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GalR Acts as a Transcriptional Activator of *galKT* in the Presence of Galactose in *Streptococcus pneumoniae*

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Key Words

Galactose · GalR · Pneumococcus · CcpA · Carbon source · Leloir pathway · Tagatose pathway

Abstract

We explored the regulatory mechanism of Leloir pathway genes in *Streptococcus pneumoniae* D39. Here, we demonstrate that the expression of *galKT* is galactose dependent. By microarray analysis and quantitative RT-PCR, we further show the role of the transcriptional regulator GalR, present upstream of *galKT*, as a transcriptional activator of *galKT* in the presence of galactose. Moreover, we predict a 19-bp regulatory site (5'-GATAGTTTAGTAAAATTTT-3') for the transcriptional regulator GalR in the promoter region of *galK*, which is also highly conserved in other streptococci. Growth comparison of D39 $\Delta galK$ with the D39 wild type grown in the presence of galactose shows that *galK* is required for the proper growth of *S. pneumoniae* on galactose.

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Introduction

Pneumonia, sepsis, meningitis, otitis media and conjunctivitis are a few of the diseases caused by *Streptococcus pneumoniae*, a major human pathogen, that result in over a million deaths worldwide each year [Ispahani et al., 2004; O'Brien et al., 2009]. In the human body, *S. pneumoniae* may come across varying environmental conditions, including carbohydrate availability. The importance of carbohydrates for *S. pneumoniae* can be realized from the fact that 30% of all transport machineries of pneumococcus are devoted to carbohydrate uptake [Tettelin et al., 2001]. *S. pneumoniae* prefers glucose as the most preferred energy source and carbon catabolite repression (CCR) is the mechanism that helps the bacteria to maintain proper energy proficiency by selecting a preferred carbon source over a nonpreferred one [Carvalho et al., 2011; Iyer et al., 2005]. Nonpreferred carbon source utilization genes are switched off by CCR until the preferred carbon source is utilized. CcpA (carbon catabolite protein A) is a transcriptional factor that controls CCR in the presence of a preferred carbon source, i.e. glucose [Buckwalter and King, 2012; Gorke and Stulke, 2008; Titgemeyer and Hillen, 2002; Zomer et al., 2007]. Catabolite repression elements (*cre* boxes) are commonly pres-

Fig. 1. Graphical representation of the putative tagatose and Leloir pathways in *S. pneumoniae*.

ent in the promoter regions of genes involved in the utilization of nonpreferred sugars and CcpA binds to these *cre* boxes to mediate the repression of these genes in the presence of a preferred sugar [Fridovich-Keil, 2006; Russell et al., 1992]. Regulatory mechanisms of some of the carbon utilization systems have already been studied in *S. pneumoniae* [Afzal et al., 2014, 2015b; Nieto et al., 1997; Shafeeq et al., 2011a, 2013], but many of them still deserve attention.

Galactose, a monosaccharide, is a slowly metabolized nonpreferred sugar that pneumococcus can experience in the nasopharynx [King, 2010; Yesilkaya et al., 2008]. In *S. mutans*, it has been shown that galactose and lactose can be metabolized by the tagatose-6-phosphate pathway, while galactose can also be utilized through the Leloir pathway [Zeng et al., 2010]. The tagatose and Leloir pathways for the utilization of lactose and galactose are shown in figure 1. Lactose and galactose induce the expression of the tagatose pathway genes in *S. mutans*, whereas galactose can also induce the expression of the Leloir pathway genes [Ajdic et al., 1996; Hamilton and Lo, 1978]. Lactose and galactose have also been shown to induce the expression of the tagatose pathway genes in *S. pneumoniae* [Afzal et al., 2014]. The *lac* gene cluster (consisting of *lac* operon-I and II) comprises the tagatose pathway genes in *S. pneumoniae*. *lac* operon-I consists of four

genes (*lacABCD*), whereas *lac* operon-II consists of five genes (*lacF*, *lacE*, *lacG*, *hp* and *lacT*). *lacAB* encode for galactose-6-P isomerase which converts galactose-6-P into tagatose-6-P. *lacC* encodes for tagatose-6-P kinase, which converts tagatose-6-P into tagatose-1,6-bP, whereas *lacD* codes for the tagatose-1,6-bP aldolase which converts tagatose-1,6-bP into glycerone-P and D-glyceraldehyde-3-P [Afzal et al., 2014; Zeng et al., 2010]. *lacFE* encode for the A and BC components of the lactose-specific phosphotransferase system (PTS) EII, *lacG* encodes the 6-phospho-β-galactosidase and *lacT* encodes a BglG-family transcriptional antiterminator. Most likely, in *S. pneumoniae*, lactose is transported inside the cell by the phosphoenolpyruvate-dependent lactose-specific PTSs (*lacFE*) like in other Gram-positive bacteria, producing lactose-6-phosphate (Lac-6-P), which is then further hydrolyzed to glucose and galactose-6-phosphate (Gal-6-P) by LacG, and the Gal-6-P is catabolized through the tagatose pathway [de Vos and Vaughan, 1994; Jagusztyn-Krynicka et al., 1992]. The genome of *S. pneumoniae* D39 also encodes Leloir pathway proteins GalKT-E. However, the regulatory mechanism of the Leloir pathway genes has not yet been explored in *S. pneumoniae*. In this study, we explore the regulatory mechanism of the Leloir pathway genes and show that GalR, a transcriptional regulator present upstream of *galKT*, acts as a transcriptional acti-

vator of *galKT*. We also predict a 19-bp putative regulatory site (5'-GATAGTTTAGTAAAATTT-3') for GalR in the promoter region of *galK*. We further show that growth of *S. pneumoniae* is hampered in the presence of galactose when *galK* is deleted.

Results and Discussion

Organization and Localization of the Leloir Pathway Genes in *S. pneumoniae* D39

Lactose enters the cell through *lacFE* (PTSs) and gets phosphorylated. Lactose-6-P is then converted into galactose-6-P by a phospho- β -galactosidase (LacG). Similarly, pneumococcus may utilize a couple of PTSs to import galactose. These PTSs are *spd_0262-3-4* (*manLMN*) and *spd_0559-60-61* [Bidossi et al., 2012]. *spd_0066-7-8-9* is another PTS that may be involved in galactose transport [Bidossi et al., 2012]. In addition to the PTS, there is an ABC transporter that has been suggested to be involved in galactose transport (*spd_0088-89-90*) [Bidossi et al., 2012]. Galactose is converted into galactose-6-P after entering the cell. This galactose-6-P can further be metabolized by the tagatose-6-P pathway. Incoming galactose can be further metabolized via the Leloir pathway, which is omnipresent in bacteria and can be used as a catabolic pathway for the utilization of galactose as an energy and carbon source. The Leloir pathway signifies the only source of galactose by biological interconversion from glucose to galactose in the absence of an external galactose reservoir [Frey, 1996]. The Leloir pathway is comprised of the *galKTE* operon in *S. mutans*. *galK* encodes for a galactokinase that converts galactose into galactose-1-P. *galT* encodes for galactose-1-P uridylyltransferase, which converts galactose-1-P to UDP-galactose that is then converted into UDP-glucose by the UDP-glucose 4-epimerase encoded by *galE*. BLAST searches with protein sequences of GalKT-E of *S. mutans* revealed the presence of GalKT-E in *S. pneumoniae* D39. The organization of the Leloir pathway genes is different in *S. pneumoniae* D39 compared to the known organization of the Leloir pathway genes in other bacteria. *galKTE* is present as an operon (*galKTE*) in *S. thermophilus*, *S. mutans*, *S. salivarius*, *S. equi* and *Lactococcus lactis*, whereas in *S. pneumoniae* these genes are present as *galKT* (*spd-1634-33*) and *galE* (*spd-1432*) genes. Bioinformatics analysis suggests that *galE* is present downstream of a glycosyltransferase coding gene in an operon and is under the control of the promoter of the transferase coding gene [Baerends et al., 2004]. The genome of *S. pneumoniae* also

Fig. 2. The relative increase/decrease in expression of *galKT-E*. *S. pneumoniae* D39 wild type and D39 $\Delta galR$ were grown in GM17 (0.5% glucose + M17) or GalM17 (0.5% galactose + M17). The expression of the *galK*, *galT* and *galE* was normalized with house-keeping gene *gyrA*. Results represent the mean and standard deviation of three independent replications.

codes for a second copy of GalET (SPD-1612-13). Interestingly, in *Lactobacillus helveticus*, the *galE* gene is linked with *lacLM* genes like many lactic acid bacteria, and lactose metabolism genes are found organized with the *gal* genes [de Vos and Vaughan, 1994]. Moreover, our bioinformatics analysis showed that the *galKT* and *galE* genes are highly conserved in all the pneumococcal strains available in the KEGG database [Kanehisa et al., 2014]. Conservation of the Leloir pathway genes in all these pneumococcal strains indicates its important function in the physiology of *S. pneumoniae*. Therefore, we decided to further study the regulation of these genes in the presence of galactose.

The Leloir Pathway Genes Are Functional and Respond to Galactose in *S. pneumoniae* D39

To observe the effect of galactose on the Leloir pathway genes, we performed quantitative reverse transcription (RT)-PCR for *galKT* and *galE* on RNA isolated from D39 wild type grown in the presence of glucose or galactose. The expression of *galK* and *galT* was significantly higher in the GalM17 (0.5% galactose + M17) compared to that in GM17 (0.5% glucose + M17) medium, whereas no change in the expression of *galE* was observed (fig. 2). This data suggests that the Leloir pathway in *S. pneumoniae* is most likely composed of *galKT* and is functional.

Table 1. Summary of transcriptome comparison of *S. pneumoniae* D39 wild type and D39 $\Delta galR$ grown in GM17 (0.5% glucose + M17) or GalM17 (0.5% galactose + M17)

D39 tag ¹	Function ²	GM17	GalM17
<i>spd_1632</i>	Hypothetical protein	-2.4	-1.5
<i>spd_1633</i>	Galactose-1-phosphate uridylyltransferase, GalT	-2.4	-1.8
<i>spd_1634</i>	Galactokinase, GalK	-3.6	-2.2
<i>spd_1635</i>	Galactose operon repressor, GalR	-10.9	-2.4

GM17 and GalM17 represent the fold-decrease in the expression of genes in D39 $\Delta galR$ compared to the D39 wild type.

¹ Gene numbers refer to D39 locus tags.

² D39 annotation [Lanie et al., 2007].

Similar observations were noticed in *S. mutans*, where galactose was demonstrated to be the most efficient inducer of the *gal* operon [Ajdic et al., 1996]. Comparable results were observed in a transcriptome study of *S. pneumoniae* D39 grown in the presence of galactose in the chemically defined medium (CDM) [Carvalho et al., 2011]. *galKT* was upregulated around 25-fold in the presence of galactose in CDM [Carvalho et al., 2011]. However, no effect on the expression of *galE* was observed in the presence of galactose/lactose in M17 medium or in the presence of galactose in CDM [Afzal et al., 2014; Carvalho et al., 2011]. Similarly, no significant change in the expression of the second copies of *galT* and *galE* were observed in the mentioned studies [Afzal et al., 2014; Carvalho et al., 2011]. In another study, raffinose was also observed to induce the *gal* operon in *S. mutans* [Ajdic et al., 1996]. Raffinose is a galactose-containing carbohydrate and this property of raffinose was speculated to be the reason for the induction of the *gal* operon. This observation was further confirmed by electrophoretic mobility shift assays in the presence of a number of sugars, which showed that galactose is the true intracellular inducer of the *gal* operon in *S. mutans* [Ajdic et al., 1996].

GalR Acts as a Transcriptional Activator of the Leloir Pathway Genes in S. pneumoniae

A LacI-family transcriptional regulator, GalR, is encoded by the gene that is present upstream of the *galKT* (fig. 3). The LacI family of transcriptional regulators is mostly involved in the regulation of catabolic pathways (e.g. pathways utilizing lactose, galactose, ribose, fructose, gluconate, maltose, etc.) [Nguyen and Saier, 1995], and sugars or their phosphorylated counterparts act as effector molecules for LacI-family transcriptional regula-

tors. LacI-family transcriptional regulators are mostly repressors, but some may act both as repressors and activators. The DNA-binding affinity of a LacI-family transcriptional regulator alters after binding with an effector ligand [Swint-Kruse and Matthews, 2009]. The presence of GalR next to *galKT* might indicate its role in the regulation of *galKT*. GalR has been demonstrated to be the transcriptional repressor of the *gal* operon in *S. mutans* and *Escherichia coli* in the absence of galactose. To elucidate the role of GalR in the regulation of the Leloir pathway genes and to study the impact of *galR* deletion on the transcriptome of *S. pneumoniae*, the D39 wild type was compared to the D39 $\Delta galR$ grown in GM17 (0.5% glucose + M17). Based on the function of GalR as a transcriptional repressor in *S. mutans* and *E. coli*, we anticipated that the deletion of *galR* would lead to derepression of *galKT* in the absence of galactose. However, after applying a Bayesian p value <0.001 and a fold-change cut-off of 1.5, our transcriptome results revealed that deletion of *galR* led to the inactivation of the *galKT*. *galR* was downregulated about 10-fold, confirming the deletion of *galR* in the D39 $\Delta galR$. Downregulation of *galKT* in the D39 $\Delta galR$ suggests the role of *galR* as a transcriptional activator of *galKT* in *S. pneumoniae* D39. To further confirm this, we decided to perform a transcriptome comparison of the D39 wild type against D39 $\Delta galR$ grown in GalM17 (0.5% galactose + M17) and the results were consistent with our glucose microarray suggesting that GalR acts as a transcriptional activator of *galKT* (table 1). $\Delta galR$ has a 10-fold reduction in the expression of *galR* when grown on glucose and only 2.4-fold reduction when grown on galactose. This could be due to growth differences when grown on different carbon sources as D39 grows to an optical density (OD) of ~0.6 in GM17 and ~0.2 in GalM17. No change in the expression of the second copies of *galT* and *galE* were observed in our $\Delta galR$ microarray studies. These microarray data further conclude that the regulatory role of GalR in *S. pneumoniae* differs from that in *S. mutans* and *E. coli*. GalR acts as a transcriptional repressor of the *gal* operon in the absence of galactose in *S. mutans* and *E. coli*, whereas in *S. pneumoniae* GalR acts as a transcriptional activator of *galKT* in the presence of galactose.

Quantitative RT-PCR of galKT in $\Delta galR$

We performed quantitative RT-PCR for *galK* and *galT* on the RNA isolated from D39 wild type and D39 $\Delta galR$ grown in GalM17 (0.5% galactose + M17) or GM17 (0.5% glucose + M17) to confirm our microarray results. The expression of *galK* and *galT* was significantly downregu-

Fig. 3. Genetic organization of the *galKT* genes and the transcriptional regulator *galR* in *S. pneumoniae* D39. The dotted rectangle represents the putative *cre* box in *PgalK*, whereas the solid rectangle represents the putative GalR regulatory site.

Table 2. Number of genes significantly affected in D39 $\Delta ccpA$ compared to the D39 wild type grown in GM17 (0.5% glucose + M17) or GalM17 (0.5% galactose + M17) medium

Functional categories	Total	GM17		Total	GalM17	
		up	down		up	down
C: Energy production and conversion	10	6	4	2	0	2
D: Cell cycle control, cell division, chromosome partitioning	–	–	–	–	–	–
E: Amino acid transport and metabolism	4	4	0	1	0	1
F: Nucleotide transport and metabolism	2	2	0	1	0	1
G: Carbohydrate transport and metabolism	34	28	6	7	2	5
H: Coenzyme transport and metabolism	1	1	0	–	–	–
I: Lipid transport and metabolism	–	–	–	–	–	–
J: Translation, ribosomal structure and biogenesis	2	2	0	7	7	0
K: Transcription	5	4	1	2	1	1
L: Replication, recombination and repair	1	0	1	4	2	2
M: Cell wall/membrane/envelope biogenesis	2	1	1	–	–	–
O: Posttranslational modification, protein turnover, chaperones	2	2	0	1	1	0
P: Inorganic ion transport and metabolism	1	1	0	–	–	–
Q: Secondary metabolites biosynthesis, transport and catabolism	–	–	–	2	2	0
R: General function prediction only	8	6	2	7	2	5
S: Function unknown	20	20	0	17	13	4
T: Signal transduction mechanisms	4	4	0	–	–	–
U: Intracellular trafficking, secretion and vesicular transport	–	–	–	–	–	–
V: Defense mechanisms	3	3	0	–	–	–
Total number of genes	99	84	15	51	30	21

Genes affected more than 2-fold in D39 $\Delta ccpA$ as compared to the D39 wild type are shown in COG functional categories.

lated in D39 $\Delta galR$ compared to D39 wild type (fig. 2), which not only confirms our microarray results, but also confirms the role of GalR as a transcriptional activator of *galKT* in the presence of galactose or glucose.

Role of CcpA in the Regulation of the Leloir Pathway Genes

To find the possible role of CcpA in the regulation of *galKT*, we examined the promoter region of *galK* and found a putative *cre* box (5'-AAGAAAACGATTACAC-3') in *PgalK* (fig. 3). The presence of a *cre* box in *PgalK* might indicate the role of CcpA in the regulation of *galKT*. Therefore, we performed transcriptome com-

parison of the D39 wild type against the D39 $\Delta ccpA$ grown in the presence of glucose or galactose as a sole carbon source in M17 medium. Expression of several genes and operons was altered in these transcriptome comparisons (table 2). After applying the criteria of a ≥ 2.0 -fold difference as the threshold change and a p value < 0.001 , these genes were grouped into COG (clusters of orthologous groups) functional categories on the basis of the putative functions of their corresponding proteins. In the presence of glucose, the number of genes with altered expression was double compared to that in the presence of galactose. Most of the genes regulated in our glucose microarray were carbohydrate transport and metabolism

Fig. 4. Identification of the GalR regulatory site in *PgalK*. **a** Weight matrix of the identified GalR regulatory sites in *PgalK* of different streptococci. **b** Position of the GalR regulatory sites in *PgalK* of different streptococci. Core promoter sequences are underlined, and translational start sites and putative GalR regulatory sites are bold. SP = *S. pneumoniae*; SL = *S. mitis*; SL = *S. gallolyticus*; SE = *S. equi*; SM = *S. mutans*; SF = *S. thermophilus*; SG = *S. gordonii*; SS = *S. suis*; SN = *S. sanguinis*.

genes. The expression of genes involved in energy production and conversion of intermediates were also altered (table 2). The expression of some genes encoding hypothetical proteins and with unknown functions belonging to the COG S category was also altered. Similarly, in galactose microarray, expression of genes belonging to the COG S category was altered. Carbohydrate transport and metabolism genes were also regulated in galactose microarray, although their number was much less than that in glucose microarray. Most of these genes have *cre* boxes in their promoter regions and the absence of CcpA appears to be the reason for their altered expression in the presence of galactose/glucose. We could not observe any change in the expression of *galKT* in our microarrays (neither in GM17 nor in GalM17), suggesting no role of CcpA in the regulation of *galKT* under our tested conditions.

Quantitative RT-PCR was performed for *galK* and *galT* on the RNA isolated from D39 wild type and D39 Δ ccpA grown in GalM17 (0.5% galactose + M17) or GM17 (0.5% glucose + M17) in order to verify the microarray results. No significant change in expression of *galKT* was observed in D39 Δ ccpA compared to D39 wild type in the

presence of galactose or glucose (data not shown). These quantitative RT-PCR results corroborated our microarray results mentioned above. However, in a transcriptome comparison of the D39 Δ ccpA against D39 wild type grown in the presence of glucose and galactose in CDM, the expression of *galKT* was upregulated in Δ ccpA strain [Carvalho et al., 2011]. We speculate here that the difference of medium might be the reason why we could not observe a derepression of *galKT* in Δ ccpA under our tested conditions. Further studies need to be performed to address this question.

Prediction of a GalR Regulatory Site in *PgalK*

A 19-bp sequence (5'-GATAGTTTAGTAAAATT-3') located in the promoter region of *galK* of *S. pneumoniae* D39 wild type was predicted by using a MEME motif sampler search (fig. 4a) [Bailey and Elkan, 1994]. This DNA stretch might act as a GalR regulatory site. Our in silico analysis with this predicted GalR regulatory site indicates that the GalR regulatory site is highly conserved in other sequenced strains of *S. pneumoniae* available in the KEGG database (<http://www.genome.jp/kegg/>). *S. equi*, *S. gordonii*, *S. mitis*, *S. mutans*, *S. sanguinis*, *S. gal-*

Fig. 5. Growth comparison of the D39 wild type and the D39 $\Delta galK$ strains grown in GalM17.

lolyticus, *S. thermophilus* and *S. suis* also possess putative Leloir pathway genes. To find the level of conservation of the GalR regulatory site in these streptococci, we analyzed the *galK* promoter region in these species for the presence of the GalR regulatory site. The promoter analysis revealed that the GalR regulatory sequence is highly conserved in all the streptococci mentioned above. *S. equi*, *S. gordonii*, *S. mitis*, *S. mutans*, *S. sanguinis* and *S. suis* contain a putative GalR regulatory site in *PgalK*, whereas *S. gallolyticus* and *S. thermophilus* also contain an additional putative GalR regulatory site in *PgalM* and *PlacS*, respectively. We further generated a weight-matrix of a 19-bp-wide putative GalR regulatory site (5'-RAWAK-TTTAGTAAAWTWT-3') by aligning the predicted GalR regulatory sequences in the *PgalK* of *S. equi*, *S. gallolyticus*, *S. gordonii*, *S. mitis*, *S. mutans*, *S. sanguinis*, *S. suis* and *S. thermophilus* (fig. 4b).

GalK Is Required for Proper Growth of *S. pneumoniae* D39 in the Presence of Galactose

GalK is a galactokinase that converts galactose into galactose-1-P, which is further metabolized by other enzymes of the Leloir pathway. It has been shown previously in *S. mutans* that the *galK*-deficient strain was not able to grow in the presence of galactose as a sole carbon source [Abranches et al., 2004]. However, the *galK*-deficient strain showed a normal pattern of growth compared to the wild type in the presence of glucose and lactose in *S. mutans* [Abranches et al., 2004]. It was further demonstrated that lactose can also induce the expression of lactose PTS (*lacF*) in the *galK*-deficient strain of *S. mutans*, strengthening the notion that lactose metabolism does

Table 3. List of strains used in this study

Strain/ plasmid	Description	Source
<i>S. pneumoniae</i>		
D39	Serotype 2 strain, <i>cps 2</i>	Laboratory of P. Hermans
$\Delta ccpA$	D39 $\Delta ccpA$; Spec ^R	Carvalho et al. [2011]
MA601	D39 $\Delta galR$; Spec ^R	This study
MA602	D39 $\Delta galK$; Spec ^R	This study

not need the Leloir pathway [Abranches et al., 2004]. To assess the significance and involvement of GalK in the metabolism of galactose, we constructed a $\Delta galK$ strain by replacing the *galK* gene with a spectinomycin resistance cassette in *S. pneumoniae* D39. Growth comparison of the D39 wild type and the D39 $\Delta galK$ revealed that growth of D39 $\Delta galK$ was affected in the presence of galactose (fig. 5), suggesting the requirement of *galK* in *S. pneumoniae* to grow on galactose as a sole carbon source. However, the growth patterns of the D39 wild type and D39 $\Delta galK$ were similar in the presence of glucose. These data indicate that GalK in *S. pneumoniae* D39 contributes to the proper growth in the presence of galactose as a sole carbon source.

Materials and Methods

Bacterial Strains, Growth Conditions and DNA Manipulation

The bacterial strains and plasmids used in this study are listed in table 3. *S. pneumoniae* D39 strain [Lanie et al., 2007] was grown as described before [Afzal et al., 2014]. All bacterial strains used in this study were stored in 10% (v/v) glycerol at -80°C , and all DNA manipulations were done as described previously [Kloosterman et al., 2006]. For PCR amplification, chromosomal DNA of *S. pneumoniae* D39 [Lanie et al., 2007] was used. The primers used in this study are based on the sequence of the D39 genome [Lanie et al., 2007] and are listed in table 4.

Construction of *galR* and *galK* Mutants

galR and *galK* deletion mutants were made by allelic replacement with a spectinomycin-resistance marker. Briefly, primers *galR*-1/*galR*-2 and *galR*-3/*galR*-4 were used to generate PCR fragments of the left and right flanking regions of *galR*. Similarly, primers *galK*-1/*galK*-2 and *galK*-3/*galK*-4 were used to generate PCR fragments of the left and right flanking regions of *galK*. PCR products of the left and right flanking regions of *galR* and *galK* contain *AscI* and *NotI* restriction enzyme sites, respectively. The spectinomycin-resistance marker was amplified with the primers Spec-F/Spec-R from plasmid pORI38. The spectinomycin-resistance marker also contains *AscI* and *NotI* restriction enzyme sites on its ends. Then, by restriction and ligation, the left and right flanking

Table 4. List of primers used in this study

Name	Nucleotide sequence (5'→3')	Restriction site
galR-1	GAAGACAAGCCAGAACCATTG	–
galR-2	GCTATGGCGCGCCAATGTCTTTAAGGTAGCC	AscI
galR-3	GCTAAGCGGCGCGTACCCTAAATCAAGAATAG	NotI
galR-4	CAAGGTTGTTGTTATCGGTGATGGGGC	–
galK-1	GCCCTGTTAGAATCCCGATGC	–
galK-2	GCTATGGCGCGCGCGAAGAGTTTCAGCAGTAAGATG	AscI
galK-3	GCTAAGCGGCGCGCACTCGCGTCCTTGACTAGTC	NotI
galK-4	CCTGGAAACTGCTCTACGATAGCC	–
<i>Quantitative RT-PCR primers</i>		
galK-F	GATTGGTGAACACACGGAC	–
galK-R	CAGCAAGAGGCACTTCG	–
galT-F	GGATCGAATCTACCTGACC	–
galT-R	GTCTGACTATCCTCAATCG	–
galE-F	CATCAAGTTGTTGTGGTGG	–
galE-R	GACACCAGTCGGTCTTCC	–
gyrA-F	CGAGGCACGTATGAGCAAGA	–
gyrA-R	GACCAAGGGTTCCCGTTCAT	–

regions of *galR* and *galK* were fused to the spectinomycin-resistance gene. The resulting ligation product was transformed to *S. pneumoniae* D39 wild type, resulting in MA601 and MA602, respectively. Selection of the *galR* and *galK* mutant strains was done using the appropriate concentration of spectinomycin (150 µg ml⁻¹). The deletion of *galR* and *galK* was further confirmed by PCR and DNA sequencing.

RNA Extraction, RT-PCR and Purification for Quantitative RT-PCR

Total RNA was isolated from *S. pneumoniae* D39 wild type and $\Delta galR$ grown in GM17 (0.5% glucose + M17) or GalM17 (0.5% galactose + M17) as previously described [Shafeeq et al., 2011b]. The RNA sample was treated with 2 U of RNase-free DNase I (Invitrogen, Paisley, UK) to remove any DNA contamination. First, strand cDNA synthesis was performed on RNA [Shafeeq et al., 2011b; Yesilkaya et al., 2008]. cDNA (2 µl) was amplified in a 20-µl reaction volume that contained 3 pmol of each primer (table 4) and the reactions were performed in triplicate [Shafeeq et al., 2011b]. The transcription level of specific genes was normalized to *gyrA* transcription, amplified in parallel with *gyrA*-F and *gyrA*-R primers. The results were interpreted using the comparative C_T method [Schmittgen and Livak, 2008]. Differences in expression of 2-fold or greater relative to the control were considered as significant.

Microarray Analysis

To analyze the effect of the *galR* deletion on the transcriptome of *S. pneumoniae*, the D39 wild-type strain and its mutant ($\Delta galR$) strain were grown in replicates in GalM17 (0.5% galactose + M17) or GM17 (0.5% glucose + M17) medium and harvested in their respective mid-exponential growth phase. All other procedures regarding the DNA microarray experiment were performed as described previously [Afzal et al., 2015a; Shafeeq et al., 2015].

Similarly, to analyze the effect of the *ccpA* deletion on the transcriptome of *S. pneumoniae*, the D39 wild-type strain and its mutant ($\Delta ccpA$) strain were grown in replicates in GalM17 (0.5% galactose + M17) medium and harvested in their respective mid-exponential growth phase. Wild-type cells were harvested at an OD₆₀₀ ~0.15 when grown in GalM17, whereas $\Delta ccpA$ was harvested at an OD₆₀₀ ~0.1 when grown in GalM17. All other procedures regarding the DNA microarray experiment were performed as described previously [Afzal et al., 2015b; Shafeeq et al., 2011b].

Microarray Data Analysis

DNA microarray data were analyzed as previously described [Afzal et al., 2015a; Shafeeq et al., 2015]. For the identification of differentially expressed genes a Bayesian p value <0.001 and a fold-change cut-off of 1.5 was applied. Microarray data have been submitted to GEO (Gene Expression Omnibus) database under the accession number GSE74502.

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