



RESEARCH ARTICLE

REVISED Signaling mechanism by the *Staphylococcus aureus* two-component system LytSR: role of acetyl phosphate in bypassing the cell membrane electrical potential sensor LytS [version 2; referees: 2 approved]

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Abstract

The two-component system LytSR has been linked to the signal transduction of cell membrane electrical potential perturbation and is involved in the adaptation of *Staphylococcus aureus* to cationic antimicrobial peptides. It consists of a membrane-bound histidine kinase, LytS, which belongs to the family of multiple transmembrane-spanning domains receptors, and a response regulator, LytR, which belongs to the novel family of non-helix-turn-helix DNA-binding domain proteins. LytR regulates the expression of *cidABC* and *lrgAB* operons, the gene products of which are involved in programmed cell death and lysis. *In vivo* studies have demonstrated involvement of two overlapping regulatory networks in regulating the *lrgAB* operon, both depending on LytR. One regulatory network responds to glucose metabolism and the other responds to changes in the cell membrane potential. Herein, we show that LytS has autokinase activity and can catalyze a fast phosphotransfer reaction, with 50% of its phosphoryl group lost within 1 minute of incubation with LytR. LytS has also phosphatase activity. Notably, LytR undergoes phosphorylation by acetyl phosphate at a rate that is 2-fold faster than the phosphorylation by LytS. This observation is significant in lieu of the *in vivo* observations that regulation of the *lrgAB* operon is LytR-dependent in the presence of excess glucose in the medium. The latter condition does not lead to perturbation of the cell membrane potential but rather to the accumulation of acetate in the cell. Our study provides insights into the molecular basis for regulation of *lrgAB* in a LytR-dependent manner under conditions that do not involve sensing by LytS.

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REVISED Amendments from Version 1

The major differences between version 1 and this version of the manuscript are as follows:

1. A correction was made in the introduction, paragraph 3 to reflect the fact that *cid* and *lrg* operons are not regulated by LytR, instead, LytR regulates the *lrgAB* operon, and *cidABC* operon is regulated by CidR. In addition, in the same paragraph, we have clarified that CidR is activated by accumulation of acetate under glucose excess conditions.
2. In the Discussion section, paragraph 1, we have made a correction to reflect the fact that LytR regulates *lrgAB* operon, not *cidABC* operon.
3. We have cited the work by Lehman *et al.* (2015) which show *in vivo* that LytR is indeed activated through phosphorylation by acetyl phosphate under the condition of glucose excess in the growth medium.

See referee reports

Summary statement

The molecular basis of signal transduction by LytSR is unknown. We show that LytS has kinase and phosphatase activity. LytR undergoes rapid phosphorylation by acetyl phosphate. Activity of LytR is regulated either through LytS or acetyl phosphate. LytSR is at the interface of two regulatory pathways that respond to excess glucose metabolism and cell membrane electrical potential, respectively.

Introduction

The two-component system LytSR of *Staphylococcus aureus* is reported to function as a sense-response system for detecting subtle changes in the electrical potential of the cell membrane. It is also involved in adaptation of *S. aureus* to cationic antimicrobial peptides (CAMPs)¹⁻³. CAMPs are bactericidal agents released by the host innate immune system during colonization by *S. aureus*^{4,5}. Their mechanism of action is believed to involve perturbation of cell membranes which, in turn, alters the electrical potential of the cell membrane^{3,6}. The function of LytSR in the cell is reported as a regulator of cell wall lysis during programmed cell death and biofilm formation⁷⁻¹².

A typical two component system (TCS) consists of a membrane bound histidine kinase (HK) which intercepts an environmental cue and through an act of auto-phosphorylation transduces the signal intracellularly. The response to the cue is mediated through a phosphotransfer process whereby a second protein, in response to a regulator protein, receives the phosphoryl group from the cognate histidine kinase (HK) at a conserved aspartate residue. The response regulator (RR) protein is often a transcription factor and in some cases an enzyme¹³. LytSR is comprised of the membrane-bound sensor HK LytS and the RR protein LytR. LytS belongs to a family of bacterial receptor proteins that contain five transmembrane-spanning domains. LytR is a transcription factor that falls into a novel family of proteins that contain non-helix-turn-helix DNA-binding domains, known as LytTRs^{14,15}.

LytR regulates *lrgAB* operon in response to alterations in the electrical potential of the cell membrane¹⁶. The *lrgAB* operon together with *cidABC* operon are involved in the control of programmed

cell death and lysis during biofilm formation^{12,17,18}; the gene products of the *cid* operon enhance murein hydrolysis activity and antibiotic tolerance¹⁹ while the *lrg* genes inhibit these processes⁹. Interestingly, both operons were also shown to be induced by carbohydrate metabolism¹⁶ and proposed to be regulated through a *cidR*-dependent signaling pathway¹. The *cidR* gene encodes a LysR-type transcription factor which has been proposed to be activated by accumulation of acetate during metabolism of excess glucose by *S. aureus* at logarithmic growth^{20,21}. Recent work by Sharma-Kuinkel *et al.*² demonstrated that *lrgAB* is instead regulated only through LytR, either in response to carbohydrate metabolism (e.g. excess of glucose) or as a result of disruption of cell membrane electrical potential. However, the molecular basis for this observation remained obscure.

To examine the signal transduction mechanism of LytSR and probe its involvement in the regulation of *lrgAB* in response to carbohydrate metabolism as a result of a phosphorylation-induced activation of LytR by acetyl phosphate, we cloned and purified LytS and LytR and investigated the autokinase activity of LytS, the kinetics of the phosphotransfer between LytS and LytR, and the kinetics of phosphorylation of LytR by acetyl phosphate. Our study shows that LytSR is capable of mediating signaling either through LytS in response to cell membrane electrical potential or through LytR in response to carbohydrate metabolism. Furthermore, phosphorylation-induced activation of LytR by either LytS or acetyl phosphate is likely to involve dimerization of LytR at the receiver domain.

Materials and methods

Chemical reagents and materials

Chemicals and antibiotics were purchased from Sigma (Oakville, Canada) or Thermo-Fisher (Whitby, Canada), unless otherwise stated. Chromatography media and columns were purchased from GE Healthcare (Quebec, Canada). Growth media were purchased from Fisher. *Escherichia coli* strains, NovaBlue and BL21 (DE3), and cloning and expression plasmids were purchased from EMD4 Biosciences (New Jersey, USA). The pGEX-4T vector was purchased from GE Healthcare (Quebec, Canada). Restriction enzymes were obtained from New England Biolabs (Pickering, Canada) or Thermo-Fisher. The [γ -³²P] Adenosine triphosphate (ATP) was purchased from Perkin Elmer LAS Canada Inc. (Toronto, Canada) or GE Healthcare. The Proteo Extract All-in-One Trypsin Digestion Kit was purchased from EMD4 Bioscience. The genome of the *S. aureus* strain Mu50 was obtained from Cedarlane (Burlington, Canada).

Cloning of the *lytS* gene that encodes the cytoplasmic domain into an over-expression plasmid

The gene sequence of *lytS* (SAV0260, as per gene numbering in *S. aureus* Mu50 strain) encoding the cytoplasmic region of the protein (amino acid residues 355–584) was amplified from *S. aureus* Mu50 genome using the primers: Dir 5'- CACCGCAGAAGGATTGGCAAAT-3' and Rev 5'-TTATTCCTCCTCTTG TCTTT CA-3'. To enable directional cloning, the forward primer contained a specific 4 base pair (bp) sequence (italicized) at the 5' end of the primer. The 701 bp *lytS* gene was amplified using Phusion High-Fidelity DNA polymerase with the following PCR conditions: initial denaturation at 98°C for 30 s, followed by 30 cycles at 98°C at 10 s, annealing at 61°C for 20 s, extension at 72°C for 20 s and

final extension at 72°C for 10 min. The blunt ended amplicon was then ligated to pET151/D-TOPO vector using the Champion™ pET Directional TOPO® Expression Kit. The ligated *pET151/D-TOPO::lytS* construct was used to transform *E. coli* NovaBlue cells for further amplification. The correct sequence of *lytS* was confirmed by DNA sequencing (The Centre for Applied Genomics, the Hospital for Sick Children, Toronto, Canada). The *pET151/D-TOPO::lytS* plasmid was used to transform the *E. coli* BL21 (DE3) to facilitate protein expression. This cloning strategy resulted in introduction of an NH₂-terminous 6xHis tag upstream of *lytS*.

Production and purification of His-LytS

E. coli BL21(DE3) cells carrying the construct *pET151/D-TOPO::lytS* were used to inoculate 5 mL of Luria Bertani (LB) medium supplemented with 100 µg/mL final concentration of ampicillin and allowed to grow overnight at 37°C by shaking. An aliquot of 1 mL of the overnight cell culture was used to inoculate 1 L of Terrific Broth (TB) medium supplemented with 100 µg/mL of ampicillin. Cells were allowed to grow at 37°C by shaking at 200 rpm until the cell culture reached an optical density of 0.6 to 0.8 absorbance units at 600 nm (OD_{600nm}). At this point, the cell culture was cooled to 4°C and protein production initiated by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.1 mM. Cell culture was shaken at 200 rpm at 18°C for 12 hrs. Cells were harvested by centrifugation at 3,300 × *g* for 20 min.

For isolation of His-LytS, the cell pellet was resuspended in 50 mM sodium phosphate pH 7.5, supplemented with 300 mM NaCl and 10 mM Imidazole. The cellular content was liberated by sonication while cooling on ice for 10 min (10s on/15s off) and cell debris was removed by centrifugation at 18,000 × *g* for 1 h at 4°C. Purification of His-LytS was carried out by loading the supernatant onto a self-assembled affinity column (Generon, UK) packed with 8 mL of Ni-NTA resin. All purification steps were carried out at 4°C using the Äkta purifier 10 (GE Healthcare). The unbound protein was removed by washing with buffer for three column volumes (CV). The protein of interest was eluted using a step gradient of 10%, 40%, 70% and 100% of 300 mM imidazole in 50 mM sodium phosphate pH 7.5 buffer, supplemented with 300 mM NaCl, at a flow rate of 1.5 mL/min in three CV. Fractions of 5 mL containing the protein were concentrated using Amicon Ultra-10K concentrator (Millipore) followed by dialysis into the storage buffer: 50 mM Tris pH 7.5, supplemented with 150 mM NaCl and 5 mM MgCl₂. The homogeneity of protein was assessed by loading samples onto a 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) apparatus and staining the gel with Coomassie blue.

Cloning of the full length *lytR* gene into an over-expression plasmid

The gene sequence of *lytR* (SAV0261) was amplified from the *S. aureus* Mu50 genome using the primers: Dir 5'-GGAATCCAT-ATGAAAGCATAATCATAGATGATG-3' and Rev 5'-CGGAAT-TCTTAT TAAAGTAATCCTA TCGACG-3'. The primers were designed to introduce the *NdeI* and *EcoRI* restriction sites (italicized sequences) at 5' and 3' of *lytR*, respectively. Amplification of the 741 bp *lytR* gene was carried out using Phusion® High-Fidelity DNA polymerase following these conditions: initial denaturation

at 98°C for 30 s, followed by 30 cycles at 98°C at 10 s, annealing at 62°C for 20 s, extension at 72°C for 20 s and final extension at 72°C for 10 min. The resulting amplicon was purified and together with the host vector, pET26b, was digested with *NdeI* and *EcoRI*. The digestion products were gel purified from 1% agarose gel using the QIAquick Gel Extraction Kit (Qiagen) and subjected to ligation using T4 DNA ligase (NOVAGEN). The subsequent construct was referred to as *pET26b::lytR* and was used to transform *E. coli* NovaBlue cells for further amplification. The correct sequence of the *lytR* gene was confirmed by DNA sequencing (The Centre for Applied Genomics, the Hospital for Sick Children, Toronto, Canada). The *pET26b::lytR* plasmid was used to transform the *E. coli* BL21 (DE3) expression cells. This cloning strategy resulted in introduction of no tags or additional amino acids to LytR.

Cloning of the *lytR* gene that encodes for the receiver domain (*lytR*^N) into an over-expression plasmid

To clone the receiver domain of the LytR protein, LytR^N (residues 1-134), a stop codon after the 134th amino acid residue was introduced using the QuikChange® Site-Directed Mutagenesis method (Thermo Fisher). The process of site directed mutagenesis was carried out using the *Pfu Turbo*® DNA polymerase and the following mutagenic primers: 5'-GCGAATGATATGTCGATAGAAATTTTGATCAAAGC-3' and 3'-GCTTTGATCAAATTTCTA CGACATAT-CATTCGC-5' (mutated nucleotides are italicized). The construct *pET26b::lytR* was used as the template. The amplified mutagenic construct, *pET26b::lytR*^N was treated with the restriction endonuclease *DpnI* and used to transform *E. coli* NovaBlue cells. Successful insertion of the stop codon was confirmed by DNA sequencing (The Centre for Applied Genomics, the Hospital for Sick Children, Toronto, Canada) and the *pET26b::lytR*^N vector was used to transform *E. coli* BL21 (DE3).

Cloning of *lytR* fused to the COOH-terminus of the Glutathione S-Transferase protein (GST) and construction of the Asp53Ala mutant of GST-LytR

The following primer set was used to clone the full length *lytR* into pGEX-4T-1 to enable fusion of the protein to the C-terminal of GST, Dir 5'-AGTCGGGATCCATGAAAGCATTAATCATA GATG-3' and Rev 5'-CG GAATTCCTATTAAAGTAATCCTATCG ACG-3'. The primers were designed to introduce *BamHI* and *EcoRI* restriction sites (italicized sequences) at 5' and 3' of *lytR* ends respectively, to enable cloning.

The Asp-53 residue of LytR was mutated to an Ala residue using QuikChange® Site-Directed Mutagenesis (Thermo Fisher). Briefly, the process of site directed mutagenesis was carried out using the *Pfu Turbo*® DNA polymerase with the designed mutagenic primers: 5'-AC ATTATATTTTTAGCTGTCAATTTAATGG-3' and 3'-CCATTAAATTGACAGCTAAAA AT ATAATGTC- 5' (mutated nucleotides are italicized). The construct *pGEX-4T1::lytR* was used as a template. The amplified mutagenic construct, referred to as *pGEX-4T::lytR*Asp53Ala, was treated with the restriction endonuclease *DpnI* and used to transform *E. coli* NovaBlue. Successful mutation of Asp to Ala was confirmed by DNA sequencing (The Centre for Applied Genomics, the Hospital for Sick Children, Toronto, Canada). The *pGEX-4T::lytR*Asp53Ala plasmid was used to transform the *E. coli* BL21 (DE3).

Production and purification of LytR

In general, *E. coli* BL21 (DE3) carrying the appropriate plasmid was used to inoculate 5 mL of LB in the presence of 50 µg/mL of kanamycin. An aliquot of 1 mL of the overnight cell culture was used to inoculate 1 L of TB supplemented with 50 µg/mL of kanamycin. The cells were allowed to grow to $OD_{600nm} = 0.6-0.8$ with shaking at 200 rpm at 37°C. Once desired growth was achieved the media was cooled to 4°C and protein production was induced by adding IPTG to a final concentration of 0.1 mM. Cell culture was allowed to shake for an additional 12 h at 18°C. Cells were harvested by centrifugation at $3,300 \times g$ for 20 min.

To isolate LytR the method of protein precipitation by ammonium sulfate was employed. All purifications steps were carried out at 4°C. Cell pellet was suspended in 1:10 (w/v) of 50 mM Tris, pH 8.0, 100 mM NaCl, 5 mM MgCl₂ supplemented with 10% glycerol. The cellular content was liberated by sonication and cell debris was removed by centrifugation at $18,000 \times g$ for 1 h at 4°C. Total volume of supernatant was adjusted to be 50 mL (for 5 g cell) and 2.67 g of ammonium sulfate was added gently while stirring, to achieve saturation of 10%. The solution was incubated while stirring at 4°C for 30 min. The precipitated protein was collected by centrifugation at $3,300 \times g$ for 5 min. The protein pellet was dissolved in 10 mL of 50 mM Tris, pH 8.0, 100 mM NaCl, 5 mM MgCl₂ supplemented with 10% glycerol. The purity of the protein was evaluated by 15% SDS-PAGE stained with Coomassie blue. The protein solution was dialysed to remove ammonium sulfate.

Production and purification of LytR^N

E. coli BL21 (DE3) harboring *pET26b::lytR^N* was used to inoculate 5 mL of LB in the presence of 50 µg/mL kanamycin. An aliquot of 1 mL of the overnight cell culture was used to inoculate 1 L of TB supplemented with 50 µg/mL of kanamycin. The cells were allowed to grow to an $OD_{600nm} = 0.6-0.8$ while shaking at 200 rpm at 37°C. The cell culture was cooled to 4°C and protein production initiated by addition of 0.5 mM IPTG. The cell culture was allowed to shake for an additional 12 h at 25°C for. Cells were harvested by centrifugation at $3,300 \times g$ for 20 min.

To isolate LytR^N, cell pellet was suspended in 1:10 (w/v) of 20 mM Tris pH 7.5, supplemented with 5 mM MgCl₂. The cellular content was liberated by sonication and cell debris was removed by centrifugation at $18,000 \times g$ for 1 h at 4°C. The supernatant was loaded onto a DEAE-Sepharose™ column (GE Healthcare) and mounted into the Äkta purifier 10. The protein was eluted over ten column volumes in a linear gradient of 20–500 mM Tris (pH 7.5) (supplemented with 5 mM MgCl₂) at a flow rate of 3 mL/min. Elution fractions containing protein were pooled together and concentrated by centrifugation using Amicon Ultra-3K concentrator (Millipore) to a final volume of 5 mL. The protein was loaded onto a HiPrep 26/60 Sephacryl S-200 HR gel-filtration column (GE Healthcare). The homogeneity of the protein was determined using 18% SDS-PAGE.

Production and purification of GST-LytR and GST-LytR-Asp53Ala

E. coli BL21 (DE3) carrying the desired plasmid were used to inoculate 5 mL of LB in the presence of 100 µg/mL ampicillin. An aliquot of 1 mL of the overnight grown seed culture was used to

inoculate 1 L of TB supplemented with 100 µg/mL of ampicillin. Cells were allowed to grow to an $OD_{600nm} = 0.6-0.8$ at 37°C while shaking at 200 rpm. Then the cell culture was cooled to 4°C and protein production was initiated by addition of 0.5 mM IPTG and incubated further at 18°C for 12 h. The cells were harvested by centrifugation at $3,300 \times g$ for 20 min.

For purification of the wild type or mutant GST-LytR protein, the cell pellet was suspended in 1:10 (w/v) of 50 mM Tris pH 7.5, supplemented with 100 mM NaCl and 5 mM MgCl₂. The cellular content was liberated by sonication and cell debris was removed by centrifugation at $18,000 \times g$ for 1 hour at 4°C. Purification was carried out by loading the supernatant onto a self-assembled affinity column packed with 5 mL of GST affinity resin (Generson). The protein of interest was eluted at a flow rate of 1.5 mL/min with 10 mM reduced glutathione in 50 mM Tris-HCl pH 8.0 buffer over three CV. Fractions containing the protein were concentrated using Amicon Ultra-10K concentrator (Millipore) followed by dialysis to exchange the buffer into 50 mM Tris pH 7.5, supplemented with 100 mM NaCl and 5 mM MgCl₂. The homogeneity of protein was assessed by 12.5% SDS-PAGE stained with Coomassie blue.

The identities of the all proteins isolated in this study were confirmed by cutting the protein band from the SDS-PAGE gel, subjecting this to trypsin digestion and submitting the digest for mass spectrometry analysis. The molecular mass of the purified proteins was determined by electrospray ionization mass spectrometry (ESI-MS). All the mass spectrometry analyses were done at the Advanced Protein Technology Centre, Hospital for Sick Children (Toronto, Canada).

Assessment of the autokinase activity of LytS

His-LytS at 5 µM was equilibrated in phosphorylation buffer (PB: 50 mM Tris, pH 7.4, 50 mM KCl, 5 mM MgCl₂) supplemented with 10 mM CaCl₂. The phosphorylation reaction was initiated by the addition of [γ -³²P]-ATP (10 Ci/mmol) mixed with cold ATP to prepare reaction mixtures at different ATP final concentrations. Samples were incubated at room temperature. The reaction was stopped at different time intervals by adding 5 × SDS sample buffer (125 mM Tris, pH 6.8, 2.5% SDS, 25% glycerol, 100 mM dithiothreitol (DTT), 0.0025% bromophenol blue). Samples were loaded onto a 15% SDS-PAGE. The SDS-PAGE gels were washed in water containing 2% (v/v) glycerol and were exposed to a phosphor screen (GE Healthcare) overnight and imaged using a Typhoon Trio+ imager (GE Healthcare). The radioactive gels were stained by Coomassie blue dye to analyze protein content of the samples.

Each time-dependent phosphorylation experiment was repeated twice. The progress of the reaction was assessed by analyzing the phosphor-image of the radioactive gels using NIH ImageJ software (Version 1.45s) (freely available at <http://imagej.nih.gov/ij/download.html>). The progress curves were fitted to the first-order rate Equation 1, where I is the band intensity quantified by NIH ImageJ, k_{obs} is the observed rate constant, t is the incubation time and A is the proportionality constant relating intensity with concentration of phosphorylated His-LytS.

$$I = A \times \{1 - \exp(-k_{obs} \times t)\} \quad (1)$$

The data were fitted using Erithacus GraFit software (version 5.0.10) (available at http://www.erithacus.com/grafit/Software_Updates.htm). The observed phosphorylation rate constant was calculated for each ATP concentration and was plotted against each ATP concentration to determine the rate constant of autophosphorylation of LytS using the equation (2), where k_{obs} is the observed rate constant measured at each ATP concentration, k is the autophosphorylation rate constant for LytS, K_s is the dissociation constant of ATP and $[S]$ the ATP concentration.

$$k_{obs} = k \times \frac{[S]}{K_s + [S]} \quad (2)$$

To study the effect of salt ions on the phosphorylation of His-LytS, we looked at the effect of K^+ and Ca^{2+} . His-LytS at 3 μ M was equilibrated in the phosphorylation buffer (PB: 50 mM Tris, pH 7.4, 5 mM $MgCl_2$) with varying concentrations of either KCl or $CaCl_2$. The phosphorylation reaction was initiated by the addition of [γ - ^{32}P]-ATP (10 Ci/mmol) to a final concentration of 20 μ M. The reaction was incubated for 90 min at RT then quenched by adding 5 \times SDS sample buffer. Samples were loaded onto a 15% SDS-PAGE. The radioactive gels were washed in water containing 2% (v/v) glycerol and gels were exposed to a phosphor screen (GE Healthcare) overnight. The screen was imaged using a Typhoon Trio⁺ imager (GE Healthcare).

To investigate the stability of the phosphorylated state of LytS, His-LytS at 10 μ M was allowed to undergo autophosphorylation for 90 min by adding [γ - ^{32}P]-ATP (10 Ci/mmol) to a final concentration of 20 μ M. Excess [γ - ^{32}P]-ATP was removed by desalting using the Zeba Spin Desalting column (Pierce, Thermo Scientific) equilibrated with PB. The reaction mixture was further incubated at room temperature and aliquots were removed at different time intervals and quenched by adding 5 \times SDS sample buffer. Samples were loaded onto 15% SDS-PAGE and the gel was analyzed as described above.

Phosphorylation of LytR by LytS and small molecule phosphate donors

The ability of LytS to phosphorylate LytR was examined as described earlier²². Briefly, His-LytS at 15 μ M was phosphorylated for 90 min. Excess [γ - ^{32}P]-ATP was removed by desalting using the Zeba Spin Desalting column which was equilibrated with PB. Phosphorylated His-LytS (4 μ M) was incubated with GST-LytR (10 μ M) in the PB buffer at room temperature. Aliquots of 10 μ L were removed at different time intervals and quenched by adding 10 μ L of 5 \times SDS-PAGE sample buffer. Samples were loaded onto a 15% SDS-PAGE. The radioactive gels were washed with water containing 2% (v/v) glycerol and gels were exposed to phosphor screen (GE Healthcare) overnight and imaged using a Typhoon Trio⁺ imager (GE Healthcare). The radioactive gels were stained by Coomassie blue dye. The phosphor images of the radioactive gels were quantified using NIH ImageJ software (Version 1.45s). Similar experiments were carried out using GST-LytR-D53A mutant and LytR^N.

To investigate phosphorylation of LytR by small molecule phosphate donors, lithium potassium acetyl phosphate was used as described earlier²². Briefly, LytR at 10 μ M or LytR^N at 20 μ M was equilibrated in PB20 buffer (PB: 50 mM Tris, pH 7.4, 50 mM KCl, 20 mM $MgCl_2$) and phosphorylation was initiated by addition of lithium

potassium acetyl phosphate to a final concentration of 50 mM. The reaction mixture was incubated at 37°C and 15 μ L aliquots were removed and quenched by adding 5 \times SDS sample buffer. The phosphorylated species were separated from unphosphorylated species using a 15% SDS-PAGE containing Acrylamide-pendant Phos-tagTM AAL-107 at 50 μ mol/L (Wako chemical USA, inc., Cedarlane)²³. The gels were stained by Coomassie blue dye. Band intensities of the phosphorylated species were quantified using NIH ImageJ and plotted against incubation time. These data were fitted to Equation 1 using Erithacus GraFit software (version 5.0.10) to determine the phosphorylation rate constants of LytR and LytR^N by acetyl phosphate.

To investigate the effect of phosphorylation on oligomerization state of LytR or LytR^N, each protein was phosphorylated by acetyl phosphate as described above. Unphosphorylated and phosphorylated samples of LytR^N (10 μ M, 20 μ M and 40 μ M) were loaded onto a 10% native-PAGE. The internal temperature of the buffer during the gel electrophoresis was maintained at 4°C. The gels were stained with Coomassie blue to visualize the protein bands. We analyzed phosphorylated and unphosphorylated LytR^N (80 μ M) also by size exclusion chromatography TSKgel G2000SW_{XL} (7.8 \times 300 mm, 5 μ m, TOSOH Biosciences LLC) on HPLC.

Phosphatase activity of LytS

To assess the phosphatase activity of LytS, LytR^N was phosphorylated by acetyl phosphate as described above. Excess acetyl phosphate was removed by the desalting column. LytR^N-P (80 μ M) was incubated with 5 μ M of LytS at different time intervals, in the absence or presence of 200 μ M ATP, at 37°C. Samples were quenched with native-PAGE loading buffer and loaded onto a 15% native-PAGE. Native-PAGE was stained with Coomassie blue to visualize the protein bands which were quantified by NIH ImageJ.

Circular dichroism spectroscopy

Far UV circular dichroism (CD) spectra (200–260 nm) of LytR and LytR^N were recorded using a Jasco J-180 instrument at 20°C. The buffer composition in these experiments was 20 mM Tris, 5 mM $MgCl_2$, pH 8.0. Two spectra scans were averaged for the sample and the buffer. Later, buffer contribution was subtracted from each protein spectrum. To assess the thermal stability of LytR and LytR^N, thermal melting of each protein was recorded by monitoring the change in the CD signal at 222 nm by ramping up the temperature from 20°C to 90°C at a rate of 3°C/min. Typically for these experiments, 20 μ M of the respective protein was buffer exchanged into 10 mM sodium phosphate buffer at pH 7.4.

Results

Modular architectures of LytS and LytR

The predicted domain architectures of LytS and LytR are shown in Figure 1. LytS belongs to the family of LytS-YhcK multi-transmembrane domain bacterial receptors which carry at their intracellular C-terminal a GAF (cyclic Guanosine Monophosphate-specific phosphor-diesterases, Adenylyl cyclases and the FhlA proteins²⁴) domain, and a kinase domain²⁵. The amino acid analysis of LytS by InterPro (EMBL-EBI; available online at <http://www.ebi.ac.uk/interpro/>) predicted that this protein has five transmembrane regions (TMs), an extracellular NH₂-terminus, and an intracellular GAF domain and a kinase domain. The intracellular kinase

domain harbors the dimerization and histidine phosphotransfer (DHp) domain and the catalytic and ATP-binding (CA) domain.

LytR consists of two domains, the conserved N-terminal receiver domain (LytR^N) and the variable C-terminal DNA-binding domain referred to as the effector domain (LytR^C). In general, the receiver domain of the RRs harbors a conserved Asp residue that undergoes a reversible phosphorylation by the cognate HK^{26,27}. The sequence analysis of LytR reveals that the receiver domain is homologous to the receiver domains of the OmpR/NarL protein families. Based on the sequence alignments with these RRs, we determined the phosphorylation site in LytR to be Asp-53. Sequence analysis of LytR also indicates that the DNA-binding domain is homologous to that found in the novel family of non-helix-turn-helix DNA-binding domains, known as LytTR^{14,15}. This family of proteins consists of AlgR and AgrA transcription factors¹⁵ which are involved in regulation of important virulence factors in pathogenic bacteria²⁸. These groups of effector domains are unique in their ability to bind to DNA and account for ~2.7% of all prokaryotic RRs²⁸.

Expression and purification of His-LytS and LytR proteins

The cloning strategy of *lytS* aimed to clone the cytoplasmic region of LytS spanning the amino acids 355-584, which harbors the DHp and CA domains. The cytoplasmic domain of LytS was fused at the C-terminus of a six-histidine tag (calculated molecular mass of the His-LytS is 28,359 D). The (His)₆-tag aided purification of LytS to homogeneity as assessed by SDS-PAGE.

Cloning of *lytR* (246 amino acids) led to the expression of LytR without tags or additional amino acids (calculated molecular mass 28,221 D). Expression of LytR was good but its purification was challenging. Conventional chromatographic methods failed, and it was evident that the actual isoelectric point (pI) was different from the theoretical one (pI ~5.68). We resorted to the use of ammonium sulfate precipitation to purify the protein. The protein precipitated out at 10% ammonium sulfate and the purity was assessed to be 80%. The protein was prone to aggregation at concentrations higher than 3 mg/mL. To facilitate purification and solubility of the protein, we cloned LytR fused to the COOH-terminus of GST. GST-LytR was purified to homogeneity by affinity chromatography.

Cloning of the receiver domain of LytR, LytR^N, spanning amino acids 1-134, led to the expression of a soluble protein with a molecular mass of 15,028 D without additional amino acids or tags. The protein was expressed at a higher level than LytR or GST-LytR. Moreover the protein was purified by conventional chromatographic methods based on the theoretical value of pI ~4.44. The protein was very stable and soluble at concentrations as high as 10 mg/mL.

The thermal melting points of LytR and LytR^N were measured to be 55°C and 70°C, respectively. The 15 degree difference in the thermal stabilities between these two proteins is an indication that the effector domain may destabilize the N-terminal domain in the context of the full-length protein.

Kinetics of the autokinase activity of His-LytS

We monitored the level of autophosphorylation of His-LytS at different time intervals and at different ATP concentrations (Figure 2, Dataset 1). (The preliminary data were partially published as part

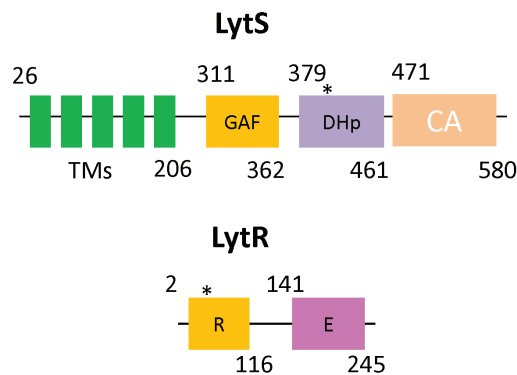


Figure 1. Domain organizations of LytS (P60612) and LytR (P60609) as predicted by InterPro (EMBL-EBI) ("TM" stands for transmembrane regions; "*" denotes the phosphorylation sites, His-390 in LytS and Asp-53 in LytR; "R" stands for receiver domain; "E", stands for the effector domain).

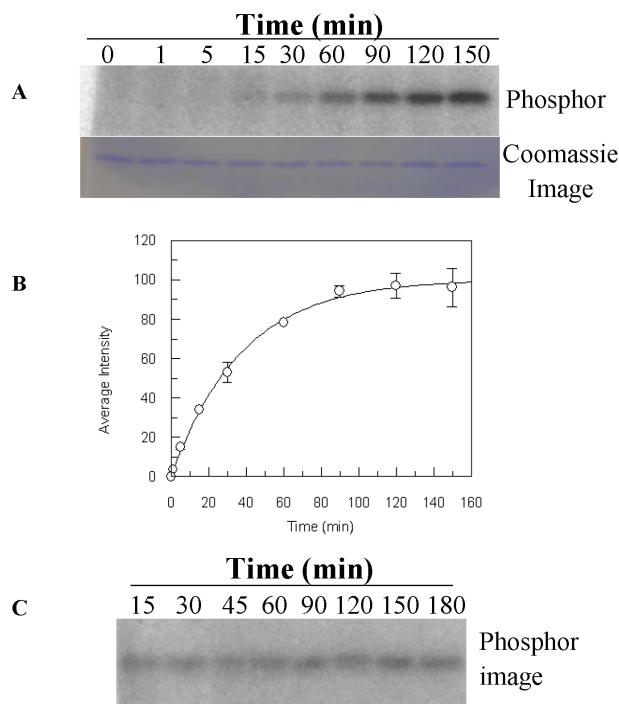


Figure 2. The autokinase activity of His-LytS. (A) His-LytS at 5 μ M was incubated with [γ -³²P]-ATP (100 μ M) in PB at room temperature. Reaction was quenched at different time intervals and samples were analyzed by 15% SDS-PAGE. (B) Progress curve of His-LytS autophosphorylation. The quantified band intensities of phosphorylation were plotted against time. The data were fitted using Origin software to pseudo first order equation (1) to calculate the rate constant. Errors are the standard deviation from two independent trials (Dataset 1). (C) Stability of the phosphorylated His-LytS species. His-LytS at 5 μ M was phosphorylated for 90 min in PB buffer (50 mM Tris, pH 7.4, 5 mM MgCl₂) at room temperature. Excessive ATP was removed by desalting and stability was monitored over 3 hours at different time intervals. Samples were analyzed by 15% SDS-PAGE. All gels were exposed to phosphor-screen (GE Healthcare) overnight and imaged using a Typhoon Trio+ imager (GE Healthcare). The gel in panel (A) was also stained with Coomassie blue to view the protein bands.

of a poster presentation in ASBMB in April 2013²⁹.) The pseudo first-order rate constant of the autokinase activity of His-LytS was determined to be $0.030 \pm 0.001 \text{ min}^{-1}$. The autophosphorylation rate constant of LytS is smaller compared to the *S. aureus* cell wall damage sensing HK VraS (0.07 min^{-1})²², *Enterococci faecium* vancomycin resistance factor HK VanS of (0.17 min^{-1})³⁰ or *S. aureus* essential HK WalK (0.36 min^{-1})³¹. However, it is similar to the autophosphorylation rate constants for other HKs such as *E. coli* nitrate sensing HK NarQ (0.014 min^{-1})³², and *E. coli* citrate sensing HK DcuS (0.043 min^{-1})³³. The binding affinity of LytS for ATP ($K_s = 7.9 \pm 0.6 \mu\text{M}$) is higher in comparison to other kinase such as, VanS ($K_m^{\text{ATP}} = 620 \pm 42 \mu\text{M}$)³⁰, VraS ($K_m^{\text{ATP}} = 230 \pm 42 \mu\text{M}$ (Belcheva *et al.* unpublished data)) and WalK ($K_m^{\text{ATP}} = 130 \mu\text{M}$)³¹.

Phosphorylation of LytR

We investigated phosphorylation of LytR through its cognate HK, LytS, and the small molecule phosphate donor, acetyl phosphate. Our efforts to investigate the phosphotransfer between His-LytS and LytR were hampered by the fact that the molecular masses of His-LytS and LytR were similar and this affected their separation by SDS-PAGE. To remove this obstacle, LytR was fused to GST which increased the molecular mass of LytR by 26 kD. When P³²-labeled His-LytS was incubated with GST-LytR, we observed a time-dependent reduction in signal from P³²-labeled His-LytS, which was associated with an increase in P³²-labeling of GST-LytR (Figure 3A). The observed rate constant for the phosphotransfer process is $0.3 \pm 0.1 \text{ min}^{-1}$. When incubation of P³²-labeled His-LytS was done in the presence of GST-LytRAsp55Asn, no reduction in signal from phosphorylated His-LytS was observed (Figure 3B). These experiments show that LytS is capable of phosphorylating LytR for as long as the phosphorylation site in LytR is available.

Incubation of LytR with a small molecule phosphate donor such as acetyl phosphate resulted in rapid phosphorylation of LytR (Figure 4A, Dataset 2). The observed phosphorylation rate constant for LytR was $0.6 \pm 0.1 \text{ min}^{-1}$. This rate is about 30-fold faster than phosphorylation of VraR by acetyl phosphate (0.022 min^{-1})²², or MtrA (0.014 min^{-1}) and PrrA (0.028 min^{-1}), 10-fold faster than DrrD (0.10 min^{-1}), and comparable to PhoB (0.45 min^{-1})³⁴. In the case of the stand-alone receiver domain, LytR^N, the observed phosphorylation rate constant was $0.9 \pm 0.2 \text{ min}^{-1}$ (Figure 4B). The higher phosphorylation rate constant measured for the LytR^N (1.5-fold compared to LytR) is another indication that the effector domain may perturb the receiver domain and very likely it does so through an interdomain interaction. Barbieri *et al.* recently reported a correlation between a higher phosphorylation rate constant for the receiver domains compared to the full-length RRs in cases where domains in RRs were engaged in interdomain interactions³⁴.

Investigation of the oligomerization state of LytR was not possible through native-PAGE (Tris-Glycine, pH 8.3) as the protein was not resolved under this buffer condition. Instead, we analyzed the oligomerization state of the phosphorylated LytR^N, which resolved well in native-PAGE. These experiments showed that phosphorylation of LytR^N led to dimerization (Figure 5). These results can be used to predict the oligomerization state of phosphorylated LytR and it is very likely this protein dimerizes upon phosphorylation, and it does so at the receiver domain.

The propensity of LytR^N to dimerize upon phosphorylation was used to monitor the phosphatase activity of LytS; dephosphorylation of LytR^N will lead to disintegration of the dimer which can readily be monitored in a native-PAGE system. Indeed, incubation of the phosphorylated LytR^N with LytS led to conversion of

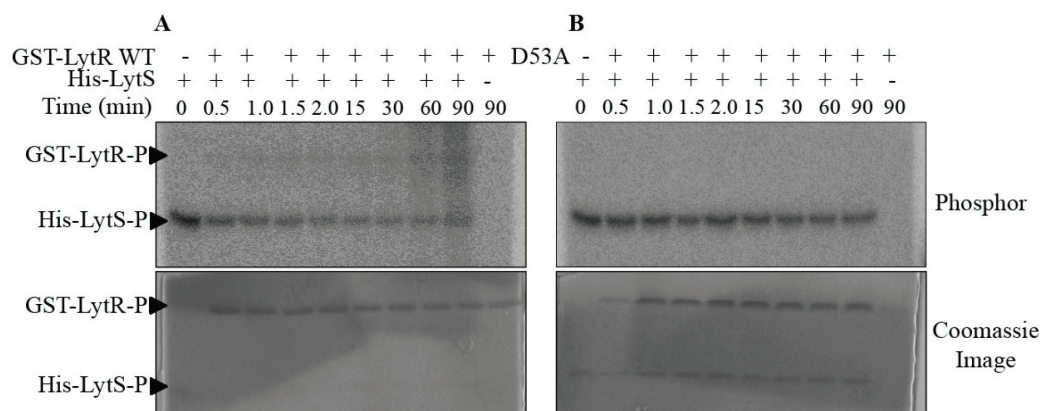


Figure 3. The phosphotransfer between His-LytS and GST-LytR or GST-LytRAsp53Ala variant. (A) Phosphorylated His-LytS at $4 \mu\text{M}$ was incubated with GST-LytR at $10 \mu\text{M}$ in PB at room temperature. The reaction was quenched at various time intervals and samples were analyzed by 15% SDS-PAGE. (B) Similar reaction as shown in (A), performed with GST-LytRAsp53Ala. The reaction was quenched at various time intervals and samples were analyzed by 15% SDS-PAGE. Gels were exposed to phosphor screen (GE Healthcare) overnight and imaged (top gels) using a Typhoon Trio⁺ imager (GE Healthcare) followed with Coomassie blue staining (bottom gels).

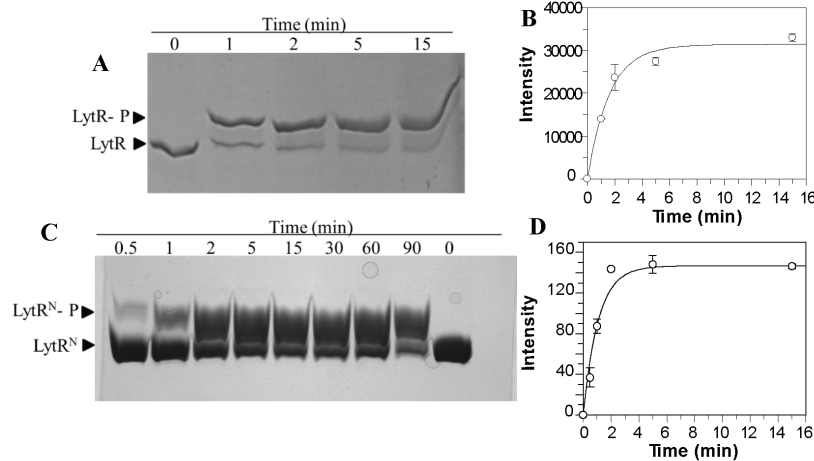


Figure 4. Phosphorylation of LytR and LytR^N by acetyl phosphate. (A) LytR at 10 μ M was phosphorylated by acetyl phosphate (50 mM) in PB for 1 h at 37°C. (B) Quantitative analysis of the data using NIH ImageJ (Dataset 2). The data were fitted using Origin software to pseudo first order equation (1) to calculate the rate constant. Errors are the standard deviation from two independent trials. (C) LytR^N at 20 μ M was phosphorylated by acetyl phosphate (50 mM) in PB buffer at 37°C and reactions were quenched at various time intervals. The phosphorylated proteins were separated from the unphosphorylated protein by 15% Phos-tag gel. The gels were stained with Coomassie blue to view the protein bands. (D) Quantitative analysis of the data using NIH ImageJ. The data were fitted using Origin software to pseudo first order equation (1) to calculate the rate constant. Errors are the standard deviation from two independent trials (Dataset 3).

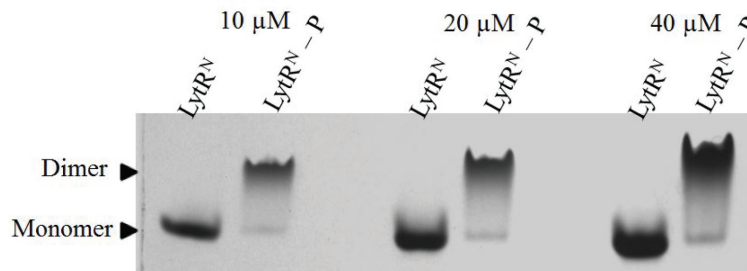


Figure 5. The effect of phosphorylation on the oligomerization states of LytR^N. LytR^N and LytR^N-P at 10 μ M, 20 μ M and 40 μ M were analyzed by 15% native-PAGE and stained with Coomassie blue.

the dimer to monomer species indicating that LytS has phosphatase activity (Figure 6A, Dataset 4). Interestingly, the phosphatase activity of LytS was more prominent in the presence of ATP similar to the observations made with *E. coli* osmosensor HK EnvZ; this phenomenon was proposed to be due to the allosteric effect that binding of ATP to CA domain had on the phosphatase activity of the DHP domain of EnvZ³⁵.

LytR^N underwent slow dephosphorylation at 37°C (Figure 6B, Dataset 4). This is an indication that LytR has autophosphatase activity. However, the rate of auto-dephosphorylation of LytR is about 10% of the dephosphorylation rate by LytS (Figure 6C) hence, it may not be relevant *in vivo*.

Raw data for the role of acetyl phosphate in bypassing the cell membrane electrical potential sensor LytS

4 Data Files

<http://dx.doi.org/10.6084/m9.figshare.1339843>

Discussion

LytSR is involved with sense-response to alterations of the cell electrical membrane potential due to cell membrane perturbations^{2,3}. Its function is related to regulation of genes controlling cell apoptosis, autolysin activity and biofilm formation^{9,19}. LytR regulates the *lrgAB operon*⁷ whereby the *lrg* genes products encode antiholin-like proteins that inhibit murein hydrolysis activity and cell lysis⁹.

To the present day, molecular details and functionality of the LytSR signal transduction pathway have only been presumed. We undertook *in vitro* characterization of LytS and LytR and probed their involvement in the signal transduction process. Signal transduction processes mediated by TCSs involve two reversible phosphorylation-mediated processes, autophosphorylation of the HK and transfer of its phosphoryl group to the cognate RR. Quite often the role of HK is to control the phosphorylation level of RRs, which in turn regulates the transcriptional activity of RR. It does so by possessing phosphatase activity toward RR, in addition to the kinase activity¹³. The phosphorylation level of RRs, however, can also be regulated through phosphorylation by small molecule phosphate donors such as acetyl phosphate and the autophosphatase activity of RRs²⁶.

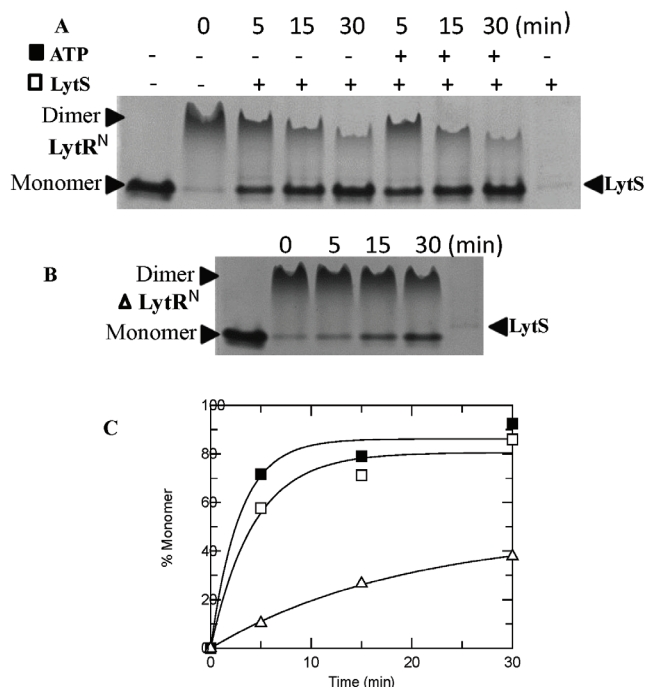


Figure 6. The phosphatase activity of LytS and autophosphatase activity of LytR^N. (A) LytS at 5 μ M was incubated with LytR^N-P at 80 μ M at different time intervals, in the absence or presence of 200 μ M ATP, at 37°C. (B) The stability of phosphorylated LytR^N-P at 37°C. (C) Time-dependence of the phosphatase activity of LytS in the absence of ATP (empty squares) or presence of ATP (solid squares) and autophosphatase activity of LytR (empty triangles). The data points were taken from the analyses of panels A and B using NIH ImageJ (Dataset 4).

Our study demonstrates that the cytoplasmic domain of LytS has autokinase activity ($k = 0.030 \pm 0.001 \text{ min}^{-1}$). The LytS phosphorylation rate constant is comparable to other HKs. The unusually low dissociation constant measured for ATP with LytS in comparison to other RRs, such as VraR, WalK, or VanS, suggests that the autophosphorylation efficiency for LytS is high and the kinase is well positioned to participate directly in the signalling process induced by changes in the cytoplasmic membrane electrical potential. The fast phosphotransfer process that we observed between LytS and LytR (0.3 min^{-1}) suggests that any alteration in the cell membrane electrical potential sensed by LytS is efficiently transduced intracellularly.

It is well established that most RRs are also equipped with the ability to catalyze their own phosphorylation, independently of their cognate kinases, using endogenous low molecular weight phosphoryl group donors³⁶. In fact, phosphorylation of RRs by low molecular weight phosphoryl group donors such as acetyl phosphate, carbamyl phosphate or phosphoramidate appears to be more common than phosphorylation by non-cognate HKs³⁷. Intracellular concentration of acetyl phosphate ranges from 1 mM to 3 mM *in vivo*, suggesting that this phosphate group donor is available in the cell in similar quantities as ATP^{34,36,37}. Herein, we show that LytR undergoes rapid and quantitative phosphorylation by acetyl phosphate ($k = 0.6 \text{ min}^{-1}$). Further, phosphorylation of the receiver domain by acetyl phosphate leads to dimerization of this domain demonstrating that phosphorylation-induced activation of LytR involves formation of dimers at the receiver domain. The differences in the thermal denaturation and phosphorylation rates by acetyl phosphate of LytR and LytR^N provide evidence that the effector domain has a

destabilizing effect on the receiver domain, which could plausibly be due to an interdomain interaction.

The rapid phosphorylation of LytR by acetyl phosphate observed in our study (about 2-fold faster than phosphorylation by LytS) strongly suggests that this pathway is important *in vivo*. Interestingly, *in vivo* studies have demonstrated presence of two overlapping regulatory networks in regulation of the *lrgAB* operon^{1,20}. One regulatory network responds to excess glucose metabolism and the other responds to changes in the cell membrane potential². Induction of *lrgAB* in response to glucose metabolism was shown *in vivo* to rely on LytR², however, metabolism of excess glucose does not lead to changes in the cytoplasmic membrane electrical potential¹, hence it is less likely that signaling occurs through LytS in this case. The fast kinetics of LytR phosphorylation by acetyl phosphate makes this pathway an efficient signaling and regulatory mechanism of *lrgAB* in response to the glucose metabolism. Moreover, the phosphatase activity of LytS toward phosphorylated-LytR, which otherwise is stable during the cell division time, provides the regulatory means to shut-down this pathway when the glucose level in the medium goes down.

In summary, our study shows that LytSR can participate in two signal transduction pathways through two phosphorylation processes: phosphorylation of LytR by LytS and phosphorylation of LytR by acetyl phosphate (Figure 7). Further, our findings provide the molecular mechanism for the *in vivo* observation that regulation of *lrgAB* operon is LytR dependent, either in response to excess of glucose metabolism or perturbation of cell membrane electrical potential.

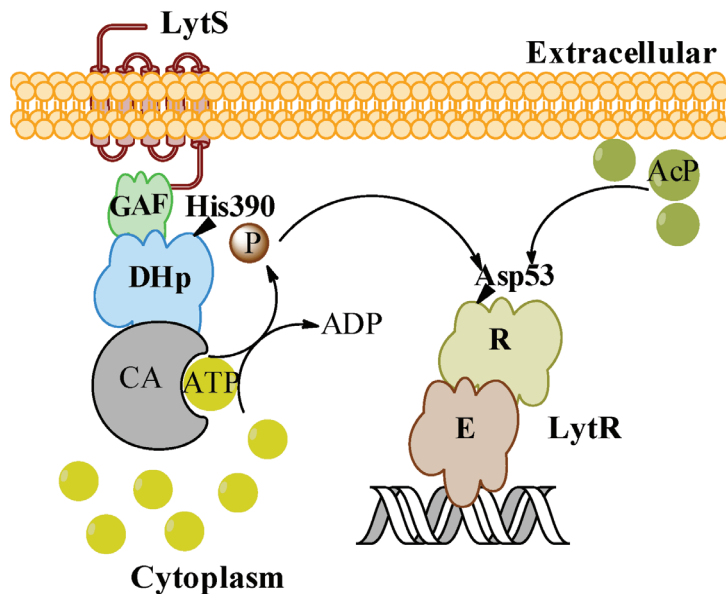


Figure 7. The schematic illustration of the signal transduction by LytSR. Our data support two pathways of activation of LytR: through LytS as a result of perturbation of the membrane electric potential, and through acetyl phosphate as a result of accumulation of acetate during metabolism of excess glucose.

A recent report by Lehman *et al.* showed that phosphorylation of LytR by acetyl phosphate is relevant *in vivo* and it is important for regulation of *lrgAB* operon under high level of glucose³⁸.

Data availability

Figshare: Raw data for the role of acetyl phosphate in bypassing the cell membrane electrical potential sensor LytS doi: [10.6084/m9.figshare.13398439](https://doi.org/10.6084/m9.figshare.13398439)

Author contributions

KP designed, carried out the experiments, analyzed the data and revised the manuscript. DGK designed the project and prepared the manuscript. All authors have agreed to the final content of the manuscript.

Competing interests

No competing interests were disclosed.

Grant information

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I confirm that the funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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References

- Patton TG, Yang SJ, Bayles KW: **The role of proton motive force in expression of the *Staphylococcus aureus cid* and *lrg* operons.** *Mol Microbiol.* 2006; **59**(5): 1395–1404.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Sharma-Kuinkel BK, Mann EE, Ahn JS, *et al.*: **The *Staphylococcus aureus* LytSR two-component regulatory system affects biofilm formation.** *J Bacteriol.* 2009; **191**(15): 4767–4775.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Yang SJ, Xiong YQ, Yeaman MR, *et al.*: **Role of the LytSR two-component regulatory system in adaptation to cationic antimicrobial peptides in *Staphylococcus aureus*.** *Antimicrob Agents Chemother.* 2013; **57**(8): 3875–3882.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Peschel A: **How do bacteria resist human antimicrobial peptides?** *Trends Microbiol.* 2002; **10**(4): 179–186.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Voyich JM, Braughton KR, Sturdevant DE, *et al.*: **Insights into mechanisms used by *Staphylococcus aureus* to avoid destruction by human neutrophils.** *J Immunol.* 2005; **175**(6): 3907–3919.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Peschel A, Jack RW, Otto M, *et al.*: ***Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with L-lysine.** *J Exp Med.* 2001; **193**(9): 1067–1076.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Brunskill EW, Bayles KW: **Identification of LytSR-regulated genes from *Staphylococcus aureus*.** *J Bacteriol.* 1996; **178**(19): 5810–5812.
[PubMed Abstract](#) | [Free Full Text](#)
- Brunskill EW, Bayles KW: **Identification and molecular characterization of a putative regulatory locus that affects autolysis in *Staphylococcus aureus*.** *J Bacteriol.* 1996; **178**(3): 611–618.
[PubMed Abstract](#) | [Free Full Text](#)
- Groicher KH, Firek BA, Fujimoto DF, *et al.*: **The *Staphylococcus aureus* *IrgAB***

- operon modulates murein hydrolase activity and penicillin tolerance.** *J Bacteriol.* 2000; **182**(7): 1794–1801.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
10. Rice KC, Bayles KW: **Death's toolbox: examining the molecular components of bacterial programmed cell death.** *Mol Microbiol.* 2003; **50**(3): 729–738.
[PubMed Abstract](#) | [Publisher Full Text](#)
 11. Bayles KW: **Are the molecular strategies that control apoptosis conserved in bacteria?** *Trends Microbiol.* 2003; **11**(7): 306–311.
[PubMed Abstract](#) | [Publisher Full Text](#)
 12. Rice KC, Mann EE, Endres JL, *et al.*: **The *cidA* murein hydrolase regulator contributes to DNA release and biofilm development in *Staphylococcus aureus*.** *Proc Natl Acad Sci U S A.* 2007; **104**(19): 8113–8118.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 13. Stock AM, Robinson VL, Goudreau PN: **Two-component signal transduction.** *Annu Rev Biochem.* 2000; **69**: 183–215.
[PubMed Abstract](#) | [Publisher Full Text](#)
 14. Nikolskaya AN, Galperin MY: **A novel type of conserved DNA-binding domain in the transcriptional regulators of the AlgR/AgrA/LytR family.** *Nucleic Acids Res.* 2002; **30**(11): 2453–2459.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 15. Sidote DJ, Barbieri CM, Wu T, *et al.*: **Structure of the *Staphylococcus aureus* AgrA LytTR domain bound to DNA reveals a beta fold with an unusual mode of binding.** *Structure.* 2008; **16**(5): 727–735.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 16. Rice KC, Nelson JB, Patton TG, *et al.*: **Acetic acid induces expression of the *Staphylococcus aureus* *cidABC* and *IrgAB* murein hydrolase regulator operons.** *J Bacteriol.* 2005; **187**(3): 813–821.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 17. Bayles KW: **The biological role of death and lysis in biofilm development.** *Nat Rev Microbiol.* 2007; **5**(9): 721–726.
[PubMed Abstract](#) | [Publisher Full Text](#)
 18. Rice KC, Bayles KW: **Molecular control of bacterial death and lysis.** *Microbiol Mol Biol Rev.* 2008; **72**(1): 85–109.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 19. Rice KC, Firek BA, Nelson JB, *et al.*: **The *Staphylococcus aureus* *cidAB* operon: evaluation of its role in regulation of murein hydrolase activity and penicillin tolerance.** *J Bacteriol.* 2003; **185**(8): 2635–2643.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 20. Yang SJ, Rice KC, Brown RJ, *et al.*: **A LysR-type regulator, *CidR*, is required for induction of the *Staphylococcus aureus* *cidABC* operon.** *J Bacteriol.* 2005; **187**(17): 5893–5900.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 21. Patton TG, Rice KC, Foster MK, *et al.*: **The *Staphylococcus aureus* *cidC* gene encodes a pyruvate oxidase that affects acetate metabolism and cell death in stationary phase.** *Mol Microbiol.* 2005; **56**(6): 1664–1674.
[PubMed Abstract](#) | [Publisher Full Text](#)
 22. Belcheva A, Golemi-Kotra D: **A close-up view of the *VraSR* two-component system. A mediator of *Staphylococcus aureus* response to cell wall damage.** *J Biol Chem.* 2008; **283**(18): 12354–12364.
[PubMed Abstract](#) | [Publisher Full Text](#)
 23. Kinoshita E, Kinoshita-Kikuta E, Koike T: **A single nucleotide polymorphism genotyping method using phosphate-affinity polyacrylamide gel electrophoresis.** *Anal Biochem.* 2007; **361**(2): 294–298.
[PubMed Abstract](#) | [Publisher Full Text](#)
 24. Cann MJ: **Sodium regulation of GAF domain function.** *Biochem Soc Trans.* 2007; **35**(pt 5): 1032–1034.
[PubMed Abstract](#) | [Publisher Full Text](#)
 25. Anantharaman V, Aravind L: **Application of comparative genomics in the identification and analysis of novel families of membrane-associated receptors in bacteria.** *BMC genomics.* 2003; **4**(1): 34.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 26. Gao R, Mack TR, Stock AM: **Bacterial response regulators: versatile regulatory strategies from common domains.** *Trends Biochem Sci.* 2007; **32**(5): 225–234.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 27. Gao R, Stock AM: **Molecular strategies for phosphorylation-mediated regulation of response regulator activity.** *Curr Opin Microbiol.* 2010; **13**(2): 160–167.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 28. Galperin MY: **Telling bacteria: do not LytTR.** *Structure.* 2008; **16**(5): 657–659.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 29. Patel KH, Golemi-Kotra D: ***In vitro* Characterization of the LytSR two component system in *Staphylococcus aureus*.** *Faseb J.* 2013; **27**.
[Reference Source](#)
 30. Wright GD, Holman TR, Walsh CT: **Purification and characterization of VanR and the cytosolic domain of VanS: a two-component regulatory system required for vancomycin resistance in *Enterococcus faecium* BM417.** *Biochemistry.* 1993; **32**(19): 5057–5063.
[PubMed Abstract](#) | [Publisher Full Text](#)
 31. Clausen VA, Bae WH, Throup J, *et al.*: **Biochemical characterization of the first essential two-component signal transduction system from *Staphylococcus aureus* and *Streptococcus pneumoniae*.** *J Mol Microbiol Biotechnol.* 2003; **5**(4): 252–260.
[PubMed Abstract](#) | [Publisher Full Text](#)
 32. Noriega CE, Schmidt R, Gray MJ, *et al.*: **Autophosphorylation and dephosphorylation by soluble forms of the nitrate-responsive sensors NarX and NarQ from *Escherichia coli* K-12.** *J Bacteriol.* 2008; **190**(11): 3869–3876.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 33. Janausch IG, Garcia-Moreno I, Uden G: **Function of DcuS from *Escherichia coli* as a fumarate-stimulated histidine protein kinase *in vitro*.** *J Biol Chem.* 2002; **277**(42): 39809–39814.
[PubMed Abstract](#) | [Publisher Full Text](#)
 34. Barbieri CM, Mack TR, Robinson VL, *et al.*: **Regulation of response regulator autophosphorylation through interdomain contacts.** *J Biol Chem.* 2010; **285**(42): 32325–32335.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 35. Zhu Y, Qin L, Yoshida T, *et al.*: **Phosphatase activity of histidine kinase EnvZ without kinase catalytic domain.** *Proc Natl Acad Sci U S A.* 2000; **97**(14): 7808–7813.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 36. Lukat GS, McCleary WR, Stock AM, *et al.*: **Phosphorylation of bacterial response regulator proteins by low molecular weight phospho-donors.** *Proc Natl Acad Sci U S A.* 1992; **89**(2): 718–722.
[PubMed Abstract](#) | [Free Full Text](#)
 37. McCleary WR, Stock JB, Ninfa AJ: **Is acetyl phosphate a global signal in *Escherichia coli*?** *J Bacteriol.* 1993; **175**(10): 2793–2798.
[PubMed Abstract](#) | [Free Full Text](#)
 38. Lehman MK, Bose JL, Sharma-Kuinkel BK, *et al.*: **Identification of the amino acids essential for LytSR-mediated signal transduction in *Staphylococcus aureus* and their roles in biofilm-specific gene expression.** *Mol Microbiol.* 2015; **95**(14): 723–737.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
 39. Patel K, Golemi-Kotra D: **Raw data for the role of acetyl phosphate in bypassing the cell membrane electrical potential sensor LytS.** *Figshare.* 2015.
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I have read the revised version of the manuscript by K. Patel K and D. Golemi-Kotra entitled "Signaling mechanism by the Staphylococcus aureus two-component system LytSR: role of acetyl phosphate in bypassing the cell membrane electrical potential sensor LytS" and approve the changes made.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Referee Report 29 March 2016

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In this revised manuscript, the authors addressed most of my previous concerns. But, two concerns are still remaining.

Abstract

The authors still indicate that LytR regulates cidABC.

Abstract & Discussion

In this revised manuscript, the authors say that the phosphotransfer reaction from LytS-P to LytR is fast. "LytS... can catalyze a fast phosphotransfer reaction, with 50% of its phosphoryl group lost within 1 min of incubation with LytR."

"The fast phosphotransfer process that we observed between LytS and LytR (0.3 min⁻¹) suggests that..."

I admit that "fast" is a relative terminology and can be interpreted differently, depending on how you see it. In addition, the reaction condition is rather artificial in that variant proteins (i.e., cytoplasmic domain of LytS and GST-fusion form of LytR) were used, and the results might not reflect what is happening in the bacterial membrane. Nonetheless, I still think the presented data do not support the notion of "fast

phosphotransfer” based on the following reason.

As the author indicated, the rate constant of phosphotransfer reaction was calculated from “dephosphorylation of LytS-P”, not from “phosphorylation of LytR. If all phosphoryl groups detached from LytS-P are transferred to LytR, that would not matter. However, according to the results in Fig. 3A, that seems not to be the case. In the reaction, the LytR concentration is 2.5 times higher than that of LytS (10 μ M LytR vs 4 μ M LytS). According to the authors, 50% of 32 P signals were lost from LytS-P within 1 min. However, at 1 min, the 32 P signal of LytR-P is barely visible, indicating that most of the phosphoryl groups detached from LytS-P were NOT transferred to LytR. It might be argued that the low 32 P signal of LytR-P is due to the phosphatase activity of LytS. In this case, we can expect a fast disappearance of LytS-P due to the futile phosphorylation/dephosphorylation of LytR. However, as Fig. 3A shows, LytS-P is very stable, and a significant amount of LytS-P is still seen at 90 min, even in the presence of 2.5 times excess LytR. If majority of phosphor-transfer occurs within 1 min, as the authors indicated in their response, we should see a saturation of LytR-P at 1 min. But, Fig. 3A shows that the LytR-P increases gradually, and its level is highest at 90 min.

So, I recommend the authors to draw a graph for appearance of LytR-P and disappearance of LytS-P like the one in Fig. 4, which might help to clear up this issue.

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Version 1

Referee Report 07 March 2016

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Kenneth W Bayles

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The authors of this paper sought to further characterize the signal mechanism of the *Staphylococcus aureus* LytSR two-component system. Previous studies have identified two signals that LytSR responds to: dissipation of membrane potential and excess glucose. Using purified protein, the authors characterize the kinetics of the autophosphorylation of LytS and the phosphorylation of LytR by LytS and acetyl-phosphate. The findings include a higher first-order rate constant for the phosphorylation of LytR by acetyl-phosphate as compared to its cognate kinase, LytS. In addition, the first-order rate constant of LytS autophosphorylation indicates a slow reaction as compared to other kinases. It was also demonstrated that LytR forms a dimer once phosphorylated, that LytS has phosphatase activity which is much more efficient than the auto-dephosphorylation of LytR.

The results of the paper confirm and add greater insight into the molecular signaling mechanism of LytSR as previously described by Lehman *et al*, 2015. Overall, manuscript is well written and the experiments nicely executed. However, as can be seen from the comments below, the primary concern is that the authors seemed to have completely missed the work of Lehman *et al*, 2015, which describes much of

what is presented in the current manuscript. Thus, the authors should include references to this work as indicated below.

Abstract

The closing statement of the abstract seems to be a bit overstated as this is not the “first time” these results have been generated. Indeed, very similar results were presented by Lehman *et al* 2015 that acetyl-phosphate is responsible for the LytS-independent phosphorylation of LytR. In fact, the Lehman *et al* paper goes further using *in vivo* studies of mutants affecting acetyl-phosphate levels to show that under conditions of excess glucose acetyl-phosphate contributes to the signaling of LytSR.

Introduction

Paragraph 3: The authors state “both operons were also shown to be induced by carbohydrate metabolism and proposed to be regulated through a CidR-dependent signaling pathway²⁰.” This is an inaccurate reference, as *cidABC* has only been shown to be regulated by CidR, whereas *IrgAB* expression is dependent on LytSR.

Paragraph 3: The authors should include a reference to the Lehman *et al* 2015 paper, which followed up the work performed by Sharma *et al* (from the same lab). As stated above, the Lehman *et al* 2015 paper demonstrated that acetyl-phosphate does influence LytS-independent phosphorylation of LytR. Thus, the statement that the “molecular basis for this observation remains obscure” (referring to the Sharma *et al.* paper) is misleading.

Paragraph 4: The authors state “Our study shows that LytSR is capable of mediating signaling either through LytS in response to cell membrane electrical potential or through LytR in response to carbohydrate metabolism.” This is overstated as there were no experiments to demonstrate this conclusion.

Results

Paragraph 2: It was published by Brunskill and Bayles 1996 that Asp-53 is the likely site of LytR phosphorylation. This observation was confirmed in Lehman *et al.* 2015.

Discussion

Paragraph 1: LytR does not regulate the *cidABC* operon as stated.

Paragraphs 2 and 5: See Lehman *et al* 2015 which characterized the LytSR signal transduction pathway *in vitro* and *in vivo*.

References

1. Lehman MK, Bose JL, Sharma-Kuinkel BK, Moormeier DE, Endres JL, Sadykov MR, Biswas I, Bayles KW: Identification of the amino acids essential for LytSR-mediated signal transduction in *Staphylococcus aureus* and their roles in biofilm-specific gene expression. *Mol Microbiol.* 2015; **95** (4): 723-37 [PubMed Abstract](#) | [Publisher Full Text](#)

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

Author Response 11 Mar 2016

Dasantila Golemi-Kotra, York University, Toronto, Canada

We are very thankful to the reviewer's comments and suggestions. We have addressed them point-by-point below:

The authors of this paper sought to further characterize the signal mechanism of the Staphylococcus aureus LytSR two-component system. Previous studies have identified two signals that LytSR responds to: dissipation of membrane potential and excess glucose. Using purified protein, the authors characterize the kinetics of the autophosphorylation of LytS and the phosphorylation of LytR by LytS and acetyl-phosphate. The findings include a higher first-order rate constant for the phosphorylation of LytR by acetyl-phosphate as compared to its cognate kinase, LytS. In addition, the first-order rate constant of LytS autophosphorylation indicates a slow reaction as compared to other kinases. It was also demonstrated that LytR forms a dimer once phosphorylated, that LytS has phosphatase activity which is much more efficient than the auto-dephosphorylation of LytR.

The results of the paper confirm and add greater insight into the molecular signaling mechanism of LytSR as previously described by Lehman et al, 2015. Overall, manuscript is well written and the experiments nicely executed. However, as can be seen from the comments below, the primary concern is that the authors seemed to have completely missed the work of Lehman et al, 2015, which describes much of what is presented in the current manuscript. Thus, the authors should include references to this work as indicated below.

Abstract

The closing statement of the abstract seems to be a bit overstated as this is not the "first time" these results have been generated. Indeed, very similar results were presented by Lehman et al 2015 that acetyl-phosphate is responsible for the LytS-independent phosphorylation of LytR. In fact, the Lehman et al paper goes further using in vivo studies of mutants affecting acetyl-phosphate levels to show that under conditions of excess glucose acetyl-phosphate contributes to the signaling of LytSR.

To acknowledge the study by Lehman et al. we have included this reference in the new version of the manuscript. We have removed "first time" from abstract as to not divert the attention from the essence of this study.

Introduction

Paragraph 3: The authors state "both operons were also shown to be induced by carbohydrate metabolism and proposed to be regulated through a CidR-dependent signaling pathway²⁰." This is an inaccurate reference, as cidABC has only been shown to be regulated by CidR, whereas IrgAB expression is dependent on LytSR.

Paragraph 3 has been modified now to address the reviewers concerns. Of note, it is proposed in several papers that CidR is involved in regulation of both operons. We have cited the work by Patton et al. 2006. The figure 6 in this work indicates the proposed model. We have replaced Ref 20 with Patton et al. report. We have also added the work by Yang et al 2006.

Paragraph 3: The authors should include a reference to the Lehman et al 2015 paper, which

followed up the work performed by Sharma et al (from the same lab). As stated above, the Lehman et al 2015 paper demonstrated that acetyl-phosphate does influence LytS-independent phosphorylation of LytR. Thus, the statement that the “molecular basis for this observation remains obscure” (referring to the Sharma et al. paper) is misleading.

We have included Lehman et al. paper in the new version of the manuscript.

Paragraph 4: The authors state “Our study shows that LytSR is capable of mediating signaling either through LytS in response to cell membrane electrical potential or through LytR in response to carbohydrate metabolism.” This is overstated as there were no experiments to demonstrate this conclusion.

We have been very careful with the use of the language here. We wrote that our study shows that “LytSR is **capable of** mediating signaling...”. Indeed, autophosphorylation activity of kinase LytS, indicates that LytS is capable of participating directly (with no need for accessory) in signal transduction, and fast phosphorylation of LytR by acetyl phosphate indicates that LytR is capable of participating in signal transduction independently of LytS.

Results

Paragraph 2: It was published by Brunskill and Bayles 1996 that Asp-53 is the likely site of LytR phosphorylation. This observation was confirmed in Lehman et al. 2015.

Because the phosphorylation site in all response regulators is conserved, the purpose of mutating Asp53 to Ala in this study was to confirm that signal can be transduced from LytS to LytR as an act of phosphotransfer between LytS and LytR. Kindly refer to Figure 3.

Discussion

Paragraph 1: LytR does not regulate the cidABC operon as stated.

We agree. We have removed this statement.

Paragraphs 2 and 5: See Lehman et al 2015 which characterized the LytSR signal transduction pathway in vitro and in vivo.

We have recognized the work by Lehman et al. in the new version of the manuscript.

Competing Interests: There is no competing interests.

Referee Report 24 February 2016

doi:10.5256/f1000research.6666.r12500



Taeok Bae

Department of Microbiology and Immunology, Indiana University School of Medicine-Northwest, Gary, IN, USA

In *Staphylococcus aureus*, transcription of the IrgAB operon is positively regulated by the LytSR two component system. Previous studies demonstrated that LytS, the sensor histidine kinase, is activated by two different kinds of signals: the loss of membrane potential and glucose/acetic acid. In this paper, using purified proteins, the authors assessed the enzymatic activities of LytS and phosphorylation of LytR by

LytS and acetyl phosphate. The autophosphorylation of LytS was rather slow, and the LytS-P was stable. The phosphotransfer from LytS-P to LytR was very slow while the LytR phosphorylation by acetyl phosphate was very efficient. When phosphorylated, the receptor domain of LytR formed a dimer. The phosphatase activity of LytS was also demonstrated. Based on these results, the authors concluded that acetyl phosphate is the signal transducer from the glucose signal.

This manuscript is well-written and, overall, experiments are properly conducted. However, I have concerns on the interpretation of the experiment results. Those concerns are listed below.

Abstract

“Herein, we show that LytS has autokinase activity and can catalyze a fast phosphotransfer reaction, with 50% of its phosphoryl group lost within 1 minute of incubation with LytR.”

: Indeed, in Fig. 3, the His-LytS-P signal at 1 min was significantly reduced as compared with that at 0 min. However, the reduction was not accompanied by increase of GST-LytR-P, indicating that the disappeared phosphoryl group was not transferred to GST-LytR. In addition, from 0.5 min to 90 min, the decrease of His-LytS-P signal was very slow. Therefore, the phosphotransfer reaction appears very slow in the condition employed.

Introduction

3rd paragraph: The references 20 and 21 do NOT say that CidR is activated by acetyl phosphate. Ref 20 shows that the acetate (glucose) activates the transcription of cidABC operon through CidR. Ref 21 reports that CidC is a pyruvate oxidase.

The last paragraph: The in vitro phosphorylation of LytR by acetyl phosphate does not guarantee that acetyl phosphate phosphorylates LytR in vivo. To my understanding, acetyl phosphate can phosphorylate many response regulators whose phosphorylation is not affected by carbohydrate metabolism.

Materials and methods

2nd paragraph, 4th line from bottom: ... for Sick Kids, Toronto, Canada. -> ..for Sick Kids, Toronto, Canada).

Page 5, 1st paragraph, 2nd line from bottom: delete “for” after 25C.

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Page 7, 1st sentence: “cognate” might be a better word than “conjugated”.

Page 8, Figure 3: The results, in particular GST-LytR-P signals, are not clear. Nonetheless, the phosphotransfer from LytS-P to LytR is very slow: even at 90 min, a significant amount of His-LytS-P still remains. The authors reported that the observed rate constant for the reaction is 0.3 min^{-1} . Although I am not an expert in biochemistry, the rate constant seems too high for the slow reaction. In addition, I wonder how the rate constant was calculated: was it based on the phosphorylation of GST-LytR or dephosphorylation of His-LytS-P?

Page 8, the last sentence: (Figure 4B) -> (Figure 4C & D).

Page 8 & Figure 4: The observed phosphorylation rate constant for LytR was 0.6 min^{-1} while it was 0.9 min^{-1} for LytRN. However, Fig. 4A shows that a majority of LytR was phosphorylated at 1 min while less than half of LytRN was phosphorylated at the time point (Fig. 4C). I understand that two different

concentrations (10 μM for LytR and 20 μM for LytRN) were used. But still, to me, the LytR seems to be phosphorylated faster than LytRN.

Page 9, last sentence and Page 10, the first sentence: The authors say **“the phosphatase activity of LytS was more prominent in the presence of ATP..”**. However, to me, the dephosphorylation rates are very similar, regardless of ATP (Fig. 6A). I wonder whether the difference shown in Fig. 6C at 5 min is statistically significant.

Discussion

Page 10 (right column), top sentence: The authors say **“The fast phosphotransfer process that we observed between LytS and LytR (0.3 min^{-1}) suggests that any alteration in the cell membrane electrical potential sensed by LytS is efficiently transduced intracellularly.”** In my view, the phosphotransfer process is rather slow, and the rate constant (0.3 min^{-1}) might be miscalculated. If I understand the rate constant correctly, 30% of LytR would be phosphorylated within 1 min (or 30% of LytS-P will be dephosphorylated within 1 min?). Nonetheless, Fig. 3A shows that either reaction does not proceed that fast.

Page 10 (right column), the second paragraph from bottom: The authors say **“The rapid phosphorylation of LytR by acetyl phosphate observed in our study (about 2-fold faster than phosphorylation by LytS) strongly suggests that this pathway is important in vivo.”** Although I also think it is likely, the in vitro phosphorylation of LytR by acetyl phosphate cannot serve as a definitive evidence for the in vivo phosphorylation of LytR by acetyl phosphate. To provide direct evidence, the authors can grow wild type and the mutants of *pta* (phosphate acetyltransferase) and *ackA* (acetate kinase) in the presence of glucose; then they can compare the transcript levels of the *IrgAB* operon. Since *Pta* synthesizes acetyl phosphate, no acetyl phosphate will be present in the *pta* mutant. On the other hand, *ackA* is converting acetyl phosphate into ATP; therefore, in the *ackA* mutant, the level of acetyl phosphate will be higher than that in wild type. If acetyl phosphate is indeed the in vivo mediator of the glucose signal, the transcript level of *IrgAB* will be lower in the *pta* mutant while higher in the *ackA* mutant, as compared with wild type cells.

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Fig. 7. The model.

To me, the following model fits better to the data presented in the paper.

1. No glucose (nor acetate), full membrane potential

: In this condition, LytS will have a net phosphatase activity due to slow autokinase activity (Fig. 2A), very inefficient phosphotransferase activity (Fig. 3A), and relatively higher phosphatase activity (Fig. 6), resulting in a low expression of *IrgAB*.

2. No glucose, loss of membrane potential (e.g., gramicidin, CCCP etc)

: The loss of membrane potential will activate the kinase activity of LytS, converting LytS from a phosphatase to kinase. The level of LytR-P will increase, resulting in higher expression of *IrgAB*.

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: The efficient phosphorylation of LytR by acetyl phosphate will overcome the phosphatase activity of LytS, resulting in higher expression of *IrgAB*.

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I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

Author Response 11 Mar 2016

Dasantila Golemi-Kotra, York University, Toronto, Canada

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: Indeed, in Fig. 3, the His-LytS-P signal at 1 min was significantly reduced as compared with that at 0 min. However, the reduction was not accompanied by increase of GST-LytR-P, indicating that the disappeared phosphoryl group was not transferred to GST-LytR. In addition, from 0.5 min to 90 min, the decrease of His-LytS-P signal was very slow. Therefore, the phosphotransfer reaction appears very slow in the condition employed.

When comparing panels A and B of Figure 3 (top figures), it is clear that majority of phosphor-transfer, as with any other histidine kinase, occurs within 1 min, followed by a slow decrease in phosphor-signal of LytS-P as LytR undergoes dephosphorylation (by LytS) and then, probably, phosphorylated back by LytS-P. We acknowledge that the LytR-P band is faint under the current quality of the picture in this print.

The experimental data in Fig3A, the dephosphorylation of LytS-P, were fitted into first-order rate kinetics to determine the rate constant of the phosphor-transfer. However, a quick view at the

phosphor image (although it is only an estimation method) (Fig 3A top) shows that in about 1 min, 50% of the ^{32}P -signal is lost from LytS-P. Dephosphorylation of LytS follows first-order rate kinetics, hence, $t_{1/2} = \ln 2/k$ (where $t_{1/2}$ is the time that it takes for 50% of the reaction to complete, and k is the observed first-order rate constant), then $k = \ln 2/t_{1/2}$, from which one can readily determine the k value.

Introduction

3rd paragraph: The references 20 and 21 do NOT say that CidR is activated by acetyl phosphate. Ref 20 shows that the acetate (glucose) activates the transcription of *cidABC* operon through CidR. Ref 21 reports that CidC is a pyruvate oxidase.

Ref. 21 shows among others that *cidABC* operon is involved in generation of acetate in cell under high content of glucose, and Ref 20 shows that CidR regulates the *cidABC* operon in response to accumulation of acetate at high concentrations of glucose. It is known that (Ref 35 in the discussion) synthesis of acetyl phosphate from acetate and ATP are reversible processes and the enzymes that catalyse these reactions, are encoded by genes (*pta* and *ackA*) that are nearly constitutive. In addition, it is known that levels of acetyl phosphate vary with the carbon source in the growth medium (Ref 35). We have made the connection here that the loner response regulator CidR (it is not part of a two-component signal transduction) is activated by acetate through acetyl phosphate. However, we have replaced “acetyl phosphate” with “acetate” as to remain closer to the results in Refs. 20 and 21.

The last paragraph: The in vitro phosphorylation of LytR by acetyl phosphate does not guarantee that acetyl phosphate phosphorylates LytR in vivo. To my understanding, acetyl phosphate can phosphorylate many response regulators whose phosphorylation is not affected by carbohydrate metabolism.

This is a good point. It is true that acetyl phosphate is present in the cell. The notion that it could serve as a phosphorylation agent in the response regulator proteins it may seem to contradict the working of two-component systems. However, the kinetics of phosphorylation of response regulators by their cognate kinases and acetyl phosphate will determine whether phosphorylation by acetyl phosphate is relevant *in vivo*. In the case of LytR, our study shows that phosphorylation by acetyl phosphate is faster than phosphorylation by LytS (and faster than phosphorylation of other response regulator). Hence, it is likely to play a role *in vivo*. A recent paper by Lehman et al shows that our findings have correctly predicted what happens *in vivo*.

Materials and methods

2nd paragraph, 4th line from bottom: ... for Sick Kids, Toronto, Canada. -> ..for Sick Kids, Toronto, Canada).

Corrected.

Page 5, 1st paragraph, 2nd line from bottom: delete “for” after 25C.

Corrected.

Results

Page 7, 1st sentence: “cognate” might be a better word than “conjugated”.

Corrected.

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The rate constant of phosphotransfer from LytS to LytR is 0.3 min^{-1} . It was measured from monitoring the loss of P32-signal from LytS-P in Figure 3A (LytR-P signal is relatively low to be measured accurately). In comparison to the phosphorylation by acetyl phosphate, this process is slower, indeed.

Page 8, the last sentence: (Figure 4B) -> (Figure 4C & D).

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We have shown one representative gel for each case. The data, obtained from quantification of the band intensities are provided in the datasets 2 and 3. These data are grafted in Figs 4B and D. Although from the gel it seems as if that at 0.5 min the signal for LytR-P is higher than LytRN-P, overall the fitting of all the data points shows that for LytRN-P the signal increases faster when considering all the data points.

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To provide objectivity on analysing these data, we have plotted the data in Fig. 6C. To us it was interesting that others have observed similar phenomenon as we did. The data are reproducible (but we only have three different trials to comment on statistical significance). Whether it has any relevance *in vivo*, it needs to be investigated further.

Discussion

Page 10 (right column), top sentence: The authors say **“The fast phosphotransfer process that we observed between LytS and LytR (0.3 min^{-1}) suggests that any alteration in the cell membrane electrical potential sensed by LytS is efficiently transduced intracellularly.”** In my view, the phosphotransfer process is rather slow, and the rate constant (0.3 min^{-1}) might be miscalculated. If I understand the rate constant correctly, 30% of LytR would be phosphorylated within 1 min (or 30% of LytS-P will be dephosphorylated within 1 min?). Nonetheless, Fig. 3A shows that either reaction does not proceed that fast.

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We agree. The recent work by Lehman et al. have carried out the above experiments (Ref 37 in the new version of manuscript).

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Yes, that is correct.

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4. Glucose, loss of membrane potential (e.g., gramicidin, CCCP etc)

*: LytR will be phosphorylated by both LytS and acetyl phosphate, resulting in maximal expression of *IrgAB*.*

We agree. Ref. 1 study supports the above models, in the addition to the recent work by Lehman et al. (Ref 37 in the new version of the manuscript).

Competing Interests: There is no competing interests.
