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The CiaRH System of *Streptococcus pneumoniae* Prevents Lysis during Stress Induced by Treatment with Cell Wall Inhibitors and by Mutations in *pbp2x* Involved in β-Lactam Resistance

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The two-component signal-transducing system CiaRH of *Streptococcus pneumoniae* plays an important role during the development of beta-lactam resistance in laboratory mutants. We show here that a functional CiaRH system is required for survival under many different lysis-inducing conditions. Mutants with an activated CiaRH system were highly resistant to lysis induced by a wide variety of early and late cell wall inhibitors, such as cycloserine, bacitracin, and vancomycin, and were also less susceptible to these drugs. In contrast, loss-of-function CiaRH mutants were hypersusceptible to these drugs and were apparently unable to maintain a stationary growth phase in normal growth medium and under choline deprivation as well. Moreover, disruption of CiaR in penicillin-resistant mutants with an altered *pbp2x* gene encoding low-affinity PBP2x resulted in severe growth defects and rapid lysis. This phenotype was observed with *pbp2x* genes containing point mutations selected in the laboratory and with highly altered mosaic *pbp2x* genes from penicillin-resistant clinical isolates as well. This documents for the first time that PBP2x mutations required for development of beta-lactam resistance are functionally not neutral and are tolerated only in the presence of the CiaRH system. This might explain why *cia* mutations have not been observed in penicillin-resistant clinical isolates. The results document that the CiaRH system is required for maintenance of the stationary growth phase and for prevention of autolysis triggered under many different conditions, suggesting a major role for this system in ensuring cell wall integrity.

Streptococcus pneumoniae contains 13 two-component signal transduction systems (30, 48). The CiaRH system has been identified as the first two-component signal transduction system for this organism in spontaneous beta-lactam-resistant laboratory mutants that were isolated in order to investigate the development of beta-lactam resistance (14). Independent mutant families were selected from the laboratory strain *S. pneumoniae* R6, with each family consisting of a series of spontaneous mutants with stepwise-increasing resistance to the two beta-lactam antibiotics cefotaxime and piperacillin, respectively (28). As expected, the resistant mutants contained mutations in penicillin binding proteins (PBPs), the targets of beta-lactam antibiotics. However, mutations in non-PBP genes were also identified that conferred beta-lactam resistance (18). In cefotaxime-resistant mutants, mutations were characterized in the *ciaH* gene encoding a histidine protein kinase (14, 55). This showed for the first time that beta-lactam resistance in *S. pneumoniae* can be the result of non-PBP mutations.

The *ciaH* gene is part of an operon with the preceding *ciaR* gene encoding the cognate response regulator CiaR. CiaH belongs to the EnvZ subgroup of histidine kinases with an N-terminal extracytoplasmic sensor domain flanked by two short putative transmembrane segments and a C-terminal cytoplasmic kinase domain. In every cefotaxime-resistant mutant family, mutations in the histidine protein kinase CiaH conferring a two- to threefold increase in resistance occurred after from one to four selection steps (55). One mutation, T230→P, that occurred during the third selection step in the mutant C306 was located close to the conserved histidine residue His226 and was shown recently to activate the CiaRH system (14, 36). Another *ciaH* mutation, A203→V, located at the end of the second transmembrane region connecting the external receiver domain with the internal kinase domain, was found in first-step mutants (55). The finding that these two *ciaH* mutations mediated decreased cefotaxime sensitivity by itself without additional mutations in *pbp* genes documented a completely PBP-independent contribution to resistance. CiaR target genes were identified using a solid-phase DNA binding assay in combination with a genomewide transcript analysis (36). Comparison of the transcription profile of a *ciaR*^{null} mutant (OFF mutant) with the *ciaH* ON mutant R6*ciaH*^{C306} containing the CiaH mutation T230→P (ON mutation) revealed the scope of the *cia* regulon, and it was suggested that the CiaRH system is activated during cell wall damage induced by treatment with beta-lactam antibiotics (36).

Another phenotype revealed in the *cia* ON mutant R6*ciaH*^{C306} was a complete transformation deficiency (14). A point mutation in CiaR reverted the competence-negative phenotype of the *ciaH* ON mutation completely (10), and consequently, loss-of-function *ciaR* mutants have been shown to be competent (8, 15, 35), suggesting that the CiaRH system controls directly or indirectly genetic competence.

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TABLE 1. Properties of the mutant lineage C003 and derivatives

Strain	Mutation in CiaR/H	Mutation in PBP2x	MIC of CTX[a] (μg/ml)	Competence	Generation time (min)	Growth (N[b])	Stat. phase (h)[g]
R6			0.02	+	43	130	5
R6*ciaR*^{null}[c]	*ciaR*^{null}		0.02	+	57	110	4
C103	CiaH V203		0.04	(+)[d]	45	130	5
R6*ciaH*^{C103}	CiaH V203		0.04	(+)[d]	ND[e]	ND	ND
C103*ciaR*^{null}	*ciaR*^{null}		0.03	+	66	110	3.5
C203	CiaH V203	D597	0.2	–[f]	39	135	7
C203*ciaR*^{null}	*ciaR*^{null}	D597	0.1	(+)[d]	65	105	2
C303	CiaH V203	D597/A550	0.8	–	39	125	7
R6*pbp2x*^{C303}		D597/A550	0.2	+	40	130	5
R6*ciaH*^{C103} *pbp2x*^{C303}	CiaH V203	D597/A550	0.8	–	ND	ND	ND
C303*ciaR*^{null}	*ciaR*^{null}	D597/A550	0.2	(+)[d]	92	70	0

[a] CTX, cefotaxime.
[b] N, Nephelo units reached in stationary phase.
[c] Similar results were obtained with constructs using the spectinomycin resistance cassette for gene disruption.
[d] Transformation efficiency of 10^{-1} to 10^{-2} compared to that of the R6 strain.
[e] ND, not determined.
[f] Transformation efficiency of $<10^{-5}$ compared to that of the R6 strain.
[g] Length of stationary phase.

Regulation of genetic competence in *S. pneumoniae*, a quorum sensing mechanism, involves another two-component system, ComDE, and a peptide pheromone, CSP, the secreted derivative of the *comC* gene (for a review, see reference 4). CSP is targeted by the sensor kinase ComD, a process that results in the activation of the response regulator ComE, which in turn induces the cascades of early and late competence genes required for genetic transformation (41).

The complicated interconnection between the two regulatory systems ComDE and CiaRH has become evident from several studies since. Microarray-based transcription analyses showed that the entire competence regulon including the *comCDE* operon was turned off in the R6*ciaH*^{C306} strain, whereas loss-of-function CiaR mutants were perfectly competent under growth conditions that prevented competence development in the wild type (36, 44). On the other hand, the CiaR regulon was shown to be induced during competence within the group of "delayed" competence genes, i.e., it is itself regulated indirectly by the ComDE system (41), and evidence was presented that the CiaRH system might be required for the cells to exit normally from the competent state (6).

Meanwhile, a variety of other phenotypes associated with mutations in *ciaH* and *ciaR* have been described. The CiaRH system is an important virulence factor in systemic infections in mice and contributes to colonization of the mouse lung and the nasopharynx of infant rats (34, 44, 48). One of the genes controlled by CiaR, the protease/chaperone HtrA, has recently been analyzed in detail and appears to be the main mediator of the virulence phenotype and competence inhibition of *cia* mutants (21, 22, 44, 45). Furthermore, increased autolysis of *ciaR* mutant cells has been observed under many conditions, including stationary-phase lysis and lysis triggered by deoxycholate or upon addition of CSP (6, 10, 18, 30).

During investigations of the cefotaxime-resistant laboratory mutants, we observed that the presence of a mutated *pbp2x* gene apparently greatly enhanced the tendency of *cia* mutants to autolyze. The aim of the present study was a detailed examination of the role of the CiaRH system in the context of cefotaxime resistance conferred by *pbp2x* mutations and under lysis-inducing or protective conditions. We will show that the CiaRH system is apparently activated in mutants that are beta-lactam resistant due to mutations in *pbp2x*, whereas disruption of the *cia* system in *pbp2x* mutants is highly deleterious to the cells and leads to enhanced autolysis. Furthermore, we investigated *cia* mutants under a variety of conditions that affect autolysis in *S. pneumoniae*, such as treatment with antibiotics that inhibit different steps during cell wall biosynthesis. Evidence will be presented that lysis is greatly enhanced in *cia*^{null} mutants during treatment with cell wall inhibitors independently of their mode of action and that the *cia* ON mutation is highly protective under such conditions.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Streptococcus pneumoniae* R6 is a nonencapsulated penicillin-sensitive derivative of the Rockefeller University strain R36A (1). C103, C203, C303, and C206 are spontaneous cefotaxime-resistant laboratory mutants derived from the R6 strain (28). The three-digit number specifies the selection step (first numeral) and the mutant lineage (last numeral). Properties of the mutants and derivatives are listed in Table 1. The strains were grown at 37°C without aeration in C medium (26) supplemented with 0,2% yeast extract. Growth was monitored by nephelometry. For growth at defined choline concentrations, a chemically defined medium (53), which was kindly provided by Werner Fischer, was used and supplemented with choline to obtain the final concentrations as indicated in the Results section. *Escherichia coli* InvaF′ (Invitrogen) was grown in LB medium, and pCR2.1 and derivates were selected with ampicillin and kanamycin (50 μg/ml each).

MICs were determined on blood agar plates (3% sheep blood) containing a narrow range of antibiotic concentrations and incubated at 37°C for 24 h. MICs are the means from at least three independent experiments.

Microscopic techniques. Samples were observed and photographed with a Nikon *Eclipse* E600 microscope equipped with a ×40/numerical aperture 0.75 and a ×100/numerical aperture 1.4 oil objective. Viability of the cells was determined in growing cultures using the LIVE/DEAD*Bac*Light bacterial viability kit (Molecular probes) according to the manufacturer. For the parallel observations of SYTO-9 and propidium iodide fluorescent stains, a B-2A green longpass filter (EX 450 to 490; DM 505; BA 520; Nikon) was used. Photographs were taken with a U-III photo unit and a Nikon FDX-35 camera and automatic exposure time settings using Kodachrome 64 diapositive films.

Transformation. To obtain competent cells, *S. pneumoniae* strains were cultured in C medium supplemented with 0.07% bovine serum albumin and aliquots stored in the presence of 10% glycerol at −80°C. Cells were transformed essentially according to published procedures by 30 min of incubation in the presence of DNA at 30°C, followed by 2 h of phenotypic expression at 37°C (29, 49). Transformation efficiency was determined using the *S. pneumoniae* AmiA9 high-level streptomycin resistance marker as standard donor DNA (43). In some experiments, CSP was used at a concentration of 100 ng/ml. R6 transformants containing mosaic blocks of the penicillin-resistant clinical isolates *S. pneumoniae* 8249 (type 19) and 681 (6B) have been described (46). The *pbp2x* fragments containing the point mutations of the mutants C103 and C203 were amplified by PCR using the oligonucleotide primers 5′-A_{1501}TCGTTTTAAATTTGG or 5′-C_{1709}AAATGATC AAACTGC and 5′-T_{2345}CTGGAACCTCCTCTG according to the sequence available under GenBank accession number X16367, and transformation of the *pbp2x* mutations into the R6 strain was verified by sequencing of the transformants. Antibiotic concentrations used for selection of transformants were as follows: streptomycin, 200 μg/ml, final concentration; erythromycin, 1 μg/ml; spectinomycin, 100 μg/ml. Cefotaxime-resistant transformants of the R6 strain were isolated using cloned *pbp2x* genes as donor DNA and cefotaxime concentrations for selection as described below. The *pbp2x* mutations in the transformants were verified by DNA sequence analysis.

Construction of *ciaR* and *ciaH* mutants. R6*ciaH*^{C306} was obtained by transformation of R6 with the *ciaH* gene of the mutant C306 carrying the mutation T230P (14). Two classes of loss-of-function *ciaR* mutants were constructed. Insertion-duplication mutagenesis using the pJDC9-derivative pEGR91 containing an internal fragment of the *ciaR* gene has been described (14). Alternatively, the gene was disrupted by insertion of the spectinomycin resistance gene *aad9* from pDL278 (31), and no differences in phenotypes from the pJDC9 derivative was noted. The R6*ciaR*^{null} (Spc^r) mutant (R6*ciaR*::*aad9*) has been described (36). No CiaH protein could be detected in the *ciaR*^{null} R6*ciaR*::pEGR91 mutants with CiaH-specific antiserum (55).

RNA extraction and real-time reverse transcriptase PCR (RT-PCR). *S. pneumoniae* R6 and mutant strains were grown exponentially in C medium to the same cell density (n = 40) and harvested by centrifugation, and RNAs were prepared using hot phenol essentially as described by Mascher et al. (36). Seven hundred fifty nanograms of purified RNA was reverse transcribed into single-stranded cDNA using the 1st Strand cDNA synthesis kit for RT-PCR (Roche) following the manufacturers instructions. After a 1:50 dilution of the cDNA, 5 μl was used in a RT-PCR using the LightCycler Fast Start DNA Master^{Plus} SYBR Green I kit (Roche). The reaction was performed according to the manufacturer's instructions using 10 pmol of each primer in a total volume of 20 μl. The reaction mix was placed into a LightCycler capillary, which was then centrifuged at 735 × *g* for 15 s in the LC Carousel 2.0 centrifuge (Roche) and loaded into the LightCycler 2.0 thermocycler. The thermocycler conditions were as follows: 10 min 95°C for activation of the polymerase; 45 cycles of 10 s at 95°C, 10 s at 54°C, and 20 s at 72°C. SYBR green fluorescence was measured at the end of each cycle. Melting-curve analysis was performed after the PCR cycles as follows: the temperature was dropped to 65°C, followed by a 0.1°C s^{-1} increase in temperature until 95°C was reached. SYBR Green fluorescence was measured continually during the melting-curve analysis.

Gene-specific amplifications from cDNA were carried out with the following primer combinations: 16sF (GGTGAGTAACGCGTAGGTAA) and 16sR (ACGATCCGAAAACCTTCTTC), htrA_fwd (GCAAAGTAGATA TTCGATTGTC) and htrA_rev (GAGTGACAGTATTTGCATATTC), and RT_0931_for (ACCGAAATGCAGCCGTAACT) and RT_0931_rev (CTG GAATGGGTGCTGCTACA), yielding products of 325 bp, 209 bp, and 65 bp, respectively. Total cDNA abundance between test samples was normalized using the 16S rRNA gene as a housekeeping control. Each measurement was performed in duplicate, and the mean was taken for calculating the differences of expression levels, in Cp values. The experiment was performed with two independently grown cultures.

RESULTS

C003 family of cefotaxime-resistant mutants. Previous investigations of CiaH mutants concentrated on the mutation T230→P of the cefotaxime-resistant mutant C306. This mutant was isolated after three successive selections on increasing concentrations of cefotaxime. The *ciaH* mutation occurred during the third selection step, after the introduction of one *pbp2x* mutation each during selection steps 1 and 2 (Fig. 1).

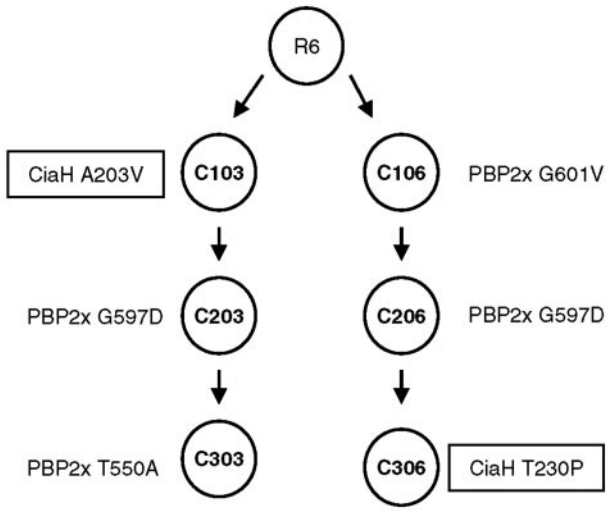

FIG. 1. Cefotaxime-resistant laboratory mutants of the two lineages C003 and C006. Mutants were obtained from the parental R6 strain by successive selection on increasing cefotaxime concentrations. During each step, single point mutations in CiaH or PBP2x were selected; the amino acid changes are indicated.

Transformation of the $ciaH_{C306}$ allele into the parental strain, R6, resulted in the transformant R6$_{ciaH\text{C}306}$ with a twofold increase in the cefotaxime MIC compared to that for the R6 strain. Surprisingly, this transformant was completely noncompetent, documenting that this particular *ciaH* allele conferred competence deficiency as well (10, 14, 18). The P230 mutation in CiaH was later shown by genomic transcription analysis to activate the CiaRH system and was hence referred to as ON mutation (36). In other words, activation of the CiaRH system is reflected by the absence of competence for genetic transformation. In fact, since all high-level cefotaxime-resistant mutants were noncompetent and all of them contained mutations in *ciaH*, we suggested that in all cases the CiaRH system is activated.

During investigations of these mutants, we noticed that the CiaH mutation A203→V, which had occurred already during the first selection step in the mutant C103, did not affect competence substantially, although the mutant C503 isolated at the fifth selection step was competence deficient (55). We therefore analyzed this mutant family in detail.

The second- and third-step mutants of this family, C203 and C303, contained mutations in *pbp2x*, resulting in the amino acid changes PBP2x-G597D and T550A (27) (Fig. 1). The genotypes of the mutants were verified by successive transformation of the individual *ciaH* and *pbp2*x mutations into R6. The fact that the transformants were indistinguishable from C103, C203, and C303, respectively, confirmed the acquisition of one mutation per selection step in this mutant family and hence the presence of three, and only three, mutations in C303 (Table 1). Complete competence deficiency occurred during the third selection step in the mutant C303, but R6 derivatives containing only the two *pbp2x* mutations of C303 were still perfectly competent (Table 1). In only the mutants where both the *ciaH*^{C103} allele and *pbp2x* mutations were combined, competence was affected: the transformation efficiency was reduced 10- to 100-fold in C203 and dropped below detectability

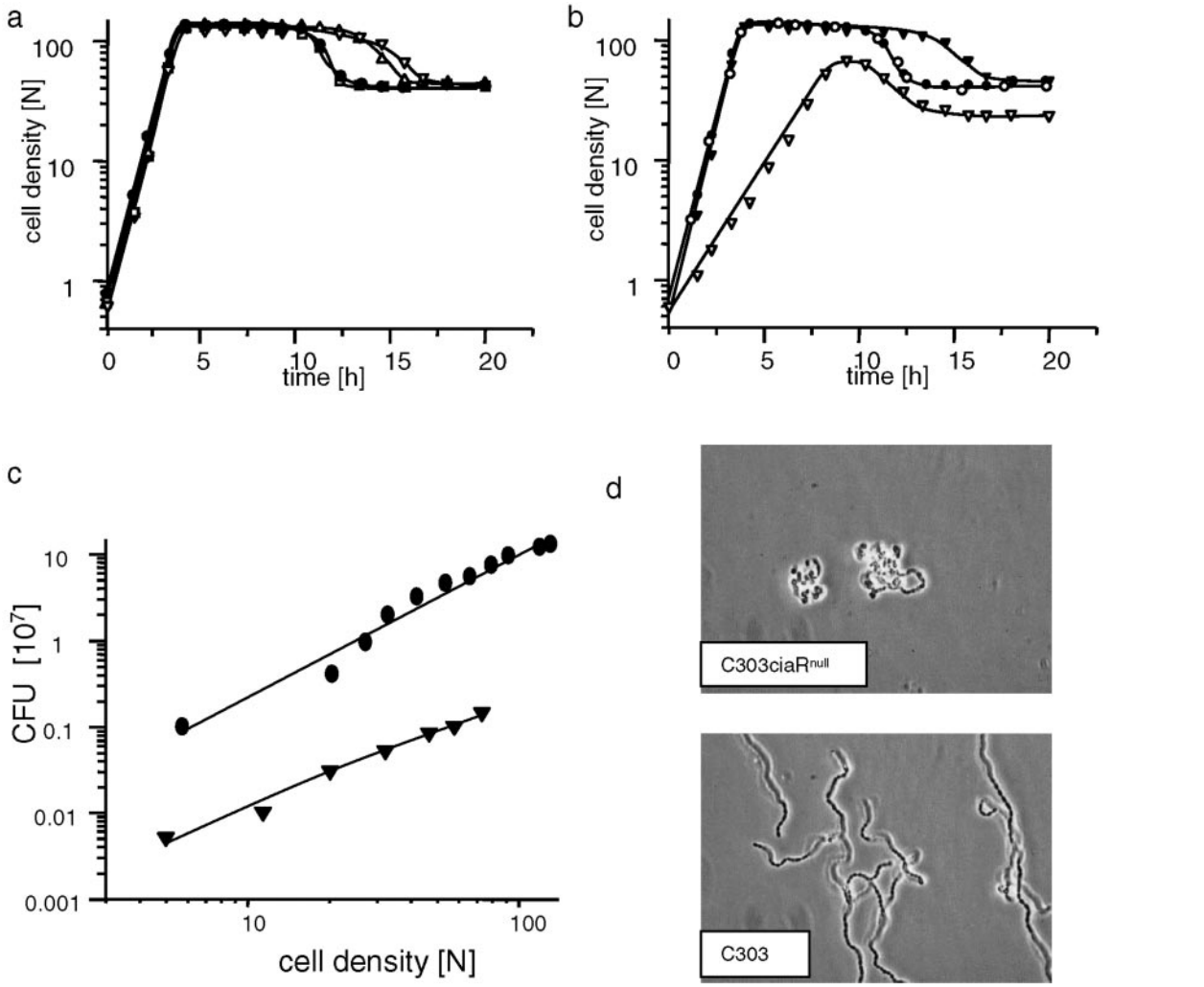

FIG. 2. Effects of *cia* and *pbp2x* mutations in the mutant lineage C003. Growth in C medium was monitored by nephelometry (N). a. Cellular growth of mutants C103, C203, and C303 in comparison with the parental R6 strain. ○, R6; □, C103; △, C203; ▽, C303. b. Cellular growth of C303 (▼), R6*pbp2x*^{C303} (○), and C303*ciaR*^{null} (▽). The R6 strain was used as a control (●). c. Viability of C303 (●) and C303*ciaR*^{null} (▼) during growth. Numbers of CFU were determined by plating appropriate dilutions on blood agar plates. d. Cell morphology. Cells were grown in C medium containing 2% choline to induce chain formation. Samples were taken during exponential growth phase, and pictures were taken with a Nikon *Eclipse* E600 microscope in phase contrast.

($<10^5$) in C303 (Table 1). We therefore concluded that the CiaRH system is activated in C203 and even more so in mutant C303 and that the degree of activation was dependent on the type of *pbp2*x mutation. In addition, the stationary phase was longer and lasted 7 h in C203 and C303 rather than the 5 h seen in R6 or C103, i.e., the onset of stationary-phase autolysis was considerably delayed, whereas growth of both C103 with the *ciaH* mutation and R6*pbp2x*^{C303} with the two *pbp2x* mutations was indistinguishable from that of the parental R6 strain (Fig. 2a and b). This suggested that activation of the CiaRH system protects the cells from autolysis that typically occurs during stationary phase in the pneumococcus to a certain degree.

The following experiments were performed in order to analyze the link between the CiaRH system and *pbp2x* mutations. Different *pbp2x* alleles were tested in combination with *cia* mutations in order to see whether the effects observed in C203 and C303 are restricted to laboratory mutants or are relevant for beta-lactam-resistant clinical strains as well. Second, loss-of-function *ciaH* and *ciaR* mutants were constructed for a variety of *pbp2x* mutants. In a second set of experiments, the impact of *cia* mutations on conditions that affect the lytic behavior was tested, such as treatment with cell wall inhibitors that induce lysis or choline starvation that prevents lysis in the wild type.

Interaction between *ciaH*^{C103} and *pbp2x* mutations. In order to see whether the phenotypes observed with C203 and C303 were restricted to these particular *pbp2x* mutations, *pbp2x* genes of another laboratory mutant as well as highly divergent mosaic *pbp2x* genes from clinical isolates were tested in combination with the *ciaH*^{C103} allele. Different *pbp2x* alleles from beta-lactam-resistant clinical isolates representing highly divergent mosaic genes were transformed into R6 and C103. The mosaic *pbp2x* genes from two genetically distinct, major penicillin-resistant *S. pneumoniae* clones from Spain and from South Africa were used which differed by between 25 (*S. pneu-*

TABLE 2. Cefotaxime resistance and competence of PBP2x and/or CiaH mutants

Strain	Mutation		MIC CTX	Competence
	CiaH	PBP2x		
C206		D597/V601	0.12	+
R6*pbp2x*^{C203}		D597	0.07	+
R6*pbp2x*^{C303}		D597/A550	0.2	+
R6*pbp2x*^{681}		Mosaic[a]	0.2	+
R6*pbp2x*^{8249}		Mosaic[a]	0.2	+
C103*pbp2x*^{681}	V203	Mosaic[a]	0.25	−[b]
C103*pbp2x*^{8249}	V203	Mosaic[a]	0.3	−
R6*ciaH*^{C306}	P230		0.04	−

[a] The mosaic *pbp2x* genes of the clinical isolates *S. pneumoniae* 681 and 8249 contained multiple alterations.
[b] Transformation efficiency of $<10^{-5}$ compared to that of the R6 strain.

moniae 8249) and 40 (*S. pneumoniae* 681) amino acids in the transpeptidase domain, corresponding to 7 to 11% in peptide sequence (46). None of these PBP2x variants contained the mutations present in the laboratory mutant C303. When transformed into the R6 strain, the *pbp2x* genes from the clinical isolates conferred cefotaxime resistance of 0.2 μg/ml, identical to that with the two point mutations in *pbp2x*^{C303}, whereas the MIC mediated by the single point mutation of *pbp2x*^{C203} was only 0.07 μg/ml (Table 2). All these constructs were competent. However, when the *pbp2x* alleles were introduced into C103 by transformation, transformants were noncompetent, similar to C303. Thus, independently of the origin of mutated *pbp2x* genes, when these genes were combined with the *ciaH*^{C103} allele, competence was reduced, suggesting that the alterations in *pbp2x* conferring beta-lactam resistance activate the CiaRH system.

A functional CiaRH system is required in cefotaxime-resistant mutants with *pbp2x* mutations. In order to examine the contribution of the CiaRH system to the observed phenotypes, loss-of-function *ciaR* mutants were constructed in the R6 strain as well as in each of the three mutants C103, C203, and C303. In the R6 strain, *ciaR* mutants showed no apparent growth deficiency in C medium (Fig. 2). Disruption of *ciaR* in the mutant C303, however, i.e., in combination with *pbp2x* containing the two mutations G597D and T550A, had a dramatic effect on cellular growth (Fig. 2b to d). The cultures grew to a lower cell density (n = 70 compared to 125), and showed no stationary phase but lysed immediately upon reaching high cell density. Furthermore, the generation time increased more than twofold to more than 90 min (Table 1). The reduced generation time for C303*ciaR*^{null} was at least partially due to cell death, as determined by plating, which showed a 50- to 100-fold reduction of viable cells throughout the growth phase (Fig. 2c), and could also be detected by a differential staining technique (LIFE-DEAD bacterial viability kit; not shown). Moreover, the cell morphology was grossly altered. In medium containing 2% choline, chain formation is induced and morphological changes can be seen more easily (3). Under these conditions, C303*ciaR*^{null} grew in shorter twisted chains of misshaped cells, in contrast to C303, where cell morphology was similar to that of the parental R6 strain (Fig. 2d).

In the mutant C203, which contains only one single point mutation, G597D, in *pbp2x*, the effect of *ciaR*^{null} was less dramatic but principally similar to that observed with C303: the generation time increased (but only 1.5-fold), the cells grew to a lower cell density (but reached N = 105) and lysed early after 2 h of stationary phase (Table 1). All these phenotypes were apparent independently of the class of *pbp2x* mutations used in combination with the *ciaR*^{null} phenotype: the *pbp2x* gene of another laboratory mutant, C206, containing the two mutations G597D and G601V, and most importantly also with highly altered mosaic *pbp2x* genes from the multiple antibiotic- and high-level penicillin-resistant 23F clone (not shown). This indicates that mutations in PBP2x that confer beta-lactam resistance do have an impact on the cellular growth that can be balanced by and therefore require a functional CiaRH system, and depending on the nature of the PBP2x mutations, the cells are affected to different degrees.

***cia*-dependent gene expression in *pbp2x* mutants.** In order to see whether the competence deficiency observed in the C303 mutant, i.e., the *ciaH*^{C103} allele, might lead to an activation of the *cia* system in the presence of *pbp2x* mutants, RT-PCR analysis was performed for two *cia*-regulated genes, *htrA* and spr0931, both belonging to strongly *cia*-inducible genes (6, 36, 44). The expression of these genes was tested in a variety of different backgrounds: C103, which contains only the *ciaH* mutation A203V; R6*pbp2x*^{C303} and R6*pbp2x*^{2349}, which contain only *pbp2x* mutations either from the laboratory mutant C303 or from the clinical isolate; and the mutant C303 with the combination of *pbp2x* and *ciaH* mutations. The data were compared to those of the R6 strain, and the *cia* ON mutant R6*ciaH*^{C306} and *cia* OFF mutant R6*ciaR*^{null} were also included. As shown in Fig. 3, *htrA* expression was not signifi-

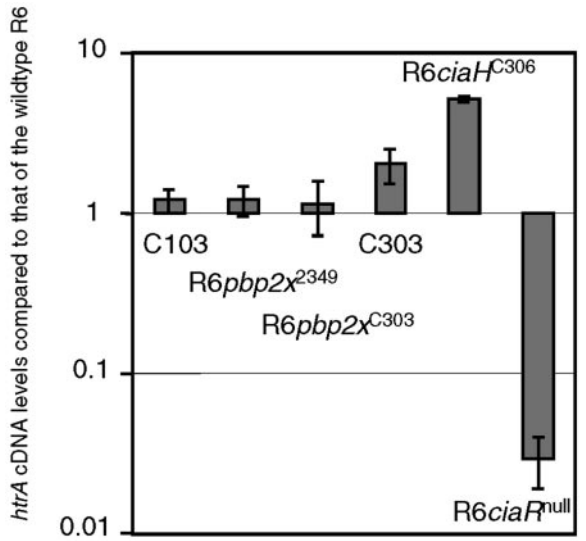

FIG. 3. Effect of *ciaH*^{C103} on *htrA* expression. Wild-type and mutant cells were grown to the same cell density (Nephelo 40). RNA was extracted and cDNA prepared and quantified by RT-PCR for *htrA* expression as described in the Materials and Methods section. The relative amounts of *htrA* cDNA of the mutants in comparison to that of the R6 strain are indicated. Error bars indicate the variance between at least two measurements obtained with cDNA isolated from two independently grown cultures.

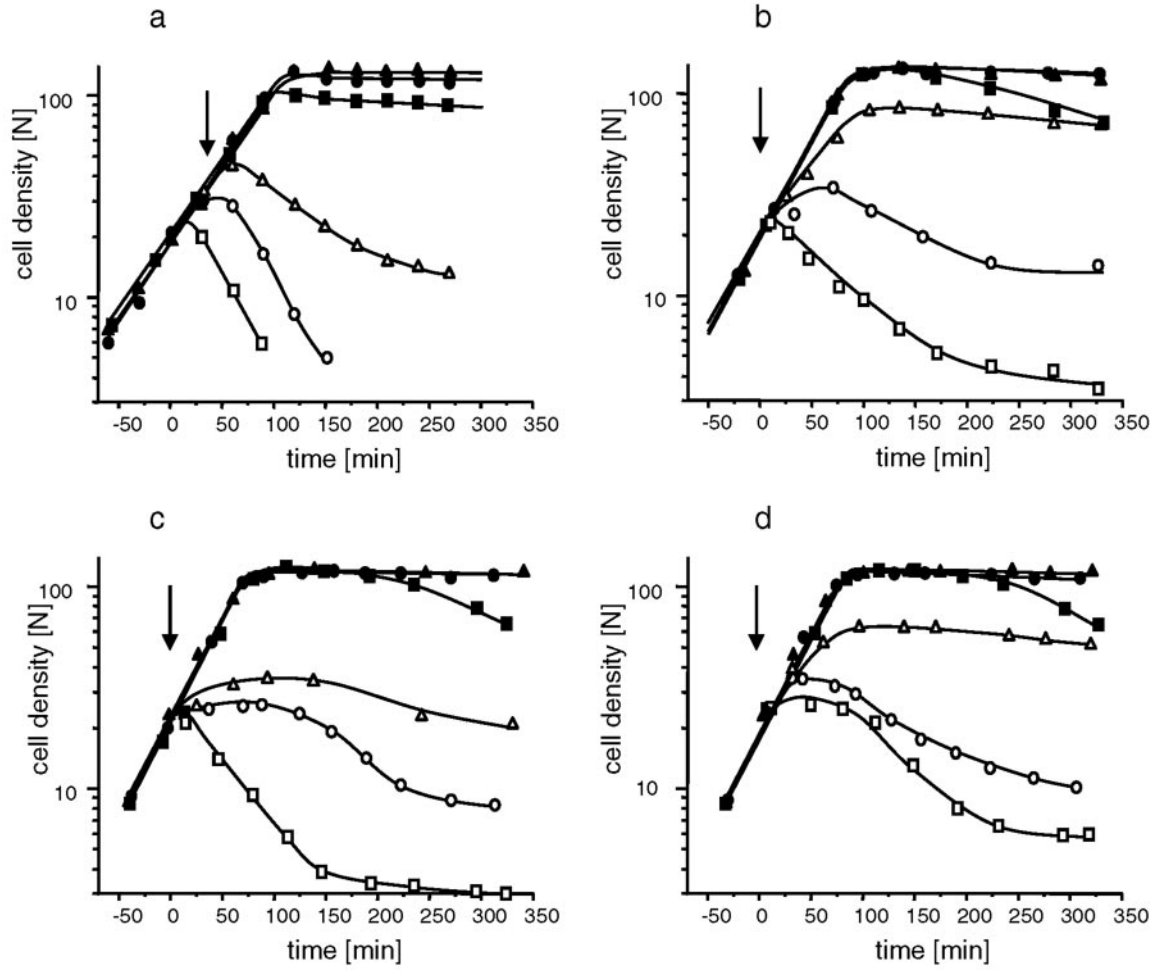

FIG. 4. Effect of cell wall inhibitors on *cia* mutants. Growth in C medium was monitored by nephelometry (N), and the addition of antibiotic is indicated by the arrow. Closed symbols, control cultures; open symbols, antibiotic-treated cultures. R6, ●; R6*ciaR*^{null}, ■; R6*ciaH*^{C306}, ▲. (a) Piperacillin was added at 0.1 μg/ml to R6 and R6*ciaR*^{null} and at 0.2 μg/ml to R6*ciaH*^{C306}; (b) vancomycin, 0.45 μg/ml; (c) bacitracin, 20 μg/ml; (d) cycloserine:, 75 μg/ml.

cantly altered compared to that in R6 in the mutants containing alterations in *pbp2x* only or in the C103 mutant. However, the mutant C303 showed an almost twofold increase in *htrA* expression, demonstrating that indeed, the combination of *pbp2x* mutations plus the *ciaH*^{C103} allele resulted in activation of the *cia*-regulated gene. *htrA* expression was still higher in the *cia* ON mutant R6*ciaH*^{C306}, whereas a dramatic decrease was observed in the R6*ciaR*^{null}. Similar results were obtained for spr0931 gene expression (not shown). In other words, the *cia* system appears to be already activated in the R6 strain, and this activation is sufficient to support the presence of *pbp2x* mutations. The data support our conclusion that the presence of the particular *ciaH*^{C103} allele in combination with *pbp2x* results in further activation of the *cia* system.

Effect of cell wall inhibitors on *cia* mutants. The lysis phenotype associated with *cia* mutants initiated a series of experiments using lysis-inducing antibiotics that inhibit cell wall biosynthesis at different stages: fosfomycin and D-cycloserine, which act upon cytoplasmic steps of murein biosynthesis and target the MurA protein (UDP-*N*-acetylglucosamine enolpyruvyl transferase) and the two enzymes D-alanine racemase and D-alanyl-D-alanine ligase, respectively; bacitracin, which prevents dephosphorylation and thereby the recyclization of the carrier lipid C_{55}-isoprenyl pyrophosphate, essential for cell wall polysaccharide biosynthesis, and inhibitors of late steps of murein biosynthesis that take place outside of the cell, such as the beta-lactam piperacillin, which inhibits *S. pneumoniae* PBPs at very low concentrations and is highly lytic; and vancomycin, which attaches to the terminal D-Ala-D-Ala moiety of the murein, preventing polymerization and cross-linking reactions. Two mutants were examined in detail: R6*ciaH*^{C306}, with an activated CiaRH system, and an R6*ciaR*^{null} mutant.

When added to growing cultures in C medium, penicillin antibiotics as well as non-beta-lactam cell wall inhibitors were highly lytic with the *ciaR*^{null} mutant, with lysis being triggered almost immediately upon addition of the antibiotic, whereas lysis with the R6*ciaH*^{C306} mutant was markedly reduced compared to results with strain R6. Examples are shown in Fig. 4. Thus, it is clear that activation of the CiaRH system is lysis protective independently of the mode of action of the cell wall inhibitors, a clear indication that it is not the antibiotic itself that is targeted by the CiaH sensor kinase.

When susceptibility was tested on blood agar plates, the MICs of the ON mutants were always higher than in the R6 strain (from 1.5- to 3-fold) but only slightly reduced in the *ciaR*^{null} background compared to that for R6 (not shown), probably since the lysis-protective effect is less evident during growth on the solid agar surface.

Effect of choline starvation in *ciaR* insertion-duplication mutant. Choline is an important structural component of the wall teichoic acid and the lipoteichoic acid, and pneumococci have an absolute growth requirement for choline (42). In contrast to the autolysis that commences within a few hours after reaching stationary phase in choline-containing growth medium, starvation of choline results in a complete halt of cellular growth and cells do not lyse at all (50). Therefore, we wanted to see whether *cia* mutations that prevented (R6*ciaR*^{null}) or extended (R6*ciaH*^{C306}) the stationary phase of growth also influenced the apparent stationary state during choline starvation. The ON mutant R6*ciaH*^{C306} stopped growing at the same cell density independently of the choline concentrations present, suggesting another choline-independent medium effect that prevented growth (Fig. 5c), and similar results have been described previously (10). The R6*ciaR*^{null} mutant grew to different cell densities depending on the choline concentration in the medium without changes in generation time, similar to the R6 strain (Fig. 5a and b). However, the R6*ciaR*^{null} mutant differed markedly from the parental R6 strain: the culture lysed immediately after reaching the highest cell density without maintaining a plateau, except at choline concentrations of 2 μg/ml and higher (Fig. 5a and b).

DISCUSSION

Since the identification of the *S. pneumoniae* CiaRH system in beta-lactam-resistant laboratory mutants, *cia* mutations have been shown to cause a variety of pleiotropic effects. In addition to the initial observations that mutations in the histidine kinase CiaH cause changes in susceptibility to cefotaxime and interfere with genetic competence (14), the CiaRH system contributes to autolysis in complex media (6, 10, 18, 30, 36), DOC-mediated lysis (10), and thermotolerance and oxidative stress tolerance (8, 22) and affects pneumococcal virulence in vivo in mouse infection models (34, 44, 48). The present analysis has extended *cia*-mediated effects to a whole battery of conditions that are related to cellular lysis: inhibition of early and late stages of peptidoglycan biosynthesis by antibiotics and prevention of teichoic acid biosynthesis by depletion of choline in the growth medium. Also, addition of the nonionic detergent Triton X-100 at concentrations as low as 0.005 to 0.01% caused a differential effect in the *cia* mutants (data not shown). Under all these conditions, *cia*^{null} mutants lysed much more rapidly than wild-type cells, whereas an activated CiaRH system reduced the lytic response considerably. Surprisingly, mutations in PBP2x that confer beta-lactam resistance cause similar phenotypes in *cia* mutants. In the absence of a functional CiaRH system, PBP2x mutants not only grew poorly but lysed immediately at the end of their exponential growth phase and showed reduced viability and altered cell morphology (see Table 1 and Fig. 2). Moreover, when PBP2x mutations—point mutations selected in the laboratory as well as highly altered mosaic genes—were combined with the *ciaH*^{C103} allele, an

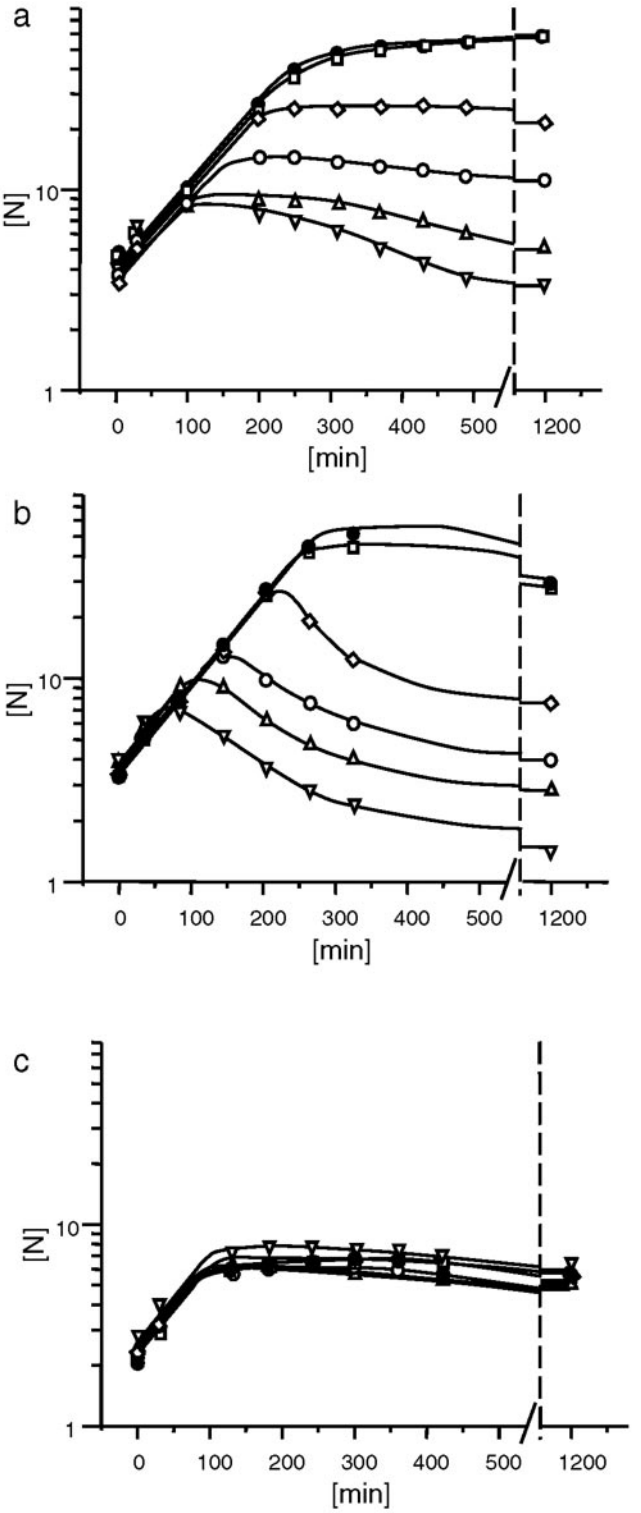

FIG. 5. Growth of *cia* mutants under limited choline concentrations. Exponentially growing cells in C medium were centrifuged and resuspended in chemically defined medium containing different concentrations of choline. (a) R6; (b) R6*ciaR*^{null}; (c) R6*ciaH*^{C306}. Choline concentrations (μg/ml): 5 (●), 2 (□), 0.5 (◇), 0.2 (○), 0.1 (△), and 0 (▽).

activation of the CiaRH system was observed, as deduced from the fact that such cells were affected in competence and by an elevated expression level of *cia*-regulated genes as determined by real-time RT-PCR, i.e., the phenotypes associated with the *cia* ON mutant (Tables 1 and 2 and Fig. 3). This documents that mutations in the penicillin target protein are not harmless to the cell but are tolerated only in the presence of a functional CiaRH system.

What is the correlation between lysis triggered by PBP2x mutations and inhibition of cell wall biosynthesis or membrane damage? One possible explanation could be that the mutations affect the specificity of PBP2x function and result in a biochemically different peptidoglycan which can be directly monitored by the CiaRH system. It could also be possible that mutations in PBP2x might have only a minor effect on its function but affect the stability of the protein structure, leading to misfolded PBP2x molecules. These proteins could be targeted by HtrA, which is part of the *cia* regulon (6, 36, 44), either in its function as a chaperone or as a protease (47), thereby ensuring a functional state of the cell wall synthesizing machinery. These two explanations, however, fail to explain why the effect of other non-beta-lactam cell wall inhibitors is also targeted by the CiaRH system. Alternatively, the activity of mutated PBP2x could be reduced rather than altered, an effect that may mimic low concentrations of other cell wall inhibitors. We will discuss PBP2x mutations and lytic conditions for *S. pneumoniae* in relation to the CiaRH system below.

PBP2x mutations. Alterations in PBP2x are the prerequisite for high resistance levels mediated by alterations in class A high-molecular-weight PBPs. Resistance is mediated by PBP mutations that cause a substantial decrease in the affinity to the inhibitor (12). The interaction of mutated PBP2x with beta-lactams as determined with purified soluble PBP2x derivatives is certainly severely affected, and hydrolysis of substrate analogues, such as depsipeptide compounds, was also reduced, but only by a factor of 10 (23, 24, 32, 39). However, since no in vitro test for the transpeptidation reaction with natural muropeptide substrates is available, the impact of mutations on its actual function remains unknown. Evidence has been provided that the natural substrates for the *S. pneumoniae* PBP2x are the branched muropeptides rather than linear molecules, making in vitro biochemical studies even more difficult (54). Nevertheless, no growth defect is apparent in most high-level-resistant clinical isolates containing multiple alterations in at least three PBP genes, and resistant transformants containing low-affinity PBP2x from clinical isolates or from laboratory mutants grow perfectly normally. Therefore, it was surprising to see that PBP2x mutations are of such severe consequences in *cia*^{null} mutants.

One should point out that the effects of *cia* mutations in combination with different *pbp2x* alleles varied considerably, an indication that the impact on protein function differs between different PBP2x mutations. In the case of the PBP2x G597D mutation, introduction of the *cia*^{null} genotype resulted in a 1.5-fold-longer generation time than that of the R6 strain, and competence was only slightly reduced in the *ciaH*^{C103} background, whereas the PBP2x^{C303}/*ciaR*^{null} (PBP2x G597D plus T550A) strain grew more than twofold slower than the wild type, and competence was reduced by $>10^6$-fold by the presence of the *ciaH*^{C103} allele; a similar phenotype was obtained with the mutant PBP2x^{C206}/*ciaR*^{null} (G597D/G601V). The T550A mutation is unusual in that it confers specific cefotaxime resistance and at the same time hypersusceptibility to penicillin beta-lactams independently of the presence of mutations in other genes relevant for resistance (5, 13), and an effect of mutations at this position on transpeptidation activity has been suggested (38). PBP2x is localized at the division zone (37), and the fact that morphology is grossly altered for the C303*ciaR*^{null} mutant containing PBP2x T550A confirms the importance of this mutation. The effect of the mutation 601V, which is located on α11 according to the high-resolution PBP2x structure (40), is not known. With the highly altered PBP2x from clinical isolates, competence was also beyond detectability when combined with the *ciaH*^{C103} allele. However, growth of the corresponding *pbp2x*^{mosaic} *ciaR*^{null} mutants was much less impaired than that of the laboratory mutants, suggesting that compensatory mutations in PBP2x have occurred during evolution of resistant strains outside the laboratory.

Autolysis and CiaRH. In the absence of a functional *cia* system, lysis proceeded much more rapidly not only for PBP2x mutants but also under a variety of conditions: during stationary phase in normal growth medium or when induced by choline depletion; and when triggered by early and late inhibitors of cell wall biosynthesis. On the other hand, with activation of the CiaRH system, e.g., in the ON mutant R6*ciaH*^{C306}, a strong lysis-protective effect was observed. The reduced lysis rate in R6*ciaH*^{C306} upon drug treatment was paralleled with a decreased susceptibility to these drugs. Although this effect of the *cia* ON mutation was quite remarkable, the MIC changes of such mutants were only within an approximately twofold range, but lysis-related effects may have more-severe consequences in liquid medium than for growth on solid agar surfaces. In agreement with this, the *cia* system appeared to be activated in a vancomycin-tolerant mutant and was thus suggested to play an important role in the bacterium's response to vancomycin stress (17).

Treatment with different cell wall antibiotics, such as bacitracin, D-cycloserine, or beta-lactams, induces a common response in *S. pneumoniae* which can be detected in autolysis-negative mutants: immediately upon addition of the drug, secretion of peptidoglycan and teichoic acid precursors into the growth medium commences and membrane vesicles are shed from the cells, demonstrating that the integrity of the cytoplasmic membrane is no longer maintained under these conditions (19, 20). The peptidoglycan and teichoic acid chains released during beta-lactam treatment in *S. pneumoniae* are not linked to one another (9), and obviously, coordinated incorporation of peptidoglycan and teichoic acid units into the cell wall growth zone does not take place under these conditions (51), resulting in induction of the autolysin via a yet-unknown mechanism.

Coordination of peptidoglycan and choline-containing teichoic acid biosynthesis is also not guaranteed in choline-deprived cells. In this case, the cells stop growing and autolysis appears completely blocked, unlike the situation in choline-containing growth media, where stationary phase is limited (50). However, for the *ciaR*^{null} mutant, lysis commenced as soon as the cells stopped growing (Fig. 5). This is in contrast to a report where choline-independent growth of the *ciaR* mutant was noted, but this could be due to differences in the growth medium (10).

Choline starvation induces the *licD2* gene (spr1152) (7), which is part of a three-gene *lic* operon and is required for the insertion of one-half of the choline residues into teichoic acids (56). It is located adjacent to another *lic* operon (spr1145 to spr1149), which is transcribed in the opposite direction and provides more functions in choline metabolism. A CiaR binding site has been mapped between these two operons, and *cia*-dependent expression of spr1145 to -1149 has been observed at the end of the exponential growth phase (36). It is possible that the CiaRH system is also involved in *licD2* expression under choline starvation, but further experiments are required to reveal the relation between CiaRH, the *licD2* product, and cellular lysis. Interestingly, cells also respond to choline deprivation with a rapid decline of cellular competence (52), another indication that the *cia* system might be activated and indeed required under these conditions.

It is curious that the CiaRH system belongs to the group of "delayed" competence genes (41), i.e., it is activated during the state of competence, where part of the cell population undergoes lysis (16, 25). In fact, Claverys and colleagues showed recently that addition of CSP induces lysis in *ciaR* mutants (6), emphasizing that competence imposes a temporary stress on the cells and that the CiaRH system is required to control competence-induced lysis and is necessary for the cells to exit normally from the competence state. Bacterial histidine protein kinases have been proposed repeatedly as targets for new antibiotics (2, 11, 33). The situation described here—a highly lytic response as the result of inactivation of a two-component system—could also be exploited during the search for histidine kinase inhibitors.

Taken together, our studies identified important functions of the CiaRH system during many different lysis-inducing conditions: (i) protection from a broad range of cell wall inhibitors and (ii) protection from detrimental effects paralleling PBP-mediated beta-lactam resistance. How this is achieved on the molecular level remains to be clarified.

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REFERENCES

1. **Avery, O. T., C. M. MacLeod, and M. McCarty.** 1944. Studies on the chemical nature of the substance inducing transformation of pneumococcal types. Induction of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III. J. Exp. Med. **79:**137–158.
2. **Barrett, J. F., and J. A. Hoch.** 1998. Two-component signal transduction as a target for microbial anti-infective therapy. Antimicrob. Agents Chemother. **42:**1529–1536.
3. **Briese, T., and R. Hakenbeck.** 1985. Interaction of the pneumococcal amidase with lipoteichoic acid and choline. Eur. J. Biochem. **146:**417–427.
4. **Claverys, J.-P., and L. S. Havarstein.** 2002. Extracellular-peptide control of competence for genetic transformation in *Streptococcus pneumoniae*. Front. Biosci. **7:**1798–1814.
5. **Coffey, T. J., M. Daniels, L. K. McDougal, C. G. Dowson, F. C. Tenover, and B. G. Spratt.** 1995. Genetic analysis of clinical isolates of *Streptococcus pneumoniae* with high-level resistance to expanded-spectrum cephalosporins. Antimicrob. Agents Chemother. **39:**1306–1313.
6. **Dagkessamanskaia, A., M. Moscoso, V. Hénard, S. Guiral, K. Overweg, M. Reuter, B. Martin, J. Wells, and J.-P. Claverys.** 2004. Interconnection of competence, stress and CiaR regulons in *Streptococcus pneumoniae*: competence triggers stationary phase autolysis of ciaR mutant cells. Mol. Microbiol. **51:**1071–1086.
7. **Desai, B. V., H. Reiter, and D. A. Morrison.** 2003. Choline starvation induces the gene *licD2* in *Streptococcus pneumoniae*. J. Bacteriol. **185:**371–373.
8. **Echenique, J. R., S. Chapuy-Regaud, and M. C. Trombe.** 2000. Competence regulation by oxygen in *Streptococcus pneumoniae*: involvement of *ciaRH* and *comCDE*. Mol. Microbiol. **36:**688–696.
9. **Fischer, H., and A. Tomasz.** 1984. Production and release of peptidoglycan and wall teichoic acid polymers in pneumococci treated with beta-lactam antibiotics. J. Bacteriol. **157:**507–513.
10. **Giammarinaro, P., A. M. Sicard, and A.-M. Gasc.** 1999. Genetic and physiological studies of the CiaH-CiaR two-component signal-transducing system involved in cefotaxime resistance and competence of *Streptococcus pneumoniae*. Microbiology **145:**1859–1869.
11. **Gilmour, R., J. E. Foster, Q. Sheng, J. R. McClain, A. Riley, P.-M. Sun, W.-L. Ng, D. Yan, T. I. Nicas, K. Henry, and M. E. Winkler.** 2005. New class of competitive inhibitor of bacterial histidine kinases. J. Bacteriol. **187:**8196–8200.
12. **Goffin, C., and J.-M. Ghuysen.** 1998. Multimodular penicillin-binding proteins: an enigmatic family of orthologs and paralogs. Microb. Mol. Biol. Rev. **62:**1079–1081.
13. **Grebe, T., and R. Hakenbeck.** 1996. Penicillin-binding proteins 2b and 2x of *Streptococcus pneumoniae* are primary resistance determinants for different classes of β-lactam antibiotics. Antimicrob. Agents Chemother. **40:**829–834.
14. **Guenzi, E., A. M. Gasc, M. A. Sicard, and R. Hakenbeck.** 1994. A two-component signal-transducing system is involved in competence and penicillin susceptibility in laboratory mutants of *Streptococcus pneumoniae*. Mol. Microbiol. **12:**505–515.
15. **Guenzi, E., and R. Hakenbeck.** 1995. Genetic competence and susceptibility to β-lactam antibiotics in *Streptococcus pneumoniae* R6 are linked via a two-component signal-transducing system, p. 125–128. *In* J. J. Ferretti, M. S. Gilmore, T. R. Klaenhammer, and F. Brown (ed.), Genetics of streptococci, enterococci and lactococci, vol. 85. S. Karger AG, Basel, Switzerland.
16. **Guiral, S., T. J. Mitchell, B. Martin, and J.-P. Claverys.** 2005. Competence-programmed predation of noncompetent cells in the human pathogen *Streptococcus pneumoniae*: genetic requirements. Proc. Natl. Acad. Sci. USA **102:**8710–8715.
17. **Haas, W., D. Kaushal, J. Sublett, C. Obert, and E. I. Tuomanen.** 2005. Vancomycin stress response in a sensitive and a tolerant strain of *Streptococcus pneumoniae*. J. Bacteriol. **187:**8205–8210.
18. **Hakenbeck, R., T. Grebe, D. Zähner, and J. B. Stock.** 1999. β-Lactam resistance in *Streptococcus pneumoniae*: penicillin-binding proteins and non penicillin-binding proteins. Mol. Microbiol. **33:**673–678.
19. **Hakenbeck, R., C. Martin, and G. Morelli.** 1983. *Streptococcus pneumoniae* proteins released into medium upon inhibition of cell wall biosynthesis. J. Bacteriol. **155:**1372–1381.
20. **Hakenbeck, R., S. Waks, and A. Tomasz.** 1978. Characterization of cell wall polymers secreted into the growth medium of lysis-defective pneumococci during treatment with penicillin and other inhibitors of cell wall synthesis. Antimicrob. Agents Chemother. **13:**302–311.
21. **Ibrahim, Y. M., A. R. Kerr, J. McCluskey, and T. J. Mitchell.** 2004. Role of HtrA in the virulence and competence of *Streptococcus pneumoniae*. Infect. Immun. **72:**3584–3591.
22. **Ibrahim, Y. M., A. R. Kerr, J. McCluskey, and T. J. Mitchell.** 2004. Control of virulence by the two-component system CiaR/H is mediated via HtrA, a major virulence factor of *Streptococcus pneumoniae*. J. Bacteriol. **186:**5258–5266.
23. **Jamin, M., C. Damblon, S. Millier, R. Hakenbeck, and J.-M. Frère.** 1993. Penicillin-binding protein 2x of *Streptococcus pneumoniae*: enzymic activities and interactions with β-lactams. Biochem. J. **292:**735–741.
24. **Jamin, M., R. Hakenbeck, and J.-M. Frère.** 1992. Penicillin binding protein 2x as a major contributor to intrinsic β-lactam resistance of *Streptococcus pneumoniae*. FEBS Lett. **331:**101–104.
25. **Kausmally, L., O. Johnsborg, M. Lunde, E. Knutsen, and L. S. Havarstein.** 2005. Choline-binding protein D (CbpD) in *Streptococcus pneumoniae* is essential for competence-induced cell lysis. J. Bacteriol. **187:**4338–4345.
26. **Lacks, S. A., and R. D. Hotchkiss.** 1960. A study of the genetic material determining an enzyme activity in pneumococcus. Biochim. Biophys. Acta **39:**508–517.
27. **Laible, G., and R. Hakenbeck.** 1991. Five independent combinations of mutations can result in low-affinity penicillin-binding protein 2x of *Streptococcus pneumoniae*. J. Bacteriol. **173:**6986–6990.
28. **Laible, G., and R. Hakenbeck.** 1987. Penicillin-binding proteins in β-lactam-resistant laboratory mutants of *Streptococcus pneumoniae*. Mol. Microbiol. **1:**355–363.
29. **Laible, G., R. Hakenbeck, M. A. Sicard, B. Joris, and J.-M. Ghuysen.** 1989. Nucleotide sequences of the *pbpX* genes encoding the penicillin-binding protein 2x from *Streptococcus pneumoniae* R6 and a cefotaxime-resistant mutant, C506. Mol. Microbiol. **3:**1337–1348.
30. **Lange, R., C. Wagner, A. de Saizieu, N. Flint, J. Molnos, M. Stieger, P. Caspers, M. Kamber, W. Keck, and K. E. Amrein.** 1999. Domain organiza-

tion and molecular characterization of 13 two-component systems identified by genome sequencing of *Streptococcus pneumoniae*. Gene **237:**223–234.
31. **LeBlanc, D. J., L. N. Lee, and J. M. Inamine.** 1991. Cloning and nucleotide base sequence analysis of a spectinomycin adenyltransferase AAD(9) determinant from *Enterococcus faecalis*. Antimicrob. Agents Chemother. **35:**1804–1810.
32. **Lu, W.-P., E. Kincaid, Y. Sun, and M. D. Bauer.** 2001. Kinetics of beta-lactam interactions with penicillin-susceptible and -resistant penicillin-binding protein 2x proteins from *Streptococcus pneumoniae*. Involvement of acylation and deacylation in beta-lactam resistance. J. Biol. Chem. **276:** 31494–31501.
33. **Lyon, G. J., P. Mayville, T. W. Muir, and R. P. Novic.** 2000. Rational design of a global inhibitor of the virulence response in *Staphylococcus aureus*, based in part on localization of the site of inhibition to the receptor-histidine kinase, AgrC. Proc. Natl. Acad. Sci. USA **97:**13330–13335.
34. **Marra, A., J. Asundi, M. Bartilson, S. Lawson, F. Fang, J. Christine, C. Wiesner, D. Brigham, W. P. Schneider, and A. E. Hromockyj.** 2002. Differential fluorescence induction analysis of *Streptococcus pneumoniae* identifies genes involved in pathogenesis. Infect. Immun. **70:**1422–1433.
35. **Martin, B., M. Prudhomme, G. Alloing, C. Granadel, and J.-P. Claverys.** 2000. Cross-regulation of competence pheromone production and export in the early control of transformation in *Streptococcus pneumoniae*. Mol. Microbiol. **38:**876–878.
36. **Mascher, T., M. Merai, N. Balmelle, A. de Saizieu, and R. Hakenbeck.** 2003. The *Streptococcus pneumoniae cia* regulon: CiaR target sites and transcription profile analysis. J. Bacteriol. **185:**60–70.
37. **Morlot, C., A. Zapun, O. Dideberg, and T. Vernet.** 2003. Growth and division of *Streptococcus pneumoniae*: localization of the high molecular weight penicillin-binding proteins during the cell cycle. Mol. Microbiol. **50:**845–855.
38. **Mouz, N., A. M. Di Guilmi, E. Gordon, R. Hakenbeck, O. Dideberg, and T. Vernet.** 1999. Mutations in the active site of penicillin-binding protein PBP2x from *Streptococcus pneumoniae*. Role in the specificity for β-lactam antibiotics. J. Biol. Chem. **274:**19175–19180.
39. **Mouz, N., E. Gordon, D.-M. Di Guilmi, I. Petit, Y. Petillot, Y. Dupont, R. Hakenbeck, T. Vernet, and O. Dideberg.** 1998. Identification of a structural determinant for resistance to β-lactam antibiotics in Gram-positive bacteria. Proc. Natl. Acad. Sci. USA **95:**13403–13406.
40. **Pares, S., N. Mouz, Y. Pétillot, R. Hakenbeck, and O. Dideberg.** 1996. X-ray structure of *Streptococcus pneumoniae* PBP2x, a primary penicillin target enzyme. Nat. Struct. Biol. **3:**284–289.
41. **Peterson, S. N., C. K. Sung, R. Cline, B. V. Desai, E. C. Snesrud, P. Luo, J. Walling, H. Li, M. Mintz, G. Tsegaye, P. C. Burr, Y. Do, S. Ahn, J. Gilbert, R. D. Fleischmann, and D. A. Morrison.** 2004. Identification of competence pheromone responsive genes in *Streptococcus pneumoniae* by use of DNA microarrays. Mol. Microbiol. **51:**1051–1070.
42. **Rane, L., and Y. Subborow.** 1940. Nutritional requirements of the pneumococcus. I. Growth factors for types I, II, V, VII, and VIII. J. Bacteriol. **40:**695–704.
43. **Salles, C., L. Creancier, J. P. Claverys, and V. Méjean.** 1992. The high level streptomycin resistance gene from *Streptococcus pneumoniae* is a homologue of the ribosomal protein S12 gene from *Escherichia coli*. Nucleic Acids Res. **20:**6103.
44. **Sebert, M. E., L. M. Palmer, M. Rosenberg, and J. N. Weiser.** 2002. Microarray-based identification of *htrA*, a *Streptococcus pneumoniae* gene that is regulated by the CiaRH two-component system and contributes to nasopharyngeal colonization. Infect. Immun. **70:**4059–4067.
45. **Sebert, M. E., K. P. Patel, M. Plotnick, and J. N. Weiser.** 2005. Pneumococcal HtrA protease mediates inhibition of competence by the CiaRH two-component signaling system. J. Bacteriol. **187:**3969–3979.
46. **Sibold, C., J. Henrichsen, A. König, C. Martin, L. Chalkley, and R. Hakenbeck.** 1994. Mosaic *pbpX* genes of major clones of penicillin-resistant *Streptococcus pneumoniae* have evolved from *pbpX* genes of a penicillin-sensitive *Streptococcus oralis*. Mol. Microbiol. **12:**1013–1023.
47. **Spiess, C., A. Beil, and M. Ehrmann.** 1999. A temperature-dependent switch from chaperone to protease in a widely conserved heat shock protein. Cell **97:**339–347.
48. **Throup, J. P., K. K. Koretke, A. P. Bryant, K. A. Ingraham, A. F. Chalker, Y. Ge, A. Marra, N. G. Wallis, J. R. Brown, D. J. Holmes, M. Rosenberg, and K. R. Burnham.** 2000. A genomic analysis of two-component signal transduction in *Streptococcus pneumoniae*. Mol. Microbiol. **35:**566–576.
49. **Tiraby, J.-G., and M. S. Fox.** 1974. Marker discrimination and mutagen-induced alterations in pneumococcal transformation. Genetics **77:**449–458.
50. **Tomasz, A.** 1967. Choline in the cell wall of a bacterium: novel type of polymer-linked choline in Pneumococcus. Science **157:**694–697.
51. **Tomasz, A., M. McDonnell, M. Westphal, and E. Zanati.** 1975. Coordinated incorporation of nascent peptidoglycan and teichoic acid into pneumococcal cell walls and conservation of peptidoglycan during growth. J. Biol. Chem. **250:**337–341.
52. **Tomasz, A., E. Zanati, and R. Ziegler.** 1971. DNA uptake during genetic transformation and the growing zone of the cell envelope. Proc. Natl. Acad. Sci. USA **68:**1848–1852.
53. **van de Rijn, I., and R. E. Kessler.** 1980. Growth characteristics of group A streptococci in a new chemically defined medium. Infect. Immun. **27:**444–448.
54. **Weber, B., K. Ehlert, A. Diehl, P. Reichmann, H. Labischinski, and R. Hakenbeck.** 2000. The *fib* locus in *Streptococcus pneumoniae* is required for peptidoglycan crosslinking and PBP-mediated beta-lactam resistance. FEMS Microbiol. Lett. **188:**81–85.
55. **Zähner, D., K. Kaminski, M. van der Linden, T. Mascher, M. Merai, and R. Hakenbeck.** 2002. The *ciaR*/*ciaH* regulatory network of *Streptococcus pneumoniae*. J. Mol. Microbiol. Biotechnol. **4:**211–216.
56. **Zhang, J. R., I. Idanpaan-Heikkila, W. Fischer, and E. I. Tuomanen.** 1999. Pneumococcal *licD2* gene is involved in phosphorylcholine metabolism. Mol. Microbiol. **31:**1477–1488.