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# Stress-triggered signaling affecting survival or suicide of *Streptococcus pneumoniae*

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ABSTRACT

*Streptococcus pneumoniae* is a major human pathogen that can survive to stress conditions, such as the acidic environment of inflammatory foci, and tolerates lethal pH through a mechanism known as the acid tolerance response. We previously described that *S. pneumoniae* activates acidic-stress induced lysis in response to acidified environments, favoring the release of cell wall compounds, DNA and virulence factors.

Here, we demonstrate that $F_0F_1$-ATPase is involved in the response to acidic stress. Chemical inhibitors (DCCD, optochin) of this proton pump repressed the ATR induction, but caused an increased ASIL. Confirming these findings, mutants of the subunit *c* of this enzyme showed the same phenotypes as inhibitors. Importantly, we demonstrated that $F_0F_1$-ATPase and ATR are necessary for the intracellular survival of the pneumococcus in macrophages.

Alternatively, a screening of two-component system (TCS) mutants showed that ATR and survival in pneumocytes were controlled in contrasting ways by ComDE and CiaRH, which had been involved in the ASIL mechanism. Briefly, CiaRH was essential for ATR (ComE represses activation) whereas ComE was necessary for ASIL (CiaRH protects against induction). They did not regulate $F_0F_1$-ATPase expression, but control LytA expression on the pneumococcal surface.

These results suggest that both TCSs and $F_0F_1$-ATPase control a stress response and decide between a survival or a suicide mechanism by independent pathways, either in vitro or in pneumocyte cultures. This biological model contributes to the current knowledge about bacterial response under stress conditions in host tissues, where pathogens need to survive in order to establish infections.



## Introduction

Q3 *Streptococcus pneumoniae* (or the pneumococcus) is one of the main human pathogens, being the causal agent of otitis and sinusitis, as well as of more severe diseases such as pneumonia, bacteremia and meningitis. During the infection process, *S. pneumoniae* has to be able to survive the environmental stress found in different host tissues. Related to this, it has been described that an acidic environment (pH 5.5–7.0) is a common characteristic of the acute inflammation produced by bacterial metabolisms and lactate production of infiltrating neutrophils and macrophages at the inflammatory foci (Park and Kim, 2013). For example, pleural effusions are frequently found in patients with acute bacterial pneumonia caused by *S. pneumoniae*, with reports of pH values close to 6.8 (Light et al., 1980). In addition, the brain interstitial pH has been observed to decrease to 6.8 in purulent experimental meningitis of *S. pneumoniae*-infected rabbits (Andersen et al., 1989). 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 minutes after phagocytosis (Bassoe and Bjerknes, 1985). Moreover, it was demonstrated that *S. pneumoniae* was able to live several hours in endothelial cells in a survival test using extracellular antibiotics (Gradstedt et al., 2013).

Many bacteria are able to survive acidic conditions using different strategies. One of the most relevant mechanisms in streptococci is the extrusion of protons from the cytoplasm by a proton pump, known as $F_0F_1$-ATPase. The $F_0$ complex, comprised of subunits *a*, *b* and *c*, is associated to the membrane and has a proton-translocating

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activity that allows this enzyme to act as a proton pump. However, this is not the only mechanism responsible for bacterial survival under acidic conditions. In fact, it has been proposed that the acidic stress response is a combination of various strategies, such as removal of protons, alkalinization of the external environment, expression of chaperones and proteases, DNA repair mechanisms and the participation of signal transduction systems to regulate gene expression (Cotter and Hill, 2003).

Some bacterial pathogens are able to survive under acidic conditions by a mechanism termed acid tolerance response (ATR), a phenomenon whereby the tolerance of an organism to lethal pH increases as a consequence of a prior exposure to a sublethal pH (Cotter and Hill, 2003). In order to survive intracellularly, bacterial pathogens have to resist the acidic environments encountered during the phagocytosis or endocytosis processes in macrophages or epithelial cells, respectively, with the phagosomal pH being low (pH 4.5–5.5) due to the activity of the vacuolar ATPase. For example, *Listeria monocytogenes* is an intracellular pathogen that is able to survive acidic stress by ATR induction. Acid-adapted cells were shown to have a nine-fold increase in invasion efficiency in the enterocyte-like cell line Caco-2 and in J774.A1 macrophages compared with non-adapted cells (Conte et al., 2000). Related to this, it was suggested that the capacity to invade Caco-2 cells depends on ATR induction (Werbrouck et al., 2009). Recently, it was reported that an acid shock (pH 5.0) caused a twelve-fold increase in the number of *L. monocytogenes* inside Caco-2 epithelial cells (Neuhaus et al., 2013).

An ATR mechanism was also reported in *S. pneumoniae*, showing that a subpopulation of pneumococci was able to survive a lethal pH of 4.4 when log phase cells of strain R6 were previously incubated at pH 5.9 (Martin-Galiano et al., 2005). For cells incubated directly at pH 4.4, the survival rate was $10^{-4}$, but cells pre-incubated for 2 h prior to lethal pH had a ten-fold increase in the survival rate. In addition, these authors also demonstrated by microarray assays that adaptation to acidic stress altered the expression of 126 genes, suggesting that this stress response is complex and involves different cellular processes, such as protein fate and the transport of manganese and iron.

In our laboratory, we described that acidic stress was also capable of inducing autolysis in *S. pneumoniae* when incubated for more than one hour at pH 5.9 (Pinas et al., 2008), and named this process acidic-stress induced lysis (or ASIL). In *S. pneumoniae*, autolysis is executed mainly by the major autolysin LytA. Acidic stress may represent for *S. pneumoniae* an alternative condition, in addition to competence and antibiotics, to assure the release by autolysis of DNA, cell wall compounds and virulence factors.

It has been described that autolysis is triggered by competence development at pH 7.8 and regulated by the two-component system ComDE (Claverys et al., 2009). The LytA autolysin is encoded by the *lytA* gene and located in the same operon as *recA*. The latter encodes a protein responsible for the homologous recombination of exogenous DNA, which belongs to the *cinA-recA-dinF-lytA* operon and is regulated by ComDE during competence development. Briefly, this TCS senses a competence-stimulating peptide (CSP) and behaves as a quorum sensing mechanism (Mortier-Barriere et al., 1998), with CSP being a pheromone derived from a precursor, ComC, which is processed and exported by the ABC transporter ComAB. It is assumed that a critical concentration of extracellular CSP is sensed by its receptor, the ComD histidine kinase, thereby leading to autophosphorylation. The transfer of the phosphate group activates ComE, its cognate response regulator, inducing the expression of *comCDE* and several other genes, such as *comX*. This gene codes for ComX (Claverys et al., 2006), a competence-specific sigma factor that induces the transcription of late genes needed for transformation (DNA uptake and recombination), such as the *cinA-recA-dinF-lytA* operon.

The ComDE-controlled regulatory circuit that leads to competence development takes place under the slightly alkaline pH of 7.8. In contrast, we reported that acidic stress triggers LytA-mediated autolysis, and curiously, this phenomenon was regulated by a CSP-independent ComE pathway, in which ComE did not require ComD phosphorylation to trigger autolysis at pH 5.9. We also investigated CiaRH, a TCS that participates in the early control of competence induction, cefotaxime resistance, stress response and virulence (Echenique et al., 2000; Giammarinaro et al., 1999; Pinas et al., 2008). Under acidic conditions, the *null* ciaR mutant showed accelerated autolysis, with the analysis of *ciaR comE* double mutants revealing that CiaRH protects cells from ASIL by a ComE-independent pathway (Pinas et al., 2008). We proposed that ComE is the principal route of the signaling pathway that determines a global stress response. Considering that the same type of stress provokes the induction of autolysis and the acid-tolerance response, we investigated the possible contribution of the pneumococcal $F_0F_1$-ATPase, the putative signaling systems that may control these contrasting mechanisms and their impact on the intracellular survival when infecting pneumocytes or macrophages. Here, we demonstrated the relevance of $F_0F_1$-ATPase as well as the TCSs ComDE and CiaRH in the control of the antagonistic processes ATR and ASIL, both in vitro and in tissue cultures, where bacterial cells must overcome the acidic pH in endosomes in order to survive or to induce autolysis and die. This is the first report on TCSs that control a stress response and decide between a survival and a suicide mechanism.

## Materials and methods

### *Bacterial strains, growth and transformation conditions*

All strains used in this study and their relevant characteristics are listed in Supplementary material (Table S1). The growth conditions and stock preparation for pneumococcal and *Escherichia coli* strains have been reported elsewhere (Pinas et al., 2008), and the transformation assays have also been previously described (Albarracin Orio et al., 2011; Echenique et al., 2000).

### *Cell lines and culture conditions*

Human lung epithelial carcinoma (A549) and murine macrophage (RAW264.7) cell lines were cultured at 37 °C, 5% $CO_2$ in Dulbecco's modified Eagle medium (DMEM) with 4.5 g/l of glucose and 10% of heat-inactivated fetal bovine serum (FBS) (Gibco BRL, Gaithersburg, Md.). Fully confluent A549 or RAW264.7 cells were split once every two or three days via trypsin/EDTA treatment and diluted in fresh media before being cultivated in Filter cap cell flasks 75 $cm^2$ (Greiner Bio-one no. 658175) until passage 6.

### *Acid tolerance response assays*

ATR experiments were carried out in THYE medium (30 g/l Todd-Hewitt, 5 g/l yeast extract). Prior to sterilization the pH was adjusted to pH 7.8 (with 1 N NaOH) or to pH 5.9 and 4.4 (with 10 N HCl). To determine survival to a lethal pH of 4.4 under non-acid-induced conditions, the pneumococcal strains were first grown at 37 °C in THYE (pH 7.8). When cultures reached $OD_{600\,nm} \sim 0.3$, 100 μl aliquots were taken and added to 900 μl of THYE (pH 4.4) and incubated for 2 h at 37 °C. Then, serial dilutions were made in THYE (pH 7.8) and plated onto 5% of sheep blood tryptic-soy agar (TSA) plates. After 48 h of incubation at 37 °C, colonies were counted to determine the number of survivors, with the total CFU being obtained by plating serial dilutions of cells grown THYE pH 7.8 onto 5% sheep blood TSA, made just before being switched



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