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FEEDBACK CONTROL OF THE TWO-COMPONENT REGULATORY SYSTEM CiaRH IN *STREPTOCOCCUS PNEUMONIAE* R6

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ABSTRACT : The CiaRH - system is a classical two-component system and has been identified as the first TCS in *S. pneumoniae*, which is consisting of the membrane-bound histidine CiaH and the cytoplasmic response regulator CiaR. This system is significantly involved in the β-lactam resistance, genetic competence, growth and autolysis of the bacterium. The CiaR- regulon comprising 25 genes, including it. To explore conditions altering the activity of CiaRH, possible feedback regulation of the system by the strongly regulated genes *ccnA-E* and *htrA* was investigated. For that were tested systematically whether members of the CiaR regulon could influence CiaR activity. Data showed that the activation of the response regulator occurs mainly independently from the bacterial growth media. Deletion of *ccnA-E* and *htrA* genes has led to a strong activation of the CiaR-dependent genes. Thus, the translation of CaiR and CiaH was increased. In addition, our study has confirmed that the *htrA* and *csRNAs* genes affected the CiaR activity via CiaH regulation. Accordingly, we postulated that a part of the feedback regulation relies on a mechanism influencing the CiaH activity.

Key words : *Streptococcus pneumoniae*, CiaRH, protease HtrA, csRNAs.

INTRODUCTION

The ability to sense and respond to environmental changes is crucial for bacteria. One of the most prevalent mechanisms to perceive stimuli and launch adaptive responses is by two-component regulatory systems (TCS) (Laub and Goulian, 2007; Gao and Stock, 2009; Casino *et al*, 2010). A prototypical TCS is composed of a histidine kinase (HK), which is involved in stimulus perception and phosphotransfer to a response regulator (RR) (Gao and Stock, 2009). Since the majority of RRs have DNA binding effector domains (Gao and Stock, 2009), the most common output is the alteration of gene expression. This mode of regulation implies that substantial switches in expression levels occur, depending on the availability of the signal(s). In contrast, the essential TCS WalRK (YycFG, VicRK) in low-GC Gram-positive bacteriais a striking example that a TCS is not only conditionally needed (Fabret and Hoch, 1998; Winkler and Hoch, 2008). Its activity is required throughout growth.

In addition to input-dependent regulation, TCS may be subject to autoregulation or feedback control (Groisman, 2016). The majority of TCS regulatory pairs is encoded in operons, which are preceded by promoters quite often regulated by the RR. Autoregulation may help to increase a response to a given stimulus. Moreover, gene products of the RR-controlled regulon may exert positive or negative feedback regulation of the TCS activity (Groisman, 2016). Positive feedback regulation will also strengthen a response, while a negative feedback loop serves to stabilize the expression state of a system.

In the human pathogen *Streptococcus pneumoniae* the TCS CiaRH (Guenzi *et al*, 1994) has been studied in some detail (Halfmann *et al*, 2007b; Halfmann *et al*, 2011; Marx *et al*, 2015). The RR CiaR controls directly 16 promoters and thereby around 30 genes (Halfmann *et al*, 2007b; Denapaite *et al*, 2012). Major phenotypes associated with CiaRH, reduction of competence and enhanced β-lactam resistance are indirectly affected by components of the CiaR regulon. Five small non-coding csRNAs (*cia*-controlled sRNAs) and the protease HtrA, whose genes are strongly activated by CiaR, have been shown to be responsible for the above mentioned phenotypes (Sebert *et al*, 2005; Schnorpfeil *et al*, 2013; Laux *et al*, 2015). CiaRH was shown to be active under a variety of growth conditions in the laboratory (Kumar *et al*, 2010; Tsui *et al*, 2010; Halfmann *et al*, 2011), but also in animals (Marra *et al*, 2002; Sebert *et al*, 2002; Ibrahim *et al*, 2004). Therefore, CiaRH has apparently evolved to maintain elevated, rather constant gene

expression. How this is achieved is unknown and signals to which CiaH could respond have not been defined.

Under certain growth conditions, CiaH is even dispensable to maintain high CiaR activity (Martin *et al*, 2000; Halfmann *et al*, 2011; Marx *et al*, 2015). Experiments in *S. pneumoniae* mutant strains altered in acetyl phosphate (AcP) production indicated that AcP could be the alternative phosphodonorfor CiaR (Marx *et al*, 2015). By this route of phosphorylation, CiaR activity could be linked to the metabolism of the cell without the need of signal(s) stimulating phosphotransfer from the kinase CiaH.

A TCS such as CiaRH in *S. pneumoniae* evolved to maintain a rather constant expression level under a variety of conditions would be a prime candidate to include feedback control in its regulatory circuit. In this communication, we describe extensive feedback regulation within the CiaRH regulon of *S. pneumoniae*.

MATERIALS AND METHODS

Bacterial strains, plasmids, growth conditions, transformation, β–Galactosidase Assay and Western blot analysis.

S. pneumoniae strains in this work are derivatives of *S. pneumoniae* R6 (Ottolenghi and Hotchkiss, 1962). They are listed in Table 1. The promoter probe plasmid pPP2 derivative containing P*htrA* and P*ccnA*, respectively, have been described previously (Halfmann *et al*, 2007a). All plasmids were used in this work. They are listed in Table 2. *S. pneumoniae* strains were grown at 37°C in brain heart infusion medium (BHI) (Alloing *et al*, 1996) was used to grow *S. pneumoniae* on agar platesand select transformants. Plasmids were multiplied in *Escherichia coli* DH5α [Φ80d*lacZ*ÄM15 Ä(*lacZYA-argF*) *recA1 endA1 hsdR17supE44 thi-1 gyrA96 phoA relA1*]. *E. coli* strains were grown in LB medium.

S. pneumoniae was grown in brain heart infusion medium (BHI). *E. coli* transformation followed standard procedures.

In a â-galactosidase assay, the measurement of â-galactosidase activity was done to determine the strength of the promoter activities.

The promoters found in the integrative promoter probe plasmid pPP2 (Table 2) were fused in front of a promoterless *E. coli* lacZ gene (Halfmann *et al*, 2007a, 2007b) and were integrated within the endogenous β-galactosidase gene *bgaA* in the genome of the *S. pneumoniae* strain. Subsequently, β-galactosidase activities were measured in BHI medium.

The synthetic compound o-nitrophenyl-β-D-galactoside (ONPG) is also recognized as alternative substrate and cleaved to yield galactose and o-nitrophenol, which has a yellow colour. When ONPG is in excess over the enzyme in a reaction, the production of o-nitrophenol per unit time is proportional to the concentration of β-Galactosidase; thus, the production of yellow colour can be used to determine enzyme concentration. Enzyme activity can be measured by the rate of appearance of yellow colour using a photometer (Thermo Scientific UV-10 UV-Vis).

It is often useful to measure the development of gene expression over time in order to identify expression changes that are influenced by the cell density. For this purpose, the following approach was performed:

About 14ml of BHI medium was inoculated (1:100) with glycerol stock culture and incubated at 37°C in a water bath. Growth was measured after every 30 min. Following growth measurement, samples (640 µl) were taken and mixed with 160 µl lysis buffer. The mixture was then incubated at 37°C in water bath for 15 min until lysis completed. The lysate was kept on ice until it was used. The mixture was transferred into a cuvette and shortly before the measurement 200 µl of pre-warmed (30°C) ONPG were added. The measurement of the Enzyme activity is the end point assay done each 40 seconds over a period 15 min at an optical density of 420 nm.

Evaluation of β-galactosidase activity is calculated over time. The β-galactosidase activity is stated as Miller units in nmol released o-nitrophenol (ONP) per mg protein per minute.

In the Western blot analysis: *S. pneumoniae* was grown in brain heart infusion medium (BHI) at 37°C until $OD_{600} = 0.8$, then 4 ml of the culture was aliquoted into two 2 ml Eppendorf tubes and then centrifuged at 14,000 rpm for 5 min. The supernatant was discarded and cell pellet was resuspended in 20mM sodium phosphate buffer using 0.2% Triton X-100. The following formula was used to calculate the buffer amount:

Amount of Na phosphate buffer [ìl] = culture volume [ml] × (actualNephelo / 20) × 5 ìl.

The CiaR and CiaH western blot bands were detected using primary anti-CIAR, anti-CIAH and secondary anti-rabbit antibodies and western blot followed standard procedures.

Inactivation of genes of the CiaR regulon

In this work, the inactivation of *htrA* and *ciaH* genes performed by PCR using pairs of oligonucleotide primers (Table 3). *ciaH::aad9* (Halfmann *et al*, 2011) and

htrA::aphIII (Schnorpfeil *et al*, 2013) were amplified by PCR from the strain R6 *ciaH::aad9* and *ciaH::aad9*.

RESULTS AND DISCUSSION

Feedback regulation of CiaRH activity by the protease HtrA

The expression of *htrA* in *S. pneumoniae* is regulated by the CiaR/CiaH two-component system (Sebert *et al*, 2002; Mascher *et al*, 2003), which in turn influences genetic competence, autolysis, bacteriocin production and virulence (Halfmann *et al*, 2011).

Expression of the gene *htrA* is under strict control of the TCS CiaRH was studies. It has been reported that the deletion of the response regulators CiaR results in almost no activity of P*htrA* (Half Man *et al*, 2007b). However, only partly a media-dependent activation of the Cia system was investigated.

No significant differences were noticed in activity of *htrA* and *ccnA* promoters in the R6 background at OD_{600} =0.4 and OD_{600} = 0.8, while the promoters in the *htrA* deleted strains showed significant increase in their activity (Fig. 1).

The promoters in the *htrA* deleted strains showed significant increase in their activity. The activity of promoter P*htrA* which corresponds to a 1.7-fold increased by deletion of *htrA* and the activity of promoter P*ccnA* 1.8-fold was also increased by deletion of the *htrA*gene in BHI medium. This indicates a negative regulation by *htrA*on CiaRH system (Fig. 1).

The promoters P_{htrA} and P_{ccnA} were cloned in front of lacZ and β-galactosidase. Activities were measured in strains grown in BHI medium at an OD_{600} of 0.4 and 0.8. The mean values determined from at least three independent experiments Units (U) are shown along with the standard deviation. The β-galactosidase activity (U) is defined as nmol ONP released/min/mg of total protein. For comparison are shown the promoter activity of the wild type (RKL43 wt P*htrA*-lacZ) compared to strain (RKL197 Ä*htrA PhtrA-lacZ*) and the promoter activity of the wild type (RKL20 wt P*ccnA*-lacZ) compared to strain (RKL312 Ä*htrA* P*ccnA*-lacZ).Blue column: WT. Green Column: mutant R6.

Feedback regulation of CiaRH activity by small non-coding csRNAs

The non-coding csRNAs harbor sequences complementary to translational initiation regions and act therefore most likely as base-pairing RNAs to repress translation of genes. In addition, sRNAs may also reduce mRNA stability (Storz *et al*, 2012). Provided the csRNAs could also do so, a transcriptome analysis could reveal new csRNA targets.

The small regulatory *csRNAs* (*CCNA-E*) are among the most controlled and expressed genes of CiaR regulon (Half Man *et al*, 2007b). Recently, it has been elaborated that csRNAs are the main effectors of the Cia associated phenotypes resistance, competence and autolysis (Müller, 2011).

Fig. 2 clearly shows significant activation (1.3 fold increase) in P*htrA* and in P*ccnA*(1.4 fold) were recorded by deletion of the *csRNAs* in comparison to the wild type grown in BHI medium.

β-Galactosidase assays has indicated a slightly increase in the promoters activity during the bacterial growth at OD 600 = 0.8 in comparison to the earlier stage OD600 = 0.4 of growth. The results confirmed the impact of regulatory *csRNAs*on CiaR activity. This result also indicates a negative regulation of the CiaRH system by *csRNAs* (Fig. 2).

The promoters P_{htrA} and P_{ccnA} were cloned in front of lacZ and β-galactosidase activities were measured in strains grown in BHI medium at an OD_{600} of 0.4 and 0.8. The mean values determined from at least three independent experiments Units (U) are shown along with the standard deviation. The β-galactosidase activity (U) is defined as nmol ONP released/min/mg of total protein, comparison is shown the promoter activity of the wild type (RKL43 wt P*htrA*-lacZ) compared to strain (RKL364 Ä*ccnA-E PhtrA-lacZ*) and the promoter activity of the wild type (RKL20 wt P*ccnA*-lacZ) compared to strain (RKL650 Δ*ccnA-E PccnA-lacZ*). Orange column: WT R6. Blue Column: mutant R6.

Feedback regulation of CiaRH activity by both HtrA and small non-coding csRNAs

Activation of the Cia system in double deletion of *htrA* and *CCNA-E* is clearly confirmed in this work. This raises the question of how far these genes products are involved in the regulatory mechanisms of each other's. Therefore activation of the Cia system was checked through measuring β-galactosidase activity of the two promoters P*htrA* and P*ccnA*, which are known to be strongly controlled by CiaR system.

The resulted data showed that double inactivation of *htrA* and *csRNAs* lead very strongly to the activation of CiaRH system (Fig. 3) in which high increase of the promoter P*htrA* and P*ccnA* activity was recorded.

The promoter P*htrA* shows a nearly 9-fold increase activity by deletion of the genes *csRNAs*, *htrA* and the promoter P*ccnA* activity shows a nearly 6.5-fold increase in the *csRNAs*, *htrA* double deletion background.

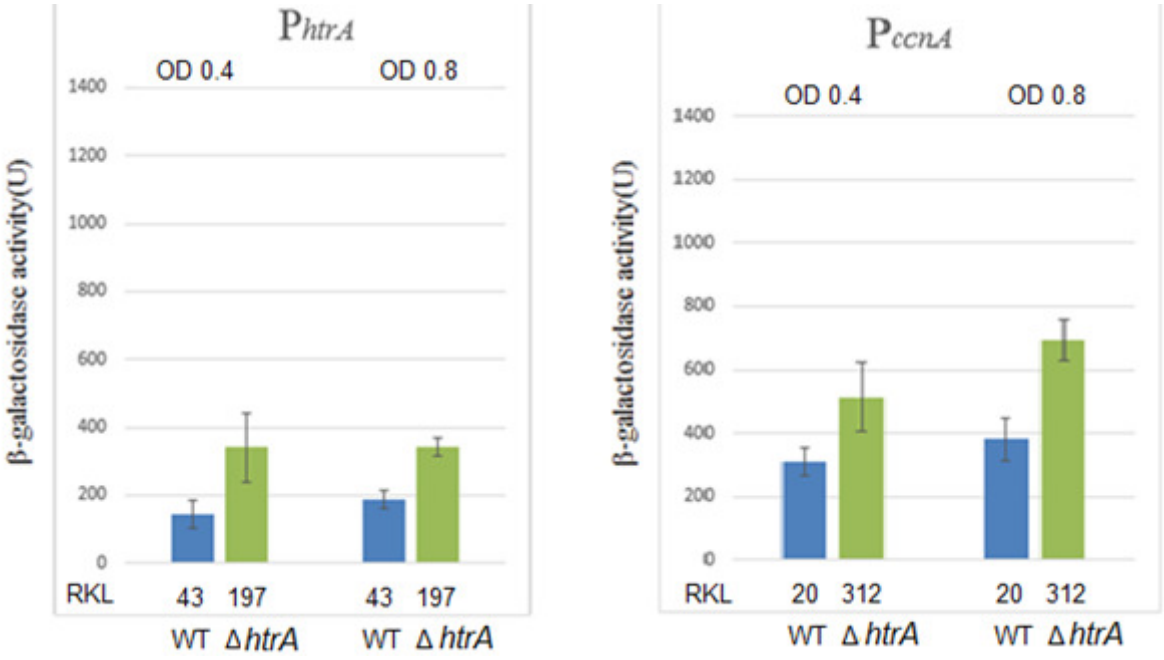

Fig. 1 : Feed-back regulation by deletion of *htrA* component of the CiaR regulon.

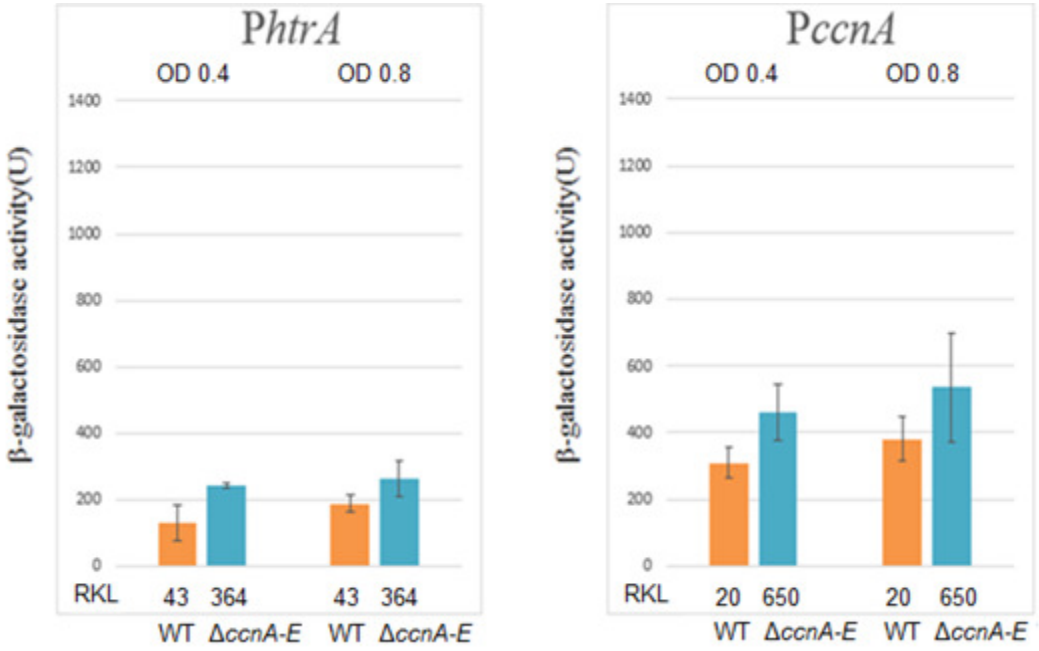

Fig. 2 : Feed-back regulation by deletion of *ccnA-E* component of the CiaR regulon.

Accordingly the activities of the cia-dependent promotersare significantly higher in the double deletion Δ*csRNA*, Δ*htrA* compared to that of the single deletion (Δ*csRNAs*) or (Δ*htrA*). This result confirms a negative regulation of the CiaRH system by the *csRNAs*, *htrA* products. Thus, the protease *htrA* and the non-coding *RNAs* act additively in feedback regulation.

The promoters P_{htrA} and P_{ccnA} were cloned in front of lacZ and β-galactosidase activities were measured in strains grown in BHI medium at an OD_{600} of 0.4 and 0.8. The mean values determined from at least three independent experiments Units (U) are shown along with the standard deviation. The β-galactosidase activity (U) is defined as nmol ONP released / min / mg of total protein, comparisons are shown the promoter activity of the wild type (RKL43 wt P*htrA*-lacZ) compared to strain (RKL652 Δ*ccnA-E*, Δ*htrA*, *PhtrA-lacZ*) and the promoter activity of the wild type (RKL20 wt *PccnA*-lacZ) compared to strain (RKL651 Δ*ccnA-E*, Δ*htrA*, *PccnA-lacZ*). Blue column: WT R6. Red Column: mutant R6.

Table 1 : *S. pneumoniae* strains used in this study.

RKL No.	Straina	Genotype	Reference
	R6 *S.pneumoniae*	Wild-type, without capsule, competent	Ottolenghi and Hotchkiss (1962)
20	R6 pPP2*ccnA*	*bgaA*::*tetM*-P$_{ccnA}$-*lacZ*	Halfmann (2008)
43	R6 pPP2*htrA*	*bgaA::tetM*P*htrA-lacZ*	Halfmann (2008)
86	R6 pPP2*htrA* Δ*ciaH*	*bgaA::tetM*P*htrA-lacZ;ciaH::aad9*	Halfmann (2008)
197	R6 pPP2htrA Δ*htrA*	*bgaA*::*tetM*-P$_{htrA}$-*lacZ;htrA::aphIII*	Muller *et al* (2011)
312	R6 pPP2$_{ccnA}$Δ*htrA*	*bgaA*::*tetM*-P$_{ccnA}$-*lacZ;htrA::aphIII*	Muller (2011)
316	R6 pPP2htrA Δ*htrA* Δ*ciaH*	*bgaA*::*tetM*-P$_{htrA}$-*lacZ;htrA::aphIII, ciaH::aad9*	Muller (2011)
364	R6 pPP2$_{htrA}$ Ä*ccnCDEAB*	*bgaA*::*tetM*-P$_{htrA}$-*lacZ; ccnC::lox72, ccnD::lox72; ccnE::lox72; ccnAB::lox72*,	Muller (2011)
650	R6 pPP2$_{ccnA}$ Ä*ccnCDEAB*	*bgaA::tetM-PccnA-lacZ;ccnC::lox72, ccnD::lox72; ccnE::lox72; ccnAB::lox72*	Shambhu (2013)
651	R6 pPP2$_{ccnA}$ Δ*ccnCDEABhtrA*	*bgaA::tetM-PccnA- lacZ;ccnC::lox72,ccnD::lox72; ccnE::lox72; ccnAB::lox72;htrA::aphIII*	Shambhu (2013)
652	R6 pPP2$_{htrA}$ Δ*ccnCDEABhtrA*	*bgaA::tetM-PhtrA-lacZ;accnC::lox72,ccnD::lox72; ccnE::lox72; ccnAB::lox72;htrA::aphIII*	Shambhu (2013)
985	R6 pPP2$_{htrA}$Δ*htrA* Δ*ccnCDEBA*	*bgaA::tetM-PhtrA-lacZ; bgaAΔ:tmp-pccnA-'spr056;ÄccnC::lox72, ÄccnD::lox72; ÄccnE::lox72;ÄccnAB::lox72; htrA::aphIII*	This work
986	R6 pPP2$_{htrA}$ Δ*htrA*Δ*ccnCDEBA*	*bgaA::tetM-PhtrA-lacZ; bgaA": tmp-pccnB-'spr056 ;ÄccnC::lox72, ÄccnD::lox72;ÄccnE::lox72;ÄccnAB::lox72; htrA::aphIII*	This work
987	R6 pPP2$_{htrA}$ Δ*htrA*Δ*ccnCDEBA*	*bgaA::tetM-PhtrA-lacZ; bgaA": tmp-pccnC-'spr056 ;ÄccnC::lox72, ÄccnD::lox72;ÄccnE::lox72;ÄccnAB::lox72; htrA::aphIII*	This work
988	R6 pPP2$_{htrA}$ Δ*htrA*Δ*ccnCDEBA*	*bgaA::tetM-PhtrA-lacZ;bgaA":tmp-pccnD-'spr056; ÄccnC::lox72, ÄccnD::lox72;ÄccnE::lox72; ÄccnAB::lox72 ; htrA::aphIII*	This work
989	R6 pPP2$_{htrA}$ Δ*htrA*Δ*ccnCDEBA*	*bgaA::tetM-PhtrA-lacZ; bgaA": tmp-pccnE-'spr056 ;ÄccnC::lox72, ÄccnD::lox72;ÄccnE::lox72;ÄccnAB::lox72; htrA::aphIII*	This work
991	R6 pPP2$_{htrA}$Δ*ccnCDEAB,* Δ*htrA*Δ*ciaH*	*bgaA::tetM-PhtrA -lacZ ;ÄccnC::lox72, ÄccnD::lox72; ÄccnE::lox72;ÄccnAB::lox72; htrA::aphIII; ciaH::aad9*	This work
1024	R6 pPP2$_{htrA}$ Ä*ccnCDEAB*Ä*ciaH*	*bgaA*::*tetM*-P$_{htrA}$-*lacZ; ccnC::lox72, ccnD::lox72; ccnE::lox72; ccnAB::lox72*, *ciaH::aad9*	This work

a.All strains are R6 derivatives

Contribution of the CiaH kinase to feedback regulation

The *htrA* and *csRNAs* genes in *S. pneumoniae* are regulated by the CiaR/CiaH two-component systems and belong to the strongest CiaR-dependent genes (Halfmann *et al*, 2007b; Halfmann *et al*, 2011). The CiaRH system is unlike any other known two-component systems constitutively "turned on", thus reaching even higher expression levels of the CiaR regulon when activated (Halfmann *et al*, 2011).

It has been observed that that P*htrA* promoter activity dropped by 10 fold in the absence of functional CiaH protein studied in the strain deletion *ciaH* compared to the wild type. However, presence of *ciaH* and absence

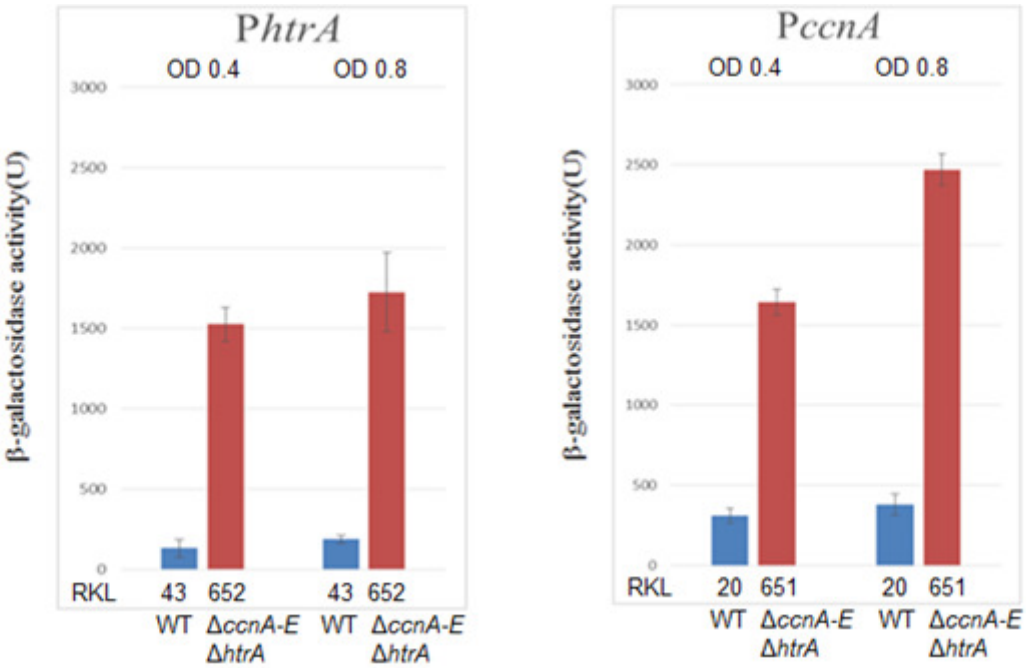

Fig. 3 : Feed-back regulation by double deletion of *ccnA-E* and *htrA* component of the CiaR regulon.

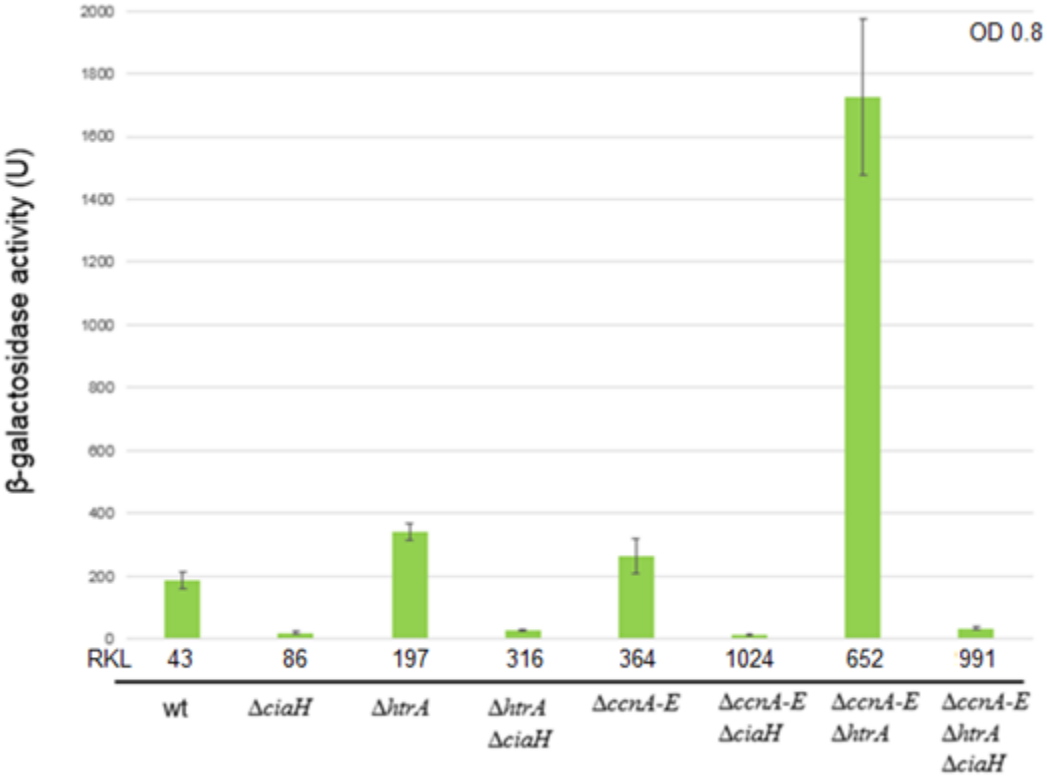

Fig. 4 : Influence of *ciaH* on feed-back regulation by *htrA* and *csRNAs* at an OD_{600} of 0.8.

of Δ*htrA*, Δ*ccnA-E* resulted in higher P*htrA* promoter activity (nearly 10 fold) compared to the wild type with the intact *ciaH* gene. Moreover, absence of Δ*ciaH*, Δ*htrA*, Δ*ccnA-E*, showed 10 fold inhibition of the P*htrA* promoter activity in comparison to the recombinant strain (Δ*htrA*, Δ*ccnA-E*) containing *ciaH* gene (Fig. 4).

This confirms the significant influence of *ciaH* on *htrA* and *csRNA* expression. In addition, the results of the experiments showed that the *htrA* and *csRNAs* gene affected the CiaR activity via CiaH regulation. Accordingly, we postulated that a part of the feed-back regulation relies on a mechanism influencing the CiaH activity.

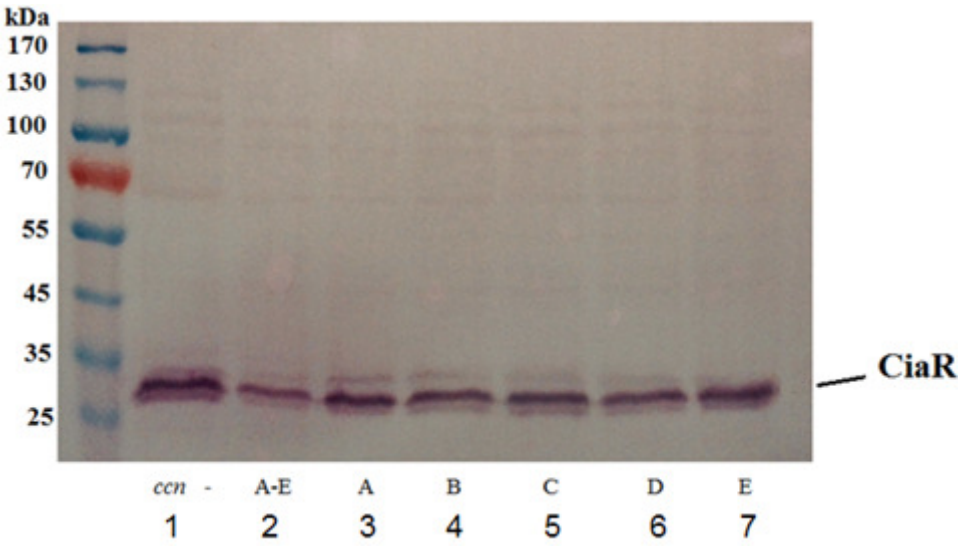

Fig. 5 : Western blot analysis representing theeffect of small *csRNAs* on CiaR (molecular weight of about 26 kDa) regulation in *S. pneumonia* (lane1) (RKL652 Ä*ccnA-E,* Ä*htrA*), (lane 2) (RKL197 Ä*htrA* with intact *ccnA-E*), (lane 3) (RKL985 Ä*ccnBCDE*, Ä*htrA*), (lane 4) (RKL986 Ä*ccnACDE*, Ä*htrA*), (lane 5) (RKL987 Ä*ccnABDE*, Ä*htrA*), (lane 6) (RKL988 Ä*ccnABCE*, Ä*htrA*) and (lane 7) (RKL989 Ä*ccnABCD*, Ä*htrA*) grown in BHI medium at 37°C in OD_{600} = 0.8. The CiaR western blot bands were detected using primary anti-CIAR and secondary anti-rabbit antibodies.

Table 2 : Plasmids used in this work.

Plasmid	Description	Selection condition	Reference
pPP2-*htrA*	$pPP2_{htrA}$-*lacZ*	Ampicillin 100 ìg/ml in *E. coli*. Tetracyclin 3 ìg/ml in *S. pneumoniae*	Halfmann *et al* (2007a)
pPP2-*ccnA*	$pPP2_{ccnA}$-*lacZ*	Ampicillin 100 ìg/ml in *E.coli*. Tetracyclin 3 ìg/ml in *S. pneumoniae*	Halfmann *et al* (2007a)
pPP2-*ccnA*	Derivative fromp SW1, native *ccnA* was introduced	Ampicillin 100ìg/ml in *E.coli.* Trimethoprim15 ìg/mlin *S.pneumoniae*	Brunner (2010)
pPP2-*ccnB*	Derivative from pSW1, native *ccnB* was introduced	Ampicillin 100 ìg/ml in *E. coli.* Trimethoprim 15 ìg/mlin *S.pneumoniae*	Brunner (2010)
pPP2-*ccnC*	Derivative from pSW1, native *ccnC* was introduced	Ampicillin 100 ìg/ml in *E.coli.* Trimethoprim 15 ìg/ml in *S.pneumoniae*	Brunner (2010)
pPP2-*ccnD*	Derivative from pSW1, native *ccnD* was introduced	Ampicillin 100 ìg/ml in *E.coli.* Trimethoprim 15 ìg/mlin *S.pneumoniae*	Brunner (2010)
pPP2-*ccnE*	Derivative from pSW1, native *ccnE* was introduced	Ampicillin 100 ìg/ml in *E.coli.* Trimethoprim 15 ìg/ml in *S.pneumoniae*	Brunner (2010)

Table 3 : Oligonucleotides for producing the *htrA* deletion (*apHIII* cassette) and *ciaH* deletion (*aad9* cassette) were used in this study.

Primer name	Sequence (5' to 3')
htrA htrA-ko-6	GAACAATAACCGTCCCAAATCC
SpoOJI	GAGATATTTTACCATAGATAGC
ciaH CiaRup_f	ATTGATGAAGGAACGGATGCTGAAACAGCC
ciaH_down_r	ATTGACCGCAACGAGCAATTCCTTACCAGC

The promoters P_{htrA} was cloned in front of *lacZ* and β-galactosidase activities were measured in strains grown in BHI medium. The mean values determined from at least three independent experiments Units (U) are shown along with the standard deviation. The β-galactosidase activity (U) is defined as nmol ONP released/min/mg of total protein.

Feedback regulation and Effect of amount *HtrA* and small non-coding *csRNAs* on *CiaRH* system

The investigations by means of a Western blot showed that *htrA* deletion, *csRNAs* deletion and double deletions (*htrA*, *csRNAs*) lead to a significantly increased CiaR and CiaH amount compared with the wild type in whichfeedback regulation takes place. It has been

investigated that the mutations Δ*htrA* and Δ*ccnA-E* respectively showed approximately equal induction's effect on the Cia system expression, while inactivation of both genes together showed douplicated effect on CiaR and CiaH expression.

Furthermore, expression of the CiaR protein was checked under effect of single *csRNA* to determine the feedback effect of the *csRNAs* together, or each *csRNA* individually on the protein production. In which collection of the *csRNAs* showed the higher repression effect on the *CiaR* protein synthesis as shown in Fig. 5.

As shown in Fig. 5 single small *csRNA* genes showed negative effect in the *ciaR* expression, however the impact of *csRNA* genes individually was lower in comparison to that of the whole *csRNAs* (A-E). Thus, *csRNAs* gene in the bacteria repress the Cia system in *S. pneumoniae*.

Strains (RKL43 wt), (RKL197 Δ*htrA*), (RKL 364Δ*ccnA-E*) and (RKL652 Δ*ccnA-E*, Δ*htrA*), were grown in BHI medium at 37°C until $OD_{600} = 0.8$. **A.** The CiaR western blot bands were detected using primary anti-CIAR, and secondary anti-rabbit antibodies. **B.** The CiaH western blot bands were detected using primary anti-CIAH and secondary anti-rabbit antibodies.

CONCLUSION

The *htrA* and *csRNAs* genes in *S. pneumoniae* are most effective genes playing role in feedback regulation.

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