Strain-specific impact of PsaR of *Streptococcus pneumoniae* on global gene expression and virulence

Wouter T. Hendriksen,† Hester J. Bootsma, Angela van Diepen, Silvia Estevão, Oscar P. Kuipers, Ronald de Groot and Peter W. M. Hermans

1Department of Pediatrics, Erasmus MC-Sophia Children’s Hospital, 3000 DR Rotterdam, The Netherlands
2Laboratory of Pediatric Infectious Diseases, Radboud University Nijmegen Medical Centre, 6500 HB Nijmegen, The Netherlands
3Department of Molecular Genetics, University of Groningen, Groningen Biomolecular Sciences and Biotechnology Institute, PO Box 14, 9750 AA Haren, The Netherlands

INTRODUCTION

*Streptococcus pneumoniae* encounters different environments during its life cycle: in most cases it inhabits the human nasopharynx, where it resides asymptomatically, but it can also spread through the body, causing severe infections (Bogaert *et al.*, 2004). What exactly triggers the pneumococcus to cause infections at these sites in the body is poorly understood. In response to environmental changes, the transcriptional programme is likely to change, which is considered to be niche-specific and can result in expression of distinct virulence factors.

Manganese ions (Mn$^{2+}$) are important for bacterial life, for instance serving as cofactors for metalloenzymes. The function of these metalloenzymes is widespread among bacterial cellular processes, such as glycolysis, gluconeogenesis and oxidative stress defence (Jakubovics & Jenkinson, 2001). In the pneumococcus, involvement of Mn$^{2+}$ in competence has also been described (Dintilhac *et al.*, 1997), but most studies have focused on its involvement in oxidative stress (Ibrahim *et al.*, 2005; Johnston *et al.*, 2004; McAllister *et al.*, 2004; McCluskey *et al.*, 2004; Paterson *et al.*, 2006; Tseng *et al.*, 2002). In the latter process, Mn$^{2+}$ serves as a cofactor for superoxide...
dismutase, an important enzyme that provides defence against superoxide radicals (Archibald & Fridovich, 1981). As the concentration of manganese is much higher in saliva than in blood plasma (Chicharro et al., 1999), fluctuations in the amount of this trace element might serve as a trigger for expression of certain virulence factors.

Various transcriptional regulators have been described for the pneumococcus (Hava et al., 2003b) and several large-scale mutagenesis studies have identified a role in virulence for these regulators (Chastanet et al., 2001; Hava & Camilli, 2002; Hemsley et al., 2003; Lau et al., 2001; Polissi et al., 1998). One of them is encoded by psaR, a transcriptional regulator responsive to Mn$^{2+}$ and negatively affecting the expression of the psa operon, pcpA, rlrA and prtA (Johnston et al., 2006; Kloosterman et al., 2008). Recently, the PsaR-binding sequence was identified in the promoter region of psaBCA, prtA and pcpA, and a genome-wide screen for this binding sequence did not show any other putative targets (Kloosterman et al., 2008). Moreover, it has been reported that regulation by PsaR is opposite in reaction to two cations, namely repression in high Mn$^{2+}$ and derepression in high Zn$^{2+}$ (Kloosterman et al., 2008).

The pcpA gene encodes a choline-binding protein (Sanchez-Beato et al., 1998) and rlrA encodes a transcriptional regulator, which controls the expression of the rlrA pathogenicity islet (Hava et al., 2003a). The psaBCA operon encodes the Psa permease that transports the cations Mn$^{2+}$ and Zn$^{2+}$ into the cell (Dintilhac et al., 1997; Johnston et al., 2004, 2006; Lawrence et al., 1998; McAllister et al., 2004). The permease has been implicated in virulence and protection against pneumococcal infection (Bries et al., 2000; Tallbackton et al., 1996). Moreover, PsaA has been shown to bind to E-cadherin, a surface molecule of the host cells (Anderton et al., 2007; Romero-Steiner et al., 2003, 2006). PsaR homologues are found in other streptococci, where they regulate homologues of psaBCA and contribute to virulence (Hanks et al., 2006; Jakubovic et al., 2000; Paik et al., 2003). The role of PsaR in pneumococcal virulence has been investigated in the strain EF3030 (serotype 19F) genetic background (Johnston et al., 2006). During 7 days of colonization no difference between the wild-type and its isogenic psaR mutant was observed, while after 7 days of lung infection, the psaR mutant was significantly attenuated compared to the wild-type (Johnston et al., 2006).

In this study, we examined the effect of the deletion of psaR on global gene and protein expression in two other pneumococcal strains, D39 (serotype 2) and TIGR4 (serotype 4), using transcriptomics and proteomics. Furthermore, we investigated the contribution of PsaR to pneumococcal virulence in three murine infection models representing the major phases in the life cycle of S. pneumoniae: colonization, pneumonia and bacteraemia.

### METHODS

**Bacterial strains and media.** The pneumococcal strains used in this study are listed in Table 1; they were grown at 37 °C in Todd–Hewitt yeast broth (THY), in chemically defined medium (CDM, recipe available on request), or on Colombia base agar (Oxoid) supplemented with 5% sheep blood (Biotrading). Pneumococcal strains were maintained in 10% (v/v) glycerol, 10% skim milk at −80°C. Escherichia coli DH5α (Stratagene) was grown in Luria broth at 37°C.

**Construction of psaR mutants.** The gene encoding psaR was deleted from strain TIGR4 (sp1638) and D39 (spd1450) by allelic replacement with the dfr13 cassette conferring trimethoprim resistance (Adrian et al., 2000). To this end, psaR with 1000 bp of upstream and downstream flanking sequences was amplified from chromosomal TIGR4 DNA using primer pair psaRSacFw and psaRKnpRv (all primers are listed in Table 2). This amplicon was cloned into pBlueScript KS+, using the SacI and KpnI restriction sites. The coding sequence of psaR was deleted from the plasmid by performing an inverse PCR with primer pair psaRNofFw and psaRSalRv, amplifying the psaR-flanking sequences and pBlueScript KS+ and introducing NorI and Sall restriction sites for further cloning. This amplicon was ligated to the dfr13 cassette, which was amplified from pKOT (Hendriksen et al., 2008a) with the primers TmpSalFw and TmpNotRv, to create the knockout construct pKOpsaR-T4, and transformed into E. coli DH5α. A 2620 bp linear DNA fragment containing psaR-flanking DNA and dfr13 was amplified from pKOpsaR-T4 using primer pair psaSacFw and psaKnpRv. This PCR product was used to delete psaR from the genome of S. pneumoniae TIGR4 by CSP-2-induced (100 ng/ml) transformation. Transformants were selected on the basis of trimethoprim resistance and were checked by sequencing for recombination at the desired location on the chromosome, i.e. replacement of psaR by dfr13 (which will be transcribed in the opposite direction to psaR). Wild-type TIGR4 was subsequently transformed with chromosomal DNA isolated from these ΔpsaR transformants to rule out the possibility of any additional mutations on the chromosome. The identical procedure was performed for the construction of D39ΔpsaR, with the exception that the 3’ chromosomal region from TIGR4 of pKOpsaR-T4 was replaced by the D39-specific 3’ psaR region. This region differs between the two

### Table 1. Pneumococcal strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Gene identifier</th>
<th>Antibiotic resistance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>D39 wild-type</td>
<td>–</td>
<td>–</td>
<td>NCTC 7466; serotype 2</td>
</tr>
<tr>
<td>D39ΔpsaR</td>
<td>spd1450</td>
<td>Trimethoprim</td>
<td>This study</td>
</tr>
<tr>
<td>TIGR4 wild-type</td>
<td>–</td>
<td>–</td>
<td>ATCC BAA-334; serotype 4</td>
</tr>
<tr>
<td>TIGR4ΔpsaR</td>
<td>sp1638</td>
<td>Trimethoprim</td>
<td>This study</td>
</tr>
</tbody>
</table>
Table 2. Oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’→3’)*</th>
<th>Restriction site</th>
<th>Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>psaRSacFw</td>
<td>GCGCCCGGCGGGAATTTGACATCTCCTTTCTCC</td>
<td>SauI</td>
<td>D39/TIGR4</td>
</tr>
<tr>
<td>psaRKknRv</td>
<td>GCGCGGTACATTTGGCCGCACTAGGTTTCC</td>
<td>KpnI</td>
<td>TIGR4</td>
</tr>
<tr>
<td>psaRNotFw</td>
<td>GCGCGCGGCGGCTCCTCTGATGAGGGATT</td>
<td>NcoI</td>
<td>TIGR4</td>
</tr>
<tr>
<td>psaRSalRv</td>
<td>GCGGGTCGAGGCTAGTCTATGAGCATTTCA</td>
<td>SalI</td>
<td>TIGR4</td>
</tr>
<tr>
<td>TmpSalFw</td>
<td>GCGCGGTGGTCAGCCGAGATTTTTTGTAGGCTTGACCT</td>
<td>SalI</td>
<td>TIGR4</td>
</tr>
<tr>
<td>TmpNotFw</td>
<td>GGGGGCGGGCGGCCGTTCTACAGCGGCATAGCAG</td>
<td>NcoI</td>
<td>TIGR4</td>
</tr>
<tr>
<td>psaRKpmRv-D39</td>
<td>GAAATTTGACCAAGGCACGAGCACCTC</td>
<td>KpnI</td>
<td>D39</td>
</tr>
<tr>
<td>SeqTmpFw</td>
<td>ATAAATGCGGACCGATTCCC</td>
<td>–</td>
<td>D39/TIGR4</td>
</tr>
<tr>
<td>SeqTmpRv</td>
<td>GCGCTTCTGCCAGTGCTTAAC</td>
<td>–</td>
<td>D39/TIGR4</td>
</tr>
</tbody>
</table>

*Restriction sites on oligonucleotide primers are underlined.

Transcriptional profiling of D39 and TIGR4 psaR mutants. Microarray analysis was performed essentially as described by Hendriksen et al. (2007, 2008a). In short, 500 ml CDM was inoculated with 10–20 colonies from agar plates, and these cultures were grown statically at 37 °C. Samples for RNA isolation were taken when the cultures reached an optical density (OD600) of 0.2 (mid-exponential growth). RNA was isolated and purified using the High Pure RNA isolation kit (Roche Diagnostics) as described by Hendriksen et al. (2007, 2008a). Contaminating genomic DNA was removed by treatment with RNase-free DNase I (Roche Diagnostics). RNA was isolated from three replicate cultures. Synthesis, subsequent labelling of CDNA, and microarray hybridization were performed as previously described (Hendriksen et al., 2007; Kloosterman et al., 2006). In all cases, dye-swapping was performed with one of the three biological replicates. Microarrays used in this study were constructed as previously described (Hendriksen et al., 2007; Kloosterman et al., 2006) and contain ampiclons representing 2087 ORFs of S. pneumoniae TIGR4 and 184 ORFs unique for S. pneumoniae R6, all spotted in duplicate.

The microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) under GEO Series accession number GSE13505.

DNA microarray data analysis. Dual-channel array images were acquired with a GeneTac LS IV confocal laser scanner (Genomics Solutions) and analysed with ArrayPro 4.5 software (Media Cybernetics). Spots were screened visually to identify those of low quality. Slide data were processed using MicroPreP as previously described (Garcia de la Nava et al., 2003; Hendriksen et al., 2007; van Hijum et al., 2003). Prior to analysis, automatically and manually flagged spots and spots with very low background-subtracted signal intensity [5 % of the weakest spots (sum of Cy3 and Cy5 net signals)] were filtered out of all datasets. Spots with a signal in one channel and no signal in the other were subjected to an empty-value assignment of 1 %, after which net signal intensities were calculated using a grid-based background subtraction. A grid-based Lowess transformation was performed for slide normalization, negative and empty values were removed, and outliers were removed by the deviation test. Further analysis was performed using a Cyber-T Student’s t-test for paired data (Long et al., 2001). For identification of differentially expressed genes, only genes with a minimum of six reliable measurements, a Bayesian P-value <0.001, a false discovery rate (FDR) <0.05, and a standard deviation less than the ratio were included. Since these criteria are purely a statistical measure of differential gene expression and reproducibility across replicates, an additional fold-change cut-off of 2 was applied.

Stable isotope labelling in cell culture (SILAC). For SILAC experiments, D39 wild-type and psaR-mutant strains were inoculated in THY and grown to mid-exponential phase. These cultures were used to inoculate CDM, supplemented with both lysine and arginine as the light (12C6 L-lysine, 12C6 L-arginine; psaR mutant) or heavy (13C6 L-lysine, 13C6 L-arginine; wild-type) isotopic counterparts. When these cultures reached an OD600 of 0.2, they were diluted to an OD600 of 0.04 in fresh pre-warmed CDM with the appropriate heavy or light lysine and arginine, and grown to an OD600 of 0.2. This was repeated until the cells had been grown in heavy or light lysine- and arginine-containing CDM for at least five or six generations. After the last generation, the cells were harvested by centrifugation and washed once with PBS. Equal amounts of heavy-labelled wild-type and light-labelled mutant cells were combined and used for mass spectrometry. Bacterial pellets were lysed in lysis buffer [7 M urea, 2 M thiourea, protease inhibitor mix (Roche), pH 8.0]. Lysates were subjected to reduction and alkylation using dithiothreitol and iodoacetamide before LysC and trypsin digestion. Peptide mixtures were purified and desalted using C18-stage tips. Peptide separation and sequence determination was performed with a nano-high-performance liquid chromatography system (Agilent 1100 series) connected to a 7-T linear quadrupole ion trap ion cyclotron resonance Fourier transform mass spectrometer (Thermo Electron). Peptides were separated on a 15 cm 100 μm inner diameter PicoTip emitter for online electrospray (New Objective) packed with 3 μm C18 beads (Reprosil, Dr Maisch GmbH) with a 60 min linear gradient from 2.4 to 40 % acetonitrile in 0.5 % acetic acid at a flow rate of 300 nl min⁻¹. The four most abundant ions were sequentially isolated and fragmented in the linear ion trap by applying collisionally induced dissociation. Proteins were identified using the MASCOT search engine (Matrix Science) against the corresponding S. pneumoniae database. MSQuant was used for the quantification and determination of peptide ratios between wild-type and psaR mutant. A protein was considered differentially expressed when the results of three technical replicates showed at least a 1.5-fold change in protein abundance and a P-value <0.05 (one-sample t-test).

Infection models. Nine-week-old female outbred CD-1 mice (Harlan) were used for all infection models. Prior to the infection experiments, D39 and TIGR4 (wild-type and psaR mutants) were passaged in mice as described previously (Kerr et al., 2004). Cultures of S. pneumoniae D39 and TIGR4 (wild-type and ApsaR) were grown in THY broth to an OD600 of 0.3, and stored in aliquots in 10 % glycerol at −80 °C. Prior to infection, these aliquots were spun down and bacteria were resuspended in sterile PBS to 10⁶ c.f.u. in volumes...
Table 3. Differentially expressed genes/proteins in ΔpsaR of D39 and/or TIGR4

Ratios are given as log2-transformed expression of ΔpsaR/wild-type.

<table>
<thead>
<tr>
<th>Identifier</th>
<th>Gene</th>
<th>Annotation (<a href="http://www.kegg.com">http://www.kegg.com</a>)</th>
<th>Microarray</th>
<th>SILAC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Upregulated</strong></td>
<td></td>
<td></td>
<td>TIGR4 D39</td>
<td>TIGR4 D39</td>
</tr>
<tr>
<td>sp0232</td>
<td>spd0215</td>
<td>infA Translation initiation factor IF-1</td>
<td></td>
<td>2.4</td>
</tr>
<tr>
<td>sp0303</td>
<td>spd0277</td>
<td>bgIA 6-Phospho-β-glucosidase</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>sp0306</td>
<td>spd0280</td>
<td>Transcriptional regulator, putative</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>sp0308</td>
<td>spd0281</td>
<td>PTS system, cellobiose-specific IIA component</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>sp0310</td>
<td>spd0283</td>
<td>PTS system, cellobiose-specific IIC component</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>–</td>
<td>spd0322</td>
<td>cps2G Glycosyltransferase, group 1 family protein</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>sp0459</td>
<td>spd0420</td>
<td>pflB Formate acetyltransferase</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>sp0461</td>
<td>–</td>
<td>rlrA Transcriptional regulator</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>sp0462</td>
<td>–</td>
<td>rrgA Cell wall surface anchor family protein</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td>sp0463</td>
<td>–</td>
<td>rrgB Cell wall surface anchor family protein</td>
<td>3.7</td>
<td>2.0</td>
</tr>
<tr>
<td>sp0464</td>
<td>–</td>
<td>rrgC Cell wall surface anchor family protein</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>sp0467</td>
<td>–</td>
<td>Sortase SrtC, putative</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>sp0468</td>
<td>–</td>
<td>Sortase SrtD, putative</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>sp0641</td>
<td>spd0558</td>
<td>prtA Serine protease, subtilase family PrtA</td>
<td>3.6</td>
<td>3.0</td>
</tr>
<tr>
<td>sp0869</td>
<td>spd0764</td>
<td>sufS Cysteine desulfurases, SufS subfamily protein</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>sp1244</td>
<td>spd1101</td>
<td>ftsY Signal recognition particle-docking protein FtsY</td>
<td>7.6</td>
<td></td>
</tr>
<tr>
<td>sp1517</td>
<td>spd1345</td>
<td>greA Transcription elongation factor GreA</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>sp1531</td>
<td>spd1360</td>
<td>Hypothetical protein</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>sp1636</td>
<td>spd1448</td>
<td>Rnf2 family protein</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>sp1637</td>
<td>spd1449</td>
<td>Hypothetical protein</td>
<td>1.4</td>
<td>1.2</td>
</tr>
<tr>
<td>sp1647</td>
<td>spd1460</td>
<td>pepO Endopeptidase O</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>sp1648</td>
<td>spd1461</td>
<td>psaB Manganese ABC transporter, ATP-binding protein</td>
<td>3.6</td>
<td>3.3</td>
</tr>
<tr>
<td>sp1649</td>
<td>spd1462</td>
<td>psaC Manganese ABC-transporter permease</td>
<td>2.8</td>
<td>2.4</td>
</tr>
<tr>
<td>sp1650</td>
<td>spd1463</td>
<td>psaA ABC transporter, substrate-binding lipoprotein</td>
<td>5.4</td>
<td>5.1</td>
</tr>
<tr>
<td>sp1805</td>
<td>spd1591</td>
<td>Hypothetical protein</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>sp1856</td>
<td>spd1637</td>
<td>Transcriptional regulator, MerR family</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>sp1857</td>
<td>spd1638</td>
<td>czcD Cation-efflux system protein</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>sp2136</td>
<td>spd1965</td>
<td>pcpA Choline-binding protein PcpA</td>
<td>4.0</td>
<td>4.6</td>
</tr>
<tr>
<td>sp2210</td>
<td>spd2037</td>
<td>cysK Cysteine synthase A</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td><strong>Downregulated</strong></td>
<td></td>
<td></td>
<td>TIGR4 D39</td>
<td>TIGR4 D39</td>
</tr>
<tr>
<td>sp0112</td>
<td>spd0109</td>
<td>Polar amino acid transport system substrate-binding</td>
<td>–1.2</td>
<td></td>
</tr>
<tr>
<td>sp0138</td>
<td>spd0141</td>
<td>Hypothetical protein</td>
<td>–1.7</td>
<td></td>
</tr>
<tr>
<td>sp0139</td>
<td>spd0142</td>
<td>Hypothetical protein</td>
<td>–1.8</td>
<td></td>
</tr>
<tr>
<td>sp0140</td>
<td>spd0143</td>
<td>ugd UDP-glucose/GDP-mannose dehydrogenase</td>
<td>–1.7</td>
<td></td>
</tr>
<tr>
<td>sp0141</td>
<td>spd0144</td>
<td>mutR Positive transcriptional regulator of MutA</td>
<td>–1.2</td>
<td></td>
</tr>
<tr>
<td>sp0142</td>
<td>spd0145</td>
<td>Hypothetical protein</td>
<td>–1.0</td>
<td></td>
</tr>
<tr>
<td>sp0143</td>
<td>spd0146</td>
<td>Hypothetical protein</td>
<td>–1.9</td>
<td></td>
</tr>
<tr>
<td>sp0144</td>
<td>spd0147</td>
<td>Hypothetical protein</td>
<td>–1.5</td>
<td></td>
</tr>
<tr>
<td>sp0145</td>
<td>spd0148</td>
<td>Hypothetical protein</td>
<td>–2.0</td>
<td></td>
</tr>
<tr>
<td>sp0146</td>
<td>spd0149</td>
<td>Hypothetical protein</td>
<td>–1.7</td>
<td></td>
</tr>
<tr>
<td>sp0148</td>
<td>spd0150</td>
<td>ABC transporter, substrate-binding protein</td>
<td>–2.1</td>
<td></td>
</tr>
<tr>
<td>sp0524</td>
<td>spd0466</td>
<td>BlpT protein, fusion</td>
<td>–1.5</td>
<td></td>
</tr>
<tr>
<td>sp0525</td>
<td>spd0467</td>
<td>blpS BlpS protein</td>
<td>–1.4</td>
<td></td>
</tr>
<tr>
<td>sp0526</td>
<td>spd0468</td>
<td>blpR Response regulator BlpR (TCS13)</td>
<td>–1.2</td>
<td></td>
</tr>
<tr>
<td>sp0527</td>
<td>spd0469</td>
<td>blpH Sensor histidine kinase BlpH, putative (TCS13)</td>
<td>–1.1</td>
<td></td>
</tr>
<tr>
<td>sp0529</td>
<td>spd0471</td>
<td>blpB BlpC ABC transporter</td>
<td>–1.5</td>
<td></td>
</tr>
<tr>
<td>sp0530</td>
<td>spd0472</td>
<td>blpA BlpA, pseudogene</td>
<td>–1.5</td>
<td></td>
</tr>
<tr>
<td>sp0533</td>
<td>spd0046</td>
<td>blpK Bacteriocin BlpU (highly similar to sp0533)</td>
<td>–1.5</td>
<td></td>
</tr>
<tr>
<td>sp0541</td>
<td>spd0046</td>
<td>blpO Bacteriocin BlpO (5′ highly similar to sp0541)</td>
<td>–1.5</td>
<td></td>
</tr>
<tr>
<td>sp0545</td>
<td>spd0473</td>
<td>blpY Immunity protein BlpY</td>
<td>–2.4</td>
<td></td>
</tr>
<tr>
<td>sp0546</td>
<td>spd0474</td>
<td>blpZ BlpZ protein, fusion</td>
<td>–1.2</td>
<td></td>
</tr>
</tbody>
</table>
Bacteraemia model of infection. depending on the infection model used. Upon intranasal infection, mice were anaesthetized with 2.5% (v/v) isoflurane/O2. At predetermined time points after infection, groups of mice were sacrificed by cervical dislocation and samples of various sites were taken to determine the bacterial load. During infection, signs of disease were closely monitored. If animals reached a moribund state, they were sacrificed by cervical dislocation and excluded from the experiment prematurely. All animal experiments were performed with approval from the Animal Experimentation Committee (DEC) of Erasmus Medical Centre, Rotterdam, The Netherlands.

Colonization model of infection. In the colonization model, 10 µl PBS containing 10^6 c.f.u. bacteria was administered to the nostrils of groups of five mice as described previously (Hendriksen et al., 2008b; Kadioglu et al., 2000). Due to this small volume, only the nose of the mice becomes infected. Bacteria were recovered from the nasopharynx by flushing the nose with 2 ml sterile PBS (Kerr et al., 2004), and lungs were removed from the body and homogenized in 2 ml sterile PBS using a hand-held homogenizer (Polytron PT 1200, Kinematica AG). Viable bacteria from the nasal lavage fluid and homogenized lung samples were counted by plating serial 10-fold dilutions on Colombia blood agar plates. Time points for sampling were 30 min, 24 h, 48 h, 96 h and 192 h post-infection. The 30 min time point is considered to be the start of the infection, and is therefore referred to as t=0. Bacteriology results are expressed as geometric mean ± SEM. Comparison of bacterial loads in the time-course experiment was performed using a Student’s t-test with P<0.05 considered statistically significant.

Pneumonia model of infection. In the pneumonia model, five mice per group were infected with 50 µl PBS containing 10^8 c.f.u. pneumococci as described previously (Hendriksen et al., 2008b). Bacteria were recovered from the different sites as described above, with the addition of a blood sample obtained by cardiac puncture. Time points for sampling were 0, 12, 24 and 36 h post-infection. Viable bacteria isolated from the nasal lavage fluid, homogenized lungs and blood were quantified as described above. Bacteriology results are expressed as geometric mean ± SEM. Comparison of bacterial loads in the time-course experiment was performed using a Student’s t-test with P<0.05 considered statistically significant.

Bacteraemia model of infection. In the bacteraemia model, groups of ten mice were infected in a tail vein with 10^9 c.f.u. resuspended in 100 µl sterile PBS as described previously (Hendriksen et al., 2008b). Bacteria were recovered from the blood by a lateral tail vein puncture from the same mouse at three predetermined time points after infection (0, 12, 24 h) and by a cardiac puncture at the last time point, 36 h. In addition, mouse survival times were scored, after which analysis of survival times was performed using the log-rank test with P<0.05 considered statistically significant.

**RESULTS**

Transcriptional analyses of ΔpsaR in D39 and TIGR4

By means of microarrays analysis, we assessed which genes were affected in expression due to the mutation of psaR in two genetic backgrounds, i.e. D39 and TIGR4. To this end, transcriptional profiles of wild-type strains were compared to their isogenic ΔpsaR strains. These bacteria were grown in chemically defined medium (CDM) and harvested at mid-exponential growth phase. In all experiments, the psaR mutant strains grew like the wild-type. The concentration of Mn^2+ in CDM is 180 µM, which is sufficient for PsaR regulation (see Kerr et al., 2004, where 50 µM was used). For comprehensibility, loci of D39 are referred to by the TIGR4 gene identifiers (in Table 3 both annotations are given). Comparison of transcriptional profiles of D39 and TIGR4 wild-type with their ΔpsaR counterparts revealed 19 differentially expressed genes in TIGR4ΔpsaR, and 37 in D39ΔpsaR. Of these, five genes were upregulated in both TIGR4 and D39 psaR mutants, while only psaR itself was downregulated in both strains (Fig. 1). The genes that were differentially expressed in both serotypes were all upregulated in the psaR mutant, confirming the general role of PsaR as a transcriptional repressor (Johnston et al., 2006; Kloosterman et al., 2008). Among those were the previously described targets, the psa operon (sp1648–sp1650), pcpA (sp2136) and prtA (sp0614).

<table>
<thead>
<tr>
<th>Identifier</th>
<th>Gene</th>
<th>Annotation (<a href="http://www.kegg.com">http://www.kegg.com</a>)</th>
<th>Microarray</th>
<th>SILAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>sp0547</td>
<td>spd0475</td>
<td>Hypothetical protein</td>
<td>TIGR4</td>
<td>D39</td>
</tr>
<tr>
<td>sp0664</td>
<td>spd0577</td>
<td>ZmpB, putative</td>
<td>TIGR4</td>
<td>D39</td>
</tr>
<tr>
<td>sp0798</td>
<td>spd0701</td>
<td>CiaR, putative</td>
<td>TIGR4</td>
<td>D39</td>
</tr>
<tr>
<td>sp0925</td>
<td>spd0817</td>
<td>Hypothetical protein</td>
<td>TIGR4</td>
<td>D39</td>
</tr>
<tr>
<td>sp1024</td>
<td>spd0910</td>
<td>GlyA, putative</td>
<td>TIGR4</td>
<td>D39</td>
</tr>
<tr>
<td>sp1249</td>
<td>spd1107</td>
<td>GMP reductase</td>
<td>TIGR4</td>
<td>D39</td>
</tr>
<tr>
<td>sp1458</td>
<td>spd1287</td>
<td>Thioredoxin reductase</td>
<td>TIGR4</td>
<td>D39</td>
</tr>
<tr>
<td>sp1543</td>
<td>spd1372</td>
<td>Glyoxalase family protein</td>
<td>TIGR4</td>
<td>D39</td>
</tr>
<tr>
<td>sp1638</td>
<td>spd1450</td>
<td>Iron-dependent transcriptional regulator, PsaR</td>
<td>TIGR4</td>
<td>D39</td>
</tr>
<tr>
<td>sp1802</td>
<td>spd1588</td>
<td>Hypothetical protein</td>
<td>TIGR4</td>
<td>D39</td>
</tr>
<tr>
<td>sp1804</td>
<td>spd1590</td>
<td>General stress protein 24, putative</td>
<td>TIGR4</td>
<td>D39</td>
</tr>
<tr>
<td>sp1923</td>
<td>spd1726</td>
<td>Ply, putative</td>
<td>TIGR4</td>
<td>D39</td>
</tr>
<tr>
<td>sp2022</td>
<td>spd1831</td>
<td>PTS system, cellobiose-specific IIC component, putative</td>
<td>TIGR4</td>
<td>D39</td>
</tr>
<tr>
<td>sp2023</td>
<td>spd1832</td>
<td>PTS system, cellobiose-specific IIB component, putative</td>
<td>TIGR4</td>
<td>D39</td>
</tr>
</tbody>
</table>
Twenty-seven genes were downregulated in D39ΔpsaR (Table 3). Most pronounced were FtsY, PsaA, SufS, Cps2G, CysK and PrtA, all of which displayed expression levels at least eight times higher in the psaR mutant than in the wild-type. No proteins were identified that were less abundant in D39ΔpsaR (Table 3).

In TIGR4ΔpsaR, six proteins were more abundant than in the wild-type. Most pronounced were PcpA, PsaA and RrgB, all with at least a fourfold increase in expression in the psaR mutant (Table 3). Three proteins were less abundant in TIGR4ΔpsaR, i.e. an ABC transporter of a putative bacteriocin system (sp0148), TrxB and ZmpB (Table 3).

PsaR does not contribute to pneumococcal colonization

To assess the contribution of PsaR regulation to pneumococcal virulence, we examined the phenotypes of the two wild-type strains and their psaR mutants in three murine models of infection. In the colonization model, both wild-type D39 and TIGR4 were capable of extended colonization of the murine nasopharynx for a period of 192 h. The level of colonization was fairly consistent during this period, varying between 10^4 and 10^6 c.f.u. ml^−1. No significant differences in bacterial load were observed throughout the entire experiment (data not shown).

ΔpsaR counterparts were cultured in the presence of stable isotope-labelled (heavy, ^13C_6) or normal (light, ^12C_6) L-lysine and L-arginine, respectively. Equal amounts of bacteria were mixed and analysed by mass spectrometry, after which changes in protein expression between wild-type and isogenic ΔpsaR strains were derived from the corresponding heavy to light peptide ratios.

In D39ΔpsaR, nine proteins were found to be more abundant than in the wild-type (Table 3). In TIGR4ΔpsaR, six proteins were more abundant than in the wild-type. Most pronounced were PcpA, PsaA and RrgB, all with at least a fourfold increase in expression in the psaR mutant (Table 3). Three proteins were less abundant in TIGR4ΔpsaR, i.e. an ABC transporter of a putative bacteriocin system (sp0148), TrxB and ZmpB (Table 3).

Fig. 1. Number of genes differentially expressed in D39ΔpsaR and TIGR4ΔpsaR.

In addition, sp1637, encoding a hypothetical protein of unknown function, was found to be upregulated in the psaR mutants of both strains (Table 3).

Four genes were found to be repressed by PsaR in D39 (i.e. upregulated in D39ΔpsaR) only: sp0303, encoding 6-phospho-β-glucosidase; sp0306, encoding a putative transcriptional regulator; and two genes of a putative operon encoding a cellulose-specific phosphotransferase system (PTS) (sp0308 and sp0310).

Twenty-seven genes were downregulated in D39ΔpsaR. This set of genes contained sp0112, predicted to encode an amino acid substrate-binding protein; the transcriptional regulator mutR (sp0141); a putative bacteriocin system (sp0142–sp0146); the blp two-component system (TCS13; sp0526–sp0527); the gene encoding response regulator CiaR (sp0798); and glyA (sp1024) encoding serine hydroxymethyltransferase. The full set of differentially expressed genes in D39 is listed in Table 3.

In addition to the common gene targets, nine genes were specifically upregulated in the psaR mutant in TIGR4 (Fig. 1). These included the rlrA pathogenicity islet (sp0461–sp0468) as reported previously (Johnston et al., 2006); sp1636 (encoding an Rf2 family protein); and two adjacent genes, encoding a MerR family transcriptional regulator (sp1856) and czcD (sp1857), a Zn^{2+}-efflux pump (Table 3).

Four genes were downregulated: guaC, encoding GMP reductase (sp1249); ply, encoding pneumolysin (sp1923); and two genes encoding the B and C components of a putative cellobiose-specific PTS (sp2022 and sp2023). The complete set of differentially expressed genes in TIGR4ΔpsaR is given in Table 3.

Proteome analysis of ΔpsaR in D39 and TIGR4

To examine if the observed PsaR-mediated differences in gene expression corresponded to changes in protein expression, we performed SILAC for both wild-type strains and their psaR mutants. To this end, wild-type and isogenic D39ΔpsaR mutants. To this end, wild-type and isogenic D39ΔpsaR mutants.
Furthermore, bacterial loads in the nasopharynx were comparable to those of the first 36 h in the colonization model (data not shown). We did not observe any clear difference in the bacterial survival in the lungs between wild-type and psaR mutants (Fig. 3a). However, immediately after infection (0 h) and 12 h post-infection, the lung homogenates of D39ΔpsaR-infected mice had significantly higher bacterial loads than those of wild-type infected mice: 1.2×10^6 vs 6.3×10^5 c.f.u. ml⁻¹ at 0 h, and 1.5×10^5 vs 5.8×10^4 c.f.u. ml⁻¹ at 12 h. This suggests that there might be an initial positive effect of the psaR mutation, as the inoculum of the wild-type contained (more than) twice as many bacteria as that of the psaR mutant (1.2×10^6 and 4.5×10^5 c.f.u. ml⁻¹, respectively).

In contrast, mice infected with the TIGR4 psaR mutant had lower bacterial loads in the lungs compared to mice infected with TIGR4 wild-type, although these differences were only statistically significant at 24 h post-infection (P=0.0401) (Fig. 3b).

The number of bacteria that were able to reach the systemic circulation was not significantly different between wild-type and ΔpsaR in either D39 or TIGR4 (data not shown), indicating that PsaR regulation is not required for the transition from the lungs to the systemic circulation.

**Psar contributes to survival of pneumococci during bacteraemia**

The most prominent phenotype of the psaR mutants was observed in the bacteraemia model of infection. D39 wild-type-infected mice had significantly more bacteria in the blood than the D39ΔpsaR-infected mice during the first 24 h of infection (Fig. 4a), while variation in the inocula was marginal (1.6×10^6 for wild-type and 1.4×10^6 for psaR mutant). In contrast, no differences were seen between mice infected with TIGR4 wild-type and TIGR4ΔpsaR during the first 24 h of infection. At 36 h post-infection, however, TIGR4 wild-type-infected mice had twice as many bacteria in the bloodstream (P=0.0396) (Fig. 4b).

In addition to the differences in bacterial load, we also observed a difference in murine survival after infection with D39 or TIGR4 in our bacteraemia model: all TIGR4-infected mice (wild-type and ΔpsaR) survived, in contrast to D39 wild-type infected mice, which died within 30 h. Of the D39ΔpsaR-infected mice, three survived the experiment. Consequently, the median survival time of D39ΔpsaR-infected mice was significantly longer than that of mice infected with its wild-type parental strain (the median survival for ΔpsaR was 24 h and for wild-type 30 h, with P=0.0405 and hazard ratio=1.866).

---

**Fig. 2.** Colonization model. Bacterial loads in the nasal lavage fluid of mice infected with (a) D39 wild-type (■) and D39ΔpsaR (□), (b) TIGR4 wild-type (■) and TIGR4ΔpsaR (□). *, P<0.05.

**Fig. 3.** Pneumonia model. Bacterial loads in lungs and blood of mice infected with (a) D39 wild-type (■) and D39ΔpsaR (□), (b) TIGR4 wild-type (■) and TIGR4ΔpsaR (□). *, P<0.05.
transcriptomics and proteomics. In addition, we found et al.
operon, lation. The previously reported PsaR targets, the Psa expression, protein instability, or post-translational regu-
result of several factors, such as low levels of gene
discrepancies between these two approaches could be the
analyses for the identification of PsaR targets. Observed
We used a combination of transcriptional and proteome
strains, TIGR4 and D39.

In D39ΔpsaR, the gene ciaR, involved in competence
development, was upregulated. It has been reported that Mn2+
is required for genetic transformation (Dintilhac et al., 1997), suggesting that downregulation of this gene
might be an indirect effect of the psaR mutation. Several
other systems were downregulated in D39ΔpsaR alone,
such as a putative bacteriocin system (sp0142–sp0146), the
blp bacteriocin system, and the blp two-component system
(TCS13). The genes sp0142–sp0146 have also been shown
to be regulated by the nutritional regulator CodY in D39
(Hendriksen et al., 2008a). Possibly, the downregulation of the
blp bacteriocin genes is due to downregulation of the
blp two-component system (Dawid et al., 2007; de Saizieu et al., 2000).

We used a combination of transcriptional and proteome
analyses for the identification of PsaR targets. Observed
discrepancies between these two approaches could be the
result of several factors, such as low levels of gene
expression, protein instability, or post-translational regu-
lation. The previously reported PsaR targets, the Psi
operon, pcPA and prtA (Johnston et al., 2006; Kloosterman et al., 2008), were confirmed in TIGR4 and D39 by both
transcriptomics and proteomics. In addition, we found sp1637, encoding a hypothetical protein, to be upregulated
in both psaR mutants. Since the latter gene is located
directly upstream of psaR (sp1638), we cannot entirely
exclude the possibility that the derepression of sp1637 is
caused by transcriptional read-through of the trimetho-
prim-resistance cassette used to create the psaR knockout.
We have previously shown that the common PsaR target
pcPA is required for adherence to the human Detroit
epithelial cell line and that the expression of this gene is
also directly positively regulated by the nutritional
regulator CodY (Hendriksen et al., 2008a). The inability of the codY mutant to colonize the murine nasopharynx
underscored, albeit indirectly, the involvement of PcpA in
adherence and colonization. However, a recent study
showed that PcpA is not involved in colonization but has
a role in invasive disease (Glover et al., 2008). These
contradictory results are possibly due to different experi-
mental set-ups; however, the exact role of PcpA during
pneumococcal pathogenesis remains unclear.

In addition to the shared PsaR targets (i.e. the overlapping
genes between D39 and TIGR4), several genes were
differentially expressed in D39 or TIGR4 only. This
strain-specific differential expression might be due to
direct regulation by PsaR, but is more likely to be either an
indirect effect caused by an imbalance in Mn2+/Zn2+
homeostasis due to the lack of PsaR or, possibly,
downstream signalling of other regulators. Moreover, it
seems that this effect was more severe for D39, since more
genes and proteins were affected in their expression level in
this strain. Downstream regulation or a disturbed balance
in other cellular processes might be the cause of this
differential expression, since manganese cations have been
shown to be required for several bacterial cellular processes
(Jakubovics & Jenkinson, 2001). For example, manganese
cations have been shown to function as cofactors for
enzymes in glycolysis, amongst others 6-phospho-β-
glucosidase (Varrot et al., 1999). The genes encoding
6-phospho-β-glucosidase and a PTS downstream of it
(sp0303, sp0306 and sp0308), were upregulated in
D39ΔpsaR, in line with indirect regulation. However, in
D39 these genes are strongly downregulated in the presence
of Zn2+, which is not in agreement with the opposite effect
of Mn2+ and Zn2+ on PsaR regulation (Kloosterman et al.,
2008).

DISCUSSION

During colonization and infection of the human host, S.
pneumoniae encounters fluctuating amounts of free Mn2+
in the different niches where the bacterium resides. Since
Mn2+ is required for several cellular processes, proper
regulation of manganese homeostasis is vital for pneumo-
coccal physiology and virulence. The transcriptional
repressor PsaR has been described to play an important
role in this process, at least in the genetic background of
strain EF3030 (serotype 19F) (Johnston et al., 2006). However, we and others have previously shown that, even
though transcriptional regulators themselves appear to be
conserved between S. pneumoniae strains, they often have a
strain-specific impact on global transcription and virulence
(Hendriksen et al., 2007; Blue & Mitchell, 2003; McCluskey
et al., 2004). To examine whether a similar strain specificity
holds true for PsaR, we identified its targets and its
contribution to experimental virulence in two additional
strains, TIGR4 and D39.

We used a combination of transcriptional and proteome
analyses for the identification of PsaR targets. Observed
discrepancies between these two approaches could be the
result of several factors, such as low levels of gene
expression, protein instability, or post-translational regu-
lation. The previously reported PsaR targets, the Psi
operon, pcPA and prtA (Johnston et al., 2006; Kloosterman et al., 2008), were confirmed in TIGR4 and D39 by both
transcriptomics and proteomics. In addition, we found sp1637, encoding a hypothetical protein, to be upregulated
in both psaR mutants. Since the latter gene is located
directly upstream of psaR (sp1638), we cannot entirely
exclude the possibility that the derepression of sp1637 is
caused by transcriptional read-through of the trimetho-
prim-resistance cassette used to create the psaR knockout.
We have previously shown that the common PsaR target
pcPA is required for adherence to the human Detroit
epithelial cell line and that the expression of this gene is
also directly positively regulated by the nutritional
regulator CodY (Hendriksen et al., 2008a). The inability of the codY mutant to colonize the murine nasopharynx
underscored, albeit indirectly, the involvement of PcpA in
adherence and colonization. However, a recent study
showed that PcpA is not involved in colonization but has
a role in invasive disease (Glover et al., 2008). These
contradictory results are possibly due to different experi-
mental set-ups; however, the exact role of PcpA during
pneumococcal pathogenesis remains unclear.

In addition to the shared PsaR targets (i.e. the overlapping
genes between D39 and TIGR4), several genes were
differentially expressed in D39 or TIGR4 only. This
strain-specific differential expression might be due to
direct regulation by PsaR, but is more likely to be either an
indirect effect caused by an imbalance in Mn2+/Zn2+
homeostasis due to the lack of PsaR or, possibly,
downstream signalling of other regulators. Moreover, it
seems that this effect was more severe for D39, since more
genes and proteins were affected in their expression level in
this strain. Downstream regulation or a disturbed balance
in other cellular processes might be the cause of this
differential expression, since manganese cations have been
shown to be required for several bacterial cellular processes
(Jakubovics & Jenkinson, 2001). For example, manganese
cations have been shown to function as cofactors for
enzymes in glycolysis, amongst others 6-phospho-β-
glucosidase (Varrot et al., 1999). The genes encoding
6-phospho-β-glucosidase and a PTS downstream of it
(sp0303, sp0306 and sp0308), were upregulated in
D39ΔpsaR, in line with indirect regulation. However, in
D39 these genes are strongly downregulated in the presence
of Zn2+, which is not in agreement with the opposite effect
of Mn2+ and Zn2+ on PsaR regulation (Kloosterman et al.,
2008).

In D39ΔpsaR, the gene ciaR, involved in competence
development, was upregulated. It has been reported that Mn2+
is required for genetic transformation (Dintilhac et al., 1997), suggesting that downregulation of this gene
might be an indirect effect of the psaR mutation. Several
other systems were downregulated in D39ΔpsaR alone,
such as a putative bacteriocin system (sp0142–sp0146), the
blp bacteriocin system, and the blp two-component system
(TCS13). The genes sp0142–sp0146 have also been shown
to be regulated by the nutritional regulator CodY in D39
(Hendriksen et al., 2008a). Possibly, the downregulation of the
blp bacteriocin genes is due to downregulation of the
blp two-component system (Dawid et al., 2007; de Saizieu et al., 2000).
Notably in TIGR4, a MerR family regulator (sp1856) and czcD (sp1857), encoding a Zn\(^{2+}\)-efflux system, were upregulated in the psaR mutant. These two genes have also been shown to be upregulated in the presence of Zn\(^{2+}\), underscoring the reported opposite effect of Mn\(^{2+}\) and Zn\(^{2+}\) on PsaR regulation (Kloosterman et al., 2008). The czcD gene has recently been shown to be regulated by SczA (sp1858) in reaction to increasing cellular zinc concentrations (Kloosterman et al., 2007), but this efflux system might also be involved in Mn\(^{2+}\) homeostasis. This again indicates that these regulatory systems of cation homeostasis are intertwined (Kloosterman et al., 2008).

The expression of ply (encoding the pneumococcal toxin pneumolysin) was downregulated as a result of the psaR mutation during in vitro growth in TIGR4 only. If this downregulation also occurred during our infection experiments, it did not have a large effect on experimental virulence of the TIGR4 strain, as the ΔpsaR mutant was as virulent as the wild-type in all our infection models. Only at 24 h post-infection during experimental pneumonia did mice infected with the psaR mutant have significantly lower bacterial loads in the lungs compared to those infected with the wild-type.

A role for PsaR in virulence has been reported in a strain EF3030 genetic background (Johnston et al., 2006). During 7 days of colonization no difference between the wild-type and isogenic psaR mutant was observed, which is in agreement with our results. Johnston et al. (2006) reported that during lung infection the psaR mutant had significantly lower bacterial loads than the wild-type after 7 days of infection. However, in our pneumonia model, we observed higher bacterial loads in D39ΔpsaR-infected mice at the beginning of the experiment (the first 12 h). The TIGR4 ΔpsaR-infected mice had significantly lower bacterial loads at 24 h only. Taking the results together, we did not observe any clear role for PsaR during lung infection in our study.

Upon intravenous infection, we observed the most pronounced effect of deletion of psaR. The psaR mutant was attenuated at the early stages of blood infection. In line with this, PsaR was identified by a large signature-tagged mutagenesis study to be required for full virulence of a mutant (Kloosterman et al., 2008). The observed differences in PsAR-regulated gene expression between D39 and TIGR4 might be the indirect effect of an unbalanced Mn\(^{2+}\) homeostasis, which suggests that physiological differences might reflect genetic differences present in these strains. A clear example of the genetic difference is the rlrA pathogenicity islet, which is strongly upregulated in TIGR4ΔpsaR. However, the genetic diversity between strains only varies as much as 10 %, most of which can be attributed to the capsular genes (Hakenbeck et al., 2001; Lanie et al., 2007). In conclusion, PsaR does not contribute to colonization of S. pneumoniae, but it is involved in invasive disease where it has a strain-specific impact during both pneumonia and bacteremia.

ACKNOWLEDGEMENTS

W. T. H. is supported by the Sophia foundation for Medical Research (SSWO 356, Rotterdam, The Netherlands) and H. J. B. is supported by IOP Genomics grant IGE03002 of the Dutch Ministry of Economic Affairs. We thank Theo Hoogenboezem, Bedette van de Zande, Anne de Jong and Lambert Lambooy for technical assistance and Tomas Kloosterman for helpful discussion.

REFERENCES


Edited by: T. Msadek