> *	<i>PpcpA</i>	<i>PpsaB</i>	<i>PprptA</i> *
CDMchelex						
Ni ²⁺ [0.0]	29 (7)	68 (8)	0.57 (0.06)	1363 (35)	1290 (30)	2.1 (0.2)
Ni ²⁺ [0.1]	48 (4)	118 (11)	0.92 (0.06)	1226 (42)	1230 (40)	2.1 (0.3)
Ni ²⁺ [0.3]	73 (6)	218 (20)	1.38 (0.1)	1195 (52)	1220 (24)	2.2 (0.4)
Ni ²⁺ [0.5]	101 (8)	419 (15)	1.57 (0.1)	1190 (23)	1202 (55)	2.0 (0.2)
CDMchelex-Mn²⁺						
Ni ²⁺ [0.0]	84 (10)	565 (40)	0.74 (0.2)	ND	ND	ND
Ni ²⁺ [0.1]	160 (12)	628 (43)	1.24 (0.2)	ND	ND	ND
Ni ²⁺ [0.3]	360 (36)	873 (50)	1.62 (0.1)	ND	ND	ND
Ni ²⁺ [0.5]	571 (30)	1072 (102)	1.87 (0.1)	ND	ND	ND

doi:10.1371/journal.pone.0142839.t004

Table 5. Summary of transcriptome comparison of *S. pneumoniae* D39 wild-type strain with Δ psaR grown in CDM with 0.3 mM Ni²⁺.

Gene tag ^a	Function ^b	Ratio ^c	P-value
<i>spd_0616</i>	Amino acid ABC transporter, ATP-binding protein	-4.40	1.32E-05
<i>spd_0617</i>	Amino acid ABC transporter, permease protein	-5.99	7.46E-07
<i>spd_0618</i>	Amino acid ABC transporter, permease protein	-6.19	4.27E-07
<i>spd_0558</i>	Cell wall-associated serine protease PrtA	3.02	6.65E-05
<i>spd_1461</i>	Manganese ABC transporter, ATP-binding protein	2.39	7.47E-05
<i>spd_1462</i>	Manganese ABC transporter, permease protein, putative	2.52	1.46E-04
<i>spd_1450</i>	Iron-dependent transcriptional regulator (PsaR)	-4.43	7.45E-07
<i>spd_1632</i>	Hypothetical protein	-2.22	9.00E-04
<i>spd_1965</i>	Choline binding protein PcpA	14.42	2.65E-09

^aGene numbers refer to D39 locus tags.

^bD39 annotation/TIGR4 annotation. [34,62]

^cRatios >2.0 or <2.0 (Δ psaR / wild-type).

doi:10.1371/journal.pone.0142839.t005

CDMchelex with 0, 0.1, 0.3 or 0.5 mM Ni²⁺. The expression of *PpcpA-lacZ*, *PpsaB-lacZ*, and *PprtA-lacZ* was highly derepressed in the *psaR* mutant. We did not observe significant difference in the expression of *PpcpA-lacZ*, *PpsaB-lacZ*, and *PprtA-lacZ* in the *psaR* mutant strain at different concentrations of Ni²⁺ (Table 4), indicating that PsaR mediates the Ni²⁺-dependent expression of the PsaR regulon.

To analyze the impact of *psaR* deletion on the global gene expression of *S. pneumoniae* and find more targets of PsaR in the presence of Ni²⁺, transcriptome of *psaR* mutant strain was compared with *S. pneumoniae* D39 wild-type strain grown in CDMchelex with 0.3 mM Ni²⁺. The expression of *psaR* was significantly downregulated, confirming the inactivation of *psaR* in the *psaR* deletion strain. The expression of *pcpA*, *psaBCA*, and *prtA* was highly upregulated in the *psaR* mutant (Table 5). This data further confirms our β -galactosidase data mentioned above indicating Ni²⁺-dependent derepression of the PsaR regulon. We did not find any new target of PsaR in the presence of Ni²⁺. Notably, an operon (*spd_0616-spd_618*) encoding amino acid ABC transporter proteins was downregulated in our transcriptomic analysis, but in our β -galactosidase assay we did not observe any activity of the respective promoter of this operon in the *psaR* mutant (Data not shown here).

Opposite effect of Ni²⁺ and Mn²⁺ in the regulation of the PsaR regulon

Previous studies showed that the PsaR-mediated expression of the PsaR regulon depends on the balance between Mn²⁺, Co²⁺ and/ or Zn²⁺ [9,21,31]. In this study, we observed that the expression of the PsaR regulon was highly derepressed in response to various Ni²⁺ concentrations. Therefore, we decided to explore the influence of Ni²⁺ and Mn²⁺ together on the expression of the PsaR regulon. The expression of *PpcpA-lacZ*, *PpsaB-lacZ*, and *PprtA-lacZ* in *S. pneumoniae* D39 wild-type was measured at different concentrations of Ni²⁺ and Mn²⁺ in CDMchelex and CDMchelex-Mn²⁺ (Table 6). β -galactosidase data (Miller units) showed that high expression of *PpcpA-lacZ*, *PpsaB-lacZ*, and *PprtA-lacZ* at 0.1 or 0.3 mM of Ni²⁺ was nullified by the addition of 0.02 or 0.05 mM Mn²⁺ (Table 6). However, Mn²⁺ repression was higher in CDMchelex compared to CDMchelex-Mn²⁺. This might be due to the fact that CDMchelex contains 5–7 μ M of Mn²⁺ which is enough to cause the repression of the PsaR regulon [21]. These results suggest that the Mn²⁺-dependent repression of the PsaR regulon is derepressed by the addition of Ni²⁺.

Table 6. Expression level (in Miller units) of *PpcpA-lacZ*, *PpsaB-lacZ*, and *PprtA-lacZ* in D39 wild-type in CDMchelex and CDMchelex-Mn²⁺ supplemented with different concentrations of Ni²⁺ and Mn²⁺ (mM). Standard deviation of three independent replicates is indicated in bars.

Medium	β-galactosidase Activity (Miller Units)		
	<i>PpcpA</i>	<i>PpsaB</i>	<i>PprtA</i>
CDMchelex	29 (3)	72 (9)	0.50 (0.07)
Ni ²⁺ [0.1]	32 (4)	99 (10)	0.91 (0.08)
Ni ²⁺ [0.3]	66 (6)	200 (28)	1.20 (0.2)
Ni ²⁺ [0.1] + Mn ²⁺ [0.02]	20 (5)	79 (7)	0.55 (0.05)
Ni ²⁺ [0.3] + Mn ²⁺ [0.02]	36 (27)	142 (12)	0.69 (0.1)
Ni ²⁺ [0.1] + Mn ²⁺ [0.05]	20 (5)	79 (7)	0.30 (0.05)
Ni ²⁺ [0.3] + Mn ²⁺ [0.05]	36 (27)	142 (12)	0.35 (0.1)
CDMchelex-Mn ²⁺	90 (15)	550 (50)	0.70 (0.2)
Ni ²⁺ [0.1]	180 (18)	640 (48)	1.10 (0.2)
Ni ²⁺ [0.3]	390 (60)	890 (106)	1.40 (0.1)
Ni ²⁺ [0.1] + Mn ²⁺ [0.02]	100 (10)	450 (70)	0.80 (0.05)
Ni ²⁺ [0.3] + Mn ²⁺ [0.02]	280 (47)	565 (120)	0.90 (0.1)
Ni ²⁺ [0.1] + Mn ²⁺ [0.05]	50 (10)	210 (70)	0.30 (0.05)
Ni ²⁺ [0.3] + Mn ²⁺ [0.05]	120 (47)	335 (120)	0.60 (0.1)

doi:10.1371/journal.pone.0142839.t006

Ni²⁺ counteracts the Mn²⁺-PsaR interaction with *PpcpA*, *PpsaBCA*, and *PprtA*

To find out whether the observed opposite effects of Ni²⁺ and Mn²⁺ on the expression of *pcpA*, *psaBCA*, and *prtA* are mediated by the direct DNA binding activity of the PsaR protein, the effects of these metal ions on the binding of PsaR-Strep tag to ³³P-labeled promoters of *pcpA*, *psaB*, and *prtA* were studied *in vitro*. The promoter region of *phtB* was used as a negative control. Due to the metal-ion chelating ability of EDTA, we decided to exclude it from all buffers used to perform EMSAs. PsaR-Strep tag was not able to bind with the promoter regions of *pcpA*, *psaB*, and *prtA* without the addition of any metal ion (Fig 1A, 1B and 1C. Lane 2) which is in agreement with the previous study [9]. First of all, we checked the DNA binding activity of PsaR-Strep to the promoter regions of *pcpA*, *psaB*, and *prtA* with different concentrations of Mn²⁺. We observed that 0.05 and 0.1 mM Mn²⁺ were able to stimulate the binding of PsaR-Strep tag to the promoter region of *pcpA*. However, only 0.1 mM Mn²⁺ was able to stimulate the binding of PsaR-Strep to the promoter regions of *psaB* and *prtA* (Fig 1A, 1B and 1C. Lane 4). No binding of PsaR-Strep to the promoter regions of *psaB*, and *prtA* was observed at 0.05 mM Mn²⁺ (Fig 1A, 1B and 1C. Lane 3). Interestingly, no shift in the promoter regions of *pcpA*, *psaB*, and *prtA* was observed with 0.2 or 0.4 mM Ni²⁺ (Fig 1A, 1B and 1C. Lane 5 and 6), suggesting that Ni²⁺ does not stimulate the binding of PsaR with *pcpA*, *psaB*, and *prtA* promoters. Previously, it has been shown that Zn²⁺ binds to the PsaR in such a way which leads to the inactivation of Mn²⁺-PsaR interaction with the promoter regions of *pcpA*, *psaB*, and *prtA* [9]. We hypothesized that like Zn²⁺, Ni²⁺ also interferes in the Mn²⁺-dependent binding of PsaR-Strep to the promoter regions of *pcpA*, *psaB*, and *prtA*. Therefore, we decided to explore the influence of Ni²⁺ on the *in vitro* Mn²⁺-PsaR-Strep tag interaction. Interestingly, the binding of PsaR to all three promoters in the presence of Mn²⁺ was impaired with the addition of Ni²⁺ (Fig 1 Lanes 7–10). This data suggests that the Mn²⁺-PsaR interaction with *pcpA*, *psaB*, and

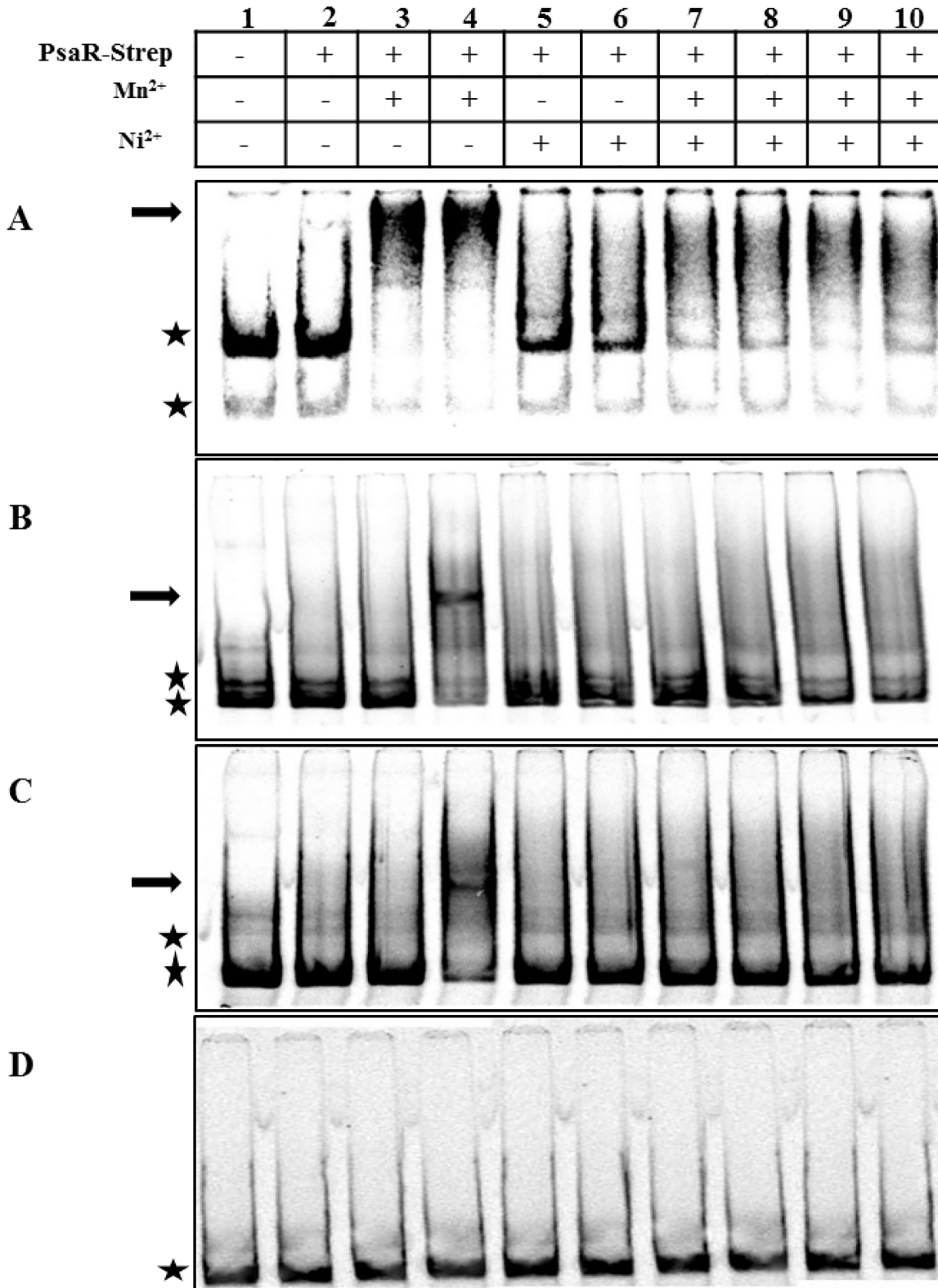


Fig 1. *In vitro* interaction of PsaR-Strep tag with the promoter regions of *pcpA* (A), *psaB* (B), *prtA* (C), and *phtB* (D). PsaR-Strep was added at concentration of 30 nM as indicated above panel, while lane 1 is without added protein. Arrows indicate the position of shifted probe and asterisks indicate the position of free probe. Mn²⁺ was added with concentrations of 0.05 mM in lanes 3, 7, and 9, and 0.1 mM in lane 4, 8, and 10. Ni²⁺ was added with concentrations of 0.2 mM in lanes 5, 7, and 9, and 0.4 mM in lanes 6, 8, and 10.

doi:10.1371/journal.pone.0142839.g001

prtA promoters is competed away in the presence of Ni²⁺, indicating a direct role of Ni²⁺ in the regulation of the PsaR regulon through PsaR.

A high concentration of Ni²⁺ in the medium leads to Mn²⁺ deficiency in the cells

To determine the cell-associated concentrations of metal ions, we performed an ICP-MS analysis on the cells grown in CDMchelex either with 0 or 0.3 mM of Ni²⁺. ICP-MS data revealed that the cells grown in the presence of 0.3 mM Ni²⁺ accumulate 10-fold ($P < 0.01$, One way ANOVA) more Ni²⁺ (Fig 2A) compared to cells grown in the absence of Ni²⁺. No significant difference in the concentrations of other metal ions was observed in our ICP-MS analysis except for Mn²⁺. The concentration of Mn²⁺ was reduced by 1.5-fold ($P < 0.01$, One way ANOVA) in the presence of Ni²⁺ (Fig 2A). This data indicates that high concentration of Ni²⁺ leads to Mn²⁺ deficiency in the cell. To study this in more details, we have checked the impact of various concentrations of Ni²⁺ on the cell-associated Mn²⁺. Cells were grown in CDMchelex with the addition of 0.02 mM Mn²⁺, and 0, 0.1, 0.3 or 0.5 mM Ni²⁺. As expected, addition of Ni²⁺ in medium leads to an increased cell-associated Ni²⁺ concentration. The cell-associated Ni²⁺ concentration was increased by 2-fold ($P < 0.01$, One way ANOVA) at 0.1 mM Ni²⁺, 13-fold at 0.3 mM Ni²⁺, and 16-fold at 0.5 mM Ni²⁺ when compared to 0 mM Ni²⁺ (Fig 2B). ICP-MS analyses data further revealed that an increasing concentration of Ni²⁺ leads to a decrease in the concentrations of Mn²⁺. The cell-associated concentration of Mn²⁺ was decreased by 1.25-fold ($P < 0.01$, One way ANOVA) at 0.1 mM Ni²⁺, 3.52-fold ($P < 0.01$, One way ANOVA) at 0.3 mM Ni²⁺, and 7.4-fold ($P < 0.01$, One way ANOVA) at 0.5 mM Ni²⁺ (Fig 2B) when compared to the Mn²⁺ concentration at 0 mM Ni²⁺. Notably, the cell-associated concentration of other metal ions (Zn²⁺, Fe²⁺, and Co²⁺) was not affected (Fig 2). This data demonstrate that Ni²⁺ has ability to cause Mn²⁺ starvation which ultimately leads to the high expression of the PsaR regulon in the presence of Ni²⁺.

Discussion

Adherence to epithelial cells of human nasopharynx is the primary step of *S. pneumoniae* towards the pathogenesis [40]. The pneumococcal surface adhesion protein, PsaA and choline binding protein, PcpA are among those proteins that promote pneumococcal adherence in nasopharyngeal epithelial cells and colonization in mice [14,41,42]. Similarly, PrtA, a serine protease containing an LPXTG-anchor motif, is expressed on the surface of nearly all virulent pneumococcal strains and is required for full virulence in animal models [43,44]. The *pcpA*, *psaBCA*, and *prtA* genes comprise the PsaR regulon and their expression is regulated by transcriptional regulator PsaR [9]. The role of Mn²⁺, Zn²⁺, and Co²⁺ in the regulation of *pcpA*, *psaBCA*, and *prtA* (PsaR regulon) has already been established [9,21,45]. In this study, we investigated the role of Ni²⁺ on the expression of the PsaR regulon. The expression of the PsaR regulon was increased with the increasing concentrations of Ni²⁺ and this increased expression of the PsaR regulon is directly linked with cell-associated Mn²⁺ deficiency caused by a high concentration of Ni²⁺. Moreover, Mn²⁺ and Ni²⁺ have opposite regulatory effects on the expression of the PsaR regulon in *S. pneumoniae*. Where, Mn²⁺-binding represses the expression of the PsaR regulon, Ni²⁺ derepresses the repression caused by Mn²⁺.

Mn²⁺ is an important transition metal ion that is a cofactor for many pneumococcal proteins which are involved in the colonization, virulence, and resistance to oxidative stress in *S. pneumoniae* [15]. Mn²⁺ accumulation shows significant flexibility and cells can survive even at a 3% concentration of the normal accumulation level [45,46]. *S. pneumoniae* has a dedicated system for Mn²⁺ transport (PsaBCA) that consists of two ABC transporters (PsaBC) and a cell

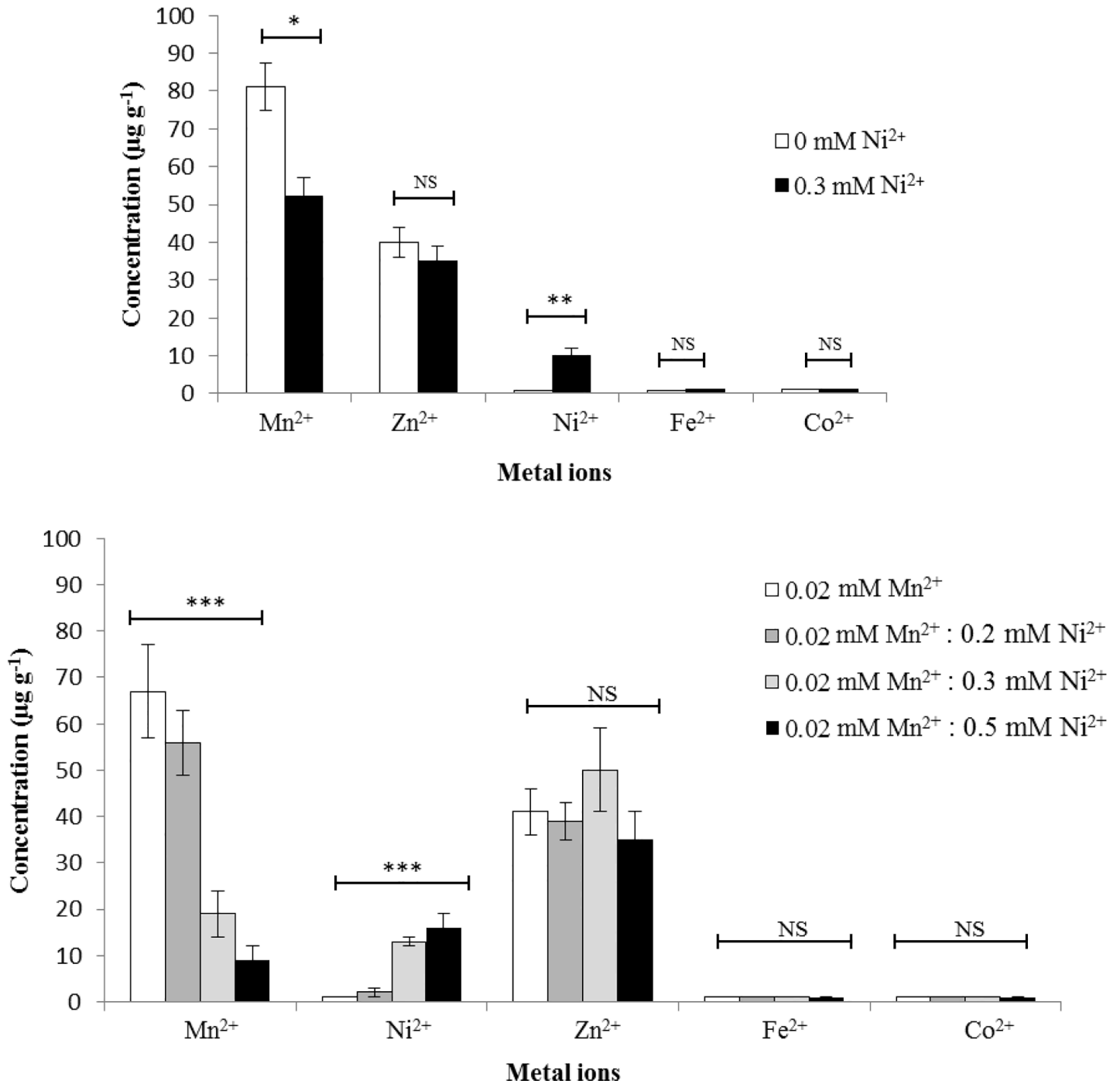


Fig 2. (A) Cell-associated metal ion concentrations (expressed $\mu\text{g g}^{-1}$) of *S. pneumoniae* D39 wild type when grown in CDMchelex with either 0 mM or 0.3 mM Ni²⁺. (B) Metal ions contents of *S. pneumoniae* D39 wild-type when grown in CDMchelex containing 0.02 mM Mn²⁺ with addition of 0, 0.1, 0.3 or 0.5 mM Ni²⁺. The statistical significance of the differences in the mean metal concentrations was determined by one-way ANOVA (NS not significant, *P<0.01, and *P<0.0001)**

doi:10.1371/journal.pone.0142839.g002

surface salute binding protein (PsaA) [47–49]. Previous studies have shown that PsaA is not only important for virulence [14,41], but also has a direct role in the accumulation of cell

associated Mn²⁺ [48,49]. PsaA has the ability to bind Zn²⁺ and Mn²⁺ [46,48]. The binding affinity of PsaA to Zn²⁺ is much higher compared to that of Mn²⁺, and PsaA-Zn²⁺ interaction led to the ~40% decrease in cell associated Mn²⁺ accumulation [31,46]. Structural studies of PsaA have revealed that Cd²⁺ can also bind to PsaA and ultimately results in the reduction of cell-associated Mn²⁺ [30]. Recently, it was shown that PsaA can also bind to other *d*-block elements including Ni²⁺ [50]. This unique property of PsaA to bind with different metal ions makes its role very important in the life style of *S. pneumoniae*. In our ICP-MS analysis, we observed a cell-associated Mn²⁺ deficiency in the presence of relatively high concentrations of Ni²⁺. Therefore, based on our ICP-MS data, we can speculate that most likely Ni²⁺ interacts with PsaA, which leads to Mn²⁺ deficiency.

Biochemical studies of transcriptional regulator PsaR of *S. pneumoniae* showed that PsaR harbors two pairs of metal binding sites where Mn²⁺ or Zn²⁺ can bind [51]. Similarly, Mn²⁺-responsive regulators DtxR from *Corynebacterium diphtheria* and MntR from *Bacillus subtilis*, which are homologous of PsaR, also have two metal binding sites [52,53]. The binding of DtxR to the *tox* operon in *C. diphtheria* not only depends on the availability of Mn²⁺ but also on Co²⁺, Fe²⁺, and Ni²⁺ [54]. Similarly, The Mn²⁺-dependent DNA binding activity of MntR in *B. subtilis* is diminished in the presence of Ni²⁺, Zn²⁺, and Fe²⁺ [55–57]. The metal responsive transcriptional regulators, ScaR of *Streptococcus gordonii* and SloR of *Streptococcus mutants* also belongs to DtxR family, and are homologous to PsaR [58–60]. Interestingly, the PsaR binding site is similar to the operator sequences of ScaR and SloR [61]. This might suggest that PsaR uses a similar mechanism of metal ion competition for regulatory metal ion homeostasis as other member of DtxR family regulators adopt.

It has been previously demonstrated that PsaR represses the expression of the PsaR regulon in the presence of Mn²⁺ whereas Zn²⁺ and Co²⁺ relieved this repression [21,61]. Moreover, the *in vitro* studies of the interaction of PsaR to its target promoters showed that both Zn²⁺ and Co²⁺ could bind to PsaR in a different way [21]. When Zn²⁺ interacts with PsaR, it relieves the PsaR interaction with the promoter regions of the PsaR regulon, whereas Co²⁺, just like Mn²⁺, stimulates the interaction of PsaR with the promoter regions of the PsaR regulon [9,21]. Here, we demonstrated that Mn²⁺-PsaR interaction leads to the binding of PsaR to the promoter regions of *pcpA*, *psaBCA*, and *prtA* which is an agreement with previous studies [9]. However, the Mn²⁺-PsaR interaction with *pcpA*, *psaB*, and *prtA* promoters was alleviated by the addition of Ni²⁺ which suggests that the observed transcriptional response of the PsaR regulon is directly linked to the interaction of Ni²⁺ and Mn²⁺ on the PsaR-promoter interactions. In conclusion, we have shown that the interaction of PsaR to Ni²⁺ plays a similar role as Zn²⁺, to induce derepression by PsaR in competition with Mn²⁺.

Author Contributions

Conceived and designed the experiments: IM SS OPK. Performed the experiments: IM SS. Analyzed the data: IM SS. Contributed reagents/materials/analysis tools: IM SS OPK. Wrote the paper: IM SS OPK.

References

1. McGeer A, Low DE. Is resistance futile? *Nat Med*. 2003; 9: 390–392. doi: [10.1038/nm0403-390](https://doi.org/10.1038/nm0403-390) PMID: [12669054](https://pubmed.ncbi.nlm.nih.gov/12669054/)
2. Saha SK, Naheed A, Arifeen S El, Islam M, Al-Emran H, Amin R, et al. Surveillance for invasive *Streptococcus pneumoniae* disease among hospitalized children in Bangladesh: antimicrobial susceptibility and serotype distribution. *Clin Infect Dis Off Publ Infect Dis Soc Am*. 2009; 48 Suppl 2: S75–81. doi: [10.1086/596544](https://doi.org/10.1086/596544)

3. Zaidi AKM, Thaver D, Ali SA, Khan TA. Pathogens associated with sepsis in newborns and young infants in developing countries. *Pediatr Infect Dis J*. 2009; 28: S10–18. doi: [10.1097/INF.0b013e3181958769](https://doi.org/10.1097/INF.0b013e3181958769) PMID: [19106757](https://pubmed.ncbi.nlm.nih.gov/19106757/)
4. Mitchell TJ. The pathogenesis of streptococcal infections: from tooth decay to meningitis. *Nat Rev Microbiol*. 2003; 1: 219–230. doi: [10.1038/nrmicro771](https://doi.org/10.1038/nrmicro771) PMID: [15035026](https://pubmed.ncbi.nlm.nih.gov/15035026/)
5. Bogaert D, De Groot R, Hermans PWM. Streptococcus pneumoniae colonisation: the key to pneumococcal disease. *Lancet Infect Dis*. 2004; 4: 144–154. doi: [10.1016/S1473-3099\(04\)00938-7](https://doi.org/10.1016/S1473-3099(04)00938-7) PMID: [14998500](https://pubmed.ncbi.nlm.nih.gov/14998500/)
6. Lynch JP, Zhanel GG. Streptococcus pneumoniae: epidemiology, risk factors, and strategies for prevention. *Semin Respir Crit Care Med*. 2009; 30: 189–209. doi: [10.1055/s-0029-1202938](https://doi.org/10.1055/s-0029-1202938) PMID: [19296419](https://pubmed.ncbi.nlm.nih.gov/19296419/)
7. Obaro S, Adegbola R. The pneumococcus: carriage, disease and conjugate vaccines. *J Med Microbiol*. 2002; 51: 98–104. PMID: [11863272](https://pubmed.ncbi.nlm.nih.gov/11863272/)
8. Gupta R, Shah P, Swiatlo E. Differential gene expression in Streptococcus pneumoniae in response to various iron sources. *Microb Pathog*. 2009; 47: 101–109. doi: [10.1016/j.micpath.2009.05.003](https://doi.org/10.1016/j.micpath.2009.05.003) PMID: [19464356](https://pubmed.ncbi.nlm.nih.gov/19464356/)
9. Kloosterman TG, Witwicki RM, van der Kooi-Pol MM, Bijlsma JJE, Kuipers OP. Opposite effects of Mn²⁺ and Zn²⁺ on PsaR-mediated expression of the virulence genes pcpA, prtA, and psaBCA of Streptococcus pneumoniae. *J Bacteriol*. 2008; 190: 5382–5393. doi: [10.1128/JB.00307-08](https://doi.org/10.1128/JB.00307-08) PMID: [18515418](https://pubmed.ncbi.nlm.nih.gov/18515418/)
10. Shafeeq S, Kloosterman TG, Kuipers OP. Transcriptional response of Streptococcus pneumoniae to Zn²⁺ limitation and the repressor/activator function of AdcR. *Met Integr Biometal Sci*. 2011; 3: 609–618. doi: [10.1039/c1mt00030f](https://doi.org/10.1039/c1mt00030f)
11. Wakeman CA, Skaar EP. Metalloregulation of Gram-positive pathogen physiology. *Curr Opin Microbiol*. 2012; 15: 169–174. doi: [10.1016/j.mib.2011.11.008](https://doi.org/10.1016/j.mib.2011.11.008) PMID: [22155062](https://pubmed.ncbi.nlm.nih.gov/22155062/)
12. Bayle L, Chimalapati S, Schoehn G, Brown J, Vernet T, Durmort C. Zinc uptake by Streptococcus pneumoniae depends on both AdcA and AdcAll and is essential for normal bacterial morphology and virulence. *Mol Microbiol*. 2011; 82: 904–916. doi: [10.1111/j.1365-2958.2011.07862.x](https://doi.org/10.1111/j.1365-2958.2011.07862.x) PMID: [22023106](https://pubmed.ncbi.nlm.nih.gov/22023106/)
13. Jomaa M, Terry S, Hale C, Jones C, Dougan G, Brown J. Immunization with the iron uptake ABC transporter proteins PiaA and PiuA prevents respiratory infection with Streptococcus pneumoniae. *Vaccine*. 2006; 24: 5133–5139. doi: [10.1016/j.vaccine.2006.04.012](https://doi.org/10.1016/j.vaccine.2006.04.012) PMID: [16707196](https://pubmed.ncbi.nlm.nih.gov/16707196/)
14. Rajam G, Anderton JM, Carlone GM, Sampson JS, Ades EW. Pneumococcal surface adhesin A (PsaA): a review. *Crit Rev Microbiol*. 2008; 34: 163–173. doi: [10.1080/10408410802383610](https://doi.org/10.1080/10408410802383610) PMID: [18819028](https://pubmed.ncbi.nlm.nih.gov/18819028/)
15. Rosch JW, Gao G, Ridout G, Wang Y-D, Tuomanen EI. Role of the manganese efflux system mntE for signalling and pathogenesis in Streptococcus pneumoniae. *Mol Microbiol*. 2009; 72: 12–25. doi: [10.1111/j.1365-2958.2009.06638.x](https://doi.org/10.1111/j.1365-2958.2009.06638.x) PMID: [19226324](https://pubmed.ncbi.nlm.nih.gov/19226324/)
16. Shafeeq S, Yesilkaya H, Kloosterman TG, Narayanan G, Wandel M, Andrew PW, et al. The cop operon is required for copper homeostasis and contributes to virulence in Streptococcus pneumoniae. *Mol Microbiol*. 2011; 81: 1255–1270. doi: [10.1111/j.1365-2958.2011.07758.x](https://doi.org/10.1111/j.1365-2958.2011.07758.x) PMID: [21736642](https://pubmed.ncbi.nlm.nih.gov/21736642/)
17. Kloosterman TG, van der Kooi-Pol MM, Bijlsma JJE, Kuipers OP. The novel transcriptional regulator SczA mediates protection against Zn²⁺ stress by activation of the Zn²⁺-resistance gene czcD in Streptococcus pneumoniae. *Mol Microbiol*. 2007; 65: 1049–1063. doi: [10.1111/j.1365-2958.2007.05849.x](https://doi.org/10.1111/j.1365-2958.2007.05849.x) PMID: [17640279](https://pubmed.ncbi.nlm.nih.gov/17640279/)
18. Ulijasz AT, Andes DR, Glasner JD, Weisblum B. Regulation of iron transport in Streptococcus pneumoniae by RitR, an orphan response regulator. *J Bacteriol*. 2004; 186: 8123–8136. doi: [10.1128/JB.186.23.8123-8136.2004](https://doi.org/10.1128/JB.186.23.8123-8136.2004) PMID: [15547286](https://pubmed.ncbi.nlm.nih.gov/15547286/)
19. Plumtre CD, Hughes CE, Harvey RM, Eijkelkamp BA, McDevitt CA, Paton JC. Overlapping Functionality of the Pht Proteins in Zinc Homeostasis of Streptococcus pneumoniae. *Infect Immun*. 2014;
20. Johnston JW, Briles DE, Myers LE, Hollingshead SK. Mn²⁺-dependent regulation of multiple genes in Streptococcus pneumoniae through PsaR and the resultant impact on virulence. *Infect Immun*. 2006; 74: 1171–1180. doi: [10.1128/IAI.74.2.1171-1180.2006](https://doi.org/10.1128/IAI.74.2.1171-1180.2006) PMID: [16428766](https://pubmed.ncbi.nlm.nih.gov/16428766/)
21. Manzoor I, Shafeeq S, Kloosterman TG, Kuipers OP. Co²⁺-dependent gene expression in Streptococcus pneumoniae: Opposite effect of Mn²⁺ and Co²⁺ on the expression of the virulence genes psaBCA, pcpA and prtA. *Microb Physiol Metab*. 2015; 6: 748. doi: [10.3389/fmicb.2015.00748](https://doi.org/10.3389/fmicb.2015.00748)
22. Anjem A, Imlay JA. Mononuclear iron enzymes are primary targets of hydrogen peroxide stress. *J Biol Chem*. 2012; 287: 15544–15556. doi: [10.1074/jbc.M111.330365](https://doi.org/10.1074/jbc.M111.330365) PMID: [22411989](https://pubmed.ncbi.nlm.nih.gov/22411989/)
23. Anjem A, Varghese S, Imlay JA. Manganese import is a key element of the OxyR response to hydrogen peroxide in Escherichia coli. *Mol Microbiol*. 2009; 72: 844–858. doi: [10.1111/j.1365-2958.2009.06699.x](https://doi.org/10.1111/j.1365-2958.2009.06699.x) PMID: [19400769](https://pubmed.ncbi.nlm.nih.gov/19400769/)

24. Harvie DR, Andreini C, Cavallaro G, Meng W, Connolly BA, Yoshida K, et al. Predicting metals sensed by ArsR-SmtB repressors: allosteric interference by a non-effector metal. *Mol Microbiol*. 2006; 59: 1341–1356. doi: [10.1111/j.1365-2958.2006.05029.x](https://doi.org/10.1111/j.1365-2958.2006.05029.x) PMID: [16430705](https://pubmed.ncbi.nlm.nih.gov/16430705/)
25. Ong C-LY, Potter AJ, Trappetti C, Walker MJ, Jennings MP, Paton JC, et al. Interplay between manganese and iron in pneumococcal pathogenesis: role of the orphan response regulator RitR. *Infect Immun*. 2013; 81: 421–429. doi: [10.1128/IAI.00805-12](https://doi.org/10.1128/IAI.00805-12) PMID: [23184523](https://pubmed.ncbi.nlm.nih.gov/23184523/)
26. Couñago RM, McDevitt CA, Ween MP, Kobe B. Prokaryotic substrate-binding proteins as targets for antimicrobial therapies. *Curr Drug Targets*. 2012; 13: 1400–1410. PMID: [22664093](https://pubmed.ncbi.nlm.nih.gov/22664093/)
27. Ma Z, Jacobsen FE, Giedroc DP. Coordination chemistry of bacterial metal transport and sensing. *Chem Rev*. 2009; 109: 4644–4681. doi: [10.1021/cr900077w](https://doi.org/10.1021/cr900077w) PMID: [19788177](https://pubmed.ncbi.nlm.nih.gov/19788177/)
28. Waldron KJ, Rutherford JC, Ford D, Robinson NJ. Metalloproteins and metal sensing. *Nature*. 2009; 460: 823–830. doi: [10.1038/nature08300](https://doi.org/10.1038/nature08300) PMID: [19675642](https://pubmed.ncbi.nlm.nih.gov/19675642/)
29. Irving H, Williams RJP. 637. The stability of transition-metal complexes. *J Chem Soc Resumed*. 1953; 3192–3210. doi: [10.1039/JR9530003192](https://doi.org/10.1039/JR9530003192)
30. Begg SL, Eijkelkamp BA, Luo Z, Couñago RM, Morey JR, Maher MJ, et al. Dysregulation of transition metal ion homeostasis is the molecular basis for cadmium toxicity in *Streptococcus pneumoniae*. *Nat Commun*. 2015; 6: 6418. doi: [10.1038/ncomms7418](https://doi.org/10.1038/ncomms7418) PMID: [25731976](https://pubmed.ncbi.nlm.nih.gov/25731976/)
31. Jacobsen FE, Kazmierczak KM, Lisher JP, Winkler ME, Giedroc DP. Interplay between manganese and zinc homeostasis in the human pathogen *Streptococcus pneumoniae*. *Met Integr Biometal Sci*. 2011; 3: 38–41.
32. Sun X, Yu G, Xu Q, Li N, Xiao C, Yin X, et al. Putative cobalt- and nickel-binding proteins and motifs in *Streptococcus pneumoniae*. *Met Integr Biometal Sci*. 2013; 5: 928–935. doi: [10.1039/c3mt00126a](https://doi.org/10.1039/c3mt00126a)
33. Avery OT, MacLeod CM, McCarty M. 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*. 1944. *Mol Med Camb Mass*. 1995; 1: 344–365. PMID: [8521292](https://pubmed.ncbi.nlm.nih.gov/8521292/)
34. Lanie JA, Ng W-L, Kazmierczak KM, Andrzejewski TM, Davidsen TM, Wayne KJ, et al. Genome sequence of Avery's virulent serotype 2 strain D39 of *Streptococcus pneumoniae* and comparison with that of unencapsulated laboratory strain R6. *J Bacteriol*. 2007; 189: 38–51. doi: [10.1128/JB.01148-06](https://doi.org/10.1128/JB.01148-06) PMID: [17041037](https://pubmed.ncbi.nlm.nih.gov/17041037/)
35. Afzal M, Shafeeq S, Kuipers OP. LacR Is a Repressor of lacABCD and LacT Is an Activator of lacTFEG, Constituting the lac Gene Cluster in *Streptococcus pneumoniae*. *Appl Environ Microbiol*. 2014; 80: 5349–5358. doi: [10.1128/AEM.01370-14](https://doi.org/10.1128/AEM.01370-14) PMID: [24951784](https://pubmed.ncbi.nlm.nih.gov/24951784/)
36. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*. 2001; 29: e45. PMID: [11328886](https://pubmed.ncbi.nlm.nih.gov/11328886/)
37. Afzal M, Manzoor I, Kuipers OP. A Fast and Reliable Pipeline for Bacterial Transcriptome Analysis Case study: Serine-dependent Gene Regulation in *Streptococcus pneumoniae*. *J Vis Exp*. 2015; doi: [10.3791/52649](https://doi.org/10.3791/52649) PMID: [25938895](https://pubmed.ncbi.nlm.nih.gov/25938895/)
38. Shafeeq S, Afzal M, Henriques-Normark B, Kuipers OP. Transcriptional profiling of UlaR-regulated genes in *Streptococcus pneumoniae*. *Genomics Data*. 2015; 4: 57–59. doi: [10.1016/j.gdata.2015.02.004](https://doi.org/10.1016/j.gdata.2015.02.004) PMID: [26484177](https://pubmed.ncbi.nlm.nih.gov/26484177/)
39. Kuipers OP, de Ruyter PGG., Kleerebezem M, de Vos WM. Quorum sensing-controlled gene expression in lactic acid bacteria. *J Biotechnol*. 1998; 64: 15–21. doi: [10.1016/S0168-1656\(98\)00100-X](https://doi.org/10.1016/S0168-1656(98)00100-X)
40. Rosch JW, Mann B, Thornton J, Sublett J, Tuomanen E. Convergence of Regulatory Networks on the Pilus Locus of *Streptococcus pneumoniae*. *Infect Immun*. 2008; 76: 3187–3196. doi: [10.1128/IAI.00054-08](https://doi.org/10.1128/IAI.00054-08) PMID: [18443093](https://pubmed.ncbi.nlm.nih.gov/18443093/)
41. Frolet C, Beniazza M, Roux L, Gallet B, Noirclerc-Savoye M, Vernet T, et al. New adhesin functions of surface-exposed pneumococcal proteins. *BMC Microbiol*. 2010; 10: 190. doi: [10.1186/1471-2180-10-190](https://doi.org/10.1186/1471-2180-10-190) PMID: [20624274](https://pubmed.ncbi.nlm.nih.gov/20624274/)
42. Sánchez-Beato AR, López R, García JL. Molecular characterization of PcpA: a novel choline-binding protein of *Streptococcus pneumoniae*. *FEMS Microbiol Lett*. 1998; 164: 207–214. PMID: [9675866](https://pubmed.ncbi.nlm.nih.gov/9675866/)
43. Mirza S, Wilson L, Benjamin WH, J., Novak J, Barnes S, Hollingshead SK, et al. Serine protease PrtA from *Streptococcus pneumoniae* plays a role in the killing of *S. pneumoniae* by apolactoferrin. *Infect Immun*. 2011; 79: 2440–2450. doi: [10.1128/IAI.00489-10](https://doi.org/10.1128/IAI.00489-10) PMID: [21422179](https://pubmed.ncbi.nlm.nih.gov/21422179/)
44. Bethe G, Nau R, Wellmer A, Hakenbeck R, Reinert RR, Heinz HP, et al. The cell wall-associated serine protease PrtA: a highly conserved virulence factor of *Streptococcus pneumoniae*. *FEMS Microbiol Lett*. 2001; 205: 99–104. PMID: [11728722](https://pubmed.ncbi.nlm.nih.gov/11728722/)
45. Eijkelkamp BA, Morey JR, Ween MP, Ong CY, McEwan AG, Paton JC, et al. Extracellular Zinc Competitively Inhibits Manganese Uptake and Compromises Oxidative Stress Management in *Streptococcus pneumoniae*. *PLoS ONE*. 2014; 9.

46. McDevitt CA, Ogunniyi AD, Valkov E, Lawrence MC, Kobe B, McEwan AG, et al. A Molecular Mechanism for Bacterial Susceptibility to Zinc. *PLoS Pathog.* 2011; 7: e1002357. doi: [10.1371/journal.ppat.1002357](https://doi.org/10.1371/journal.ppat.1002357) PMID: [22072971](https://pubmed.ncbi.nlm.nih.gov/22072971/)
47. Dintilhac A, Alloing G, Granadel C, Claverys JP. 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.* 1997; 25: 727–739. PMID: [9379902](https://pubmed.ncbi.nlm.nih.gov/9379902/)
48. Lawrence MC, Pilling PA, Epa VC, Berry AM, Ogunniyi AD, Paton JC. The crystal structure of pneumococcal surface antigen *PsaA* reveals a metal-binding site and a novel structure for a putative ABC-type binding protein. *Struct Lond Engl* 1993. 1998; 6: 1553–1561.
49. McAllister LJ, Tseng H-J, Ogunniyi AD, Jennings MP, McEwan AG, Paton JC. Molecular analysis of the *psa* permease complex of *Streptococcus pneumoniae*. *Mol Microbiol.* 2004; 53: 889–901. doi: [10.1111/j.1365-2958.2004.04164.x](https://doi.org/10.1111/j.1365-2958.2004.04164.x) PMID: [15255900](https://pubmed.ncbi.nlm.nih.gov/15255900/)
50. Couñago RM, Ween MP, Begg SL, Bajaj M, Zuegg J, O'Mara ML, et al. Imperfect coordination chemistry facilitates metal ion release in the *Psa* permease. *Nat Chem Biol.* 2014; 10: 35–41. doi: [10.1038/nchembio.1382](https://doi.org/10.1038/nchembio.1382) PMID: [24212134](https://pubmed.ncbi.nlm.nih.gov/24212134/)
51. Lisher JP, Higgins KA, Maroney MJ, Giedroc DP. Physical Characterization of the Manganese-Sensing Pneumococcal Surface Antigen Repressor from *Streptococcus pneumoniae*. *Biochemistry (Mosc).* 2013; 52: 7689–7701. doi: [10.1021/bi401132w](https://doi.org/10.1021/bi401132w)
52. D'Aquino JA, Tetenbaum-Novatt J, White A, Berkovitch F, Ringe D. Mechanism of metal ion activation of the diphtheria toxin repressor *DtxR*. *Proc Natl Acad Sci U S A.* 2005; 102: 18408–18413. doi: [10.1073/pnas.0500908102](https://doi.org/10.1073/pnas.0500908102) PMID: [16352732](https://pubmed.ncbi.nlm.nih.gov/16352732/)
53. Golynskiy MV, Gunderson WA, Hendrich MP, Cohen SM. Metal binding studies and EPR spectroscopy of the manganese transport regulator *MntR*. *Biochemistry (Mosc).* 2006; 45: 15359–15372. doi: [10.1021/bi0607406](https://doi.org/10.1021/bi0607406)
54. Tao X, Murphy JR. Binding of the metalloregulatory protein *DtxR* to the diphtheria toxin operator requires a divalent heavy metal ion and protects the palindromic sequence from DNase I digestion. *J Biol Chem.* 1992; 267: 21761–21764. PMID: [1400485](https://pubmed.ncbi.nlm.nih.gov/1400485/)
55. Golynskiy MV, Davis TC, Helmann JD, Cohen SM. Metal-induced structural organization and stabilization of the metalloregulatory protein *MntR*. *Biochemistry (Mosc).* 2005; 44: 3380–3389. doi: [10.1021/bi0480741](https://doi.org/10.1021/bi0480741)
56. Lieser SA, Davis TC, Helmann JD, Cohen SM. DNA-binding and oligomerization studies of the manganese(II) metalloregulatory protein *MntR* from *Bacillus subtilis*. *Biochemistry (Mosc).* 2003; 42: 12634–12642. doi: [10.1021/bi0350248](https://doi.org/10.1021/bi0350248)
57. Que Q, Helmann JD. Manganese homeostasis in *Bacillus subtilis* is regulated by *MntR*, a bifunctional regulator related to the diphtheria toxin repressor family of proteins. *Mol Microbiol.* 2000; 35: 1454–1468. PMID: [10760146](https://pubmed.ncbi.nlm.nih.gov/10760146/)
58. Jakubovics NS, Smith AW, Jenkinson HF. Expression of the virulence-related *Sca* (Mn²⁺) permease in *Streptococcus gordonii* is regulated by a diphtheria toxin metalloregulator-like protein *ScaR*. *Mol Microbiol.* 2000; 38: 140–153. PMID: [11029696](https://pubmed.ncbi.nlm.nih.gov/11029696/)
59. Kitten T, Munro CL, Michalek SM, Macrina FL. Genetic Characterization of a *Streptococcus mutans* *LraI* Family Operon and Role in Virulence. *Infect Immun.* 2000; 68: 4441–4451. doi: [10.1128/IAI.68.8.4441-4451.2000](https://doi.org/10.1128/IAI.68.8.4441-4451.2000) PMID: [10899841](https://pubmed.ncbi.nlm.nih.gov/10899841/)
60. Oram DM, Avdalovic A, Holmes RK. Analysis of Genes That Encode *DtxR*-Like Transcriptional Regulators in Pathogenic and Saprophytic *Corynebacterial* Species. *Infect Immun.* 2004; 72: 1885–1895. doi: [10.1128/IAI.72.4.1885-1895.2004](https://doi.org/10.1128/IAI.72.4.1885-1895.2004) PMID: [15039307](https://pubmed.ncbi.nlm.nih.gov/15039307/)
61. Kloosterman TG, Witwicki RM, van der Kooi-Pol MM, Bijlsma JJ, Kuipers OP. Opposite effects of Mn²⁺ and Zn²⁺ on *PsaR*-mediated expression of the virulence genes *pcpA*, *prtA*, and *psaBCA* of *Streptococcus pneumoniae*. *J Bacteriol.* 2008; 190: 5382–5393. doi: [10.1128/JB.00307-08](https://doi.org/10.1128/JB.00307-08) PMID: [18515418](https://pubmed.ncbi.nlm.nih.gov/18515418/)
62. Hoskins J, Alborn WE Jr, Arnold J, Blaszczyk LC, Burgett S, DeHoff BS, et al. Genome of the bacterium *Streptococcus pneumoniae* strain R6. *J Bacteriol.* 2001; 183: 5709–5717. doi: [10.1128/JB.183.19.5709-5717.2001](https://doi.org/10.1128/JB.183.19.5709-5717.2001) PMID: [11544234](https://pubmed.ncbi.nlm.nih.gov/11544234/)