CelR-mediated activation of the cellobiose-utilization gene cluster in *Streptococcus pneumoniae*

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The human pathogen *Streptococcus pneumoniae* harbours many genes encoding phosphotransferase systems and sugar ABC (ATP-binding cassette) transporters, including systems for the utilization of the β -glucoside sugar cellobiose. In this study, we show that the transcriptional regulator CelR, which has previously been found to be important for pneumococcal virulence, activates the expression of the cellobiose-utilization gene cluster (*cel* locus) of *S. pneumoniae*. Expression directed by the two promoters present in the *cel* locus was increased in the presence of cellobiose as sole carbon source in the medium, while expression decreased in the presence of glucose in the medium. Furthermore, we have predicted a 22 bp putative CelR regulatory site (5'-YTTTCCTAWCAWTWAGGAAAA-3') in the promoters of *celA* and *celB*, and *in silico* analysis showed that it is highly conserved in other pathogenic streptococci as well. Promoter truncations of *celA* and *celB*, where the half or full CelR regulatory site was deleted, confirmed that the CelR-binding site in *PcelA* and *PcelB* is functional. Transcriptome studies with the *celR* mutant and *in silico* prediction of the CelR regulatory site in the entire D39 genome sequence show that the *cel* locus is the only cluster of genes under the direct control of CelR. Therefore, CelR is a regulator dedicated to the cellobiose-dependent transcriptional activation of the *cel* locus.

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INTRODUCTION

The major Gram-positive human pathogen *Streptococcus pneumoniae* has the ability to colonize the nasopharynx (Bogaert *et al.*, 2004) and from there to spread to different parts of the human body, causing serious infections like pneumonia, meningitis, otitis media and sepsis (Kadioglu *et al.*, 2008; Obaro & Adegbola, 2002). For *S. pneumoniae*, a big challenge for survival in the host environment is adaptation to the changing nutritional conditions encountered in the different niches inside the host, according to which it has to tune the expression of the appropriate genes (Brown *et al.*, 2008; Mitchell, 2003; Shelburne *et al.*, 2008).

Glucose is the preferred source of energy for many bacteria, but the presence of different sugar-specific phosphotransferase systems (PTSs) gives them the ability to utilize other carbon sources as well (Deutscher *et al.*,

2006). Bacteria are able to ferment different β -glucosides such as cellobiose, aesculin, arbutin and salicin, which are commonly present in plants (Kiliç *et al.*, 2004). Cellobiose can be a potential source of energy for different streptococci as well (Keyhani & Roseman, 1997; McKessar & Hakenbeck, 2007; Zeng & Burne, 2009). In *S. pneumoniae*, the gene cluster for cellobiose utilization (*cel* locus) comprises seven genes, encoding a 6-phospho- β -glucosidase (*CelA*), a putative DNA-binding transcriptional regulator (*CelR*), the A, B and C domains of a cellobiose PTS EII^{Cel} (*CelB*, *CelC* and *CelD*), and two hypothetical proteins. It has been shown that *S. pneumoniae* lacking *celR*, *celD* or the hypothetical gene *spr0281* (*SPD_0282*) was hardly able to grow in a chemically defined medium (CDM) supplemented with cellobiose as a sole carbon source (McKessar & Hakenbeck, 2007). A similar study has been performed in *Streptococcus mutans* (Zeng & Burne, 2009), where strains with deletion of *celA*, *celB*, *celC*, *celD* or *celR* were unable to grow in medium with cellobiose as a sole source of energy.

Interestingly, the extracellular matrix (ECM) of mammalian tissues is rich in glycosaminoglycans that contain repeating units of β -linked disaccharides (Kiliç *et al.*, 2004). The degradation of glycosaminoglycans from the mammalian ECM may release structural analogues of cellobiose

Abbreviations: CRE, catabolite-responsive element; PTS, phosphotransferase system.

The microarray data reported have been deposited with the Gene Expression Omnibus (GEO) with the accession number GSE30891.

A supplementary table, listing PCR primers used in this study, is available with the online version of this paper.

(Kiliç *et al.*, 2004). Although it is unlikely that *S. pneumoniae* encounters cellobiose in the human body, it is possible that it can utilize structurally similar β -glucosides that are derived by degradation of the mammalian ECM (Kiliç *et al.*, 2004; King, 2010).

The two-component system TC08 is one of the 13 two-component systems (Throup *et al.*, 2000) found in *S. pneumoniae* and has been shown to be involved in the regulation of the pilus locus in strain TIGR4 (Song *et al.*, 2009). In strain R6, it regulates the *cel* locus: a mutant producing a hyperactive variant of HK08 displayed high repression of transcription of the *cel* genes (McKessar & Hakenbeck, 2007). Moreover, this *hk08* mutant was not able to grow in the presence of cellobiose as sole energy source (McKessar & Hakenbeck, 2007). On the other hand, in *S. mutans* the *cel* locus was activated by the transcriptional regulator CelR and a strain lacking *celR* was unable to grow in medium with cellobiose as sole energy source (Zeng & Burne, 2009). Furthermore, the *celA* gene in the *cel* locus encodes one of the three β -glucosidases present in *S. mutans*, and a strain in which *celA* was deleted was unable to ferment cellobiose, amygdalin, gentiobiose and salicin (Old *et al.*, 2006). Deletion of the other two β -glucosidases in *S. mutans* had no effect on the fermentation of these four β -glucosides (Old *et al.*, 2006). These studies demonstrate the importance of CelA in the fermentation of cellobiose and the role of CelR as an activator of the *cel* locus in *S. mutans*.

In this study, we show that CelR acts as a cellobiose-dependent transcriptional activator of the *cel* locus in *S. pneumoniae* as well. Furthermore, in the presence of cellobiose or glucose, the expression of the *cel* locus was not affected by deletion of either *hk08* or *ccpA*. Investigation of the *celR* deletion strain by means of DNA microarray analyses demonstrated that, besides the *cel* locus, CelR has no other targets under the conditions tested. By MEME motif search, a putative regulatory site of CelR was identified in the promoter regions of *celA* and *celB*, which is conserved in *PcelA* and *PcelB* of other streptococci as well. The functionality of this CelR regulatory site was proven by means of promoter truncation, showing that deletion of the half or full regulatory sequence leads to total inactivation of the *celA* and *celB* promoters.

METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. M17 broth (Terzaghi & Sandine, 1975) supplemented with 0.5 % (w/v) glucose was used for growing *S. pneumoniae* D39 wild-type (Lanie *et al.*, 2007) on blood agar plates supplemented with 1 % (v/v) defibrinated sheep blood in micro-aerophilic conditions at 37 °C. For β -galactosidase assays, derivatives of *S. pneumoniae* D39 were grown in M17 medium supplemented with different sugars (glucose or cellobiose at the concentrations described in Results), and cells were harvested at OD₆₀₀ 0.3. For selection on antibiotics, media were supplemented

Table 1. Strains and plasmids used in this study

Strain/plasmid	Description	Source
<i>S. pneumoniae</i>		
D39	Serotype 2 strain, <i>cps</i> 2	Lanie <i>et al.</i> (2007)
Δ celR	D39 Δ celR::Spec ^R	This study
Δ ccpA	D39 Δ ccpA::Spec ^R	S. M. Carvalho
Δ hk08	D39 Δ hk08::Ery ^R	This study
SS401	D39 Δ bgaA::PcelA-lacZ; Tet ^R	This study
SS402	D39 Δ bgaA::Pspd_0278-lacZ; Tet ^R	This study
SS403	D39 Δ bgaA::PcelB-lacZ; Tet ^R	This study
SS404	D39 Δ bgaA::PcelR-lacZ; Tet ^R	This study
SS405	D39 Δ bgaA::PcelD-lacZ; Tet ^R	This study
SS406	Δ celR Δ bgaA::PcelA-lacZ; Tet ^R	This study
SS407	Δ celR Δ bgaA::PcelB-lacZ; Tet ^R	This study
SS408	Δ ccpA Δ bgaA::PcelA-lacZ; Tet ^R	This study
SS409	Δ ccpA Δ bgaA::PcelB-lacZ; Tet ^R	This study
SS410	Δ hk08 Δ bgaA::PcelA-lacZ; Tet ^R	This study
SS411	Δ hk08 Δ bgaA::PcelB-lacZ; Tet ^R	This study
SS412	D39 Δ bgaA::PcelA F-lacZ; Tet ^R	This study
SS413	D39 Δ bgaA::PcelA H-lacZ; Tet ^R	This study
SS414	D39 Δ bgaA::PcelA N-lacZ; Tet ^R	This study
SS415	D39 Δ bgaA::PcelB F-lacZ; Tet ^R	This study
SS416	D39 Δ bgaA::PcelB H-lacZ; Tet ^R	This study
SS417	D39 Δ bgaA::PcelB N-lacZ; Tet ^R	This study
<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
Plasmids		
pPP2	Amp ^R Tet ^R ; promoterless <i>lacZ</i> , for replacement of <i>bgaA</i> with promoter- <i>lacZ</i> fusion; derivative of pTP1	Halfmann <i>et al.</i> (2007)
pSS401	pPP2 <i>PcelA</i>	This study
pSS402	pPP2 <i>PSPD_0278</i>	This study
pSS403	pPP2 <i>PcelB</i>	This study
pSS404	pPP2 <i>PcelR</i>	This study
pSS405	pPP2 <i>PcelD</i>	This study
pSS406	pPP2 <i>PcelA</i> F	This study
pSS407	pPP2 <i>PcelA</i> H	This study
pSS408	pPP2 <i>PcelB</i> N	This study
pSS409	pPP2 <i>PcelB</i> F	This study
pSS410	pPP2 <i>PcelB</i> H	This study
pSS411	pPP2 <i>PcelB</i> N	This study

with the following concentrations of antibiotics: erythromycin, 0.25 μ g ml⁻¹ for *S. pneumoniae* and 120 μ g ml⁻¹ for *Escherichia coli*; spectinomycin, 150 μ g ml⁻¹; tetracycline, 2.5 μ g ml⁻¹ for *S. pneumoniae*; and ampicillin, 100 μ g ml⁻¹ for *E. coli*. All bacterial strains used in this study were stored in 10 % (v/v) glycerol at -80 °C.

DNA isolation and manipulation. All DNA manipulations in this study were done as described previously (Kloosterman *et al.*, 2006a). For PCR amplification, chromosomal DNA of *S. pneumoniae* D39 wild-type (Lanie *et al.*, 2007) was used. Primers used in this study are listed in Supplementary Table S1, available with the online version of

this paper, and based on the sequence of the D39 genome (Lanie *et al.*, 2007).

Construction of deletion mutants of *hk08* and *celR*. *hk08* and *celR* deletion mutants were made by allelic replacement with an erythromycin- and a spectinomycin-resistance marker, respectively. Briefly, primers HK08-1/HK08-2, HK08-3/HK08-4, SPD0280-1/SPD0280-2 and SPD0280-3/SPD0280-4 were used to generate PCR fragments of the left and right flanking regions of *hk08* and *celR*, respectively. PCR products of the erythromycin- and spectinomycin-resistance markers were generated with primers Ery-F/Ery-R and Spec-F/Spec-RP-New from plasmids pORI28 and pORI38 (Leenhouts *et al.*, 1996), respectively. Then, by means of overlap extension PCR, the left and right flanking regions of *hk08* and *celR* were fused to these resistance genes. The resulting PCR products were transformed to *S. pneumoniae* D39 wild-type and selection of the mutant strains was done on the appropriate antibiotic. Erythromycin- and spectinomycin-resistant clones were further examined for the presence of the $\Delta hk08$ and $\Delta celR$ deletion by PCR.

Construction of *lacZ* fusions and β -galactosidase assays. Chromosomal transcriptional *lacZ* fusions to the *celA*, *celB*, *celR*, *celD* and *SPD_0278* promoters were constructed in the integration plasmid pPP2 (Halfmann *et al.*, 2007) via double crossover in the *bgaA* gene with primer pairs listed in Supplementary Table S1, leading to plasmids pSS401–405. These *lacZ* fusion constructs were introduced into D39 wild-type cells, resulting in strains SS401–405. *PcelA-lacZ* and *PcelB-lacZ* were also transformed to the *celR*, *ccpA* and *hk08* deletion strains, resulting in SS406–411. All plasmid constructs were checked by sequencing.

Specific β -galactosidase activity was measured as described previously (Kloosterman *et al.*, 2006a) using cells grown in M17 with appropriate sugars (exact concentrations are given in Results) which were harvested in the mid-exponential phase of growth.

Construction of *celA* and *celB* promoter subclones in pPP2. The following promoter subclones of the *celA* and *celB* promoters were made in pPP2 (primer pairs are listed in Supplementary Table S1): *PcelA-F* (truncated just a few bases upstream of the *CelR* regulatory site), *PcelA-H* (with the deletion of half of the *CelR* regulatory site), *PcelA-N* (full *CelR* regulatory site deleted), *PcelB-F* (truncated just a few bases upstream of *CelR* regulatory site), *PcelB-H* (with the deletion of half of the *CelR* regulatory site) and *PcelB-N* (full *CelR* regulatory site deleted), resulting in plasmids pSS406–411. These *lacZ* fusion constructs were introduced into D39 wild-type, resulting in strains SS412–417.

Microarray analysis. For transcriptome analysis the *S. pneumoniae* D39 wild-type strain and its isogenic *celR* mutant were grown in three biological replicates in CM17 (M17 + 0.5 % cellobiose) medium and harvested in the mid-exponential phase. All other procedures regarding the DNA microarray experiment were performed as described previously (Kloosterman *et al.*, 2006b; Shafeeq *et al.*, 2011b).

Microarray data analysis. DNA microarray data were analysed as described previously (Kloosterman *et al.*, 2006b; Shafeeq *et al.*, 2011a). In brief, DNA microarray slides were scanned with a Genepix 4200 laser scanner at 10 μ m resolution. Array Pro 4.5 (Media Cybernetics) was used to analyse the slides. The *MicroPrep* software package (van Hijum *et al.*, 2003) was used to obtain and analyse the microarray data. The expression ratio of the *celR* mutant over the D39 strain was calculated from the measurements of at least seven spots by Cyber-T (Long *et al.*, 2001). For identification of differentially expressed genes, only genes with a Bayesian *P*-value < 0.001 and fold change cut-off of 3 were included. The microarray data have been submitted to GEO under accession number GSE30891.

RESULTS

Organization of the cellobiose operon in *S. pneumoniae* D39

The *cel* locus of *S. pneumoniae* D39 wild-type consists of seven genes (*SPD_0277–0283*), which are organized in two putative transcriptional units starting from *PcelA* and *PcelB* (Fig. 1). The first transcriptional unit comprises two genes, encoding 6-phospho- β -glucosidase (*CelA*) and a hypothetical protein (*SPD_0278*), respectively. The second unit encodes the predicted cellobiose-specific PTS components IIBAC (*CelB*, *CelC* and *CelD*), a DNA-binding transcriptional regulator (*CelR*) and a hypothetical transmembrane protein (*SPD_0282*).

To prove that the *cel* locus is transcribed in two transcriptional units, transcriptional *lacZ* fusions to all intergenic regions in this locus were constructed in plasmid pPP2 and introduced into D39 wild-type. β -Galactosidase assays were performed with the strains containing these *lacZ* fusions grown in CM17 (M17 + 0.5 % cellobiose). *PcelA* and *PcelB* were highly active in the presence of cellobiose, while no expression from the other intergenic regions was observed (Table 2). These data demonstrate that the *cel* locus is organized in two transcriptional units originating from the promoters of *celA* and *celB*.

Cellobiose induces and glucose nullifies the expression of the *cel* locus

To investigate the transcriptional response of the *cel* locus of *S. pneumoniae* to different concentrations of cellobiose and glucose, wild-type strain D39 containing *PcelA-lacZ* or *PcelB-lacZ* was grown in M17 with different concentrations of these sugars and β -galactosidase assays were performed with these strains. The data revealed that increasing concentrations of cellobiose led to increased expression of both promoters, whereas increasing the concentration of glucose led to decreased expression of both promoters (Table 3). To study in more detail the role of cellobiose and glucose in the regulation of the *cel* locus, β -galactosidase activity was measured in strain D39 containing *PcelA-lacZ* and *PcelB-lacZ* at a constant concentration of glucose, and with increasing concentrations of cellobiose. Even with a five times higher concentration of cellobiose than glucose, the latter prevents transcription from *PcelA* and *PcelB*. These results show that cellobiose activates the expression

Fig. 1. Organization of the *cel* locus in *S. pneumoniae* strain D39. Lollipops and bent arrows indicate putative terminators and putative promoters, respectively.

Table 2. Specific β -galactosidase activity of D39 wild-type containing different transcriptional fusions grown in CM17 medium

The *PcelA-lacZ*, *PcelB-lacZ*, *PcelD-lacZ*, *PcelR-lacZ* and *PSPD_0278-lacZ* transcriptional fusions were grown in CM17 (M17 + 0.5 % cellobiose) medium. Standard deviations of three independent measurements are given in parentheses.

Promoter	Specific β -galactosidase activity (Miller units)
<i>celA</i>	805 (17)
<i>celB</i>	143 (4.0)
<i>celD</i>	8 (1.1)
<i>celR</i>	5 (1.0)
<i>SPD_0278</i>	6 (0.3)

of the *cel* locus, while glucose decreases its expression (Table 3).

Role of HK08 and CcpA in the expression of the *cel* operon

In a previous study (McKessar & Hakenbeck, 2007) it was observed that TCS08 is involved in the transcriptional regulation of the *cel* locus in *S. pneumoniae* R6. Therefore, to elucidate the putative role of HK08 in the sugar-dependent transcription of the *cel* locus in D39, expression of the *PcelA-lacZ* and *PcelB-lacZ* transcriptional fusions was measured in an *hk08* deletion strain that was created by allelic replacement with an erythromycin-resistance marker. Interestingly, despite deletion of *hk08*, *PcelA* and *PcelB* were, in the presence of cellobiose, still expressed to a similar extent as in D39 wild-type, and hardly expressed in the presence of glucose (Table 4). This shows that the

Table 3. Specific β -galactosidase activity of D39 wild-type containing the *PcelA-lacZ* or *PcelB-lacZ* transcriptional fusions grown in M17 medium with different concentrations of cellobiose (C) and/or glucose (G)

Standard deviations of three independent measurements are given in parentheses.

Medium	Specific β -galactosidase activity (Miller units)	
	<i>PcelA</i>	<i>PcelB</i>
M17	80 (3.0)	56 (5.0)
M17 + 0.2 % G	8 (1.0)	6 (0.5)
M17 + 0.2 % C	452 (21)	98 (4.0)
M17 + 0.5 % G	7 (1.0)	6 (0.6)
M17 + 0.5 % C	770 (54)	140 (4.0)
M17 + 0.1 % G + 0.1 % C	9 (0.9)	7 (1)
M17 + 0.1 % G + 0.2 % C	11 (1.2)	9 (0.8)
M17 + 0.1 % G + 0.5 % C	10 (1.1)	8 (1)

Table 4. Specific β -galactosidase activity of D39 wild-type and the $\Delta celR$, $\Delta hk08$ and $\Delta ccpA$ mutants containing the *PcelA-lacZ* or *PcelB-lacZ* transcriptional fusions grown in M17 medium plus 0.5 % glucose or cellobiose

GM17, M17 + 0.5 % glucose; CM17, M17 + 0.5 % cellobiose. Standard deviations of three independent measurements are given in parentheses.

Promoter	Specific β -galactosidase activity (Miller units)			
	WT	$\Delta hk08$	$\Delta celR$	$\Delta ccpA$
<i>PcelA</i> (GM17)	7.5 (0.5)	6 (0.1)	6 (0.2)	5 (0.4)
<i>PcelA</i> (CM17)	791 (27)	801 (33)	5 (0.1)	779 (35)
<i>PcelB</i> (GM17)	8 (0.2)	7 (0.4)	4 (0.7)	5 (0.2)
<i>PcelB</i> (CM17)	133 (4.0)	128 (7.0)	5 (0.5)	121 (2.0)

observed carbon-source-dependent regulation does not go via TCS08, but that TCS08 probably regulates the *cel* locus via an as yet unidentified signal.

CcpA is responsible for the main part of carbon catabolite regulation in Gram-positive bacteria including *S. pneumoniae*. CcpA mediates its repression by binding to catabolite-responsive element (CRE) sites present in its target promoters (Lulko *et al.*, 2007; Zomer *et al.*, 2007). To determine the putative role of CcpA in the regulation of the *cel* locus, *PcelA* and *PcelB* were analysed for the presence of a putative CRE site. Interestingly, *PcelA* contains a putative CRE box (5'-AAGAATGCGTTTTCAT-3'). However, in the presence of glucose or cellobiose we could not demonstrate an effect of CcpA on the expression of *PcelA* and *PcelB* (Table 4). Therefore, it is likely that CcpA has, as in *S. mutans* (Zeng & Burne, 2009), no role in the regulation of the *cel* locus in *S. pneumoniae* and that the CRE box present in *PcelA* is not functional, which is in agreement with a recent study (S. M. Carvalho and others, unpublished).

CelR acts as a transcriptional activator of the *cel* locus in the presence of cellobiose in *S. pneumoniae*

As the *ccpA* and *hk08* mutations have no effect on the regulation of the *cel* locus by cellobiose and glucose, we investigated the role of *celR*, the second gene of transcriptional unit 2 of the *cel* locus, which encodes a putative DNA binding transcriptional regulator. To find the effect of its mutation on the expression of the *cel* locus, *PcelA-lacZ* and *PcelB-lacZ* transcriptional fusions were introduced into a $\Delta celR$ strain. Both promoters completely lost their activity even in the presence of cellobiose (Table 4). These data suggest that *celR* acts as a transcriptional activator of the *cel* locus in the presence of cellobiose.

DNA microarray analysis of $\Delta celR$

To investigate the effect of the *celR* deletion on the transcriptome of *S. pneumoniae*, D39 wild-type was

compared with its isogenic *celR* deletion mutant grown in CM17 (M17+0.5 % cellobiose) medium. As the *celR* mutant grows more slowly than the wild-type in CM17 medium, cells were harvested in the mid-exponential growth phase according to their growth rate. Table 5 summarizes the results of the transcriptome changes induced by the deletion of *celR*. There was no broad effect of *celR* deletion on the transcriptome of *S. pneumoniae* D39, since after applying the criteria of at least a threefold difference as the threshold change and a *P*-value <0.001, the *cel* locus was the only cluster of genes that was significantly downregulated in the *celR* deletion strain and no other big responses were observed in the transcriptome. This suggests that the *cel* locus is the only target of CelR that is activated in the presence of cellobiose.

Prediction of a *celR* regulatory site in *PcelA* and *PcelB*

By applying a MEME motif sampler search (Bailey & Elkan, 1994) a 22 bp palindromic sequence (5'-YTTTCCTA-WCAWTWAGGAAAA-3') located just upstream of *celA* and *celB* was found in the promoters of *celA* and *celB* of *S. pneumoniae* D39 wild-type (Fig. 2b). This stretch of DNA might serve as a CelR regulatory site in *S. pneumoniae*. The site is conserved in other sequenced strains of *S. pneumoniae* that possess the *cel* locus. *S. mutans*, *Streptococcus gordonii*, *Streptococcus dysgalactiae*, *Streptococcus gallolyticus*, *Streptococcus pyogenes* and *Streptococcus suis* contain a *cel* locus with a similar composition as in *S. pneumoniae* as well. To find out whether the CelR regulatory site is also conserved in these streptococci the *celA* and *celB* promoters of these species were analysed for the presence of the putative CelR regulatory sequence. This showed that the CelR regulatory sequence is highly conserved in these streptococci (Fig. 2b). However, a difference in the spacing of a single base pair in

the middle of the regulatory site was present in *S. mutans* and *S. dysgalactiae*. The predicted CelR regulatory sequences in the *celA* and *celB* promoters of *S. mutans*, *S. gordonii*, *S. dysgalactiae*, *S. gallolyticus*, *S. pyogenes* and *S. suis* were aligned with that of *S. pneumoniae*. In this way, a weight matrix of a 23 bp putative CelR regulatory site (5'-WTTTCCKTW-KNCRATAMGGAAAA-3') was created (Fig. 2a).

To investigate if there are more putative CelR targets in the D39 genome, a genome-wide search with the pneumococcal CelR regulatory site was performed. No other stretch of DNA resembling the CelR regulatory site was found, supporting the observation in the microarray analysis with the *celR* mutant that the *cel* locus is the only target of CelR.

Verification of the CelR regulatory site in *PcelA* and *PcelB*

To prove the functionality of the identified CelR regulatory sequence in the regulation of the *cel* locus by CelR, transcriptional *lacZ* fusions to 5' truncations of *PcelA* and *PcelB* were constructed (Fig. 3). No expression of either of the promoters (*PcelA* and *PcelB*) was observed when the half or entire predicted CelR regulatory site was removed, in both CM17 (M17+0.5 % cellobiose) and GM17 (M17+0.5 % glucose) medium. However, when the *celA* and *celB* promoters were truncated only a few base pairs upstream of the predicted regulatory site, expression was similar to that of the full-length promoters (Fig. 3). Therefore, these data suggest that the putative predicted regulatory site is functional and acts as a CelR regulatory site. Moreover, since this predicted site is found to be highly conserved in other streptococci, it is most likely also functional as a CelR regulatory site in these species.

DISCUSSION

In this study, the expression of the *cel* locus of *S. pneumoniae*, which is important for growth on cellobiose, was shown to be activated by cellobiose and repressed by glucose via the transcriptional activator CelR. This regulatory effect depends on a 22 bp palindromic sequence present in the two promoters, *PcelA* and *PcelB*, that drive expression of the *cel* locus.

Expression of the appropriate genes necessary to make efficient use of the available energy sources is of utmost importance for the fitness of pathogenic bacteria in a fluctuating environment. In low-G+C Gram-positive bacteria, including *S. pneumoniae*, carbon catabolite protein A (CcpA) is mainly responsible for the regulation of sugar metabolic genes in response to the available carbon source(s), which ensures their optimal use (Lulko *et al.*, 2007; Zomer *et al.*, 2007; S. M. Carvalho and others, unpublished). In this study, we observed that CcpA plays no role in the regulation of the *cel* locus in *S. pneumoniae*, despite the presence of a putative CRE site in the promoter of *PcelA*. Also, there was no effect on the expression of the

Table 5. Summary of transcriptome comparison of *S. pneumoniae* D39 $\Delta celR$ and D39 wild-type grown in CM17 (M17+0.5 % cellobiose)

D39 locus tag	Function	Ratio*
SPD_0277	6-Phospho- β -glucosidase, CelA	-81.2
SPD_0278	Hypothetical protein	-2.4
SPD_0279	Cellobiose phosphotransferase system IIB component, CelB	-22.5
SPD_0280	DNA-binding transcriptional regulator, CelR	-13.0
SPD_0281	Cellobiose phosphotransferase system IIA component, CelC	-22.8
SPD_0282	Hypothetical protein	-14.1
SPD_0283	Cellobiose phosphotransferase system IIC component, CelD	-18.8

*Ratios >3.0 or >-3.0 (signal intensity of D39 $\Delta celR$ divided by that of D39 wild-type). SPD_0278 was also included, since it is in the same transcriptional unit. *P*-values all <0.001.

Fig. 2. Identification of a CelR regulatory site in different streptococci. (a) Weight matrix of the identified CelR regulatory site in *PcelA* and *PcelB* from different streptococci. (b) Position of a CelR regulatory site in *PcelA* and *PcelB* of different streptococci. PN, *S. pneumoniae*; SG, *S. gordonii*; SM, *S. mutans*; SD, *S. dysgalactiae*; Sg, *S. gallolyticus*; SP, *S. pyogenes*; SS, *S. suis*. Core promoter sequences are underlined, translational start sites are in bold italic type and putative CelR regulatory sites are in bold type.

cel locus in a recent transcriptome-wide analysis of a *ccpA* deletion mutant in glucose and galactose (S. M. Carvalho and others, unpublished). Similarly, deletion of *ccpA* has no impact on the regulation of the *cel* locus in *S. mutans* (Zeng & Burne, 2009). This means that most probably the CRE box present in *PcelA* is not functional.

A previous study showed that TCS08 activates the *cel* locus in strain R6 (McKessar & Hakenbeck, 2007). We did not find an effect of a deletion of *hk08* on the expression of *PcelA* and *PcelB* in strain D39. This means that TCS08 probably regulates the *cel* locus in response to a different

stimulus than the sugar source in strain D39. Thus, it is likely that CelR is the only regulator involved in the regulation of the *cel* locus in response to the sugar source in strain D39. In addition, our bioinformatics analysis showed that 50 % of the strains available at the KEGG website do not contain genes homologous to the *cel* locus, implying that the role of TCS08 lies outside regulation of the *cel* locus in these strains. The absence of the *cel* locus in these strains also suggests the presence of an alternative cellobiose utilization system, which, as determined by BLAST analyses, could be SPD_1830–1833, encoding a cellobiose-specific PTS. This second cellobiose system is highly conserved in all the strains

Fig. 3. Deletion analysis of *Pce/A* and *Pce/B*. Schematic diagrams of the *Pce/A* (a) and *Pce/B* (b) truncations are shown. Ovals indicate the position of the CelR regulatory site. To the right of each diagram are shown the specific β -galactosidase activities (Miller units) of the promoter truncations in D39 wild-type in GM17 (M17 + 0.5 % glucose) and CM17 (M17 + 0.5 % cellobiose) medium. Standard deviations of three independent measurements are given in parentheses.

available on the KEGG website. Therefore, it will be interesting to determine the role of this system in cellobiose metabolism in future studies.

Bioinformatic analysis revealed that CelR has two PTS regulation domains (PRDs), a helix–turn–helix (HTH) and EIIA and EIIB domains. Usually, transcriptional regulators with PRDs require phosphorylation on conserved histidine residues by one of the PTS components involved in the metabolism of a certain carbohydrate (van Tilbeurgh & Declerck, 2001). Recently, the role of CelR (CelRSM) was established in the regulation of the *cel* locus of *S. mutans* (Zeng & Burne, 2009). CelRSM-dependent regulation of the *cel* locus in the presence of cellobiose required the phosphorylation of CelRSM by CelD. Deletion of the second or both PRDs present in the CelRSM protein led to the total inactivation of expression of the *cel* locus (Zeng & Burne, 2009). It was further shown that phosphorylation at H226, H332 and H576 is required for the activation of CelRSM in the presence of cellobiose, while phosphorylation of H284 and H391 leads to inhibition of the CelRSM-dependent activation in *S. mutans* in the presence of glucose. Interestingly, alignment of CelRSM showed that all the above-mentioned histidine residues are conserved in the CelR protein (data not shown). The presence of these conserved histidines suggests that the activity of CelR is determined in the same manner as CelRSM. This is supported by a previous study, showing that *S. pneumoniae* R6 lacking *celD* is unable to grow on cellobiose (McKessar & Hakenbeck, 2007).

Previous signature-tagged mutagenesis (STM) screens highlighted the importance of CelR for the virulence of

S. pneumoniae (Hava & Camilli, 2002; Lau *et al.*, 2001). So far, however, a possible role in virulence of the other genes in the *cel* locus has never been investigated. Since CelR is dedicated exclusively to the regulation of the *cel* locus, it is likely that the attenuated virulence of a *celR* mutant is caused by deactivation of the expression of the genes present in the *cel* locus. However, since several pneumococcal strains, such as AP200, ATCC 700669-23F, G54-19F, Hungary-19A, JJA, P1031 and TCH8431/19A, lack the *cel* locus, the contribution of the *cel* locus to virulence seems to be strain-specific.

As cellobiose is unlikely to be present in the human host, the natural substrate(s) for the PTS encoded by the *cel* locus remain(s) to be determined. It is known that *S. pneumoniae* cleaves host glucosaminoglycans by the activity of many glucosidases, thereby releasing usable carbon sources (King, 2010). On the basis of their disaccharide units and linkage pattern, glycosaminoglycans are classified into three structural groups (Li *et al.*, 2000; Scott & Heatley, 1999): (i) the cellobiose type (HA: hyaluronic acid or hyaluronate); (ii) the polylactose type (chondroitin and keratan sulfates); and (iii) the polymaltose type (heparan sulfate) (Li *et al.*, 2000). In a previous study (McKessar & Hakenbeck, 2007), it was shown that deletion of *celR* has no effect on the growth of strain R6 in the presence of HA. This suggests that HA is not metabolized via the Cel system and that HA is not involved in the regulation of the *cel* locus. Therefore, it could be that β -linked disaccharides derived from degradation of other host glycosaminoglycans serve as substrates for the Cel system.

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