Acidic stress induces autolysis by a CSP-independent ComE pathway in *Streptococcus pneumoniae*

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In *Streptococcus pneumoniae*, autolysis is considered a programmed cell-death process executed principally by the major autolysin (LytA), and the underlying mechanism causing its activation is not completely understood. It is known that autolysis is triggered by competence development at alkaline pH and regulated by a two-component system, ComDE, which senses a competence-stimulating peptide (CSP) and behaves as a quorum-sensing mechanism. In this work, we found that acidic stress triggered a LytA-mediated autolysis and, curiously, this phenomenon was regulated by a CSP-independent ComE pathway. A further analysis of a hyperactive ComD mutant revealed that ComE needs to be phosphorylated to activate acidic stress-induced lysis (ASIL). The *comE* transcripts were induced by acidic culture conditions, suggesting that ComE could be sensing acidic stress. We also investigated CiaRH, a two-component system whose null mutants show a *comE* derepression and a CSP-dependent autolysis induction at alkaline pH. By analysis of *cia comE* double mutants, we demonstrated that CiaRH protected cells from ASIL by a ComE-independent pathway. Here, we propose that ComE is the principal route of the signalling pathway that determines a global stress response, and clearly regulates the induction of the LytA-mediated programmed cell death in *S. pneumoniae*.

Acidic stress may represent for *S. pneumoniae* an alternative condition, in addition to competence and antibiotics, to assure the release of virulence factors, DNA and cell-wall compounds by autolysis, favouring genetic exchange and contributing to its pathogenesis.

**INTRODUCTION**

*Streptococcus pneumoniae* is one of the main human pathogens, being the causal agent of otitis and sinusitis, as well as more severe diseases such as pneumonia, bacteraemia and meningitis. One of the most recognized phenotypes of *S. pneumoniae* is its ability to autolyse in response to antibiotics that alter peptidoglycan biosynthesis, for example penicillin and vancomycin (Lewis, 2000). Autolysis is one of the most evident mechanisms of programmed cell death, and it has been found to be involved in bacterial development processes, such as DNA transformation, sporulation and myxobacterial fruiting body formation. Autolysis is triggered by a self-digestion of the cell wall caused by peptidoglycan hydrolases, mainly by the major autolysin termed LytA (*N*-acetylmuramoyl-L-alanine amidase), which is essential for normal cell division (Sanchez-Puelles et al., 1986).

In different bacterial species, autolysis is also associated with regulation of natural transformation (Lewis, 2000). In *S. pneumoniae*, LytA is encoded by the *lytA* gene and is in the same operon as *recA*. The *recA* gene encodes a protein responsible for homologous recombination of exogenous DNA. Curiously, the *cinA-recA-dinF-lytA* operon is regulated by a two-component signal transduction system (TCS), ComDE, which controls competence development in response to a competence-stimulating peptide (CSP) (Mortier-Barriere et al., 1998). This pheromone derives from a precursor, ComC, which is processed and exported by an ABC transporter, ComAB. It is assumed that a critical concentration of extracellular CSP is sensed by its receptor, the ComD histidine kinase, leading to autophosphorylation. The transfer of the phosphate group activates ComE, its cognate response regulator, inducing the expression of *comCDE* and several other operons (for a...
review, see Claverys et al., 2006). It has been described that another signal transduction system, designated CiaRH, participates in the early control of competence induction. The cia mutants showed a derepression of the comCDE operon and a consequent increase of natural competence (Echenique et al., 2000; Martin et al., 2000). However, the relationship between ComDE and CiaRH has been shown to be indirectly due to CiaR-binding sites not being identified in the comCDE upstream region.

Microarray analysis showed the transcription of approximately 100 genes being induced by CSP (Dagkessamanskia et al., 2004; Peterson et al., 2004), and they were classified as early, late and stress (or delayed) genes. The comCDE, comAB and comX genes belong to the early class and they are involved in the induction of the competence state, with their expression depending on ComE. ComX is a competence-specific sigma factor that induces the transcription of late genes needed for transformation (DNA uptake and recombination), as well as other physiological processes. Interestingly, Peterson et al. (2004) reported that approximately 67 of 124 genes were not related to competence, suggesting that expression of CSP-responsive genes may have other functions apart from genetic transformation. For this reason, Claverys et al. (2006) proposed that the X-state (ComX-induced physiological state) is a more appropriate term than competence in S. pneumoniae.

Various authors have shown a connection between autolysis and competence in S. pneumoniae (Steinmoen et al., 2002). It has been demonstrated that DNA release is caused by CSP-induced autolysis (Moscoso & Claverys, 2004; Steinmoen et al., 2002), with the cinA-recA-dinF-lytA operon being induced by ComX (Luo et al., 2003). In addition, it has been observed that competent cells are able to autolysate non-competent cells in trans (Steinmoen et al., 2003) by a bacteriocin-mediated mechanism named allolysis, which also involves LytA (Guiral et al., 2005; Havarstein et al., 2006).

ComDE has commonly been considered to be part of a quorum-sensing mechanism that senses CSP and induces competence in response to cell density (Pestova et al., 1996). However, it was recently revealed that competence can be induced independently of cell density, showing that a shift to alkaline pH can trigger this physiological state (Claverys et al., 2006). Although it is clear that competence induction is a CSP-dependent mechanism, this peptide was proposed to be a timing device rather than a pheromone (Claverys et al., 2006). This assumption was based on the fact that competence developed at a determined time after the inoculation of cells into the alkaline culture medium. The authors hypothesized that the induction of the X-state could be considered to be a global response to alkaline stress.

Bacterial cells are able to survive acidic conditions by a mechanism termed acid tolerance response (ATR; for a review, see Cotter & Hill, 2003). Among streptococcal species, the ATR of Streptococcus mutans is one of the best characterized, and it has been shown to be regulated by a CSP-dependent ComDE pathway (Li et al., 2001). Martin-Galano et al. (2005) reported an ATR mechanism in S. pneumoniae, showing that only a low percentage of the pneumococcal population is able to survive at pH 5.6. In our work, we observed that most of the dying cells suffered autolysis when incubated for several hours under acidic conditions. The acidic stress-induced autolysis observed in pneumococcal cells prompted us to investigate the putative regulatory mechanism of this phenomenon. The objective was to find a connection between acidic stress-induced autolysis and the ComDE TCS, since competence and DNA release by autolysis have been previously found to be related when cells were cultured at pH 7.8. Here we report the effect of acidic stress on autolysis, and demonstrate the contribution of ComE to the regulation of this process by a CSP-independent pathway. The roles of ComDE and CiaRH in the general response to stress in S. pneumoniae are also discussed.

**METHODS**

**Bacterial strains, growth and transformation conditions.** All strains used in this study and their relevant characteristics are listed in Supplementary Table S1, available with the online version of this paper. Stocks of strain R801 and their mutants were routinely prepared by culturing the cells in BHI at 37 °C to OD_{s_{20}, 0} = 0.3, followed by addition of glycerol 15% (v/v, final concentration) and storage at −80 °C. For ASIL assays, we used acetate basic medium (ABM; 30 g tryptone soy broth l^{−1}, 5 g tryptone l^{−1}, 1.25 g yeast extract l^{−1} and 80 mM sodium acetate). The pH of ABM was adjusted to 7.8 (with 10 M NaOH), 5.9 and 4.4 (with 10 M HCl). After autoclaving, 2 ml 20% glucose was added. Transformation assays were performed as described previously (Echenique et al., 2000). Briefly, pre-competent cells were obtained by growing the cells in CAT (Morrison et al., 1983) pH 6.8 to an OD_{s_{20}, 0} of 0.1. The cultures were centrifuged at 15 000 g for 2 min, resuspended in complete transformation medium (CTM) pH 7.8 (McDaniel et al., 1987) containing 15% glycerol and kept frozen at −80 °C. The pre-competent cells were gently thawed on ice, centrifuged at 5000 g for 10 min, resuspended in 500 μl fresh CTM pH 7.8 medium and incubated with synthetic CSP (500 ng ml^{−1}) at 37 °C for 10 min before addition of DNA. Cells were incubated at 30 °C for 30 min for DNA uptake, diluted 1:10 with CTM pH 7.8 and incubated 2 h at 37 °C to allow chromosome segregation and phenotypic expression. Transformants were selected by plating in BHI 5% sheep-blood agar supplemented with an appropriate antibiotic.

**Construction of insertion mutants.** Insertional-duplication mutagenesis was used to create lytA and cia null mutants in the R801 genetic background. To construct R801 lytA (RC820), an internal fragment of lytA was amplified using the F_{lyt1}/R_{lyt1} primers (all the oligonucleotides used in this work are listed in Supplementary Table S2, available with the online version of this paper), and subcloned in pCR2.1-TOPO (Invitrogen) to generate the pCRlytA plasmid. The EcoRI–BamHI fragment liberated from pCRlytA was ligated to plasmid pVA891 digested with EcoRI and BamHI. The resulting plasmid, pVAlytA, was used to transform strain R801. Transformants were selected on BHI 5% sheep-blood agar plates containing erythromycin at 2.5 μg ml^{−1}. The presence of the lytA mutation was confirmed by PCR using the F_{lyt1}/R_{lyt1} primers (Supplementary Table S2).
The ciaR and ciaH mutants (RC870 and RC880, respectively) were constructed by transformation of R801 with plasmids pPICaR and pPCiaH, respectively (Supplementary Table S1), and selection of transformants was performed in BHI 5 % sheep-blood agar plates supplemented with spectinomycin at 250 μg ml⁻¹, as described previously (Echenique et al., 2000). The cia mutants were confirmed by PCR using primers Fcia/Rcia (Supplementary Table S2).

PCR-ligation mutagenesis (Lau et al., 2002) was used to construct the comA mutants (RC840 and RC850). DNA fragments flanking the comA gene were generated by PCR using the primer pairs FcomA1/RcomA1 for the 5’ fragment, and FcomA2/RcomA2 for the 3’ fragment. A kanamycin cassette (aphA3 gene) was obtained from pPJ1 by BglII/Clal digestion, and ligated to fragments 5’ and 3’ digested with BglII and Clal, respectively. The ligation mixture was used to transform strain R801, and the selection of mutants was performed in BHI 5 % sheep-blood agar plates supplemented with kanamycin at 250 μg ml⁻¹. With a similar protocol, we constructed comA-:ermB (RC840). The 5’ and 3’ fragments were digested with Sall and Xhol, respectively, and ligated to ermB liberated from pEryMCS digested with Sall. The ligation mixture was used to transform strain R801 and the selection of mutants was made in BHI 5 % sheep-blood agar plates supplemented with erythromycin at 2.5 μg ml⁻¹. The presence of the comA mutations was confirmed by PCR using primers FcomA/RcomA.

The comE mutant (RC830) was constructed by transformation of R801 with pPComE-km previously described (Echenique et al., 2000). The selection of transformants was performed in BHI 5 % sheep-blood agar plates supplemented with kanamycin at 250 μg ml⁻¹.

We constructed the comX1 mutant (RC902) by transformation of R801 with the chromosomal DNA from strain CPM8 (Lee & Morrison, 1999), and mutants were selected in BHI 5 % sheep-blood agar plates supplemented with erythromycin at 2.5 μg ml⁻¹. In a second step, strain RC902 was transformed with the same DNA to obtain the comX1 comX2 double mutant (RC903), which was selected on BHI 5 % sheep-blood agar plates supplemented with tetracycline at 0.25 μg ml⁻¹.

Construction of the cup3 mutant. The construction of the comD213E (or cup3) mutant (RC860) was accomplished in a two-step transformation protocol. First, strain R801 was transformed by an amplicon generated with primers RcomD and FcomD containing a ClaI site in the comD213E mutation. Then, cells were centrifuged, resuspended in CAT pH 6.8 and transformed in this acidic medium as described by Lacks & Greenberg (2001), using chromosomal DNA from the CBA24 strain that carried an erythromycin resistance marker. Transformants were selected in BHI 5 % sheep-blood agar plates supplemented with erythromycin at 2.5 μg ml⁻¹. The mutation was confirmed by PCR amplification, ClaI digestion and DNA sequencing.

Construction of the comD7 mutant. The comD7163X (or comD7) mutant (RC890) was constructed by transformation of strain R801 with pGEMcomD7. This plasmid carries a 0.48 kb amplicon obtained by using primers FmutcomD7 and RcomDaux. FmutcomD7 contains two base substitutions that generate a stop codon and a HindIII restriction site at position 488 of the comD gene. Two hundred colonies were picked after the transformation without antibiotic selection, as described previously (Echenique & Trombe, 2001), then pooled into groups of five and grown overnight at 37 °C in 1 ml CAT medium. DNA samples were prepared by boiling for 10 min, and PCR was performed with primers FcomDaux/RcomDaux. The presence of the comD7163X mutation was confirmed by a HindIII digestion of the PCR products and by DNA sequencing.

RNA purification and real-time RT-PCR. Total RNA was extracted from cells by using the TRIZOL Reagent (Invitrogen) according to the manufacturer’s instructions with a few modifications. Frozen stocks of pneumococcal strains were diluted in 100 ml ABM/pH 7.8 to an OD620 of 0.01, grown at 37 °C to mid-exponential phase (OD620=0.30), centrifuged at 5000 g for 5 min and then resuspended in an equal volume of ABM/pH 5.9. Aliquots of 20 ml were collected after 0, 10 and 30 min incubation at 37 °C. The samples were centrifuged at 15 000 g for 30 s at room temperature and resuspended in 500 μl 0.1 % N-lauroylsarcosine (Sigma). Cells were immediately centrifuged at 15 000 g for 30 s at room temperature, resuspended in 400 μl pre-heated lysis solution (1 % SDS, 10 mM EDTA, 50 mM sodium acetate, pH 5.1 adjusted with acetic acid) and incubated at 100 °C for 5 min. Then, 1 ml TRIZOL was added per 200 μl cell suspension and RNA extraction was performed following the manufacturer’s instructions. The RNA pellet was resuspended in 100 μl RNase-free MilliQ water (obtained by MilliQ system, Millipore Corporation). In order to reduce DNA contamination, RNA samples (approx. 100 μg) were treated for 30 min with 5 U RQ1 DNase I (Promega). RNA was extracted again with 500 μl TRIZOL and resuspended in 30 μl RNase-free MilliQ water. Samples were stored frozen at −80 °C. Before the retrotranscription reaction, RNA was treated again with RQ1 DNase I 2 (1 U (μg RNA)⁻¹). The enzymatic reaction was stopped with RQ1 DNA Stop Solution (Promega) and incubated at 65 °C for 10 min to inactivate the DNase. The absence of DNA contamination was checked by PCR. The cDNA synthesis was carried out by using M-MLV reverse transcriptase (Promega) according to the manufacturer’s instructions. Real-time PCR was performed on a 7500 Real-Time PCR System apparatus (Applied Biosystems) with 2 μl of a 1/4 dilution of cDNA (40 ng reverse-transcribed total RNA), the SYBR Green PCR Master Mix (Applied Biosystems) and 26 pmol primers in a final volume of 30 μl. Cycling conditions were as follows: 1 × 95 °C, 10 min; 40 × 95 °C, 15 s and 60 °C, 1 min. Primer efficiency was verified by using a serial dilution of cDNA ranging from 10⁻² to 10⁻⁶. Primers were designed with Primer Express Software (Applied Biosystems) in order to amplify fragments of approximately 140 bp for each of the genes in the study. The relative gene expression was calculated using the 2⁻ΔΔCT method (Livak & Schmittgen, 2001). The reference gene was gyrB (Oggoni et al., 2004) and the reference condition was time 0 min of incubation in ABM/pH 5.9 of strain RC852.

RESULTS

Acidic stress triggers a LytA-dependent autolysis

We observed that cultures of strain R801 lost their turbidity when they were incubated under acidic conditions. To produce this phenomenon, the bacterial cells were first grown to an OD620 of 0.3 (exponential phase) in ABM/pH 7.8, then incubated for 3 h at 37 °C in ABM/pH 5.9 (sublethal pH value), resulting in a 60–70 % decrease in turbidity (Fig. 1a). We also used the same basic medium but replacing sodium acetate (pKₐ 4.76) with different acids that have similar dissociation constants, such as sodium citrate (pKₐ 4.76), sodium butyrate (pKₐ 4.77) and sodium lactate (pKₐ 3.86), using 80 mM concentration for each compound. All the R801 cultures autolysed in the different media when the pH was lowered from 7.8 to 5.9 (data not shown), indicating that the autolytic phenotype was a response to a pH change and it was not dependent on a particular anion. However, when we replaced acetate with MES [2-(N-morpholino)ethane-sulfonic acid; pKₐ 6.10], the R801 cultures did not show

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autolysis, suggesting that acidic stress-induced autolysis depends on the ability of the anions used in buffers to decrease the cytoplasmic pH. Hereafter, all the experiments presented in this work were performed with ABM.

We also noticed that strain R801 autolysed at pH values ranging from 5.0 to 5.9 (data not shown). However, when the cultures were directly incubated in ABM at a lethal pH of 4.4, no autolysis was detected, suggesting that only live cells were able to trigger this process (Fig. 1a). Furthermore, ASIL was also detected in different genetic backgrounds, such as the CP1015 and R6 hexA strains, when cultured under the same conditions as mentioned above (data not shown). To determine the contribution of the pneumococcal autolysins, we constructed a 

**ComE mediates acidic stress-induced autolysis by a CSP-independent pathway**

It has been reported that autolysis in *S. pneumoniae* is induced by exposure to antibiotics, such as penicillin and vancomycin (Cotter & Hill, 2003), as well as triggered during competence development. Related to this, Moscoso & Claverys (2004) demonstrated that autolysis is responsible for DNA release after CSP addition, and this event is mediated by the LytA and LytC autolysins. It is known that competence development can be prevented by incubation in culture medium of pH 6.8 or lower (Tomasz & Mosser, 1966; Chen & Morrison, 1987), suggesting that the ComE pathway is not activated under acidic conditions. Nevertheless, we decided to investigate whether ComE could be involved in this autolytic effect induced by acidic stress. For this purpose, the *comE* mutant (constructed as described by Echenique *et al.*, 2000) was exposed to pH 5.9 and autolysis was evaluated. The *comE* strain was unable to induce ASIL (Fig. 1b), demonstrating that this process was also mediated by this response regulator. As mentioned, ComE belongs to the two-component system ComDE and is activated by CSP to induce competence at alkaline pH. Consequently, we investigated whether ASIL was also dependent on this regulatory mechanism, using a known strategy to block the CSP circuit by mutating the *comAB* genes encoding the transporters responsible for CSP export (Echenique *et al.*, 2000; Hui *et al.*, 1995; Martin *et al.*, 2000). The *comA* mutant showed the same autolytic profile as that of strain R801 (Fig. 1b), and this finding was in agreement with the fact that ASIL was constant throughout the growth curve of strain R801, independent of the cellular density (data not shown). The *comA* *comE* mutant showed inhibition of ASIL, indicating that the autolysis displayed by the *comA* mutant was also dependent on ComE. Taken together, these results indicated that ComE regulates the activation of ASIL by a CSP-independent mechanism.

**The ComE-mediated ASIL regulation is independent of ComX**

ComX, a competence-specific sigma factor encoded by the *comX* genes, which is activated by ComE, is required for the CSP-induced expression of the late *com* genes (Peterson *et al.*, 2004). To investigate whether ComX is also involved in the ComE-regulated ASIL, we constructed *comX* mutants and evaluated lysis under acidic conditions. Because there are two copies of *comX* in the chromosome, we constructed the double mutant *comX1 comX2* as described previously, using the chromosomal DNA of strain CPM8 (Lee & Morrison, 1999) to transform strain R801. This mutant strain showed no competence phenotype when cells were induced by CSP (data not shown), as

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**Fig. 1.** (a) Acidic stress triggers a LytA-dependent autolysis. Strains R801 (filled symbols) and RC820 (*lytA*; open symbols) were grown in ABM/pH 7.8 to mid-exponential phase (OD<sub>620</sub> = 0.3) and resuspended in ABM/pH 4.4 (▲, △), ABM/pH 5.9 (●, ○) and ABM/pH 7.8 (■, □). Autolysis was measured as a change in OD<sub>620</sub> over 6 h. Data are representative of at least three independent experiments. (b) The acidic stress-induced autolysis is ComE-mediated by a CSP/ComX-independent pathway. Strains R801 (■), RC840 (*comA; ●), RC843 (*comA comE; ▼), RC830 (*comE; ●) and RC903 (*comX1 comX2; ▲) were grown in ABM/pH 7.8 to mid-exponential phase (OD<sub>620</sub> = 0.3) and resuspended in ABM/pH 5.9. Autolysis was measured as a change in OD<sub>620</sub> over 6 h. Data are representative of at least three independent experiments.

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was originally described by Lee & Morrison (1999). When ASIL was assayed, the comX1 comX2 mutant exhibited the same phenotype as strain R801 (Fig. 1b), indicating that the ASIL induction is not dependent on ComX.

Hyperactive ComD increases acidic stress-induced autolysis

The fact that ComE mediates ASIL by a CSP-independent pathway does not imply that ComE acts in its unphosphorylated state. To assess the putative phosphorylation state of ComE, we constructed a hypercompetent mutant, described before, the comD^{T233I} or cup3 strain (Martin et al., 2000), which shows CSP-independent competence. This mutant was created by transformation of mutation-containing amplicons (as described in Methods), and was selected under conditions that prevented competence. The hypercompetent phenotype of cup3 was confirmed by natural competence in CTM at pH 7.8 (data not shown). When the ASIL was examined, the cup3 mutant displayed an accelerated decrease in turbidity compared with strain R801 (Fig. 2a), demonstrating that the hyperactive ComD facilitated the induction of autolysis. As proposed by Martin et al. (2000), this mutant probably mimics the competence phenotype of a phosphorylated ComD and, consequently, a phosphorylated ComE. The cup3 comE double mutant again showed an ASIL inhibition, confirming that this process was mediated by ComE.

ComE is not only activated by ComD for ASIL induction

Considering that ComE needs to be activated under acidic conditions to induce ASIL, and that the pathway activation is independent of CSP, we investigated whether ComE activation is strictly dependent on ComD, its cognate histidine kinase. To avoid polar mutations, we introduced a stop codon into the comD gene to generate the comD^{F163X} or comD^{T} mutant which encodes a truncated protein lacking the histidine kinase domain (Supplementary Table S1). The comD^{T} mutant was not able to transform DNA by natural competence or by CSP addition assays at pH 7.8 (data not shown), but it showed the same autolytic profile that R801 had when it was exposed to pH 5.9 (Fig. 2a). So far, these results suggest that ComE is activated by ComD, but it could also be activated by another mechanism, which is still unknown.

CiaRH protects cells from acidic stress-induced autolysis

As mentioned before, CiaRH is a TCS involved in competence regulation, since cia mutants showed comE derepression and a CSP-dependent autolysis induction at alkaline pH (Echenique et al., 2000). In addition, CiaRH was proposed to be an essential mechanism to support stress conditions and to prevent autolysis (Dagkessamanskaya et al., 2004; Mascher et al., 2006). Based on these results, we analysed the putative contribution of CiaRH to the ComE-regulated ASIL phenomenon. We found an enhanced autolytic profile in the ciaR (Fig. 2b) and ciaH (data not shown) mutants, similar to that shown by the cup3 mutant.

Autolysis was not induced in the ciaR lytA or ciaH lytA mutants, demonstrating that LytA was also responsible for the ciaRH-mediated autolysis (Fig. 2b). These data suggest that CiaRH is required to protect S. pneumoniae cells from the autolysis caused by acidic stress. This assumption was also confirmed by analysing cia mutants at pH values ranging from 5.6 to 6.4, which revealed a clear difference since cia mutants autolysed at pH 6.0, whereas R801 showed a constant turbidity (data not shown). The comCDE derepression shown by cia mutants and the

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**Fig. 2.** (a) Hyperactive ComD increases acidic stress-induced autolysis. Strains R801 (■), RC860 (comD^{T233I} or cup3; ▲), RC863 (cup3 comE; ●) and RC890 (comD^{T}; △) were grown in ABM/pH 7.8 to mid-exponential phase (OD\textsubscript{620}=0.3) and resuspended in ABM/pH 5.9. Autolysis was measured as a change in OD\textsubscript{620} over 6 h. Data are representative of at least three independent experiments. (b) CiaRH protects cells from acidic stress-induced autolysis by a ComE-independent mechanism. Strains R801 (■), RC870 (ciaR; ●), RC830 (comE; ○), RC873 (ciaR comE; ▲), and RC872 (ciaR lytA; ▼) were grown in ABM/pH 7.8 to mid-exponential phase (OD\textsubscript{620}=0.3) and resuspended in ABM/pH 5.9. Autolysis was measured as a change in OD\textsubscript{620} over 6 h. Data are representative of at least three independent experiments.
ComE dependence of the ASIL led us to suppose that the cia phenotype was mediated by ComE. However, both the ciaR comE (Fig. 2b) and the ciaH comE (data not shown) double mutants retained an enhanced ASIL, demonstrating that the cia mutations had an epistatic effect on the non-autolytic comE phenotype.

**Acidic stress induces transcription of the comE gene**

At alkaline pH, transcription of the comCDE operon is induced by ComE, which has been proposed to be phosphorylated by ComD in response to CSP. In the present work, we demonstrated that ASIL is regulated by a CSP-independent ComE pathway. Consequently, we suspected that transcription of the comE gene could be induced by acidic stress instead of CSP. To test this hypothesis, we assessed the comE transcript levels in different mutants exposed to pH 5.9 using a quantitative real-time RT-PCR assay. The lytA mutation was introduced into the different mutants in the R801 genetic background to avoid loss of RNA due to ASIL, and to allow for the purification of total RNA from cells cultured at pH 5.9. These assays revealed an increase in the relative expression levels of comE transcripts at 10 and 30 min of acidic pH exposure, with the same profile for the lytA and comA lytA strains (Fig. 3a). These data confirmed that acidic pH induces the comE expression by a CSP-independent pathway. We suggested before that hyperactive ComD could produce ComE phosphorylated in the cup3 mutant, and this phosphorylation state would then help to trigger ASIL. The comE transcripts obtained from the cup3 lytA mutant showed a constitutive high level rising to almost a 1000-fold increase compared with comA lytA (Fig. 3a), indicating that ComE needed to be phosphorylated to induce ASIL.

Furthermore, analysis of comE transcripts from the comD<sup>T</sup> lytA mutant revealed a similar profile to R801, although the induction was retarded in the first 10 min. The fact that comE transcripts were induced in a comD<sup>T</sup> background indicated that ComE was still activated by acidic stress, and by a mechanism different to ComD.

In addition, we have demonstrated that the ASIL induction is independent of ComX. We analysed the transcript levels of the recA and cglB genes, whose expression is mediated by ComX and which have been reported as late competence genes (Peterson et al., 2004). We observed no changes in the transcript levels of both genes, confirming that ComX is not involved in the ASIL induction (Fig. 3b and Supplementary Fig. S1).

On the other hand, we showed that the ciaR comE mutant presented a ComE-independent ASIL regulation (Fig. 2b). Then, as it is known that the comCDE operon is overexpressed in a ciaR mutant at pH 7.8 (Echenique et al., 2000), we were curious to know about the comE regulation under acidic conditions for the same genetic background. Interestingly, when comE transcripts were assessed, an initial 50-fold increase was found at time 0 min corresponding to cells previously grown at pH 7.8.

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**Fig. 3.** Kinetics of comE (a) and cglB (b) transcripts in response to acidic stress. The transcription levels were measured in cells exposed to pH 5.9. Strains RC852 (comA lytA), RC820 (lytA), RC892 (comD<sup>T</sup> lytA), RC872 (ciaR lytA) and RC862 (comD<sub>cup3</sub> lytA) were grown in ABM/pH 7.8 to mid-exponential phase and resuspended in ABM/pH 5.9 and RNA was extracted at 0 min, 10 min and 30 min. Fold change in gene expression was measured by quantitative real-time RT-PCR and was calculated using the $2^{-ΔΔCT}$ method. The gyrB gene was used as the internal control and the reference condition was time 0 min of strain RC852. Error bars indicate the standard deviation of the mean. The INSTAT software was used to perform Dunnet’s statistical comparison test for each strain with its respective basal condition (time 0 min). **P<0.01; ***P<0.001.
However, when the ciaR mutant was exposed to acidic pH, the comE transcription was downregulated by approximately 25-fold at 10 min and rose again at 30 min to the same comE transcript level shown by the R801 and comA strains (Fig. 3a). This finding suggests that the comE transcripts were only induced by acidic conditions in the ciaR mutant, which did not show the same comE increase observed under alkaline conditions.

**DISCUSSION**

We report here for the first time that autolysis is triggered by acidic stress in *S. pneumoniae*, this being a new lysis-inducing condition. *S. pneumoniae* may suffer this kind of stress in different host environments. For instance, it has been described that the brain interstitial pH decreased to 6.8 in purulent experimental meningitis of *S. pneumoniae*-infected rabbits (Andersen et al., 1989). In addition, pleural effusions are frequently found in patients with acute bacterial pneumonia caused by *S. pneumoniae*. Light et al. (1980) determined that pH values obtained from pleural fluids of the most complicated infections were close to 6.80. Notably, the lowest pH value that *S. pneumoniae* has been shown to be tolerant to is around 4.4 in phagosomal vesicles during the first few minutes after phagocytosis (Bassoe & Bjerknes, 1985). On the other hand, we think that ASIL should also be considered from a pathogenic point of view, due to *S. pneumoniae* being able to release virulence factors (pneumolysin), pro-inflammatory bacterial cell-wall compounds and nucleic acids, as suggested by different authors (Chatellier & Kotb, 2000; Nau & Eifert, 2002).

It has been reported that the undissociated forms of weak acids found at acidic pH (such as sodium acetate, used to buffer acidic cultures in this work) pass freely through the cell membrane. Once in the cytoplasm, usually at a slightly alkaline pH, the weak acid dissociates and releases a proton producing an acidification (Cotter & Hill, 2003). It is known that acidification produces an increased loss of purines and pyrimidines from DNA, compared to neutral or alkaline pH (Lindahl & Nyberg, 1985). We assume that if this proton input is prolonged in time, the acid-tolerance response may be inefficient and the cytoplasmic pH could decrease until protein denaturation and DNA damage is provoked. In the face of this particular stress situation, and probably depending on the damage levels, we propose that cells sense this signal and make a decision between either a survival or a suicidal programme.

In addition to competence development, we showed that ASIL is also regulated by ComE. This conclusion is based on the fact that ASIL was abolished in the comE mutant, with the hyperactive cup3 mutant showing an early and increased ASIL (Fig. 2a). One of the most interesting findings was that ASIL was not only independent of cellular density or quorum sensing, as suggested for competence (Claverys et al., 2006), but also independent of CSP. Furthermore, we observed an increase in comE transcripts when cells were incubated at pH 5.9. Considering this result together with that showing an increased ASIL in the hyperactive cup3 mutant, we conclude that ComE could be sensing, either directly or not, the acidic stress.

Because CSP is the typical activation signal that is sensed by ComD, the fact that ASIL could be triggered by a CSP-independent pathway led us to question whether ComD is the unique mechanism that could activate ComE. The analysis of the comD mutant revealed that ASIL was still activated, and the finding that comE transcripts were induced in this mutant under acidic culture conditions reinforced the hypothesis that ComE may also be activated in response to acidic stress by another unknown mechanism that would act in parallel to ComD.

An interconnection between ComE and autolysis has been previously established, involving another two-component system, CiaRH (Dagkessamanskaia et al., 2004). These authors showed that stationary-phase autolysis is triggered by CSP in the ciaR mutant, suggesting that CiaRH is required to support the physiological changes produced during the competence development and for a normal exit from competence. Moreover, a microarray analysis revealed a derepression of comCDE transcripts in the ciaR mutant, in agreement with other similar analyses (Mascher et al., 2003; Sebert et al., 2002) and also with Northern blots (Echenique et al., 2000; Martin et al., 2000). Dagkessamanskaia et al. (2004) also reported an enhanced induction of stress-response genes in the ciaR mutant, suggesting that this strain was less tolerant to stress conditions than strain R801. In the context of the present work, we speculate that CiaRH could have had a preventative role in the induction of autolysis in response to acidic stress. This hypothesis was supported by the early ASIL shown by the cia mutants, clearly indicating that CiaRH was playing a protective role against this particular programmed phenomenon. Taking into account the connection described for CiaRH and ComDE (Echenique et al., 2000; Moscoso & Claverys, 2004), we expected that the comE mutation could have a negative impact on the cia-triggered autolysis under acidic conditions. Curiously, both the ciaR comE and the ciaH comE double mutants showed the same early ASIL as that of the cia mutants. These results indicated that ASIL in the cia mutant is caused by a ComE-independent activation pathway, and work is currently in progress to find out the putative signalling pathway by which CiaRH exerts a protective role against ASIL induction.

As already mentioned, Peterson et al. (2004) hypothesized that CSP-induced genes may have functions other than transformation. Claverys et al. (2006) agreed with the idea that the particular state induced by ComE through ComX (named the X-state) is more extensive than competence. They also suggested that the X-state should be considered as a global response to alkaline stress rather than a quorum-sensing mechanism, despite its induction still...
being dependent on CSP. These hypotheses led us to assume that ASIL could be part of the X-state. However, we demonstrated that ComX is not involved in the induction of ASIL. Here, we report a different activation pathway of ComE, independent of CSP and ComX, which does not fit exactly into the signalling transduction scheme known so far. Our hypothesis is that ComE is the principal route of the signalling pathway that determines a global stress response, as reported for alkaline pH (Claverys et al., 2006) and antibiotics (Prudhomme et al., 2006), as well as for acidic pH in the current work.

Summing up, this study of the ASIL regulation has allowed us to analyse ComDE in a different context to competence regulation, and the ASIL model represents a new scenario to study the signal transduction systems that regulate stress response in \textit{S. pneumoniae}.

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