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Genetic and physiological studies of the CiaH–CiaR two-component signal-transducing system involved in cefotaxime resistance and competence of *Streptococcus pneumoniae*

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A mutation in the *ciaH* gene of *Streptococcus pneumoniae* induces cefotaxime resistance and transformation deficiency. *ciaH* encodes a putative sensor protein that belongs to the family of signal-transducing histidine kinases. This gene is adjacent to *ciaR*, which encodes a DNA-binding protein involved in the regulation of genes responding to environmental signals sensed by the histidine kinase. The authors have characterized a mutation that induces reversion of both cefotaxime resistance and transformation deficiency. It is a T/A deletion in the *ciaR* gene resulting in the synthesis of a truncated protein containing only 125 amino acids instead of 224. The *ciaH* mutation requires a functional CiaR protein for expression. Northern blot analysis, using *ciaR–ciaH* as a probe, revealed one mRNA from the wild-type strain, indicating that the two genes constitute an operon. Comparisons of Northern blots show that the operon is constitutively activated in the strain carrying only the *ciaH* mutation. In the wild-type strain the activation occurs when the Ca^{2+} concentration is very low, demonstrating that Ca^{2+} is the environmental signal. The pleiotropic effects caused by the *ciaH* mutation include sensitivity to antibiotics and toxins, the ability to form protoplasts and the susceptibility to lysis with deoxycholate. Null-mutants were constructed in both genes and the particular features of the *ciaR* null mutant determined. It is able to grow in choline-deprived medium, and competence development occurs in a phosphate-deprived competence medium (CH-maleate), suggesting that the CiaH–CiaR system regulates several pathways, including teiochoic acid synthesis.

Keywords: *Streptococcus pneumoniae*, PBP, phosphate, calcium, competence

INTRODUCTION

The increase of β-lactam-resistant pneumococcal clinical isolates is becoming a serious problem in antibiotic therapy. The resistance of these strains is not due to β-lactamase, but to penicillin-binding proteins (PBPs). These minor components of the bacterial membrane are often affected in their ability to bind this antibiotic, accounting for the resistance of these strains (Williamson *et al.*, 1980; Hakenbeck *et al.*, 1980). However, so far quantitative correlations between resistance levels and modification of specific PBPs have not been possible because clinical isolates are not isogenic and the wild-type strains from which they originated are not known. Moreover, sequence comparison has shown that β-lactam resistance results from horizontal transfer of several long stretches of DNA from other species (Dowson *et al.*, 1993). Thus the situation appears to be especially complex in clinical resistant strains. In order to bypass such complications, a series of independent, spontaneous mutants resistant to a high level of cefotaxime, a cephalosporin that binds to the PBPs, except PBP2B (Hakenbeck *et al.*, 1987), were isolated from *Streptococcus pneumoniae* R6, a rough laboratory strain (Laible & Hakenbeck, 1987). Genetic analysis by DNA-mediated transformation of a strain which is 64-fold more resistant than the original wild-type strain (C506) has shown that mutations of two genes, affecting respectively PBP2X and PBP3,

Abbreviation: PBP, penicillin-binding protein.

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accounted for two levels of resistance (Seroude *et al.*, 1993; Selakovich-Chenu *et al.*, 1997). A third level of resistance was obtained without any modification of a PBP. It was found that this high level of resistance resulted from a mutation in the *ciaH* gene, which encodes a histidine kinase sensor closely linked to a response regulator, CiaR; both gene products are members of the family of signal-transducing two-component systems (Guenzi *et al.*, 1994). An unexpected observation was that this highly resistant strain was no longer competent. Competence refers to a physiological state of a culture and its ability to be transformed by DNA. In the pneumococcus all bacteria become competent at the same time at the end of the exponential phase, and shortly thereafter competence disappears (Thomas, 1955). Competence requires several ions, especially Ca^{2+} (Fox & Hotchkiss, 1957; Trombe *et al.*, 1992), Mg^{2+} (Seto & Tomasz, 1976) and Zn^{2+} (Dintilhac & Claverys, 1997).

The aim of this work was to investigate the relationship between resistance to cefotaxime and loss of competence by isolating mutants affected in these genes and studying their properties under several conditions. We will show that the CiaH–CiaR system functions as an operon and that Ca^{2+} is involved in its regulation.

METHODS

Bacterial strains and plasmids. These are listed in Table 1.

Growth conditions and media. The general growth conditions of *S. pneumoniae* and the composition of CH medium were as described by Tiraby & Fox (1974). CH-maleate medium had the same composition as CH medium with the following exceptions: (1) K_2HPO_4 (0·05 M) was replaced by maleate buffer (0·05 M, pH 7), (2) the pH of the medium was adjusted to 7·8 by addition of NaOH. The composition of MS mediun was as described by Sicard (1964). MS* medium had the same composition as MS medium except for the $CaCl_2$ concentration (0·5 mM instead of 5 mM in MS). Phosphate concentration modifications of MS* were obtained by addition of potassium phosphate buffer (pH 7·6).

Transformation. Transformation of *S. pneumoniae* followed published procedures (Tiraby & Fox, 1974). Cefotaxime-resistant transformants were screened on blood agar plates containing the desired concentration of cefotaxime. Streptomycin resistance was screened with 200 μg streptomycin ml^{-1}. *Escherichia coli* was transformed by electroporation with a 'Gene-Pulser' following procedures supplied by Bio-Rad.

DNA techniques. The PCR fragments were obtained and purified as described previously (Gasc *et al.*, 1998). Sequencing was done using the Circumvent Thermal Cycle Sequencing Kit (Biolabs). The position (according to numbering of the EMBL sequence X77249) and sequence of oligonucleotide primers used for amplification and sequencing of *ciaR* and *ciaH* genes are as follows: (i) upstream primers, 5′-cgggatcc$_{219}$ATAAAAATCTTATTGGTTGAGG-3′, 5′-$_{533}$GAACTTAAAATGCGGAaTTCAGGCC-3′, 5′-ggaattcc$_{1428}$GATTGTGGTCGTGATGGC-3′; (ii) downstream primers, 5′-$_{957}$CAGGGTGAAGACACCGAAGcTTGCG-3′, 5′-$_{1665}$CAAACTCGATGCAA GCTTTCGC-3′, 5′-cccaagctt$_{2212}$ATTTTTTCTTTTTAGATGGTGTCT-3′. The small letters indicate modified bases or primer extension. The underlined letters indicate *Eco*RI or *Bam*HI restriction sites for forward primers and *Hin*dIII restriction sites for downstream primers.

Plasmid constructions and insertional inactivations of the *ciaR* and *ciaH* genes. To create the knockout mutants, the method of insertional duplication mutagenesis, a homology-directed insertion of foreign DNA, was used (Mejean *et al.*, 1981). Internal gene fragments of *ciaR* and *ciaH* were amplified using total DNA from R800 and the following pairs of oligonucleotide primers containing terminal restriction sites: 5′-cgggatcc$_{219}$ATAAAAATCTTATTGGTTGAGG-3′ and 5′-gctctaga$_{734}$CTGCGTCTTAGGCAAAATCAC-3′ for *ciaR*, 5′-gctctagag$_{900}$AACATGGTATGCGGATGACTT-3′ and 5′-cgggatcc$_{1451}$AAACTAGCCATCACGACCACAA-3′ for *ciaH*. PCR products were subsequently cloned into *Bam*HI/*Xba*I-restricted pR350. The resulting plasmids (pHG2 and pHG3 respectively) were used to transform *S. pneumoniae*. Transformants were selected using 200 μg spectinomycin ml^{-1} (Dintilhac *et al.*, 1997).

MIC determination and assay of PBPs. These were done as previously published (Selakovitch-Chenu *et al.*, 1993).

Protoplast formation. Protoplasts were produced in a buffer composed of 0·1 M Tris/HCl, 1 mM $MgCl_2$ and 3 mM β-mercaptoethanol at pH 7·6 as described by Lacks & Neuberger (1975). Samples were tested for sensitivity to osmotic shock in water before and after incubation for various periods of time.

RNA isolation and Northern blot analysis. Total RNA extractions and Northern blot analysis were as described by Mortier-Barriere *et al.* (1998). The RNA size standard was purchased from Biolabs. The *ciaR–ciaH* probe was obtained by [α-^{32}P]ATP incorporation using the Amersham Megaprime labelling system into a PCR fragment obtained with the 5′-cgggatcc-$_{219}$ATAAAAATCTTATTGGTTGAGG-3′ and 5′-cccaagctt$_{2212}$ATTTTTTCTTTTTAGATGGTGTCT-3′ primers. Photos were analysed with a phosphoimager and the band intensities were quantified with the TINA program (Moleculars Dynamics).

RESULTS

Isolation of a mutation in the *ciaR* gene

Previous experiments had shown that it was possible to obtain highly resistant bacteria by transformation of the cefotaxime-sensitive recipient strain R801 with DNA from the resistant strain C506. Forty clones were isolated at cefotaxime concentrations varying from 0·05 to 0·08 μg ml^{-1}. The transformants (801A) were all resistant to 0·15 μg ml^{-1} of this drug (Seroude *et al.*, 1993). The penicillin affinity of PBP2X was reduced in these transformants (Fig. 1). We transformed five resistant strains 801A with DNA from strain C506 and selected transformants which occurred at a frequency corresponding to the transfer of one gene. They were resistant to 0·3 μg ml^{-1} (strains 801B). The PBP electrophoretic profile shows increased labelling of PBP3 in these transformants. Indeed PBP3 was present in C506 whereas recipient sensitive strain R801 was deficient in this protein (Fig. 1) (Selakovitch-Chenu *et al.*, 1997). An 801B strain was transformed by C506 DNA. Transformants were obtained on plates containing 0·5 and

Table 1. Strains and plasmids used in this study

Bacterium or plasmid	Genotype/characteristics	Source or reference
Strains		
S. pneumoniae		
C506	R6, *pbpX penA ciaH**	Laible & Hakenbeck (1987)
R801	R6x derivative, *dacA*	Lefevre *et al* (1979)
801A	R801, *pbpX*	Seroude *et al.* (1993)
801B	801A, *dacA*$^+$	Selakovitch-Chenu *et al.* (1997)
80IC	801B, *ciaH**	This study
801Crev	801C, *ciaR*	This study
R800	R6 derivative	Lefevre *et al.* (1979)
800ciaH*	R800, *ciaH**	This study
800ciaR^{null}	R800, *ciaR*::pHG2	This study
800ciaH^{null}	R800, *ciaH*::pHG3	This study
E. coli		
DH5α	*supE44* Δ*lac* UI69(φ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	GIBCO-BRL
Plasmids*		
pR350	pSK$^+$ derivative, Ap^r Sp^r	Dintilhac *et al.* (1997)
pHG2	pR350 derivative carrying a PCR-generated 515 bp *ciaR* fragment	This study
pHG3	pR350 derivative carrying a PCR-generated 550 bp *ciaH* fragment	This study
pEG2	pJDC9 derivative carrying the *ciaH** mutation	Guenzi *et al.* (1994)

* Plasmids replicate in *E. coli* only.

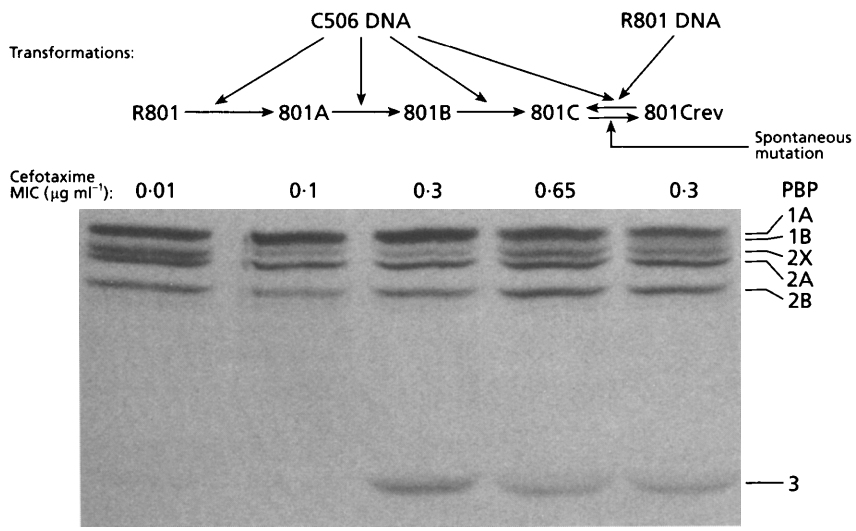

Fig. 1. PBP patterns in *S. pneumoniae* transformants resistant to various concentrations of cefotaxime. Strains 801A, 801B and 801C were derived from sensitive strain R801 by successive transformations with chromosomal DNA of cefotaxime-resistant strain C506 (MIC 1 μg ml^{-1}). Strain 801Crev is a spontaneous revertant of 801C. PBPs were detected on polyacrylamide gel by their ability to bind ^{3}H-labelled benzylpenicillin (Selakovitch-Chenu *et al.*, 1993).

0·7 μg cefotaxime ml^{-1}, at a frequency corresponding to the transfer of one gene. Eleven clones were isolated. They were resistant to 0·65 μg cefotaxime ml^{-1}, defining a third level of resistance (strains 801C). The PBP profiles were not affected in these clones. As the level of resistance of these strains was slightly lower than that of the original mutant C506 (1 μg ml^{-1}), we suspected that another mutation was present in C506 and not in 801C. We therefore tried to transform 801C with C506 DNA and subsequently plated on a concentration of cefotaxime that kills strain 801C. We failed to isolate strains more resistant to cefotaxime than 801C. As a control,

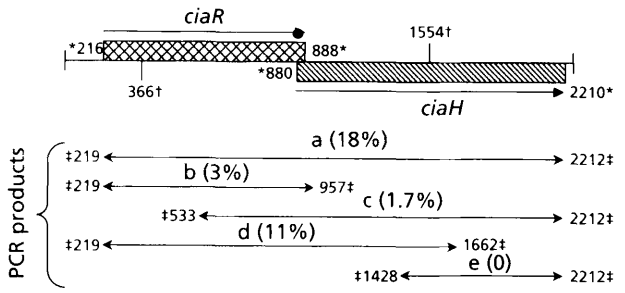

Fig. 2. Localization of the 801Crev mutation in the *ciaR–ciaH* operon. Top, *ciaR–ciaH* operon. * Initiation and stop codon positions of *ciaR* and *ciaH* genes; † phosphorylation sites of CiaR and CiaH proteins. Bottom, PCR products used to transform strain 801Crev. Transformants were selected for their resistance to cefotaxime (0·6 μg ml^{-1}); their frequencies are indicated in parentheses. ‡ Extremities of PCR products.

we transformed 801C with DNA from a cefotaxime-sensitive strain, containing the marker *str41* that confers resistance to 0·2 mg streptomycin ml^{-1}. Very few transformants resistant to streptomycin were obtained. However, these transformants had reverted to a lower level of cefotaxime resistance (0·3 μg ml^{-1}). These revertants were fully competent, whereas bacteria resistant to 0·65 μg ml^{-1} were non-transformable. The original strain C506 was also non-transformable, as already reported (Laible & Hakenbeck, 1991). These results show that there is a mutation in a gene of strain C506 that, in addition to the other two, increases resistance to 0·65 μg cefotaxime ml^{-1} and blocks competence in 801C. Guenzi *et al.* (1994) have shown that this mutation is localized in the *ciaH* gene, resulting from a Thr_{230} to Pro substitution (it will be referred to later as *ciaH** in this paper). The defect of transformation may occur at the DNA-uptake level, since plasmids could not transform the highly resistant bacteria, whereas electrotransformants were obtained at a normal frequency (Seroude *et al.*, 1993). Moreover, these non-transformable strains were unable to be induced to competence by the competence factor (Seroude *et al.*, 1993) and did not produce this competence factor themselves (Guenzi *et al.*, 1994). One such revertant (801Crev) was cultured for further study.

When the spontaneous revertant 801Crev was transformed by wild-type DNA or C506 DNA, transformants resistant to a concentration of 0·65 μg cefotaxime ml^{-1} were isolated at a frequency corresponding to the transfer of one gene. Strain 801Crev therefore contains a mutation, absent in wild-type or in C506, that reduces cefotaxime resistance and restores competence. The PBP profile of this revertant was identical to that of strain 801C (Fig. 1). A candidate region for harbouring this mutation is the *ciaR–ciaH* locus that affects cefotaxime resistance without PBP modification.

To localize the mutation that yields the revertant phenotype, several parts of the *ciaR–ciaH* locus were amplified by PCR using wild-type DNA. The revertant strain (801Crev) was transformed with these fragments, and colonies were selected at 0·6 μg cefotaxime ml^{-1}. Transformants were obtained when the fragment tested contained the region between nucleotides 533 and 957 (Fig. 2), encoding the C-terminal part of CiaR and the first 25 amino acids of CiaH. We have sequenced this region from the 801Crev strain and found one T deleted in the sequence TTTA (578 to 581), resulting in a stop codon 15 nucleotides downstream. The mutated CiaR protein, therefore, must be truncated in its C-terminal region. Since this region is required for fixation on DNA (Stock *et al.*, 1990), it is likely that the mutated protein has lost its regulatory properties. However, it cannot be excluded that this frameshift mutation in *ciaR* may block the expression of *ciaH* by a polar effect, since inactivation of *ciaH* by itself does not inhibit competence (Guenzi *et al.*, 1994).

Construction of isogenic strains carrying a mutation in the *ciaH* and *ciaR* genes

The discovery of mutations in the two-component signal-transducing system affecting cefotaxime resistance and natural competence resulted from isolation of highly resistant mutants from strains already mutated in several PBP genes. To investigate the effect of isolated mutations on the *ciaH* and *ciaR* genes, we tried to transfer these mutations to a wild-type strain. We chose as the recipient strain R800, an R6 clone isogenic to R801, because R800's spontaneous mutation rate is much lower than that of R801 (Tiraby & Sicard, 1973; Tiraby & Fox, 1973), due to efficient repair of mismatched bases in R800, and deficient repair in R801 (Hex$^-$). This precaution was undertaken to protect against unwanted mutations.

A competent culture of strain R800 was transformed with plasmid pEG2 DNA. This plasmid carries the *ciaH** mutation that confers cefotaxime resistance (Guenzi *et al.*, 1994). Selection was carried out at a concentration of 0·04 μg cefotaxime ml^{-1}. Transformants were recovered at a frequency of 3% as expected for the transfer of one gene; spontaneous mutants were very rare. One of these transformants was subcultured and the presence of the original mutation was ascertained by sequencing a PCR product of the *ciaH* region. This strain was named 800ciaH*. It is noteworthy that the increased resistance to cefotaxime resulting from the *ciaH** mutation is expressed in a wild-type strain (which is resistant to 0·02 μg cefotaxime ml^{-1}) as well as a strain carrying mutations in PBP genes.

To inactivate *ciaH*, we inserted an internal fragment of this gene into the plasmid pR350 (Dintilhac *et al.*, 1997). This plasmid is unable to replicate in the pneumococcus and carries a marker of resistance to 200 μg spectinomycin ml^{-1}. To confer resistance to this antibiotic, the plasmid has to be integrated into the chromosome by homologous recombination in the *ciaH* sequence. We transformed strain R800 with the DNA of this plasmid.

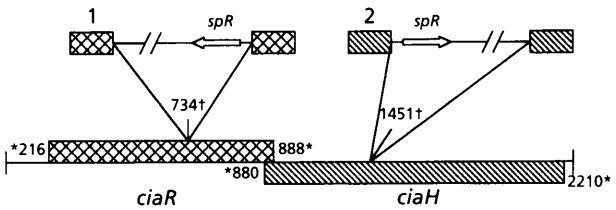

Fig. 3. Structure of 800ciaR^{null} (1) and 800ciaH^{null} (2) mutants. Mutants were obtained by insertional-duplication mutagenesis as described in Methods. * Initiation and stop codon positions of *ciaR* and *ciaH*; † positions where pHG2 (1) or pHG3 (2) plasmids were inserted. *spR*, spectinomycin-resistance gene.

Spectinomycin-resistant transformants were selected. One of them was further tested for the insertion of the plasmid genome into the *ciaH* gene. We subsequently isolated a strain carrying an insertion in the *ciaR* gene by the same procedure (Fig. 3). The isolation of such strains carrying an interrupted *ciaH* (800ciaH^{null}) or *ciaR* (800ciaR^{null}) gene shows that these are not essential genes, confirming earlier reports (Guenzi *et al.*, 1994).

Effect of *ciaH* and *ciaR* mutations on competence

We have seen previously that the *ciaH** mutation abolishes competence. Using the different mutants that we constructed, we investigated the effect of *ciaR* and *ciaH* on competence. It was suggested by Guenzi *et al.* (1994) that a homology exists between proteins CiaR and CiaH and proteins PhoB and PhoR. The PhoB/PhoR system is activated by phosphate deprivation in *E. coli* (Makino *et al.*, 1985). A possible role of phosphate on competence was also suggested by Tomasz & Hotchkiss (1964), who reported that when the phosphate buffer used in competence medium is replaced by a maleate buffer, competence is abolished. We tested the competence of the strains mutated in the *ciaR–ciaH* locus in complete competence medium (CH) and in this medium deprived of phosphate (CH-maleate). Wild-type strains (R801 and R800) as well as strains mutated in PBP genes (801B) are competent in CH medium but not in CH-maleate medium, a control confirming the observation of Tomasz & Hotchkiss (1964) (Table 2). However, inactivation of the *ciaR* gene (strains 800ciaR^{null} and 801Crev) restores competence in the absence of phosphate.

This result suggests that the CiaR/CiaH system is involved in the phosphate effect on competence. As the inactivation of either *ciaR* or *ciaH* does not affect competence in phosphate medium, the regulation of competence should be negatively controlled by this system. The *ciaH** mutation should result in a constitutive production of the CiaR and CiaH proteins. It is noteworthy that strain 800ciaH^{null} is slightly competent in CH-maleate medium. This suggests that the CiaR protein, despite being non-phosphorylated by the CiaH protein in this mutant, is able to partially inhibit competence. To test the role of phosphate in the regulation of the CiaR/CiaH system, we studied the transcription of these genes.

Table 2. Competence in CH and in CH-maleate media

Competence was estimated by determining the frequency of streptomycin-resistant transformants (see Methods). +, Competent (frequency of streptomycin-resistant transformants $\geqslant 1\%$.; −, non-competent (frequency of streptomycin-resistant transformants $\leqslant 3 \times 10^{-5}\%$).

Strain	Competence on:	
	CH	CH-maleate
R801	+	−
801B	+	−
801C	−	−
801Crev	+	+
R800	+	−
800ciaH*	−	−
800ciaR^{null}	+	+
800ciaH^{null}	+	−*

* Frequency of streptomycin-resistant transformants $3 \times 10^{-3}\%$.

Transcription of *ciaR* and *ciaH*

To study the control of several two-component signal sensing systems implicating phosphorylation in their autoregulation, we used a defined growth medium in which phosphate concentration can be modified. We reduced the Ca^{2+} concentration in the synthetic growth medium of pneumococcus (Sicard, 1964) 10-fold to avoid precipitation of calcium phosphate at high phosphate concentration (medium MS*). Total RNAs were extracted and Northern analysis was performed (see Methods). A single 2·2 kb fragment was observed (Fig. 4). This shows that the two genes are transcribed as a unique RNA, i.e. *ciaR–ciaH* is an operon. The size of the mRNA is as expected from the DNA sequences of these regions. In the wild-type the transcription increases 10-fold when the phosphate concentration is increased from 1 to 50 mM. However, transcription of the operon in the 800ciaH* strain is high whatever the phosphate concentration. The *ciaH** mutation, therefore, results in a constitutively active operon, confirming what was previously proposed (Guenzi *et al.*, 1994). The activation of the operon by an increase in the amount of phosphate in a wild-type culture shows that the CiaR/CiaH system is not analogous to the PhoB/PhoR system. In other bacteria this latter system is positively autoregulated when phosphate concentration decreases, and we observed the opposite in *S. pneumoniae*.

The phosphate concentration in the growth medium could act directly or indirectly on the CiaR/CiaH system. Calcium or magnesium phosphate precipitated in the medium might reduce the amount of available

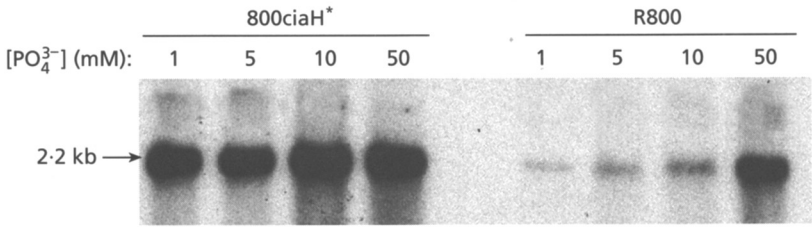

Fig. 4. Northern blot analysis of total RNA extracted from R800 and 800ciaH*. Total RNA was extracted from cells grown in MS* containing different concentrations of phosphate, as indicated at the top of the figure.

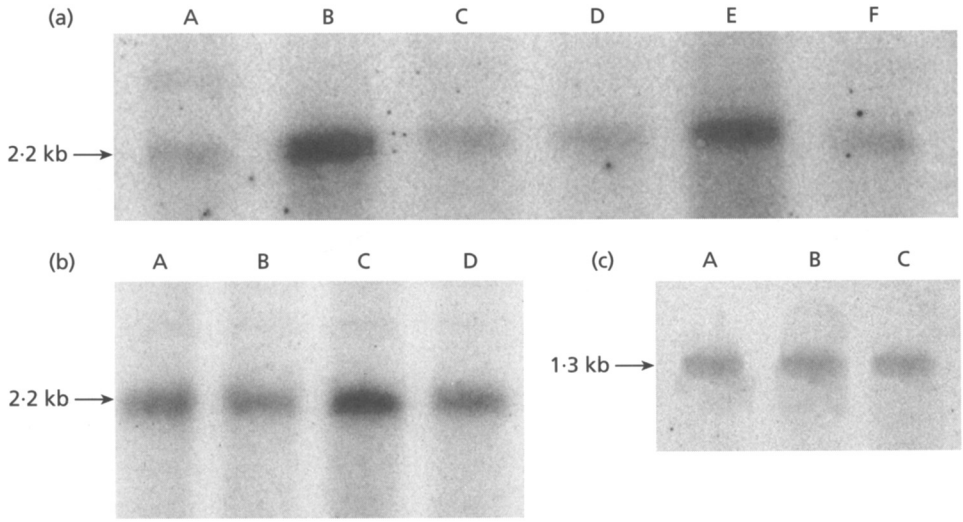

Fig. 5. Northern blot analysis of the transcription of the *ciaR–ciaH* operon. Northern blot analysis was performed as described in Methods. (a, b) Total RNAs were extracted from strain R800 grown as follows. (a) Cells were grown in MS* containing 1 mM PO_4^{3} to OD_{550} 0·3 and incubated for 10 min in different conditions: A, control; B, +0·5 mM EGTA; C, +5 mM $CaCl_2$; D, +5 mM $MgCl_2$; E, +0·5 mM EGTA and 5 mM $CaCl_2$; F, +0·5 mM EDTA. (b) Cells were grown in MS* containing 10 mM phosphate to OD_{550} 0·3 and incubated for 10 min in different conditions: A, control; B, +5 mM $CaCl_2$; C, +0·5 mM EGTA; D, +5 mM $MgCl_2$. (c) Total RNAs were extracted from strain 800ciaH^{null} grown in MS* containing 1 mM phosphate to OD_{550} 0·3 and incubated for 10 min in different conditions: A, control; B, +0·5 mM EGTA; C, +10 mM phosphate.

cations to the bacteria. We cultivated the wild-type strain R800 in MS* medium containing 1 mM phosphate to a cell density of OD_{550} 0·3. We subsequently added to the culture several combination of EGTA, EDTA, Ca^{2+} or Mg^{2+} for 10 min before extracting the RNAs and we measured the transcription of the *ciaR–ciaH* operon as described above. EGTA alone at a concentration of 0·5 mM increased the transcription threefold (Fig. 5a). Ca^{2+} addition reduced the effect of EGTA twofold. The other combinations did not significantly affect the mRNA production. Since EGTA is a chelator of Ca^{2+}, these results suggest that Ca^{2+} inhibits transcription of the *ciaR–ciaH* operon. The lack of effect of EDTA, which is also able to chelate Ca^{2+} but less efficiently than EGTA, could be explained by the low concentration of this chelator. If EGTA induces the operon by chelating Ca^{2+}, one would expect that addition of Ca^{2+} alone should inhibit this transcription. However, this is not observed (see Fig. 5a), probably because at the low phosphate concentration used (1 mM), this transcription is already fully repressed by the free Ca^{2+} in the medium. We also grew the cells in 10 mM phosphate and added, as before, Ca^{2+}, EGTA or Mg^{2+}, and noted (Fig. 5b) that Ca^{2+} addition reduced the transcription twofold.

We submitted a 800ciaH^{null} culture to a similar treatment to determine the role played by the *ciaH* gene product. Phosphate or EGTA did not affect the transcription as expected from the proposed model (see Fig. 5c). The size of the RNA fragment was 1·3 kb, confirming that the insert plasmid had interrupted the transcription in the *ciaH* gene.

These results suggest that the *ciaR–ciaH* operon is repressed by Ca^{2+}. Addition of either phosphate or EGTA would reduce the available Ca^{2+}, indirectly derepressing this system. Such regulation would depend on the integrity of the CiaH protein acting as a Ca^{2+} sensor. This protein would phosphorylate CiaR when Ca^{2+} concentration is reduced. This hypothesis led us to re-evaluate the effect of Ca^{2+} on competence and cefotaxime resistance.

Table 3. Effects of chelators and divalent cations on transformation frequency

Transformations were performed with saturating DNA concentrations. Results are expressed as the percentage of streptomycin-resistant transformants. In the control, transformants were scored in transformation medium without supplement.

Strain	Control	EGTA (0·5 mM)	EGTA (0·5 mM) + [Ca^{2+}] mM:			EGTA (0·5 mM) + [Mg^{2+}] mM:			EDTA (10 mM)	EDTA (10 mM) + Ca^{2+} (20 mM)
			0·05	0·5	5	0·05	0·5	5		
R800	1·3	0·08	0·05	0·4	0·64	0·05	0·06	0·05	0	0·05
800ciaR^{null}	1·6	0·35	0·5	2·0	1·2	0·23	0·22	0·21	0	0·2
800ciaH^{null}	1·5	0·3	0·15	1·6	1·1	0·25	0·17	0·25	0	0·23

Effect of Ca^{2+} on competence

A major role of Ca^{2+} in *S. pneumoniae* transformation is at the entry step of DNA (Fox & Hotchkiss, 1957; Seto & Tomasz, 1976). Competent cultures of several strains were treated for 10 min with chelating agents at concentrations that reduced transformation frequencies; they were then transformed with DNA carrying a streptomycin-resistance marker. In these conditions the transformation frequency of the wild-type strain was reduced 16-fold in the presence of 0·5 mM EGTA, whereas the transformation frequency of 800ciaH^{null} or 800ciaR^{null} was only reduced by a factor of 4. The simultaneous addition of 0·5 mM $CaCl_2$ abolished the inhibitory effect of this chelating agent on these two mutants whereas 5 mM $CaCl_2$ was not enough to produce the same reversal of the wild-type strain. Mg^{2+} addition was ineffective. We also treated competent cultures with 10 mM EDTA; at this concentration transformation was abolished. The addition of 20 mM $CaCl_2$ partially relieved the inhibitory effect of EDTA, and the transformation frequency of the mutants was fourfold higher than that of the wild-type (Table 3).

These results suggest that DNA uptake is controlled by Ca^{2+} through the sensing properties of the CiaR/CiaH system. Inactivation of the *ciaR–ciaH* operon reduces the requirement for Ca^{2+} without abolishing it. When the Ca^{2+} concentration is too low, the system is activated, CiaH is autophosphorylated, and phosphorylated CiaR blocks DNA entry. This correlates well with the observation that strains carrying the *ciaH** mutation are derepressed for this system and not naturally transformable, but can be electrotransformed (Seroude *et al.*, 1993).

Effect of Ca^{2+} on cefotaxime resistance

The CiaR/CiaH system was discovered by isolating bacteria resistant to cefotaxime. The genetic and molecular analysis of these strains eventually showed that cefotaxime resistance results from a missense mutation in the *ciaH* sensor gene (Guenzi *et al.*, 1994). We have seen that the sensor is most likely repressed by Ca^{2+} and becomes constitutively activated as a result of the missense *ciaH** mutation. Therefore Ca^{2+} might affect resistance to cefotaxime.

We cultivated several strains in MS medium without added Ca^{2+}, supplemented with 50 mM phosphate, or in the same defined medium containing 1 mM phosphate and 5 mM Ca^{2+}. The growth of all strains was similar in both media. When cefotaxime was added after 1 h, at a concentration of 0·04 μg ml^{-1}, the growth response was quite different with respect to the strains. The wild-type strain R800 grew in the medium at low Ca^{2+} and high phosphate concentrations in the presence of the antibiotic, but not in the medium at high Ca^{2+} concentration. Strain 800ciaH* grew in both media, and strains inactivated in *ciaH* or *ciaR* could not grow in either medium. Resistance to cefotaxime in wild-type bacteria, therefore, is increased when Ca^{2+} is at a limiting concentration. This resistance is suppressed when *ciaR* or *ciaH* are inactivated by an insertion. Constitutive activation of the operon by the *ciaH** mutation results in resistance independently of the addition of Ca^{2+} as an effector. Therefore it seemed judicious to search for proteins homologous to the CiaH protein, focusing the investigation on Ca^{2+} sensors.

Search for homologies to CiaH protein

Several proteins share homologies with CiaH. They belong to the histidine kinase two-component system family (Stock *et al.*, 1990), but their functions remain generally unknown. Homology is located in the cytoplasmic portion of the CiaH protein. In the extracytoplasmic region several fragments are conserved between CiaH and PhoQ from *Salmonella typhimurium* and *E. coli* (Fig. 6). These proteins are able to detect changes in Ca^{2+} concentration in the medium and are also protein kinases of the two-component system PhoP/PhoQ (Garcia-Vescovi *et al.*, 1997). The *ciaR* gene also shares homology with several regulatory genes belonging to the family of two-component systems, which supports the proposition that CiaR/CiaH system is a member of this family and therefore likely to be able to sense Ca^{2+} in the medium.

These results confirm the model in which the CiaH sensor is activated by Ca^{2+} starvation, resulting in autophosphorylation and phosphorylation of the CiaR protein, which in turn increases resistance to cefotaxime and blocks DNA uptake. Such a pleiotropic effect led us

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StPHOQ :  M-NKFARHFLPLSLRVRFLLATAGVVLVLSLAYGIVALVGYSVSFDKTTFRLLRGESNLF  : 59
EcPHOQ :  M-KKLLRLFFPLSLRVRFLLATAAVVLVLSLAYGMVALIGYSVSFDKTTFRLLRGESNLF  : 59
CIAH   :  MFSK-LKKTWYADDFSYFIRN-----------FGVFTLI-----FSTMTLIILQVMHSSL  : 43
          * .*  .     .    *.             .*...*.     *.. *. .*.   . .

StPHOQ :  YTLAKWENNKISVELPENLD-----------------MQRNIPWLIKSIQPEWLKTNGF  :101
EcPHOQ :  YTLAKWENNKLHVELPENIDKQSPTMTLIYDENGQLLWAQRDVPWLMKMIQPDWLKSNGF  :119
CIAH   :  YT---SVDDKLH-GLSENPQ---AVIQLAINRATEEIKDLENARADASKVE---IKPNVS  : 94
          **     ..*.   *.** .     . *  .  .  .   .  .  .    .*.*

StPHOQ :  HEIETNV-DATST-LLSEDHSAQEKLKEVREDDDDAEMTHSVAVNIYPATARMPQLTIVV  :159
EcPHOQ :  HEIEADVND-TSL-LLSGDHSIQQQLQEVREDDDDAEMTHSVAVNVYPATSRMPKLTIVV  :177
CIAH   :  SNTEVILFDKDFTQLLSGNRFLGLDKIKLEKK----ELGHIYQIQVFNSYGQEEIYRVIL  :149
           . *. . *     *** ..    .  .....    *. *   .... . ..     ...

StPHOQ :  VDT----IPIELKRSYMVWSWFVYVLAANLLLVIPLLWIAAWW--SL               :200
EcPHOQ :  VDT----IPVELKSSYMVWSWFIYVLSANLLLVIPLLWVAAWW--SL               :218
CIAH   :  METNISSVSTNIKYAAVLINTSQLEQASQKHEQLIVVVMASFWILSL               :197
          ..*    .. ..* . .. .      ...    . .. .*..*  **
```

Fig. 6. Sequence comparisons of the N-terminal parts of the CiaH and PhoQ proteins. The alignment was done using CLUSTAL. Asterisks indicate identical amino acids; dots indicate similar amino acids. The threonine residue involved in Ca^{2+} binding to PhoQ proteins is arrowed. StPHOQ, PhoQ protein of *Salmonella typhimurium*; EcPHOQ, PhoQ protein of *E. coli*; CIAH, CiaH protein of *Streptococcus pneumoniae*.

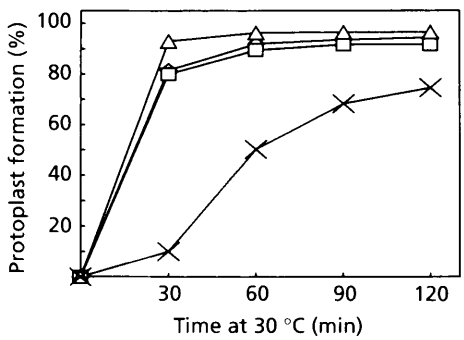

Fig. 7. Protoplast formation. The degree of protoplast formation was estimated by the sensitivity of samples to osmotic shock in water. □, 800ciaR^{null}; △, 800ciaH^{null}; ×, 800ciaH*; ◇, R800.

to suspect that other properties might be controlled by this system.

Pleiotropic effects of mutations

Growth of all strains in several complete media was very similar. We decided to compare the behaviour of the wild-type and mutated strains in their ability to form protoplasts for the following reasons: (1) antibiotic resistance and DNA uptake might involve alterations in cell-wall properties; and (2) competence activates protoplast formation (Seto & Tomasz, 1975). Moreover, non-competent clones form protoplasts with more difficulty (Lacks & Neuberger, 1975).

The strain 800ciaH* formed protoplasts less easily than the wild-type strain (Fig. 7). The 800ciaR^{null} and 800ciaH^{null} strains behaved as the wild-type. The relationship between the ability to become competent and the ability to form protoplasts is strenghtened by these observations. As it was previously suggested that the gene *lytA*, encoding a major autolysin (Garcia *et al.*, 1985), is involved in forming protoplasts (Lacks & Neuberger, 1975), we wondered whether *lytA* could be inhibited by the *ciaH** mutation. A specific phenotype of *lytA* strains is their inability to lyse during the stationary phase or because of a penicillin treatment (Tomasz *et al.*, 1970, 1988; Sanchez-Puelles *et al.*, 1986). We have observed that all strains lyse spontaneously at the end of stationary phase or after penicillin treatment (data not shown). Thus the *ciaH** mutant does not behave as a *lytA* strain. This does not argue for a repression of the *lytA* gene by the CiaR/CiaH system.

We also tested the sensitivity to lysis induced by deoxycholate. All strains lysed, although 800ciaH* was more resistant. At a concentration of 0·01% deoxycholate, 80% of a 800ciaH* culture had not lysed, whereas for the other strains only 30% of the culture had not lysed. The resistance of strain 800ciaH* to deoxycholate and to a treatment inducing protoplast formation is interesting and might suggest that derepression of the *ciaR–ciaH* operon somehow affects cell wall biosynthesis. As choline is a major component of the cell wall and required for growth by *S. pneumoniae* (Badger, 1944; Tomasz, 1967), we studied the choline requirement of these mutants in MS medium without choline. Wild-type, 800ciaH^{null} and 800ciaH* strains could not grow in this medium, whereas 800ciaR^{null} grew normally without choline, suggesting regulation of cell wall synthesis by CiaR (data not shown).

Effect of the *ciaH** mutation on susceptibility to antibiotics and toxins

We compared the resistance of the wild-type strain R800 with the resistance of the isogenic strains 800ciaR^{null} and 800ciaH^{null} to antibiotics and toxic compounds belonging to several families (arsenate, arsenite, antimonite, erythromycin, norfoflaxin, novobiocin, penicillin and vancomycin). No differences were observed for arsenite, antimonite, erythromycin and vancomycin. On the other hand the penicillin resistance of 800ciaH* was increased twofold. This is not surprising, since cefotaxime and penicillin are both β-lactams, often affecting the same target, which explains the frequent

cross-resistance. The mutant was 1·5–2-fold more resistant to arsenate and novobiocin than the wild-type, whereas it was 2-fold more sensitive to norfoflaxime.

DISCUSSION

We have investigated the function of the *ciaR–ciaH* locus. As already demonstrated by Guenzi *et al.* (1994), *ciaH* encodes a putative sensor protein that belongs to the family of signal-transducing histidine kinases. The adjacent gene, *ciaR*, encodes a protein related to the family of DNA-binding proteins involved in regulation of genes in response to environmental signals sensed by the histidine kinase.

Northern hybridizations using a *ciaR–ciaH* probe were performed on total RNA extracts from the pneumococcal strains R800 or 800ciaH*. A unique 2·2 kb band was detected, confirming the hypothesis that the two genes constitute an operon. The size of this band was as expected from the DNA sequences of these two genes. Northern hybridizations on cultures containing different concentrations of phosphate indicated that phosphate affects the expression of this operon. In strain R800 optimal expression requires 50 mM phosphate. In strain 800ciaH*, the transcripts were detected at high level, independent of the phosphate concentration. Therefore, the *ciaR–ciaH* operon is constitutively activated in this strain. These results are consistent with the observations of Guenzi *et al.* (1994), which indicated that the *ciaH** mutation does not abolish the function of the *ciaH* gene.

We isolated a mutation in *ciaR* that restores the competence lost in strain 801C. This mutation is a T deletion in position 578–581, that induces a frameshift and a stop codon 15 bases downstream. Thus the CiaR protein in this mutant is truncated and contains only 125 residues instead of 224. The protein has lost its C terminal part, where the DNA-binding domain is localized. We can conclude that the expression of the *ciaH** mutation requires a functional CiaR protein, which is what is expected for a constitutive activation of the system.

To study the function of the *ciaR* and *ciaH* genes, we constructed null mutants in a wild-type strain. The behaviour of these mutants is quite different: the *ciaR* null mutant is fully competent in phosphate-deprived medium and is able to grow in choline-free medium, whereas the *ciaH* null mutant is not competent in phosphate-deprived medium and requires choline for growth. These differences suggest that the CiaR protein is able to regulate some genes without phosphorylation by CiaH sensor protein. The difference of behaviour between *ciaR* null mutants and *ciaH* null mutants for competence in CH-maleate medium argues against the hypothesis that 801Crev phenotype is due to a polar effect of *ciaR* mutation on *ciaH*.

A major contribution of this work is the identification of the nature of the environmental signal. We have found that, at a low concentration of Ca^{2+}, CiaH is autophospholylated, and thus phosphorylates CiaR, which in turn represses genes required for DNA uptake (Fig. 8). Simultaneously, genes conferring resistance to cefotaxime are activated. The addition of phosphate will reduce the available concentration of Ca^{2+} in the medium by its reaction with this ion. Therefore the activation of the operon by the addition of phosphate would be mediated by Ca^{2+} deprivation. This proposal is also supported by the presence of amino acids conserved between the extracytoplasmic parts of PhoQ and CiaH proteins. These results are consistent with the reports by Fox & Hotchkiss (1957), Seto & Tomasz (1976) and Trombe *et al.* (1992) showing that Ca^{2+} is required for transformation in pneumococcus. In this work we have described a regulatory system mediated by this ion. The control of competence via the CiaR/CiaH system might be modulated by other genes, since when the *ciaR–ciaH* operon is inactivated, a low concentration of Ca^{2+} is required to transform *S. pneumoniae*.

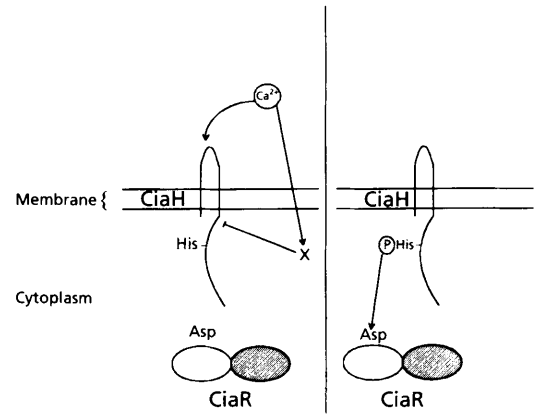

Fig. 8. Ca^{2+} sensing by the CiaR/CiaH system. (a) In the presence of Ca^{2+}, CiaH is unable to phosphorylate itself and to phosphorylate the CiaR protein. This inhibition results from a direct binding of Ca^{2+} to CiaH or indirectly by another mechanism not yet determined. (b) In Ca^{2+}-deprived medium, CiaH autophosphorylates and phosphorylates the CiaR regulatory protein. This protein can regulate genes involved in competence and antibiotic resistance. His, phosphorylation site of CiaH; Asp, phosphorylation site of CiaR. In the representation of the CiaR protein, the shaded part denotes the C-terminal region containing the DNA-binding domain; the white part denotes the N-terminal region containing the phosphorylation site (Asp).

The pleiotropic effect of the *ciaH* missense mutation that increases resistance to cefotaxime and blocks competence has been extended to other properties such as resistance to protoplast formation, to lysis induced by deoxycholate and to several antibiotics. Although it is difficult to account for the diversity of effects of the *ciaH** mutation, one possibility is a modification of the cell wall resulting from the derepression of this system.

An interesting phenotype of strain 800ciaR^{null} is the ability to grow without choline whereas the wild-type *S. pneumoniae* has an absolute requirement for this

vitamin (Badger, 1944). The role of choline in the pneumococcal cell wall is poorly understood. This vitamin, incorporated in teichoic and lipoteichoic acids (Tomasz, 1967; Mosser & Tomasz, 1970), is able to bind several proteins such as the pneumococcal surface protein (PspA) (Yother & White, 1994) and activates the major amidase that autolyses pneumococcal cells in the stationary phase (Briese & Hakenbeck, 1985; Sansz *et al.*, 1988). It has been observed that when a wild-type culture is deprived of choline, the synthesis of peptidoglycan is inhibited. Mutations that eliminate the nutritional requirement for choline may affect the regulation of the teichoic acid transferase so that the transfer of choline-free teichoic acid to the peptidoglycan polymer may permit cell viability (Yother *et al.*, 1998). The *ciaR–ciaH* operon may participate in this regulation of the cell wall biosynthesis. As a consequence, pleiotropic properties in *ciaR*^{null} mutant, such as choline independence for growth and competence extended to CH-maleate medium, could be related to the cell wall. Thus, there are at least three regulations mediated via the *cia* operon: competence induction, resistance to cefotaxime, and cell wall biosynthesis.

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