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.

Received 13 December 2007

Revised 28 January 2008

Accepted 24 February 2008

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

Abbreviations: ASIL, acidic stress-induced lysis; CSP, competence-stimulating peptide; TCS, two-component signal transduction system; X-state, ComX-induced physiological state.

Supplementary tables of strains and primers, and a supplementary figure, are available with the online version of this paper.

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 (Dagkessamanskaia *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 autolyse 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-Galiano *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 OD₆₂₀ ~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 OD₆₂₀ 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_{lyt2} and 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*-*Bam*HI fragment liberated from pCRlytA was ligated to plasmid pVA891 digested with *EcoRI* and *Bam*HI. 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 pPCiaR and pPCiaH, respectively (Supplementary Table S1), and selection of transformants was performed in BHI 5% sheep-blood agar plates supplemented with spectinomycin at 50 µg ml⁻¹, as described previously (Echenique *et al.*, 2000). The *cia* mutants were confirmed by PCR using primers F_{cia}/R_{cia} (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 F_{comA1}/R_{comA1} for the 5' fragment, and F_{comA2}/R_{comA2} for the 3' fragment. A kanamycin cassette (*aphA3* gene) was obtained from pPJ1 by *Bgl*II/*Cl*aI digestion, and ligated to fragments 5' and 3' digested with *Bgl*II and *Cl*aI, 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 *Sal*I and *Xho*I, respectively, and ligated to *ermB* liberated from pEryMCS digested with *Sal*I. 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 F_{comA}/R_{comA}.

The *comE* mutant (RC830) was constructed by transformation of R801 with pPComE-*km* as 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 *comD*^{T233I} (or *cup3*) mutant (RC860) was accomplished in a two-step transformation protocol. First, strain R801 was transformed by an amplicon generated with primers R_{comD} and F_{McomD}, which contained a *Cl*aI site in the *comD*^{T233I} 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, *Cl*aI digestion and DNA sequencing.

Construction of the *comD*^T mutant. The *comD*^{F163X} (or *comD*^T) mutant (RC890) was constructed by transformation of strain R801 with pGEMcomD^T. This plasmid carries a 0.48 kb amplicon obtained by using primers F_{mutcomD} and R_{comDaux}. F_{mutcomD} contains two base substitutions that generate a stop codon and a *Hind*III 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 F_{comDatg}/R_{comDaux}. The presence of the *comD*^{F163X} mutation was confirmed by a *Hind*III 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 [1 U (µg RNA)⁻¹]. The enzymic 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 analysed by using the 2^{-ΔΔCt} method (Livak & Schmittgen, 2001). The reference gene was *gyrB* (Oggioni *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 (pK_a 4.76) with different acids that have similar dissociation constants, such as sodium citrate (pK_a 4.76), sodium butyrate (pK_a 4.77) and sodium lactate (pK_a 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)ethanesulfonic acid; pK_a 6.10], the R801 cultures did not show

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 ($OD_{620}=0.3$) and resuspended in ABM/pH 4.4 (▲, △), ABM/pH 5.9 (●, ○) and ABM/pH 7.8 (■, □). Autolysis was measured as a change in OD_{620} 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 ($OD_{620}=0.3$) and resuspended in ABM/pH 5.9. Autolysis was measured as a change in OD_{620} over 6 h. Data are representative of at least three independent experiments.

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 *lytA* mutant, obtained by insertion-duplication mutagenesis (Supplementary Table S1). In this mutant, ASIL was completely inhibited (Fig. 1a), thus indicating that LytA was necessary for triggering this acid-induced process.

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

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 (Dagkessamanskaia *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

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 (OD₆₂₀=0.3) and resuspended in ABM/pH 5.9. Autolysis was measured as a change in OD₆₂₀ 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 (OD₆₂₀=0.3) and resuspended in ABM/pH 5.9. Autolysis was measured as a change in OD₆₂₀ 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^T 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^T* 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.

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^T lytA*), RC872 (*ciaR lytA*) and RC862 (*comD^{cup3} 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 $2^{-\Delta\Delta CT}$ 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 & Eiffert, 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, 1972). 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^T* 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 *S. pneumoniae*.

ACKNOWLEDGEMENTS

We thank Don Morrison (University of Illinois at Chicago) for encouragement during the course of this work, for his critical review of a previous version of this manuscript, and for providing us the CP1015 strain and the chromosomal DNA from the CPM8 strain. We also thank Dr L. Tao (University of Illinois at Chicago) for providing us the pVA891 plasmid, and native speaker Dr Paul Hobson (Cultura Británica-Argentina) for revising this manuscript. Financial support: Fundación Antorchas; National Council of Scientific and Technological Research (CONICET); National Agency of Scientific and Technological Promotion (ANPCYT; grant PICT 05-10894 BID 1728 OC-AR) and the Scientific and Technological Secretary of the National University of Córdoba (SECYT-UNC). G.E.P. had a PhD fellowship from SECYT-UNC and is now a PhD fellow of CONICET. A.G.A.O. had a fellowship from ANPCYT and is now a PhD fellow of CONICET. J.E. is member of the Research Career of CONICET.

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Edited by: M. Kilian