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The growth phase-dependent regulation of the pilus locus genes by two-component system TCS08 in *Streptococcus pneumoniae*☆

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ABSTRACT

The two-component system TCS08 of *Streptococcus pneumoniae* contributes to the virulence *in vivo* and regulates phosphotransferase system (PTS) genes in an avirulent strain. However, its role in pathogenic strains and virulence mechanism are largely unknown. In this study, we constructed TCS08 knockout mutants in a serotype 4 encapsulated pathogenic strain TIGR4, and investigated target genes regulated by TCS08 through transcriptional profile analysis. Compared to TIGR4, expression of the *rlrA* islet genes (SP0461–SP0468) encoding pneumococcal pili was found to be up-regulated in the *rr08* mutant (Δ*rr08*). Further quantitative real-time PCR (qRT-PCR) analysis revealed that such induction was more significant when the strains were grown to late-logarithmic phase. In phenotype analyses, disruption of both *hk08* and *rr08* genes (Δ*TCS08*) resulted in increased adherence to human lung epithelial cells (A549) at 3 h at late-logarithmic and stationary phases. However, the invasion level of Δ*TCS08* was reduced at different growth phases. Similar phenotype changes, though less significant, were also observed when the assays were performed on human nasopharyngeal epithelial cells (Detroit 562). These data suggest that TCS08 is involved in adhesion and invasion of host epithelial cells, which is likely mediated via regulation of the pilus locus genes in a growth phase-dependent manner.

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1. Introduction

Despite the existence of antibiotics and vaccines, *Streptococcus pneumoniae* is still one of major infectious disease killers (together with HIV, malaria and tuberculosis) in the world, causing respiratory tract diseases and systemic infections such as pneumonia, bronchitis, otitis media, bacteremia, sepsis and meningitis [1]. During the course of an infection, the *S. pneumoniae* bacterium encounters a number of different environmental niches such as the nasopharynx, lungs, blood, middle ear and potentially the brain. To persist and establish an infection at these various host niches, the pneumococcus needs to adapt and orchestrate its gene expression. Two-component systems (TCS) in bacteria play a central role in sensing environmental changes and regulating gene expressions accordingly. A typical TCS is composed of two different types of proteins: an *hk* encoded histidine kinase sensor protein (HK) located on the membrane, and an *rr* encoded response regulator protein (RR) located in the cytoplasm. Stimulation of HK results in activation of RR through phosphorylation, which subsequently controls expression of target genes via binding to the promoter regions of the regulated target genes. During infection, TCS modulates a variety of cellular responses in bacteria, such as osmoregulation, chemotaxis, sporulation, photosynthesis and pathogenicity [2].

In *S. pneumoniae*, a total of 13 TCSs and one orphan RR have been identified [3,4], and many of them are involved in virulence via regulating expression of different target genes [5]. For example, TCS02 and TCS06 regulate the expression of choline-binding proteins PspA [6] and CbpA [7,8], respectively. Both virulence factors are also regulated by TCS06 through different mechanisms [9]. TCS04 controls the expression of pneumococcal surface adhesin A (PsaA) [10], while TCS05 (CiaRH) is linked to HtrA, a virulence factor that contributes to nasopharyngeal colonization [11]. In animal experiments, up to 10 TCS systems have been claimed to be associated with virulence [4,5], indicating the importance of TCS in pathogenicity. Among these systems, TCS08 plays an essential role for pneumococcal growth and survival *in vivo* [4]. In an unencapsulated avirulent strain, TCS08 was found to be involved in regulation of phosphotransferase system (PTS) genes [12]. However, the roles of TCS08 in encapsulated wild-type strains and its virulence mechanism are still largely unknown.

In this study, we compared transcriptional profiles of an *rr08* knockout mutant and the parent wild-type strain, and characterized

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the target genes regulated by TCS08 through microarray and quantitative real-time PCR (qRT-PCR) analyses. One significant change was the pilus locus genes. Based on the functions of pili, we further characterized phenotype changes of the TCS08 mutant in adhesion and invasion of host epithelial cells. These studies provided more insights into the function of TCS08-mediated gene regulation and the impact on pathogen–host interactions. It also reflects the complexity of gene regulation mechanisms for the pneumococcal pilus locus genes.

2. Results

2.1. Construction and characterization of TCS08 knockout mutants

To characterize the function of TCS08, we constructed different TCS08 knockout mutants in *S. pneumoniae* TIGR4, including mutants disrupted at 100 bp–373 bp of SP0083 (Δ*rr08*), 151 bp–780 bp of SP0084 (Δ*hk08*) and the region covering both SP0083 and SP0084 (Δ*TCS08*). We did not observe apparent changes in growth rates for the constructed mutants when grown in THY media. To examine possible polar effect on the downstream genes in the constructed mutants, we determined mRNA transcription levels of selected genes in different TCS08 mutants through qRT-PCR analysis. Compared to the wild-type strain TIGR4, we observed an enhanced expression of SP0084 (*hk08*) at a fold change (*F*) of 7.6 in the Δ*rr08* strain when SP0083 (*rr08*) was disrupted, which is likely due to the upstream insertion of the erythromycin (Erm) cassette where multiple promoters were introduced at the same orientation of transcription. However, expression of SP0085 (*rpsD*) was unchanged ($F < 2.0$) in the Δ*hk08* and Δ*TCS08* mutant strains when SP0084 or both SP0083 and SP0084 were disrupted, indicating that SP0083 and SP0084 comprise one transcript, and both genes transcribe separately from the downstream SP0085. Thus, disruption of TCS08 genes is unlikely to cause a polar effect in the constructed mutant strains.

2.2. Differentially regulated genes in the Δrr08 mutant

To investigate the target genes regulated by TCS08, we examined transcriptionally changed genes in the Δ*rr08* mutant relative to the parent strain TIGR4 by performing microarray analysis. Among the differentially regulated genes, most of them were associated with regulation, cell envelope, protein fate, PTS and unknown functions (Table 1). Except for a few down-regulated genes mostly with unknown functions, one significant change was the up-regulated SP0461-SP0468 locus genes, suggesting a negative regulation mechanism (Fig. 1A). In *S. pneumoniae* TIGR4 genome (TIGR), the SP0461–SP0468 locus genes belong to the *rlrA* pathogenicity islet, encoding pneumococcal pili. It includes one regulatory gene *rlrA* (SP0461), three cell envelope genes *rrgA* (SP0462), *rrgB* (SP0463) and *rrgC* (SP0464) encoding cell wall surface anchor family proteins, and three protein fate genes *srtB* (SP0466), *srtC* (SP0467) and *srtD* (SP0468) encoding putative sortases (Fig. 1B). Although the expression change of SP0468 ($F = 1.9$) was below the selection criteria, we assume the expression of SP0468 was also changed because of the changed upstream genes (Fig. 1). In the middle of this locus, there is one small hypothetical gene (SP0465) at a size of 132 bp (Fig. 1B). The presence and expression of this gene are questionable because it was not presented in previous studies [13], and also remained unchanged ($F = 1.0$) in our microarray analysis. The SP0465 was, therefore, not pursued further in this study.

Among regulatory genes, expression of SP0083 (*rr08*) and SP0084 (*hk08*) was unexpectedly elevated in the Δ*rr08* mutant (Table 1). Sequence analysis of the mutant revealed that the microarray oligonucleotide (oligo) probe binding site for SP0083 (427 bp–496 bp), located downstream of the disrupted region, was still carried by the mutant. The enhanced expression of 3′-end SP0083 and SP0084 in microarray analysis was, therefore, because of the upstream inserted Erm cassette as above described. As the function of HK in a TCS system is usually implemented through the corresponding RR, a changed expression of *hk08* is unlikely to interfere with the TCS08-mediated gene regulation profile when *rr08* was disrupted in the Δ*rr08* mutant.

Table 1
Microarray identified genes in Δrr08 compared to wild-type strain TIGR4.

Function/ Gene	Protein	TIGR4 genome acc. ID	Fold change
Regulatory function			
rr08	DNA-binding response regulator	SP0083	9.9
hk08	Sensor histidine kinase	SP0084	3.8
rlrA	Transcriptional regulator, putative	SP0461	3.5
Cell envelope			
rrgA	Cell wall surface anchor family protein	SP0462	4.2
rrgB	Cell wall surface anchor family protein	SP0463	5.8
rrgC	Cell wall surface anchor family protein	SP0464	6.1
bgaA	Beta-galactosidase	SP0648	−2.0
Protein fate			
srtB	Sortase, putative	SP0466	3.3
srtC	Sortase, putative	SP0467	2.7
Phosphotransferase system (PTS)			
	PTS system, IIB component	SP0061	−2.0
	PTS system, mannose-specific IID component	SP0282	2.2
manL	PTS system, mannose-specific IIAB components	SP0284	2.2
Others			
celA	Competence protein CelA	SP0954	−4.2
	Alcohol dehydrogenase, zinc-containing	SP1270	−2.8
yfiA	Ribosomal subunit interface protein	SP2206	−2.1
Unknown function and hypothetical			
	Conserved hypothetical protein	SP0409	−5.8
	Conserved hypothetical protein	SP1003	−2.2
	Glutamine amidotransferase, class-I	SP2072	−10.7

Microarray data have been deposited in the ArrayExpress microarray database (http://www.ebi.ac.uk/arrayexpress) under accession No. E–FPMI-17.

2.3. Characterization of expression changes of the pilus locus genes in the TCS08 mutants

To confirm the TCS08-mediated regulation on the pilus locus genes, we analyzed the expression changes of selected genes (SP0461, SP0462 and SP0466) (Fig. 1B) in two TCS08 mutant strains (Δ*rr08* and Δ*TCS08*) in a comparison to the wild-type strain TIGR4 through qRT-PCR analysis. The RNA samples were isolated from bacteria grown in THY broth to OD_{600} 0.4. As expected, expression of all the tested genes was changed not only in the same way (up-regulation), but also in a similar scale (2–7-fold change) (Fig. 2A) compared to the microarray data (Fig. 1A). It indicates that disruption of *rr08* or both *hk08* and *rr08* resulted in similar expression changes of the pilus locus genes.

To analyze if growth factors might interfere with the TCS08-mediated gene regulation, we examined the expression levels of SP0461 and SP0466 in the Δ*rr08* and TIGR4 strains when both strains were cultured in different media (THY or BHI) to different growth densities. When bacteria were cultured in THY broth to OD_{600} 0.2, OD_{600} 0.4 and OD_{600} 1.0 or in BHI broth to OD_{600} 0.3, expression of SP0461 and SP0466 was up-regulated in the mutant (Fig. 2B) at a scale close to that described above. However, when bacteria were cultured to late-logarithmic phase at OD_{600} 0.6 in

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