RESEARCH ARTICLE

Signaling mechanism by the *Staphylococcus aureus* two-component system LytSR: role of acetyl phosphate in bypassing the cell membrane electrical potential sensor LytS

[version 1; peer review: 2 approved with reservations]

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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 for the first time the molecular basis for regulation of *lrgAB* in a LytR-dependent manner under conditions that do not involve sensing by LytS.

Keywords
LytSR, Histidine kinase, Response Regulator, Phosphotransfer, *Staphylococcus Aureus*
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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)\(^3\)-\(^6\). 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\(^1\)-\(^2\). The function of LytSR in the cell is reported as a regulator of cell wall lysis during programmed cell death and biofilm formation\(^1\)-\(^2\).

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 enzyme\(^1\). 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 LyrTRs\(^1\)-\(^2\).

LytR regulates cidABC and lrgAB operons in response to alterations in the electrical potential of the cell membrane\(^2\). Both operons are involved in the control of programmed cell death and lysis during biofilm formation\(^2\)-\(^4\),\(^1\)-\(^7\)-\(^1\). The gene products of the cid operon enhance murein hydrolysis activity and antibiotic tolerance\(^9\) while the lrg genes inhibit these processes\(^1\). Interestingly, both operons were also shown to be induced by carbohydrate metabolism and proposed to be regulated through a cidR-dependent signaling pathway\(^2\). The cidR gene encodes a LysR-type transcription factor which has been proposed to be activated by acetyl phosphate that accumulates as a result of metabolism of excess glucose by S. aureus during log-arithmic growth\(^2\)-\(^5\). Recent work by Sharma-Kinkel 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 remains 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 [\(\gamma\)-\(^3\)P] 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\'-CACCGCAGAGAATTGC-3' and Rev 5\'-TTATTCCTCCTTTGCTTTTCA-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 ampiclon was then ligated to PET151/D-TOPO vector using the Champion™ pET Directional TOPO\(^\circ\) 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 Kids, 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 NH\(_2\)-terminus 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\(_{600}\)). At this point, the cell culture was cooled to 4°C and protein production initiated by adding isopropyl \(\beta\)-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 x 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 x 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 ActiA 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 15% SDS-PAGE stained with Coomassie blue.

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

The gene sequence of lytR (S. aureus Mu50 genome) using the primers: Dir 5'-GGAATTCATGAAAGCATTAATCATA GTAGT-3' and Rev 5'-GGGAATTCCCTTTATCAAAGCATGAGTCAATTT-3' (mutated nucleotides are italicized). The construct was evaluated by 15% SDS-PAGE stained with Coomassie blue.

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Production and purification of LytRN

*E. coli* BL21 (DE3) harboring pET26b::lytRN 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 × g for 20 min.

To isolate LytRN, 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 × 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) followed by dialysis to 50 mM Tris pH 7.5, supplemented with 5 mM MgCl₂. The protein was loaded onto a HiPrep 26/60 Sephacryl S-200 HR gel-filtration column (GE Healthcare). The molecular mass of the purified proteins was determined by running gel-filtration chromatography to determine the homogeneity of 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 50 µg/mL of kanamycin. The cells were allowed to grow to an OD_{600nm} = 0.6–0.8 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 × 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 × g for 1 h 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 (Generon). The protein was eluted with a gradient of 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 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 Kids (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, 25% 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 with 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.

$$I = A \times \left[1 - \exp(-k_{obs} \times t)\right]$$

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.

$$k_{obs} = k \times \frac{[S]}{K_s + [S]}$$

To study the effect of salt ions on the phosphorylation of His-LytS, we looked at the effect of K⁺ and Ca²⁺. His-LytS at 3 µM was equilibrated in the phosphorylation buffer (PB: 50 mM Tris, pH 7.4, 5 mM MgCl₂) with varying concentrations of either KCl or CaCl₂. The phosphorylation reaction was initiated by the addition of [γ-32P]-ATP (10 Ci/mmol) to a final concentration of 20 µM. The reaction was incubated for 90 min at RT then quenched by adding 5 × 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 µM was allowed to undergo autophosphorylation...
Phosphorylation of LytR by LytS and small molecule phosphate donors

The ability of LytS to phosphorylate LytR was examined as described earlier. Briefly, His-LytS at 15 µM was phosphorylated for 90 min. Excess [γ-32P]-ATP was removed by desalting using the Zeba Spin Desalting column (Pierce, Thermo Scientific) equilibrated with PB. Phosphorylated His-LytS (4 µM) was incubated with GST-LytR (10 µM) in the PB buffer at room temperature. Aliquots of 10 µL were removed at different time intervals and quenched with adding 10 µL of 5 × 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\textsuperscript{N}.

To investigate phosphorylation of LytR by small molecule phosphate donors, lithium potassium acetyl phosphate was used as described earlier. Briefly, LytR at 10 µM or LytR\textsuperscript{N} at 20 µM was equilibrated in PB20 buffer (PB: 50 mM Tris, pH 7.4, 50 mM KCl, 20 mM MgCl\textsubscript{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 µL aliquots were removed and quenched by adding 5 × SDS sample buffer. The phosphorylated species were separated from unphosphorylated species using a 15% SDS-PAGE containing Acrylamide-pendant Phos-tag\textsuperscript{TM} AAL-107 at 50 µmol/L (Wako chemical USA, inc., Cedarlane). 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\textsuperscript{N} by acetyl phosphate.

Circular dichroism spectroscopy

Far UV circular dichroism (CD) spectra (200–260 nm) of LytR and LytR\textsuperscript{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\textsubscript{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\textsuperscript{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 µM 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 proteins\textsuperscript{20}) domain, and a kinase domain\textsuperscript{21}. 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 NH\textsubscript{2}-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\textsuperscript{N}) and the variable C-terminal DNA-binding domain referred to as the effector domain (LytR\textsuperscript{E}). In general, the receiver domain of the RRs harbors a conserved Asp residue that undergoes a

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure1.png}
\caption{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).}
\end{figure}
reversible phosphorylation by the conjugated HK. 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. This family of proteins consists of AlgR and AgrA transcription factors which are involved in regulation of important virulence factors in pathogenic bacteria. These groups of effector domains are unique in their ability to bind to DNA and account for ~2.7% of all prokaryotic RRs.

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 LytS 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 pseudo first-order rate constant of the autokinase activity of His-LytS was determined to be 0.030 ± 0.001 min⁻¹. The autophosphorylation rate constant of LytS is smaller compared to other HKs such as E. coli nitrate sensing HK NarQ (0.014 min⁻¹), and E. coli citrate sensing HK DcuS (0.043 min⁻¹). The binding affinity of LytS for ATP (Kₐ = 7.9 ± 0.6 µM) is higher in comparison to other kinase such as, VanS (Kₐ = 620 ± 42 µM), VraS (Kₐ = 230 ± 42 µM (Belcheva et al. unpublished data)) and WalK (Kₐ = 130 µM). Other HKs such as E. coli nitrate sensing HK NarQ (0.014 min⁻¹), and E. coli citrate sensing HK DcuS (0.043 min⁻¹). The binding affinity of LytS for ATP (Kₐ = 7.9 ± 0.6 µM) is higher in comparison to other kinase such as, VanS (Kₐ = 620 ± 42 µM), VraS (Kₐ = 230 ± 42 µM (Belcheva et al. unpublished data)) and WalK (Kₐ = 130 µM).

Figure 2. The autokinase activity of His-LytS. (A) His-LytS at 5 µM was incubated with [γ-32P]-ATP (100 µM) 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 phosphorylated His-LytS species. His-LytS at 5 µM 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). The gel in panel (A) was also stained with Coomassie blue to view the protein bands.

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 P²⁻labeled His-LytS was incubated with GST-LytR, we observed

![Image](image-url)
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 ± 0.1 min⁻¹. When incubation of P32-labeled His-LytS was done in the presence of GST-LytRAsp53Asn, 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 ± 0.1 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)33. In the case of the stand-alone receiver domain, LytR⁵³, the observed phosphorylation rate constant was 0.9 ± 0.2 min⁻¹ (Figure 4B).

![Figure 3. The phosphotransfer between His-LytS and GST-LytR or GST-LytRAsp53Ala variant.](image)

(A) Phosphorylated His-LytS at 4 µM was incubated with GST-LytR at 10 µM 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 LytR⁵³ by acetyl phosphate.](image)

(A) LytR at 10 µM 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) LytR⁵³ at 20 µM 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).
The higher phosphorylation rate constant measured for the LytR\textsuperscript{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 \textit{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 interactions\textsuperscript{33}.

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\textsuperscript{N}, which resolved well in native-PAGE. These experiments showed that phosphorylation of LytR\textsuperscript{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\textsuperscript{N} to dimerize upon phosphorylation was used to monitor the phosphatase activity of LytS; dephosphorylation of LytR\textsuperscript{N} will lead to disintegration of the dimer which can readily be monitored in a native-PAGE system. Indeed, incubation of the phosphorylated LytR\textsuperscript{N} with LytS led to conversion of the dimer to monomer species indicating that LytS has phosphatase activity (Figure 6A, Dataset 4). Interestingly, the phosphatase

![Figure 5. The effect of phosphorylation on the oligomerization states of LytR\textsuperscript{N}.](image)

![Figure 6. The phosphatase activity of LytS and autophosphatase activity of LytR\textsuperscript{N}.](image)
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 EnvZ.\(^2\)

LytR\(^3\) 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.

### Discussion

LytSR is involved with sense-response to alterations of the cell electrical membrane potential due to cell membrane perturbations\(^3\). Its function is related to regulation of genes controlling cell apoptosis, autolysin activity and biofilm formation\(^19\). LytR regulates the lrgAB and cidABC operons\(^1\) whereby cid genes encode holin-like proteins that enhance murein hydrolysis activity and trigger cell lysis\(^1\) and antibiotic tolerance, while the lrg genes products encode antiholin-like proteins that inhibit these processes.

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 activity\(^13\). 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 RRs\(^26\).

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 auto-phosphorylation efficiency for LytS is high and the kinase is well positioned to participate directly in the signaling process induced by changes in the cytoplasmic membrane electrical potential. The fast phosphotransfer process that we observed between LytS and LytR \(0.3 \text{ min}^{-1}\) 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 donors\(^36\). In fact, phosphorylation of RRs by low molecular weight phosphoryl group donors such as acetyl phosphate, carbamyl phosphate or phosphoramide appears to be more common than phosphorylation by non-cognate HKs\(^35\). 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\(^33,35,36\). 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\(^3\) 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\(^19\). One regulatory network responds to excess glucose metabolism and the other responds to changes in the cell membrane potential\(^1\). Induction of lrgAB in response to glucose metabolism was shown in vivo to rely on LytR\(^19\), however, metabolism of excess glucose does not lead to changes in the cytoplasmic membrane electrical potential\(^1\), 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.

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.1339843

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
This work was funded in full by a grant to DGK from Natural Sciences and Engineering Research Council of Canada and in part by the Early Researcher Award from Ontario Ministry of Research and Innovation.

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

Acknowledgements
We thank Sivadarshini Pathmanathan for her assistance in analyzing the phosphatase activity of LytS.

References


Open Peer Review

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 that 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 pathway.” 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

Competing Interests: No competing interests were disclosed.

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.

Author Response 11 Mar 2016
Dasantila Golemi-Kotra, York University, Toronto, 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 that 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 pathway.” This is an inaccurate reference, as cidABC has only been shown to be regulated by CidR, whereas lrgAB 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 out 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 my Lehman et al. in the new version of the manuscript.

**Competing Interests:** There is no competing interests.

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**Reviewer Report 24 February 2016**

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

In Staphylococcus aureus, transcription of the lrgAB 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.

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“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.

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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.

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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 uM for LytR and 20 uM 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.

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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 lrgAB 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 lrgAB will be lower in the pta mutant while higher in the ackA mutant, as compared with wild type cells.

Page 10 (right column), the second paragraph from bottom: The authors used the reference 20 to introduce two different regulation mechanism of the lrgAB operon. However, the reference 20 is about the regulation of cidABC operon by CidR. I think the reference 1 is more appropriate.

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 lrgAB.

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 lrgAB.

3. Glucose, full membrane potential
   : The efficient phosphorylation of LytR by acetyl phosphate will overcome the phosphatase activity of LytS, resulting in higher expression of lrgAB.

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 lrgAB.

**Competing Interests:** No competing interests were disclosed.

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

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“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.

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 phospho-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 32P-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 is 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.
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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 date points shows that for LytRN-P the signal increases faster when considering all the data points.

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.

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 statistically 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.

The experimental data in Fig3A, the dephosphorylation of LytS-P, were fitted into first-order rate kinetics. However, a quick view at the phosphor image shows that in about 1 min, 50% of the 32P-signal is lost from LytS-P. Dephosphorylation of LytS follows first-order rate kinetics, hence, \(t_{1/2} = \ln2/k\) (where \(t_{1/2}\) is the time that is takes for 50% of the reaction to complete, and \(k\) is the observed first-order rate constant), \(k = \ln2/t_{1/2}\). One can estimate the value of \(k\) from this single data point.

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 lrgAB 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 lrgAB will be lower in the pta mutant while higher in the ackA mutant, as compared with wild type cells.

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

Page 10 (right column), the second paragraph from bottom: The authors used the reference 20 to introduce two different regulation mechanism of the lrgAB operon. However, the reference 20 is about the regulation of cidABC operon by CidR. I think the reference 1 is more appropriate.

Yes, that is correct.

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 lrgAB.
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 lrgAB.
3. Glucose, full membrane potential
   : The efficient phosphorylation of LytR by acetyl phosphate will overcome the phosphatase activity of LytS, resulting in higher expression of lrgAB.
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 lrgAB.

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.
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