Tyrosine phosphorylation enhances activity of pneumococcal autolysin LytA

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Tyrosine phosphorylation has long been recognized as a crucial post-translational regulatory mechanism in eukaryotes. However, only in the past decade has recognition been given to the crucial importance of bacterial tyrosine phosphorylation as an important regulatory feature of pathogenesis. This study describes the effect of tyrosine phosphorylation on the activity of a major virulence factor of the pneumococcus, the autolysin LytA, and a possible connection to the Streptococcus pneumoniae capsule synthesis regulatory proteins (CpsB, CpsC and CpsD). We show that in vitro pneumococcal tyrosine kinase, CpsD, and the protein tyrosine phosphatase, CpsB, act to phosphorylate and dephosphorylate LytA. Furthermore, this modulates LytA function in vitro with phosphorylated LytA binding more strongly to the choline analogue DEAE. A phospho-mimetic (Y264E) mutation of the LytA phosphorylation site displayed similar phenotypes as well as an enhanced dimerization capacity. Similarly, tyrosine phosphorylation increased LytA amidase activity, as evidenced by a turbidometric amidase activity assay. Similarly, when the phospho-mimetic mutation was introduced in the chromosomal lytA of S. pneumoniae, autolysis occurred earlier and at an enhanced rate. This study thus describes, to our knowledge, the first functional regulatory effect of tyrosine phosphorylation on a non-capsule-related protein in the pneumococcus, and suggests a link between the regulation of LytA-dependent autolysis of the cell and the biosynthesis of capsular polysaccharide.

INTRODUCTION

Tyrosine phosphorylation is rapidly becoming a major focus of bacterial research, with studies illustrating its critical link to bacterial pathogenicity (Standish & Morona, 2014; Whitmore & Lamont, 2012). Indeed, we have been amongst those showing a link, with our long interest in the role of bacterial tyrosine kinase (BY-kinase) CpsD and protein tyrosine phosphatase (PTP) CpsB in the regulation of capsule synthesis in the major human pathogen Streptococcus pneumoniae, and as a novel target for the development of antimicrobials (Byrne et al., 2011; Ericsson et al., 2012; Morona et al., 2000, 2002, 2006; Standish et al., 2012, 2013). However, we are also interested in whether tyrosine phosphorylation plays roles outside capsule biogenesis, regulating the function of various proteins via specific tyrosine phosphorylation.

Since early in the last century, the pneumococcus has been recognized to possess a characteristic autolysin induced during the stationary phase of growth (Goebel & Avery, 1929). This has since been shown to be caused by the product of the lytA gene, an N-acetylmuramoyl-L-alanine amidase (García et al., 1985). LytA belongs to a family of proteins known as choline binding proteins (CBPs) (Rosenow et al., 1997), which while having diverse functions all share the ability to bind phosphorylcholine residues present in the pneumococcal cell wall (Rosenow et al., 1997). For LytA, binding to choline is essential for its amidase activity. LytA resides in the cytoplasm in the inactive E-form, with binding to choline present in the cell wall (Giudicelli & Tomasz, 1984), and subsequent dimerization, resulting in formation of the C-form which possesses functional amidase activity. The structure of the choline binding domain of LytA has illustrated that it contains a total of six choline binding repeats, which are characteristic of CBPs, along with a total of four choline binding sites (Fig. 1) (Fernández-Tornero et al., 2001, 2002). Unlike other CBPs, LytA does not possess a signal sequence, and to date it is unknown how it translocates to the cell wall, in order to bind phosphorylcholine and hydrolyse the cell wall.

The exact role of LytA in S. pneumoniae physiology is still unclear, with some suggestion that it is required for the release of the toxin pneumolysin (Martner et al., 2008), as well as contributing to bacterial fractricide (Eldholm et al., 2009). However, while its function is still debated, it is recognized as a virulence factor, with mutation resulting in decreased ability to cause disease in in vivo models (Berry & Paton, 2000; Dalia & Weiser, 2011).

A recent study on the phosphoproteome of S. pneumoniae identified LytA as one of 12 proteins phosphorylated on

**Abbreviations:** BY-kinase, bacterial tyrosine kinase; CBP, choline binding protein; PTP, protein tyrosine phosphatase.
tyrosine (Sun et al., 2010). With CpsD the only BY-kinase identified to date in S. pneumoniae, and both proteins known to localize to the cell septum (Henriques et al., 2011; Mellroth et al., 2012), we hypothesized that CpsD plays a role in the phosphorylation of LytA, and that phosphorylation regulates its amidase activity. This study shows for the first time an effect of tyrosine phosphorylation on protein function in S. pneumoniae encoded outside of the capsule locus, and provides a hitherto unidentified link between cell autolysis and capsular polysaccharide biosynthesis.

DNA methods and E. coli transformation. E. coli K-12 DH5α was used for all cloning. DNA manipulation, PCR and transformation into E. coli were performed as previously described (Morona et al., 1995).

Protein purification. LytA was purified essentially as described by Romero et al. (2007). Briefly, overnight cultures of the indicated strain were pelleted, washed in 20 mM phosphate buffer (pH 7) and lysed at >1000 p.s.i. (~6.9 MPa) via a French pressure cell. Insoluble material was removed by ultracentrifugation (150 000 g for 1 h) and isolated soluble fractions were incubated with DEAE Sepharose Fastflow (GE Healthcare) for 1 h at room temperature. DEAE Sepharose was washed three times in 20 mM phosphate buffer (pH 7.0) supplemented with 1.5 mM NaCl. LytA was then eluted from DEAE Sepharose with 20 mM phosphate buffer (pH 7) with 2% (w/v) choline chloride. The purity of eluted LytA was confirmed as >95% by SDS-PAGE, and LytA was stored at ~20 °C either with or without dialysis in 50 mM phosphate buffer (pH 7.0). For analysis of LytA purified from DH5α containing pG80 and pCpsCD, the protein was washed using Amicon Ultra-4 centrifugal filter units and resuspended in 50 mM phosphate buffer without choline and NaCl. Protein estimation was carried out using a BCA kit (ThermoFisher). CpsB was purified as previously described (Standish et al., 2012).

Construction of amino acid substitutions in LytA. Tyrosine 264 of LytA in pG80 was mutated to phenylalanine (oligonucleotides AS95, AS96), glutamate (AS97, AS98) and alanine (AS99, AS100) using the QuikChange Lightning Site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. Mutational alterations were confirmed by DNA sequencing.

Antibody production. To produce antibodies against LytA and CpsB, purified protein (>95%) as judged by SDS-PAGE, and LytA was stored at ~20 °C either with or without dialysis in 50 mM phosphate buffer (pH 7.0). For analysis of LytA purified from DH5α containing pG80 and pCpsCD, the protein was washed using Amicon Ultra-4 centrifugal filter units and resuspended in 50 mM phosphate buffer without choline and NaCl. Protein estimation was carried out using a BCA kit (ThermoFisher). CpsB was purified as previously described (Standish et al., 2012).

Construction of LytA amino acid substitutions in S. pneumoniae D39. To construct point mutations within lytA, first the Janus cassette (Sung et al., 2001) was inserted into the lytA gene using overlap extension PCR. The 5′ region of lytA was amplified with AS117 + AS101, a 3′ region with AS118+AS102 and the Janus cassette with AS113+AS114. These PCR products were then combined in a second round of PCR with AS101 and AS102. Overlap product was then transformed into a streptocin-resistant (Strep⁺) D39 strain (D39S), which was made resistant by transformation with a PCR product of rpsl from Strep⁺ Rx1. Transformants were selected on the basis of kanamycin resistance, and streptocin sensitivity, and was confirmed by sequencing.

LytA was mutated using overlap PCR using the following combination of oligonucleotides for 5′ and 3′ regions of lytA containing the relevant mutation; for Y264F: AS101 + AS95, AS102 + AS96; Y264E: AS97 + AS101, AS102 + AS98; Y264A: AS101 + AS99. The original PCR products were then combined in a second round of PCR using AS101 and AS102 and transformed into D39 LytAjanus, and Strep⁺ colonies selected for. Mutations were confirmed by sequencing. Transformations were carried out as described previously (Standish et al., 2005).

LytA binding assays. To investigate affinity of LytA to the choline analogue DEAE (DEAE Sepharose), 500 μl soluble lysate (0.2 mg ml⁻¹) from the indicated E. coli strain was incubated with 20 μl DEAE Sepharose for 10 min while rotating at room temperature. DEAE was

![Image](https://www.microbiologyresearch.org/article-figures/1469/1469_e01.png)

**Fig. 1.** Location of Y264 in choline binding repeat of the choline binding domain of LytA. (a) Choline binding repeats of LytA (D39; SPD_1737) showing the site of tyrosine phosphorylation (Y264; black shading) present in ChBR4. While there is significant similarity between repeats (>50% conservation highlighted in gray), Y264 is the only tyrosine present at this site. (b) Illustration of the position of Y264 (red) within the structure of the choline binding domain of LytA (Pdb: 1GVM) (Fernández-Tornero et al., 2002). Bound choline residue is highlighted in green.

**METHODS**

**Growth media and growth conditions.** *S. pneumoniae* strains (listed in Table 1) were routinely grown in Todd–Hewitt broth with 1% Bacto yeast extract (THY) or C+Y medium (McAllister et al., 2004) at 37°C as indicated or on Columbia blood agar plates supplemented with 5% (v/v) horse blood and grown at 37°C in 5% CO₂. Broth cultures were grown at 37°C without aeration. *Escherichia coli* cultures were grown in Luria–Bertani medium at 37°C with aeration. Antibiotics were used at the following concentrations: for *E. coli* ampicillin at 100 μg ml⁻¹ and erythromycin at 500 μg ml⁻¹; for *S. pneumoniae* erythromycin at 0.2 μg ml⁻¹, streptomycin at 150 μg ml⁻¹ and kanamycin at 200 μg ml⁻¹.
then washed three times in 50 mM phosphate buffer (pH 7.0) with 1.5 M NaCl and subsequently resuspended in 2x sample buffer, and the amount of LytA present was analysed by Coomassie brilliant blue staining of the SDS-PAGE gel.

**Construction of pCpsCD.** For the BY-kinase CpsD to be active it requires the polysaccharide co-polymerase protein, or kinase adaptor membrane protein, CpsC (Bender & Yother, 2001). In *Staphylococcus aureus*, fusion of the C-terminal cytoplasmic region of this homologous protein to the BY-kinase results in an active protein (Olivares-Illana et al., 2008; Soulat et al., 2006). We hypothesized this would also be the case in *S. pneumoniae*. Therefore, we fused D202–K230 of Cps4C (SP_0348) (the predicted C-terminal cytoplasmic region) to Cps4D (SP_0349) by overlap PCR. Originally we amplified D202–K230 of Cps4C with AS1 and AS2 and Cps4D with AS3 and AS4. These products were then combined in a second round of PCR and amplified with AS1 and AS4. This PCR product was ligated into pGEMT-Easy (Promega). Oligonucleotides AS68 and AS77 (Table 1) were used to amplify the DNA sequence and this PCR product was then cloned into pAL2 (Beard et al., 2002) with the lux operon deleted, as described by Trappetti et al. (2011). The transformant of *E. coli* DH5x containing the plasmid (pCpsCD) was confirmed by PCR and sequencing.

**SDS-PAGE and Western immunoblot.** Proteins were separated on 12% SDS-PAGE as described by Laemmli (1970) using a low-molecular-mass marker (Amersham). For Western immunoblot samples were transferred to either Immobilon-P (Millipore) (aPY; PY-20; Santa Cruz Biotechnology) or Nitrobind (GE Water and Process Technologies) (a-LytA). Membranes were probed with primary antibody overnight and after washes incubated as appropriate with either horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (Biomediq DPC) for 2 h. The membrane was then incubated with chemiluminescence blotting substrate (Sigma) for 5 min, followed by exposure of the membrane to X-ray film (Agfa). The film was developed using a Curix 60 automatic X-ray film processor (Agfa) or imaged with a Kodak Image Station 4000MM Pro (Carestream Molecular Imaging) to visualize the reactive bands.

| Table 1. Details of the strains, plasmids and oligonucleotides used in the present study |
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<table>
<thead>
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Non-denaturing PAGE. Non-denaturing PAGE was undertaken essentially as for SDS-PAGE, but with SDS omitted from all steps, including the PAGE, running buffer and the 2× sample buffer. 12% Gels were electrophoresed at 120 V at 4 °C and stained with Coomassie brilliant blue.

Turbidometric amidase activity assay. Amidase activity was analysed by measuring the decrease in turbidity of D39 LytAJanus (Table 1) cells after incubation with purified LytA proteins, essentially as described by Mellroth et al. (2014). D39 LytAJanus was grown to OD600 of about 0.5 in THY, then pelleted by centrifugation, washed twice in PBS and stored at −20 °C in PBS until use. To measure specific activity, thawed cells were equilibrated to an OD600 of about 1.0 and distributed into wells. They were then incubated with either purified protein at 2 μg ml−1 or the respective cell lysates at 250 μg ml−1. The initial rate of decrease in turbidity was determined for each protein, and their relative activities were calculated compared with the wt control. Results for purified proteins and cell lysates are from two and three independent experiments, respectively.

Growth curves. For growth curves, S. pneumoniae and dephosphorylated by CpsB LytA is tyrosine phosphorylated on Y264 by CpsD. As we hypothesized that this tyrosine to a phenylalanine. Purification of this protein from a strain also containing pCpsCD yielded LytAY264F, which reacted weakly with αPTyr, similar to wt LytA when purified from a strain lacking pCpsCD (Fig. 2b). This suggests that tyrosine 264 is the primary residue phosphorylated by CpsCD.

To investigate if the PTP CpsB can act on LytA-P, purified LytA-P (from E. coli DH5x containing pGL80 and pCpsCD) was incubated with purified CpsB and the level of tyrosine phosphorylation investigated by Western immunoblotting (Fig. 2c). Incubation with CpsB decreased phosphorylation by approximately 90%, suggesting the PTP can de-phosphorylate LytA-P. Thus, these data suggested that the capsule regulatory proteins CpsB and CpsD may play a role in LytA phosphorylation.

**LytA tyrosine phosphorylation enhances binding of LytA to choline analogue DEAE**

As Y264 is hypothesized to play a role in the affinity of LytA to choline, we investigated the ability of LytA, LytA-P and LytAY264F-P in E. coli-derived soluble protein fractions as described in Methods had higher levels of phosphorylation compared with LytA from the control strain containing vector alone, suggesting that CpsD can phosphorylate LytA (Fig. 2b, lanes 1 and 2).

Sun et al. (2010) reported that LytA was phosphorylated on tyrosine 264 (Fig. 1a). To confirm this finding we mutated this tyrosine to a phenylalanine. Purification of this protein from a strain also containing pCpsCD yielded LytAY264F, which reacted weakly with αPTyr, similar to wt LytA when purified from a strain lacking pCpsCD (Fig. 2b). This suggests that tyrosine 264 is the primary residue phosphorylated by CpsCD.

To investigate if the PTP CpsB can act on LytA-P, purified LytA-P (from E. coli DH5x containing pGL80 and pCpsCD) was incubated with purified CpsB and the level of tyrosine phosphorylation investigated by Western immunoblotting (Fig. 2c). Incubation with CpsB decreased phosphorylation by approximately 90%, suggesting the PTP can de-phosphorylate LytA-P. Thus, these data suggested that the capsule regulatory proteins CpsB and CpsD may play a role in LytA phosphorylation.

**LytA tyrosine phosphorylation enhances binding of LytA to choline analogue DEAE**

As Y264 is hypothesized to play a role in the affinity of LytA to choline, we investigated the ability of LytA, LytA-P and LytAY264F-P in E. coli-derived soluble protein fractions

![Fig. 2. BY-kinase CpsD and PTP CpsB act on LytA. (a) Whole-cell lysates from E. coli DH5x without (1) and with (2) expression of pCpsCD were investigated for tyrosine phosphorylation via Western immunoblotting. CpsCD is marked, while other proteins are indicated by additional increases in phosphorylation. (b) LytA (0.1 μg) purified either without (lane 1) or with (lane 2) co-expression of an active form of CpsD (pCpsCD) and LytAY264F co-expressed with CpsD (lane 3) were investigated for tyrosine phosphorylation via Western immunoblotting (αPY). Loading was confirmed by Coomassie brilliant blue-stained SDS-PAGE. (c) CpsB (2 μg) was incubated alone (lane 1) or with LytA (1 μg) (lane 2) for 1 h and phosphorylation was investigated by Western immunoblotting with αPY and αLytA.](image-url)
to bind DEAE, the choline analogue utilized to purify the autolysin. As well as being a useful method for easily purifying LytA, DEAE has been shown to result in conversion of LytA to the active E-form at similar concentrations to choline (Sanz et al., 1988). We first confirmed that LytA (lane 1), LytA-P (lane 2) and LytA$_{Y264F}$-P (lane 3) were present in the lysates at the same level (Fig. 3a). Then, we incubated lysates with DEAE Sepharose, and investigated LytA binding by SDS-PAGE and Coomassie blue staining. LytA-P was detected at approximately 1.6-fold higher levels than LytA (lane 1 vs. lane 2) (Fig. 3b, c). Furthermore, LytA$_{Y264F}$-P bound at a similar level to LytA (lane 1 vs. lane 3), suggesting specific phosphorylation of Y264 enhanced binding to DEAE.

To further confirm the specific effects of phosphorylation of Y264, a phospho-mimetic substitution was also constructed (LytA$_{Y264E}$). To control for the change in size of the residue, we also constructed a control mutation (LytA$_{Y264A}$). Investigation using Phyre2 suggested that these mutations would not influence the secondary structure of the protein (Kelley & Sternberg, 2009). Furthermore, the stability of all proteins was investigated by limited proteolysis and values were similar to wt protein (data not shown).

Incubation of the lysates containing these proteins with DEAE showed results that mirrored those seen with LytA and LytA-P. The phospho-mimetic mutation (LytA$_{Y264E}$; lane 2) enhanced affinity to DEAE while the phosphoablative mutation (LytA$_{Y264A}$; lane 3) was less than the wt control (lane 1) (Fig. 4b, c). Thus, this provided further evidence that it is specifically the tyrosine phosphorylation of Y264 that is responsible for the increased affinity.

**Phosphorylation modulates LytA dimerization**

The purification of LytA as described in Methods relies on the affinity of the amidase to choline, and thus this method purifies the C-form or active form of the enzyme. When we separated the LytA protein on non-denaturing PAGE to investigate its native oligomeric conformation, in all cases a single band was present. The C-form of LytA is dimeric (Fernández-Tornero et al., 2001), and thus we reasoned

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**Fig. 3.** LytA phosphorylation increases attachment to DEAE. (a) Soluble protein (10 μg) from *E. coli* DH5α containing LytA (lane 1), LytA-P (lane 2) and LytA$_{Y264F}$-P (lane 3) was separated on SDS-PAGE gel, and subjected to Western immunoblotting with α-LytA. (b) Each soluble lysate (0.2 mg ml$^{-1}$) was incubated with DEAE Sepharose for 30 min as described in Methods, with bound protein detected by Coomassie brilliant blue staining of the SDS-PAGE gel. Dilutions of bound protein samples were electrophoresed (Neat, 1:2 and 1:4) to help estimate differences. (c) Differences were quantified by Image J densitometric analysis of results from three separate experiments (*P<0.05, one-way ANOVA with a Tukey test).

**Fig. 4.** LytA$_{Y264E}$ increased attachment to DEAE. (a) Soluble protein (10 μg) from *E. coli* DH5α containing LytA (lane 1), LytA$_{Y264E}$ (lane 2) and LytA$_{Y264A}$ (lane 3) was separated on SDS-PAGE gel, and subjected to Western immunoblotting with α-LytA. (b) Each soluble lysate (0.2 mg ml$^{-1}$) was incubated with DEAE Sepharose for 30 min as described in Methods, with bound protein detected by Coomassie brilliant blue staining of the SDS-PAGE gel. Dilutions of bound protein samples were electrophoresed (Neat, 1:2) to help estimate differences. (c) Differences were quantified by Image J densitometric analysis of results from three separate experiments (**P<0.001, *P<0.01, one-way ANOVA with a Tukey test).
that this band represents the dimeric form of the protein (Fig. 5a). We hypothesized that as LytAY264E had increased affinity to choline, it would show an increased ability to retain its dimeric form when choline was removed by dialysis. We thus undertook dialysis in 50 mM phosphate buffer (pH 7.4) and used non-denaturing PAGE to assess the oligomeric state of LytA. Consistently, LytAY264E retained the higher order oligomeric state to a greater level than the wt, LytAY264F or LytAY264A (Fig. 5a, b). Separation of the proteins post-dialysis on denaturing SDS-PAGE resulted in one band for each LytA variant (Fig. 5c). Addition of choline resulted in only one band again being visible on the non-denaturing PAGE (data not shown). Thus, this suggested that LytAY264E had an increased ability to retain the dimeric form during dialysis. This provided further confirmation that LytAY264E substitution increased affinity to choline, which is essential for LytA dimerization.

Tyrosine phosphorylation increases LytA amidase activity

As tyrosine phosphorylation of LytA increased affinity to choline, and the ability of LytA to dimerize, we reasoned this may also enhance LytA amidase activity. To investigate this, we utilized a turbidometric amidase activity assay as described in Methods. First, we utilized purified proteins of the different LytA forms to investigate activity. However, no significant differences were apparent between the strains (Fig. 6a). We hypothesized that this was due to the fact that the enzymes had already undergone the ‘conversion’ process of pneumococcal amidases; the proteins were purified by elution with 2% choline and thus would already be converted into the C-form.

Thus, to investigate amidase activity prior to the conversion process, we used cell lysates containing LytA, as the proteins were present in lysates at equal levels (Figs 3a and 4a). These would contain LytA in the inactive E-form, as they had not been converted by binding to choline or a choline analogue. The control strain, DH5α containing vector alone, led to only minimal decrease in turbidity of D39 LytAJanus (approx. 10% of the wt control). LytA-P

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Fig. 5. Phosphorylation influences LytA dimerization. (a) LytA (lanes 1 and 5) and its variants LytAY264F (lanes 2 and 6), LytAY264E (lanes 3 and 7) and LytAY264A (lanes 4 and 8) were purified, dialysed as described in Methods, and approximately 2 μg separated by non-denaturing PAGE. (b) Relative percentages of the monomer present were determined by Image J densitometric analysis of results from three separate experiments (*P<0.05, one-way ANOVA with a Tukey test). (c) Dialysed samples were also separated on denaturing SDS-PAGE, and stained with Coomassie brilliant blue. Molecular masses (MW) are indicated (kDa).

Fig. 6. Phosphorylation increases LytA amidase activity. (a) Purified proteins (2 μg ml⁻¹) or (b) cell lysates (250 μg ml⁻¹) containing LytA (1), LytA-P (2), LytAY264F-P (3), LytAY264E (4) and LytAY264A (5) were compared for LytA amidase activity using a turbidometric assay as described in Methods. Results represent mean±SD from two (purified proteins) and three (cell lysates) independent experiments. Statistical analysis was undertaken using a one-way ANOVA with a Tukey test (**P<0.0001; ***P<0.0001).
had 215% of wt activity, with this increase lost in LytA_Y264F-P (Fig. 6b). Similarly, phospho-mimetic substitution of LytA (LytA_Y264E) had increased activity, while the phospho-ablative mutation did not. Thus, these data suggested that phosphorylation of Y264 results in increased LytA conversion capacity probably through increasing capacity to bind choline.

**Chromosomal mutation of Y264 alters autolysis of S. pneumoniae**

As *in vitro* we had seen that phosphorylation of LytA and phospho-ablative mutation altered the activity of LytA, we were interested to see whether this effect would be evident in *S. pneumoniae*. Thus, we constructed *S. pneumoniae* D39 mutants expressing chromosomally encoded LytA with phospho-ablative (D39 LytA_Y264F; D39 LytA_Y264A) and phospho-mimetic (D39 LytA_Y264E) mutations as described in Methods. These strains had similar levels of LytA, as determined by Western immunoblotting (Fig. 7a). We then investigated the growth of these strains over an extended length of time in C+Y. During exponential growth, there was no apparent difference in growth (Fig. 7b). However, consistently, the strain with the phospho-ablative mutation (D39 LytA_Y264F) showed a prolonged time to lysis compared with the wt. Conversely, the strain with the phospho-mimetic mutation (D39 LytA_Y264E) showed an earlier onset of autolysis. The strain with the additional control mutation (D39 LytA_Y264A) was similar to the wt. When we compared the rate of autolysis of the strain by comparing the slope of lysis, we saw that the phospho-mimetic mutation led to a significant increase in the rate of autolysis, with this significantly different from other strains (Fig. 7c). Thus, these results suggest that tyrosine phosphorylation of LytA on Y264 enhances activation of LytA activity in *S. pneumoniae*.

**DISCUSSION**

This is the first study, to our knowledge, to describe tyrosine phosphorylation as a regulator of non-capsule-related protein function in the major human pathogen *S. pneumoniae*. Furthermore, with the only pneumococcal BY-kinase found to date being the key capsule regulator CpsD (Morona et al., 2000), it seems possible that regulation of capsule and LytA activity is linked. Indeed, we have shown that CpsD, as well as pneumococcal PTP CpsB, can act on LytA as a substrate *in vitro*, although as yet we have been unable to detect this in *situ*, probably due to low levels of LytA tyrosine phosphorylation. Previous studies illustrated that LytA as well as CpsD and BY-kinase adaptor protein CpsC locate to the septa of *S. pneumoniae* (De Las Rivas et al., 2002; Henriques et al., 2011; Mellroth et al., 2012), suggesting the possibility that these proteins co-localize, and further that there is a link between capsular polysaccharide synthesis and autolysis.

Sun et al. (2010) performed a phosphoproteomic study of the pneumococcus in which they showed that Y264 of LytA was phosphorylated. We have confirmed this finding, showing that this is the predominant site of phosphorylation. LytA comprises two distinct domains, an N-terminal domain responsible for the N-acetyl muramyl amidase activity, and a C-terminal choline binding domain, responsible for the ability of LytA to bind to phosphorylcholine residues present in the cell wall. Y264 is present in the C-terminal choline binding domain within choline binding repeat 4. Indeed, it has been suggested that this
residue is important for the binding of choline (Fig. 1) (Fernández-Tornero et al., 2002). While there is significant homology between the choline binding repeats, Y264 is the only tyrosine at this particular site.

The forces responsible for binding of the choline in the family of CBPs are the same in all cases. While one component is hydrophobic, another is electrostatic, a cation–π interaction between the electron-rich systems of aromatic rings and the positive charge of the choline (Fernández-Tornero et al., 2001). We hypothesized that the increased negative charge of phosphorylation at Y264 may be important for the binding of LytA to phosphorylcholine, and regulation of its subsequent amidase activity, and thus set out to investigate this.

We showed that tyrosine phosphorylation increased the affinity of LytA to the choline analogue DEAE Sepharose. Furthermore, these effects were largely prevented by phospho-ablative substitution (LytAY264F). Thus, this suggested that specific phosphorylation of Y264 was responsible for the increase in affinity. Additionally, phospho-mimetic substitution (LytAY264E) also showed increased affinity to DEAE Sepharose, while the corresponding control (LytAY264A) did not, further supporting our observations.

In the cytoplasm, LytA resides in the inactive E-form, with the protein in the monomeric state (Tomasz & Westphal, 1971). Conversion to the catalytically active C-form occurs following interaction with phosphorylcholine in the cell wall, resulting in subsequent LytA dimerization. While conversion and dimerization are different processes, it is still not known whether conversion can only occur following the formation of the dimer (Romero et al., 2007). Our analysis of the oligomeric state of the LytA protein following dialysis suggested that the phospho-mimetic form (LytAY264E) retains its dimeric state to a greater extent than the wt and phospho-ablative forms (LytAY264F and LytAY264A), which correlates with an increased affinity to DEAE Sepharose. Furthermore, we also showed that this correlated with a difference in the overall activity of the enzyme. Interestingly, when we used purified proteins, having already undergone conversion due to the purification process, there were no significant amidase activity differences between the proteins (Fig. 6a). This provides evidence that we have not affected the secondary structures of the proteins through mutation or phosphorylation, as they still possess the same activity when bound to choline. However, when we used E. coli soluble cell lysates, in which LytA had not undergone previous conversion, significant differences were evident (Fig. 6b). Phosphorylation of Y264 enhanced LytA amidase activity, probably due to an increased capacity to bind choline and undergo the conversion to the C-form.

To confirm that this in vitro phenomenon played a role in vivo, we constructed LytA phospho-ablative and phospho-mimetic mutations in lytA on the chromosome of S. pneumoniae D39. The phospho-ablative substitution, D39 LytAY264F, showed prolonged time to lysis compared with the isogenic wt, suggesting that phosphorylation was occurring on the wt LytA to promote autolysis. Furthermore, the strain with the phospho-mimetic (D39 LytAY264E) substitution showed an earlier onset of autolysis. Additionally, comparison of the slope of lysis showed that the phospho-mimetic mutation led to an increased autolytic capability, correlating with our in vitro results. Thus, these data suggested that phosphorylation was responsible for both an earlier onset and faster autolysis phase.

While LytA was originally postulated to be always situated in the cell wall, evidence has recently emerged suggesting that LytA is located in the cytoplasm until the membrane is disrupted and the protein is able to gain access to peptidoglycan and cause the cell to undergo autolysis (Mellroth et al., 2012). Such a model, which seems likely, would suggest that no regulation mechanism is required. However, our work showed that phospho-mimetic and phospho-ablative mutations on the chromosome of the pneumococcus altered the time to autolysis in whole cell pneumococci, suggesting LytA tyrosine phosphorylation may alter the process whereby LytA gains access to its substrate. This may contribute in vivo to the numerous roles that LytA plays, such as in bacterial fructicide, release of pneumolysin, and control of bacterial size.

As LytA is a member of the CBP family in S. pneumoniae, it is interesting to speculate whether there are other CBPs in the pneumococcus whose affinity for choline are affected by tyrosine phosphorylation in a similar way to LytA. Indeed, another CBP, CbpC, is also phosphorylated on tyrosine, although this phosphorylation does not occur in the region of the choline binding domain of the protein, and thus its effect on function is less clear (Sun et al., 2010). Furthermore, LytA is also known to be phosphorylated on threonine (Sun et al., 2010), with further work required to determine whether this affects LytA function.

Additionally, we are interested in investigating further correlations between the phosphotyrosine regulatory system and LytA. Previous data have suggested that loss of capsule has an effect on the sensitivity of the pneumococcus to LytA amidase activity (Fernehro et al., 2004). With deletion of either the BY-kinase CpsD or the PTP CpsB resulting in strains possessing reduced capsule, this approach will be problematic. When we expressed the active CpsCD (pCpsCD) fusion in R6, an unencapsulated S. pneumoniae strain, no obvious effect on growth or lysis was evident (data not shown). It is possible that absence of the transmembrane section of this protein results in a protein unable to undergo normal localization. Furthermore, the absence of capsule and thus the hyper-sensitivity to LytA may make seeing effects difficult. Alternatively, other as-yet-unidentified BY-kinases are present that may affect the phosphorylation of the LytA. We are currently undertaking further studies to investigate this in more detail.

To date, no phosphoproteome of the pneumococcus has concentrated on the discovery of solely tyrosine-phosphorylated proteins. Indeed, the original phosphoproteomic study
on the pneumococcus found only 12 proteins phosphorylated on tyrosine, although surprisingly autophosphorylating tyrosine kinase CpsD was not amongst these (Sun et al., 2010). Thus, this would suggest that their study probably did not find the majority of tyrosine-phosphorylated proteins. Indeed, a recent study which concentrated solely on finding phosphorylated tyrosines in *E. coli* took the number of proteins known to be tyrosine phosphorylated in the bacteria from 32 to 342 (Hansen et al., 2013), suggesting that tyrosine phosphorylation is probably a much underappreciated form of post-translational regulation in bacteria as a whole, including *S. pneumoniae*. With this study illustrating that tyrosine phosphorylation can influence the activity of a major virulence factor, tyrosine phosphorylation could be a much more important form of post-translational regulation than has to date been recognized.

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