Published in final edited form as:

Biochemistry. 2018 December 11; 57(49): 6797–6805. doi:10.1021/acs.biochem.8b01072.

Two mutations commonly associated with daptomycin resistance in *Enterococcus faecium* LiaS^{T120A} and LiaR^{W73C} appear to function epistatically in LiaFSR signaling

Milya Davlieva¹, Chelsea Wu¹, Yue Zhou¹, Cesar A. Arias^{2,3,4,5,6}, Yousif Shamoo^{*,1}

¹Department of Biosciences, Rice University, Houston, TX 77005 USA

²Core for Biomolecular Structure and Function, Department of Genomic Medicine, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

³Center for Antimicrobial Resistance and Microbial Genomics and Division of Infectious Diseases, UTHealth McGovern Medical School, Houston, TX, USA

⁴Department of Microbiology and Molecular Genetics, UTHealth McGovern Medical School, Houston, TX, USA

⁵Center for Infectious Diseases, UTHealth School of Public Health, Houston, TX, USA

⁶Molecular Genetics and Antimicrobial Resistance Unit, International Center for Microbial Genomics, Universidad El Bosque, Bogota, Colombia.

Abstract

The cyclic antimicrobial lipopeptide daptomycin is now frequently used as a first-line therapy in serious infections caused by multidrug-resistant *Enterococcus faecium*. Resistance to daptomycin in *E. faecium* is mediated by activation of the LiaFSR membrane stress response pathway. Deletion of *liaR*, encoding the response regulator of the system, restores susceptibility to daptomycin, suggesting that the LiaFSR pathway is a potential target for the development of drugs that would induce hypersusceptibility to daptomycin as well as make it more difficult for enterococci to become daptomycin resistant. In clinical isolates of *E. faecium*, substitutions in the membrane-bound histidine kinase LiaS (T120A) and its response regulator LiaR (W73C) are found together, suggesting a potential epistatic relationship in daptomycin resistance. Using *in vitro* phosphorylation studies, we show that while the phosphotransfer rate of the wild type LiaS and LiaS^{T120A} to either wild type LiaR or LiaR^{W73C} remains rapid and comparable, the LiaS

* Address correspondence to Yousif Shamoo, Shamoo@rice.edu. Address: 6100 Main Street, Department of BioSciences, Houston, TX 77005, USA.

Authors Contributions

MD and YS designed the research plan, analyzed data and wrote the paper; MD and CW performed the experiments. CAA analyzed data and edited the manuscript.

Conflict of interest

Dr. Cesar Arias is on the speaker's Bureau for Forest, Theravance, Pfizer, Astra-Zeneca, Cubist, The Medicines Company, Novartis. He is also consulting for Theravance, Cubist, Bayer and grant investigator for Theravance and Scientific advisor (review panel or advisor committee) for Bayer.

Supporting Information

• Data analysis of kinase activity assays, control kinase reactions with LiaR^{D54A} and LiaS^{T120A}H164A, and circular dichroism studies.

dependent dephosphorylation rate of phosphorylated LiaR^{W73C} is markedly higher. When the two adaptive mutants LiaR^{W73C} and LiaS^{T210A} are paired, however, LiaS mediated LiaR dephosphorylation is restored back to wild type levels. Taken together with earlier work showing that LiaR^{W73C} leads to increased oligomerization and subsequently favors increased transcription of the LiaFSR regulon, the net effect of the two commonly found LiaS^{T210A} and LiaR^{W73C} alleles would be to coordinately increase the strength and persistence of LiaFSR signaling and reduce daptomycin susceptibility. The *in vitro* approaches developed in this work also provide the basis for screens to identify drug candidates that inhibit the LiaFSR pathway.

Graphical Abstract

Introduction

Antibiotic resistance within clinical pathogens continues to be a persistent and immediate threat to human health. Vancomycin-resistant enterococci (VRE) have been classified by the Centers for Disease Control and Prevention (CDC) as a serious threat and are responsible for at least 20,000 infections and ~1,300 deaths annually in the United States (<http://www.cdc.gov/drugresistance/threat-report-2013/>). Both, the CDC and World Health Organization, have included VRE as a priority for new therapies (World Health

Organization. Antibiotic-resistant priority pathogens list, Virtual press conference, 27 February 2017. <http://www.who.int/mediacentre/news/releases/2017/bacteria-antibiotics-needed/en/>). Daptomycin (DAP), a cyclic lipopeptide antibiotic whose mechanism of action requires interactions with the cell membrane of Gram-positive bacteria in a phosphatidylglycerol (PG)-dependent manner, has become an important therapy against serious infections caused by multi-drug resistant *Enterococcus faecium* (*Efm*)^{1–5}. However, in enterococci, resistance to DAP readily develops during therapy, a phenomenon that is often the result of mutations within the LiaFSR cell envelope stress response pathway^{6–8}.

The LiaFSR regulon is made up of a sensor histidine kinase LiaS; a membrane-bound protein LiaF, thought to modulate LiaS activity; and a response regulator LiaR (Lia denotes lipid II-interfering antibiotics)⁹. LiaS is a member of the HisKA_3 family and is comprised of two domains, an N-terminal transmembrane sensor domain (residues 1–80) and C-terminal catalytic kinase domain (residues 81–355)^{10–13}. Based on sequence homology, LiaS is analogous to the canonical two-component thermosensory protein DesK in *Bacillus subtilis* and VraS in *Staphylococcus aureus* that function as dimers to first autophosphorylate and then subsequently transfer the phosphate to their cognate response regulator DesR and VraR, respectively^{14–18}.

Like DesKR, the first proposed catalytic step in the membrane damage response for the LiaSR cascade is the autophosphorylation of a conserved His residue within the cytoplasmic domain of LiaS followed by transfer of the phosphate to the receiver domain of LiaR (residues 1–139) at Asp-54. Phosphorylation of LiaR leads to LiaR oligomerization and a subsequent increased affinity for specific regulatory DNA elements to produce increased gene expression of particular operons such as *liaFSR* and *liaXYZ*^{19, 20}. Thus, upregulation of signaling through LiaFSR signaling is critical to the cell membrane damage response. Deletion of *liaR* in both *Enterococcus faecalis* and *E. faecium* abolishes the adaptive response and results in hypersusceptibility not only to DAP but also to a variety of other antimicrobial peptides, including those which are produced by cells of the innate immune system, such as LL-37^{21, 22}.

Our previous studies and those of others, have consistently indicated that mutations within the LiaFSR pathway are one of the most common routes to DAP resistance in clinical isolates of *E. faecium*^{4–7, 23}. Indeed, in such isolates, the emergence of DAP resistance is frequently associated with a Trp-73 to Cys substitution in LiaR²⁴ that, to date, is always found with a Thr-120 to Ala substitution in LiaS (LiaS^{T120A}), suggesting a potential epistatic relationship²⁴ (Figure 1).

Using specific assays to measure LiaS autophosphorylation and phosphotransfer to LiaR, we have studied the *in vitro* phosphorylation kinetics of the wild type and adaptive proteins to understand the biochemical basis for the increased LiaFSR signaling and how mutations within LiaS and LiaR further increase response. Additionally, we propose that inhibition of the LiaFSR pathway could be an important and novel strategy for the discovery of new drugs whose incorporation with DAP therapy would both increase DAP sensitivity and reduce the spread of antibiotic resistance since mutations within the LiaFSR pathways commonly seen

in clinical populations would not have an easily accessible evolutionary trajectory to resistance²¹.

Materials and methods

Expression and purification of histidine kinase LiaS from *E. faecium*

A fragment lacking the first 243bp of the *liaS* (TX1330) coding sequence that corresponds to the 81 amino acid sequence encoding the two transmembrane domains was cloned into vector pET28a(+). The resulting recombinant plasmid was propagated in *E. coli* XL-1 Blue cells and then transformed into *E. coli* BL21(DE3) cells for expression. Site-direct mutagenesis of LiaS was performed by the incorporation of mutation T120A into the plasmid by inverse PCR with standard primers designed in an overlapping orientation. *E. coli* BL21 (DE3) harboring plasmids pET28a_81LiaS (TX1330) and pET28a_81LiaS^{T120A} (TX1330) were grown at 37 °C in LB medium containing 50 mg/ml kanamycin to mid-log phase. Expression was induced at 16 °C by the addition of β -d-1-thiogalactopyranoside to 0.4 mM, and the culture was incubated for an additional 16 h. After sonication (Qsonica, LLC) recombinant proteins were purified using a nickel affinity column (GE Healthcare). The column was washed with Buffer A (50 mM Tris pH 7.5, 500 mM NaCl, 20 mM Imidazole, 1mM DTT, 0.2 mM PMSF, 5% glycerol (v/v)), and protein of interest was eluted with a continuous elution gradient from 20 to 500 mM Imidazole. The fractions containing LiaS protein were pooled and dialyzed against 50 mM Tris pH 8.0, 200 mM NaCl, 1 mM DTT, 5% glycerol (v/v). The N-terminal His-tag was subsequently removed by treatment with TEV protease as described previously²⁵. The protein was further purified through Q-XL-Sepharose column (GE Healthcare) using 0.1– 1 M NaCl gradient followed by size-exclusion chromatography (50 mM Tris pH 7.5, 250 mM NaCl, 1mM DTT, 5% glycerol (v/v)). Peak fractions were pooled, and sample purity was assessed by SDS-page gel and found to be more than 95% pure and well folded (See Supplementary Information Figure S1)

Expression and purification of response regulator LiaR from *E. faecium*

A fragment of *liaR* encoding the full length of response regulator LiaR was amplified from genomic DNA of *E. faecium* R494. Site-direct mutagenesis of LiaR was performed by the incorporation of mutation W73C into the plasmid by inverse PCR with standard primers designed in an overlapping orientation. Full-length LiaR and LiaR^{W73C} were cloned, expressed and purified as described in previous work^{19, 20}.

Autophosphorylation and phosphotransfer assays

All phosphorylation assays were performed in Buffer A (50 mM Tris pH 7.5, 250 mM NaCl, 5 mM MgCl₂). The autophosphorylation assay was performed at 15 μ M LiaS or LiaS^{T120A} in Buffer A at room temperature or 37 °C. The purified putative cytoplasmic domain (residues 81–355) of LiaS or LiaS^{T120A} was incubated with 0.2 mM ATP for 30, 60, 120, 180, 240 and 420 min at 37 °C. The aliquots were withdrawn at indicated time points, and reactions were stopped by addition SDS-page sample buffer. Coomassie blue-stained gels were scanned, and quantification of the reactions was done using ImageJ software²⁶.

To perform the phosphotransferase assay, purified LiaR (15 μ M) was equilibrated in Buffer A at 37 °C. The reaction was initiated by addition of phosphorylated LiaS at 1:1 ratio (15 μ M). Note, the histidine kinase was first allowed to autophosphorylate for 60 min at 37 °C. To evaluate the phosphorylation of LiaR over time, the aliquots were withdrawn at 10, 20, 30, 60, 120 and 300 s and all reactions were stopped by adding SDS-page sample buffer. Samples were then separated by Phos-tag SDS-PAGE electrophoresis, and the amount of phosphorylated protein was quantified using ImageJ software²⁶.

The phosphorylation of LiaR by inorganic small molecule phosphoryl group donors was carried out at room temperature and 37 °C. LiaR (250 μ M) was phosphorylated by incubation with 17 mM lithium potassium phosphate (Sigma) for 20 min at 37 °C. Phosphorylated LiaR was purified using a PD-10 Sephadex G-25M column (GE Healthcare) at 25 °C. Then, in order to test P~LiaR stability reactions were stopped at different time points by adding SDS-page buffer as described above, and all samples were subjected to Phos-tag SDS-PAGE electrophoresis.

Binding affinity determination by Microscale Thermophoresis (MST)

MST experiments were performed on a Monolith NT.115 system (Nanotemper Technologies) as described previously^{19, 20, 27} using 20%, 40% and 70% LED and 90% IR-laser power. Laser on and off times were set at 30 s and 5 s, respectively. FITC labeled peptide (DVILMDLVM) was synthesized by GenScript USA Inc.

In order to measure the affinity of LiaS to phosphomimetic peptide, a solution of unlabeled protein was serially diluted in the reaction buffer (10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 2.7 mM KCl, 137 mM NaCl, 0.05% (v/v) Tween-20) to which an equal volume of FITC-labeled peptide was added to a final concentration of 84 nM.

Alternatively, LiaS was labeled in 0.01 M sodium phosphate, pH 7.4, 0.0027 M potassium chloride and 0.137 M NaCl buffer using the NT-647-NHS amine reactive dye (Monolith NT). A solution of unlabeled LiaR was serially diluted in the same reaction buffer to which an equal volume of fluorescein-labeled LiaS was added to a final concentration of 20 nM. Experiments were repeated in the presence of 0.2 mM ATP or 1mM AMP-PNP. The resulting K_d values are based on an average from at least three independent MST measurements. Data analyses were performed using Nanotemper Analysis software, v.1.5.41.

Results

In vitro autophosphorylation of the *E. faecium* LiaS cytoplasmic domain (81–355)

To reconstitute the LiaS - LiaR phosphorylation cascade, we first demonstrated that the cytoplasmic domain of *E. faecium* LiaS and the adaptive mutant LiaS^{T120A} were able to undergo autophosphorylation using ATP. To assess the phosphorylation state of LiaS, a Phos-tag sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) assay was used^{28, 29}. The amount of phosphorylated and non-phosphorylated proteins could be estimated from the intensity of the upper and lower bands in the Phos-tag assay as a function of reaction time (Figure 2a).

Interestingly, LiaS and LiaS^{T120A} both showed evidence for phosphorylation prior to the addition of ATP. Since the proteins were overexpressed and purified from *Escherichia coli*, we speculate that LiaS could be stably autophosphorylated *in vivo* prior to purification. The extent of *in vivo* phosphorylation was consistent across different protein preparations. Based on Phos-tag SDS page analysis, about 30% of purified LiaS and 10% of purified LiaS^{T120A} were estimated to be already phosphorylated presumably by the intracellular pool of ATP prior to purification. To evaluate the autophosphorylation of LiaS over time, the purified protein was incubated with 0.2 mM ATP at 37 °C for 420 minutes and the extent of autophosphorylation estimated using ImageJ²⁶. The binding curves shown in Figure 2b are the best fit of the data after the initial pool of phosphorylated LiaS was subtracted. The time course suggested that autophosphorylation of wild type LiaS consisted of two phases: a faster first 30 min followed by a second slower phase towards saturation. About 50% of LiaS autophosphorylation occurs within ~45 min. By 120 minutes autophosphorylation of LiaS was largely complete. After 360 min of incubation, about 90% of the total LiaS present in the reaction mixture was phosphorylated. The autophosphorylation activity of LiaS was slightly decreased if the temperature was reduced from 37 to 25 °C (Figure S2). The phosphorylated protein was stable at least for 2 hours at 37 °C.

The adaptive mutant LiaS^{T120A} exhibited different autophosphorylation activity. We found that ~50% of LiaS^{T120A} autophosphorylation *in vitro* occurs rapidly; however even after 420 min, only about 50% of adaptive mutant LiaS^{T120A} could be phosphorylated. Based on these results LiaS^{T120A} appears to achieve an almost 2-fold lower extent of autophosphorylation than wild type under identical assay conditions. To note, circular dichroism (CD) experiment suggests that *Efm* LiaS and adaptive mutant LiaS^{T120A} are well folded and have largely alpha-helical secondary structure (Figure S1).

Phosphotransfer from the LiaS cytoplasmic domain to LiaR is very rapid

To perform LiaS phosphotransferase activity assays, the purified putative cytoplasmic kinase domain of LiaS (LiaS^{81–355}) was first allowed to autophosphorylate with 0.2 mM ATP at 37 °C for 60 minutes and subsequently incubated with LiaR at the same temperature. As shown in Figure 3, time-dependent phosphotransfer was rapid with transfer of the phosphoryl group to LiaR occurring within seconds of incubation. It was not possible to conduct the assay faster than 10 s. As shown in Figure 3, at t=0 LiaS is largely phosphorylated, but the phosphorylated LiaS is nearly depleted by 10 s suggesting that the kinetics of transfer are largely completed by the first time point. At 20 seconds, LiaR phosphorylation reached about 35% and appeared to reach an equilibrium such that even at 5 minutes the total amount of LiaR phosphorylation was largely unchanged. At 1 to 5 minutes there was modest disappearance of phosphorylated LiaR (Figure 3). We tested all four combinations of LiaS, LiaS^{T120A}, LiaR, and LiaR^{W73C} to discern if the mutations identified in clinical settings altered either the rates or extents of phosphorylation. As shown in Figure 3 and Figure S3, phosphotransfer between LiaS^{T120A} to LiaR^{W73C}, LiaS^{T120A} to LiaR, and LiaS to LiaR^{W73C} have comparable extents and rates under these reaction conditions.

To ensure that the phosphotransfer reaction from LiaS to LiaR was occurring at Asp-54 of LiaR, we made LiaR^{D54A}. As predicted from our previous co-crystal structure of LiaR with

the small molecule phosphomimic BeF_3^- , LiaR^{D54A} cannot be phosphorylated by LiaS or by the small molecule acetyl phosphate (Figure S4). We also made the LiaS mutant LiaS^{H164A} and showed that it cannot phosphorylate LiaR suggesting that we have reconstituted the *bone fide* phosphorylation relationship of LiaS to LiaR (Figure S4). Unlike wild type LiaS or LiaS^{T120A}, upon purification LiaS^{H164A} is not a mixture of phosphorylated and unphosphorylated species again suggesting that it is not able to be phosphorylated either *in vivo* or *in vitro*.

We found that dephosphorylation of wild-type LiaR was not accompanied by a significant reverse phosphotransfer back to LiaS. In contrast, the phosphotransfer reaction using the adaptive mutant LiaR^{W73C} showed weak but clear dephosphorylation of LiaR^{W73C} followed by a modest re-emergence of the phosphorylated LiaS species (Figure 3). The adaptive mutant LiaS^{T120A} by itself did not alter the extent of the reverse reaction but together with LiaR^{W73C} showed significantly less of the reverse phosphotransfer reaction. Histidine kinase stimulated dephosphorylation of a response regulator, implying the presence of phosphatase activity, is a well-known and has been reported in *S. aureus* VraS, *B. subtilis* DesK, *E. coli* EnvZ, *Mycobacterium tuberculosis* DevS^{14, 17, 30–32}.

The total extent of *in vitro* phosphorylation of the adaptive mutant LiaR^{W73C} is much lower than wild type

We also investigated the total extent of phosphorylation of LiaR using the small molecule phosphoryl group donor acetyl phosphate. We measured the ratio of phosphorylated LiaR with respect to the total amount of protein at a fixed time point (Figure 4a). Based on our data, acetyl phosphate mediated phosphorylation was significantly lower at 25 °C than at 37 °C (Figure 4b). Also, wild-type LiaR readily acquired phosphate from acetyl phosphate and became nearly completely phosphorylated (~80%) over approximately 20 min at 37 °C. In contrast, incubation of LiaR^{W73C} with acetyl phosphate resulted in only a very modest increase in overall phosphorylation (~25%) at 37 °C, suggesting that LiaR^{W73C} is more difficult to phosphorylate. After the removal of acetyl phosphate from the reaction mixture, phosphorylated LiaR was stable at least for 30 minutes at 25 °C (Figure 5, S5).

The LiaR receiver domain (LiaR^{1–139}) is involved in LiaS:LiaR interactions

We used microscale thermophoresis (MST) to measure the binding affinity of LiaS wild-type and LiaS^{T120A} cytoplasmic domains to LiaR mimetic peptides in the presence and absence of ATP (Figure 6a). We synthesized three LiaR mimetic peptides spanning the site of Asp-54 phosphorylation to determine whether a small stretch of the LiaR receiver domain containing the site of phosphorylation could recapitulate LiaS:LiaR interactions: Peptide 1 (LMDLV), Peptide 2 (ILMDLVM) and Peptide 3 (DVILMDLVM). We found that LiaS binds to Peptide 3 at micromolar concentrations ($K_d = 208.9 \pm 59.6 \mu\text{M}$). We also observed a modest two-fold increase in affinity when the experiment was repeated in the presence of ATP ($K_d = 114.1 \pm 17.0 \mu\text{M}$). Interestingly, the binding of *E. faecium* LiaS^{T120A} to Peptide 3 did not generate a sufficient fluorescence change to obtain a reliable K_d , indicating no detectable binding. The absence of any fluorescence change suggests that the affinity of LiaS^{T120A} for Peptide 3 was very weak and was in excess of 1 millimolar. The affinity of LiaS^{T120A} for Peptide 3 in the presence of ATP was comparable to that of the wild-type LiaS

($K_d = 121.4 \pm 6.8 \mu\text{M}$). As described earlier, ~30% and ~10% of purified LiaS and LiaS^{T120A}, respectively, are already phosphorylated during expression and purification. Thus, the “no ATP” experiment is actually being performed with a heterogeneous mixture of 30% phosphorylated LiaS in the case of the wild type protein and could partly explain the difference in the observed dissociation constants between wild-type and LiaS^{T120A}. Since the extent of contamination by phosphorylated LiaS^{T120A} in the “no ATP” experiment is about three-fold less than wild type, it stands to reason that the affinity of LiaS and LiaS^{T120A} is approximately millimolar in the absence of phosphorylation.

LiaS cytoplasmic domain also shows weak binding to LiaR

We also determined the dissociation constant of the cytoplasmic domain of LiaS and full-length LiaR using MST (Figure 6b). The apparent K_d value obtained for the LiaS:LiaR interaction was $162.7 \pm 31 \mu\text{M}$. Addition of ATP resulted in an increase of the binding affinity by two-fold, consistent with our previously performed LiaR mimetic peptide binding study. As with the peptide mimetic study, the same caveats apply that the no nucleotide assays contain protein, which is already approximately 30% phosphorylated in the case of wild type LiaS. The experiments were repeated in the presence of the non-hydrolyzable ATP derivative AMP-PNP. LiaS was incubated with 2 mM AMP-PNP at 37 °C for three hours, and the K_d for LiaR estimated by MST. The K_d values in the presence of phosphorylated LiaS or in the presence of non-hydrolyzable ATP analog (AMP-PNP) were $102.0 \pm 1.2 \mu\text{M}$ and $109.0 \pm 11.9 \mu\text{M}$, respectively, indicating that the LiaS affinity for LiaR was comparable.

Discussion

In *E. faecium* and *E. faecalis*, the LiaFSR cell membrane damage response pathway is a critical modulator of DAP resistance. Importantly, mutations within LiaFSR are typically the first steps in the development of DAP non-susceptible strains in the clinic^{6, 7}. Deletion of LiaR has shown that disruption of the LiaFSR pathway leads to DAP hypersusceptibility and could form the basis of new antimicrobial strategies focused on disrupting the cell membrane adaptive response.

In the current work we established *in vitro* assay conditions to investigate the phosphorylation of the wild type and most common clinical alleles associated with DAP resistance (LiaS^{T120A} and LiaR^{W73C}). Autophosphorylation studies of LiaS and LiaS^{T120A} suggest that the overall extent of autophosphorylation readily achieved for LiaS^{T120A} is significantly reduced compared to wild-type LiaS and the reaction is much slower ($t_{1/2} \sim 45$ minutes) than the subsequent phosphotransfer to LiaR ($t_{1/2} \sim 5$ s). Our measured autophosphorylation reaction is somewhat slower than that of the *E. faecium* histidine kinase (HK) VanS (0.17 min^{-1} at room temperature, $t_{1/2} = 4 \text{ min}$)³³, *S. aureus* VraS (0.07 min^{-1} , $t_{1/2} = 10 \text{ min}$ at room temperature)¹⁷ and *E. coli* fumarate-stimulated HK DcuS (0.043 min^{-1} at room temperature, $t_{1/2} = 16 \text{ min}$)³⁴. Nonetheless, like these other systems, the $t_{1/2}$ times are on the order of minutes. The lower extent of autophosphorylation for LiaS^{T120A} is also consistent with our observation that only 10% of LiaS^{T120A}, compared to 30% of wild type LiaS, were estimated to be phosphorylated *in vivo* prior to purification. Thus, the Thr to Ala mutation appears to limit or inhibit the extent of steady-state phosphorylation although the

autophosphorylated state is relatively stable as it takes 3–5 days to purify LiaS for Phos-tag SDS page analysis. It is possible that the LiaS^{T120A} substitution may limit autophosphorylation to only one site of the presumptive LiaS dimer^{14, 15}, however, any mechanistic interpretation must take into account the caveat that our studies were performed on the cytoplasmic domain of LiaS and, therefore, the membrane context and the N-terminal sensor domain are absent. Presumably, in the context of the full-length transmembrane protein, a more robust autophosphorylation may occur during membrane stress. We measured the affinity of the LiaS cytoplasmic domain for full length LiaR as well as a peptide target that mimics the LiaR phosphorylation site in the presence of ATP or a non-hydrolyzable ATP analog and found a K_d of ~100 μ M. Since the *in vivo* concentration of LiaR is expected to be on the order of 5 μ M, the *in vitro* K_d is lower than would be expected for the full length LiaS. Importantly, we show that the LiaR mimetic peptide has about the same affinity for LiaS as LiaR, since binding is stimulated about two-fold in the presence of ATP. While our observed K_d s are lower than those for the well described DesKR system, it has been previously shown that the HK domain alone, expressed separately as a recombinant protein, was not able to form a stable complex with the response regulator protein domain either in the presence or absence of ATP³⁵. Given that the cytoplasmic domain is missing its transmembrane region, it may well be that the full-length LiaS may further increase the K_d for LiaR.

Our results indicated that while the phosphorylated form of LiaS (LiaS~P) is relatively stable, its half-life was significantly reduced in the presence of LiaR and resulted in the rapid transfer of the phosphoryl group to generate active phosphorylated species (LiaR~P), suggesting that the stability of phosphorylated LiaS~P is modulated by LiaR. The phosphorylated LiaR could be detected within seconds of incubation at 37 °C when phosphorylated LiaS was added as the phosphodonor. Our data indicate that the *in vitro* phosphotransfer rate from histidine kinase LiaS to response regulator LiaR is at least 400-fold faster than the LiaS autophosphorylation rate and is similar to the rates measured for *S. aureus* VraS, *B. subtilis* DesR, and *Listeria monocytogenes* LiaR^{17, 18, 36}.

While the overall extent of LiaR phosphorylation is about 35%, we show that our purified protein could be fully phosphorylated by small inorganic molecule phosphoryl group donors, suggesting that the reaction is at rapid equilibrium. The overall extent to which LiaR^{W73C} could be phosphorylated using acetyl phosphate as a phosphodonor was ~2.6-fold lower than wild type. In an earlier study, we demonstrated that a larger fraction of LiaR^{W73C} is in the activated oligomeric state, and thus the difficulty in reaching the higher extent of phosphorylation may be simply because more of the mutant protein is already activated and therefore recalcitrant to phosphorylation²⁰. In previous work, we were unable to measure any significant dimerization of the wild type *E. faecium* LiaR, while the LiaR^{W73C} receiver domain showed a 7-fold higher level of dimerization in the absence of phosphorylation that would potentially lead to increased constitutive signaling. Moreover, while inactivated *Efm* LiaR exists mostly as monomer in solution with a dissociation constant of $K_d > 1600 \mu$ M, the propensity towards dimerization increased at least 2 orders in magnitude by addition of BeF_3^- ($K_d = 15 \mu$ M) through the formation of the BeF_3^- :LiaR complex. In contrast, we could not detect any further increase in dimerization of the full length *Efm* LiaR^{W73C} in the presence of BeF_3^- ²⁰. Our data are also consistent with the previous model that LiaR^{W73C}

mutant would resemble our crystal structure of LiaR in the beryll fluoride-activated state and results in an activated configuration with a closed phosphate lid, reducing further phosphotransfer²⁰. Binding to LiaS could trigger phosphate lid opening and enable the transfer reaction. A similar structural regulation phenomenon was observed for the two-component system DesK-DesR from *B. subtilis*¹⁸. While we have demonstrated previously that LiaR^{W73C} has an increased propensity to form a phosphorylation independent dimer, Trp73 is also positioned at the interface of the receiver to DNA binding domains of LiaR. It is possible that the mutation could alter the interaction of the receiver domain with the DNA binding domain of the same protomer to alter signaling. Alternatively, it is possible that the molecular dynamics of the ensemble is altered in a more indirect manner to shift the LiaR ensemble towards a state that more closely resembles the phosphorylated state. Additionally, our study also suggested that adaptive mutant LiaR^{W73C} cannot be efficiently phosphorylated *in vivo* in the absence of its histidine kinase by an alternative phosphate donor, while wild-type LiaR could be subject to cross-talk from the cellular pool of acetyl phosphate in the absence of LiaS.

While the autophosphorylation and phosphotransfer to LiaR and LiaR^{W73C} are comparable, the reverse phosphotransfer to LiaS is substantially different. LiaR^{W73C} shows a substantial amount of reverse phosphotransfer to LiaS compared to wild type LiaR. However, when LiaS^{T120A} and LiaR^{W73C} are paired, the reverse reaction is substantially reduced supporting an epistatic relationship between the two alleles. Interestingly, in an earlier study we found that LiaS^{T120A} and LiaR^{W73C} have always been found together in DAP-resistant strains isolated from patients²⁴. While other LiaS variants have been observed in clinical settings such as LiaS^{D70P} and LiaS^{D251N}, LiaS^{T120A} has not been observed alone (7 of 7 observations where LiaS^{T120A} and LiaR^{W73C} are together). We speculate that LiaS^{T120A} may be compensatory to the LiaR^{W73C} adaptation to prevent the reverse phosphotransfer reaction back to LiaS that would diminish the strength and persistence of LiaFSR signaling. This model is also consistent with our observation that the half-life of LiaR^{W73C}~P is comparable with those of wild-type LiaR~P at 25 °C in the absence of LiaS (Figure 5). The estimated dephosphorylation pseudo-first order rate constant is 0.004 min⁻¹ (t_{1/2}=174 min) and 0.003 min⁻¹ (t_{1/2}=211 min) for LiaR and LiaR^{W73C}, respectively (Figure S5). Our study suggests a potential epistatic relationship for the commonly observed LiaS^{T120A} and LiaR^{W73C} variants found in clinical isolates of *E. faecium*. In addition, we have reconstituted LiaFSR phosphorylation kinetics *in vitro* in a manner that can be used for the development of drugs that could inhibit LiaFSR signaling to restore DAP susceptibility as well as to potentially reduce the evolution of future resistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by National Institutes of Health Grant [R01AI080714 to Y.S.]; [R21AI114961-01 to C.A.]; [R21/331R21AI121519-01 to C.A.].

References

- [1]. Zhang T, Muraih JK, Tishbi N, Herskowitz J, Victor RL, Silverman J, Uwumarenogie S, Taylor SD, Palmer M, and Mintzer E (2014) Cardiolipin prevents membrane translocation and permeabilization by daptomycin, *J Biol Chem* 289, 11584–11591. [PubMed: 24616102]
- [2]. Pogliano J, Pogliano N, and Silverman JA (2012) Daptomycin-mediated reorganization of membrane architecture causes mislocalization of essential cell division proteins, *J Bacteriol* 194, 4494–4504. [PubMed: 22661688]
- [3]. Mishra NN, Bayer AS, Tran TT, Shamoo Y, Mileykovskaya E, Dowhan W, Guan Z, and Arias CA (2012) Daptomycin resistance in enterococci is associated with distinct alterations of cell membrane phospholipid content, *PLoS One* 7, e43958. [PubMed: 22952824]
- [4]. Tran TT, Panesso D, Mishra NN, Mileykovskaya E, Guan Z, Munita JM, Reyes J, Diaz L, Weinstock GM, Murray BE, Shamoo Y, Dowhan W, Bayer AS, and Arias CA (2013) Daptomycin-resistant *Enterococcus faecalis* diverts the antibiotic molecule from the division septum and remodels cell membrane phospholipids, *MBio* 4, e00281–00213. [PubMed: 23882013]
- [5]. Palmer KL, Daniel A, Hardy C, Silverman J, and Gilmore MS (2011) Genetic basis for daptomycin resistance in enterococci, *Antimicrob Agents Chemother* 55, 3345–3356. [PubMed: 21502617]
- [6]. Arias CA, Panesso D, McGrath DM, Qin X, Mojica MF, Miller C, Diaz L, Tran TT, Rincon S, Barbu EM, Reyes J, Roh JH, Lobos E, Sodergren E, Pasqualini R, Arap W, Quinn JP, Shamoo Y, Murray BE, and Weinstock GM (2011) Genetic basis for in vivo daptomycin resistance in enterococci, *N Engl J Med* 365, 892–900. [PubMed: 21899450]
- [7]. Miller C, Kong J, Tran TT, Arias CA, Saxer G, and Shamoo Y (2013) Adaptation of *Enterococcus faecalis* to daptomycin reveals an ordered progression to resistance, *Antimicrob Agents Chemother* 57, 5373–5383. [PubMed: 23959318]
- [8]. Schrecke K, Jordan S, and Mascher T (2013) Stoichiometry and perturbation studies of the LiaFSR system of *Bacillus subtilis*, *Mol Microbiol* 87, 769–788. [PubMed: 23279150]
- [9]. Jordan S, Junker A, Helmann JD, and Mascher T (2006) Regulation of LiaRS-dependent gene expression in *Bacillus subtilis*: identification of inhibitor proteins, regulator binding sites, and target genes of a conserved cell envelope stress-sensing two-component system, *J Bacteriol* 188, 5153–5166. [PubMed: 16816187]
- [10]. Schultz J, Milpetz F, Bork P, and Ponting CP (1998) SMART, a simple modular architecture research tool: identification of signaling domains, *Proc Natl Acad Sci U S A* 95, 5857–5864. [PubMed: 9600884]
- [11]. Stock AM, Robinson VL, and Goudreau PN (2000) Two-component signal transduction, *Annu Rev Biochem* 69, 183–215. [PubMed: 10966457]
- [12]. Bhate MP, Molnar KS, Goulian M, and DeGrado WF (2015) Signal transduction in histidine kinases: insights from new structures, *Structure* 23, 981–994. [PubMed: 25982528]
- [13]. Zschiedrich CP, Keidel V, and Szurmant H (2016) Molecular Mechanisms of Two-Component Signal Transduction, *J Mol Biol* 428, 3752–3775. [PubMed: 27519796]
- [14]. Albanesi D, Martin M, Trajtenberg F, Mansilla MC, Haouz A, Alzari PM, de Mendoza D, and Buschiazzo A (2009) Structural plasticity and catalysis regulation of a thermosensor histidine kinase, *Proc Natl Acad Sci U S A* 106, 16185–16190. [PubMed: 19805278]
- [15]. Trajtenberg F, Albanesi D, Ruetalo N, Botti H, Mechaly AE, Nieves M, Aguilar PS, Cybulski L, Larrieux N, de Mendoza D, and Buschiazzo A (2014) Allosteric activation of bacterial response regulators: the role of the cognate histidine kinase beyond phosphorylation, *MBio* 5, e02105. [PubMed: 25406381]
- [16]. de Mendoza D (2014) Temperature sensing by membranes, *Annu Rev Microbiol* 68, 101–116. [PubMed: 24819366]
- [17]. Belcheva A, and Golemi-Kotra D (2008) A close-up view of the VraSR two-component system. A mediator of *Staphylococcus aureus* response to cell wall damage, *J Biol Chem* 283, 12354–12364. [PubMed: 18326495]

- [18]. Trajtenberg F, Imelio JA, Machado MR, Larrieux N, Marti MA, Obal G, Mechaly AE, and Buschiazzi A (2016) Regulation of signaling directionality revealed by 3D snapshots of a kinase:regulator complex in action, *Elife* 5.
- [19]. Davlieva M, Shi Y, Leonard PG, Johnson TA, Zianni MR, Arias CA, Ladbury JE, and Shamoo Y (2015) A variable DNA recognition site organization establishes the LiaR-mediated cell envelope stress response of enterococci to daptomycin, *Nucleic Acids Res* 43, 4758–4773. [PubMed: 25897118]
- [20]. Davlieva M, Tovar-Yanez A, DeBruler K, Leonard PG, Zianni MR, Arias CA, and Shamoo Y (2016) An Adaptive Mutation in *Enterococcus faecium* LiaR Associated with Antimicrobial Peptide Resistance Mimics Phosphorylation and Stabilizes LiaR in an Activated State, *J Mol Biol* 428, 4503–4519. [PubMed: 27670715]
- [21]. Tran TT, Miller WR, Shamoo Y, and Arias CA (2016) Targeting cell membrane adaptation as a novel antimicrobial strategy, *Curr Opin Microbiol* 33, 91–96. [PubMed: 27458841]
- [22]. Panesso D, Reyes J, Gaston EP, Deal M, Londono A, Nigo M, Munita JM, Miller WR, Shamoo Y, Tran TT, and Arias CA (2015) Deletion of *liaR* Reverses Daptomycin Resistance in *Enterococcus faecium* Independent of the Genetic Background, *Antimicrob Agents Chemother* 59, 7327–7334. [PubMed: 26369959]
- [23]. Reyes J, Panesso D, Tran TT, Mishra NN, Cruz MR, Munita JM, Singh KV, Yeaman MR, Murray BE, Shamoo Y, Garsin D, Bayer AS, and Arias CA (2015) A *liaR* deletion restores susceptibility to daptomycin and antimicrobial peptides in multidrug-resistant *Enterococcus faecalis*, *J Infect Dis* 211, 1317–1325. [PubMed: 25362197]
- [24]. Diaz L, Tran TT, Munita JM, Miller WR, Rincon S, Carvajal LP, Wollam A, Reyes J, Panesso D, Rojas NL, Shamoo Y, Murray BE, Weinstock GM, and Arias CA (2014) Whole-genome analyses of *Enterococcus faecium* isolates with diverse daptomycin MICs, *Antimicrob Agents Chemother* 58, 4527–4534. [PubMed: 24867964]
- [25]. Montealegre MC, Roh JH, Rae M, Davlieva MG, Singh KV, Shamoo Y, and Murray BE (2017) Differential Penicillin-Binding Protein 5 (PBP5) Levels in the *Enterococcus faecium* Clades with Different Levels of Ampicillin Resistance, *Antimicrob Agents Chemother* 6s1.
- [26]. Schneider CA, Rasband WS, and Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis, *Nat Methods* 9, 671–675. [PubMed: 22930834]
- [27]. Jerabek-Willemsen M, Wienken CJ, Braun D, Baaske P, and Duhr S (2011) Molecular interaction studies using microscale thermophoresis, *Assay Drug Dev Technol* 9, 342–353. [PubMed: 21812660]
- [28]. Kinoshita E, Kinoshita-Kikuta E, Takiyama K, and Koike T (2006) Phosphate-binding tag, a new tool to visualize phosphorylated proteins, *Mol Cell Proteomics* 5, 749–757. [PubMed: 16340016]
- [29]. Yamada S, Nakamura H, Kinoshita E, Kinoshita-Kikuta E, Koike T, and Shiro Y (2007) Separation of a phosphorylated histidine protein using phosphate affinity polyacrylamide gel electrophoresis, *Anal Biochem* 360, 160–162. [PubMed: 17092477]
- [30]. Yang Y, and Inouye M (1991) Intermolecular complementation between two defective mutant signal-transducing receptors of *Escherichia coli*, *Proc Natl Acad Sci U S A* 88, 11057–11061. [PubMed: 1662380]
- [31]. Zhu Y, Qin L, Yoshida T, and Inouye M (2000) Phosphatase activity of histidine kinase EnvZ without kinase catalytic domain, *Proc Natl Acad Sci U S A* 97, 7808–7813. [PubMed: 10884412]
- [32]. Saini DK, Malhotra V, Dey D, Pant N, Das TK, and Tyagi JS (2004) DevR-DevS is a bona fide two-component system of *Mycobacterium tuberculosis* that is hypoxia-responsive in the absence of the DNA-binding domain of DevR, *Microbiology* 150, 865–875. [PubMed: 15073296]
- [33]. Wright GD, Holman TR, and Walsh CT (1993) Purification and characterization of VanR and the cytosolic domain of VanS: a two-component regulatory system required for vancomycin resistance in *Enterococcus faecium* BM4147, *Biochemistry* 32, 5057–5063. [PubMed: 8494882]
- [34]. Janausch IG, Garcia-Moreno I, and Uden G (2002) Function of DcuS from *Escherichia coli* as a fumarate-stimulated histidine protein kinase in vitro, *J Biol Chem* 277, 39809–39814. [PubMed: 12167640]

- [35]. Sharda S, Koay MS, Kim YJ, Engelhard M, and Gartner W (2009) A non-hydrolyzable ATP derivative generates a stable complex in a light-inducible two-component system, *J Biol Chem* 284, 33999–34004. [PubMed: 19808667]
- [36]. Fritsch F, Mauder N, Williams T, Weiser J, Oberle M, and Beier D (2011) The cell envelope stress response mediated by the LiaFSRLm three-component system of *Listeria monocytogenes* is controlled via the phosphatase activity of the bifunctional histidine kinase LiaSLm, *Microbiology* 157, 373–386. [PubMed: 21030435]

Figure 1.

The sensor histidine kinase LiaS in *E. faecium* is analogous to the canonical two-component thermosensory protein DesK in *B. subtilis*, which function as a dimer to autophosphorylate and then subsequently transfer phosphate to the response regulator DesR. (a) Structural overview of the receiver domain (blue) *E. faecium* LiaR activated with BeF_3^- (PDB ID: 5HEV). BeF_3^- is bound proximal to the predicted phosphorylation site (Asp54). Mg^{2+} and BeF_3^- are shown as spheres (red) and sticks (blue-yellow), respectively. The adaptive mutation W73C (yellow/cyan sticks) is not located within the molecular surfaces that comprise the LiaR dimer. (b) Crystal structure of the *B. subtilis* DesK-DesR complex in the phosphotransfer state (PDB ID: 5IUJ) with the equivalent to T120A in *E. faecium* LiaS and with equivalent positions to W73C and D54 in *E. faecium* LiaR indicated. An approximate position 73 and position 54 in the DesR, and position 120 in DesK structures are depicted as sticks (D71-T72, D54 and E158, respectively).

Figure 2.

Autophosphorylation kinetics of *E. faecium* LiaS and adaptive mutant LiaS^{T120A} cytoplasmic domain (81–355) were examined by Phos-tag SDS-PAGE. (a) LiaS (LiaS^{T120A}) at 15 μ M was incubated with 0.2 mM ATP at 37 °C. The reaction was quenched at the indicated incubation times, and the phosphorylated proteins were visualized by Phos-tag SDS-PAGE. The bands corresponding to phosphorylated protein are indicated. One of three replicates is shown. (b) To quantify the extent of phosphorylation, the intensities of the bands corresponding to unphosphorylated and phosphorylated species were estimated using ImageJ²⁶. The experimental data for LiaS (blue diamonds) and LiaS^{T120A} (red circles) were plotted as a percentage of phosphorylated LiaS with respect to its total initial amount. The amount of phosphorylated LiaS (LiaS^{T120A}) at t=0 was subtracted. The best fits of the data of LiaS (blue) and LiaS^{T120A} (red) are presented as solid lines, and the error bars show the SEM (standard error of the mean) from the triplicates.

Figure 3.

Time-dependent phosphotransfer experiments indicated a rapid transfer of the phosphoryl group from the C-terminal kinase domain of LiaS to its cognate response regulator LiaR. Phosphorylated LiaS (top panel) or LiaS^{T120A} (bottom panel) was incubated with LiaR/LiaR^{W73C} for the indicated times and the phosphotransfer capacity of LiaS was assessed by Phos-tag SDS-PAGE. The bands corresponding to each phosphorylated protein are indicated. The amount of phosphorylated and non-phosphorylated protein were estimated from the intensity of the upper and lower bands in a phosphogel using ImageJ²⁶.

Figure 4.

Adaptive mutant LiaR^{W73C} is recalcitrant to high levels of phosphorylation with lithium acetyl phosphate *in vitro*. (a) The ability of LiaR and adaptive mutant LiaR^{W73C} to acquire a phosphate from a small molecule phosphoryl group donor was compared using Phos-tag SDS-PAGE. (b) The extent of LiaR phosphorylation was estimated by integrating the band intensity corresponding to phosphorylated LiaR (LiaR~P) and comparing it with that of LiaR in the absence of lithium acetyl phosphate using ImageJ²⁶. The phosphorylation level of LiaR is at least 3-fold higher at the physiological temperature (37 °C) compared to that of LiaR^{W73C}. The error bars indicate the SEM (standard error of the mean) calculated from four independent experiments.

Figure 5.

In vitro rates of dephosphorylation for LiaR and LiaR^{W73C} suggest a long half-life for phosphorylated LiaR. (a) Phosphorylated species of LiaR were made by incubation of LiaR with acetyl phosphate as in Figure 4. Excess unreacted acetyl phosphate was removed using a PD-10 Sephadex G-25M column (GE Healthcare). In order to test P~LiaR stability, reactions were stopped at different time points, and samples were subjected to Phos-tag SDS-PAGE. Three independent experiments were performed. (b) The experimental data obtained in A using ImageJ²⁶ were plotted against incubation time, LiaR (green diamonds) and LiaR^{W73C} (pink circles), and used to estimate the pseudo-first rates (k , min^{-1}) of dephosphorylation. The error bars were calculated from three independent experiments and represent the standard deviation. The initial point on Y-axis reflects the amount of

phosphorylated LiaR by acetyl phosphate at 20 min after the purification of phosphorylated species.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 6.

Phosphorylation of LiaS increases affinity for LiaR. Microscale Thermophoresis was used to measure *E. faecium* LiaS binding to a LiaR mimetic peptide and to full length LiaR. The resulting K_d values were based on the average from at least three independent measurements. Bars indicate the SEM (standard error of the mean). The fraction bound was plotted on the y-axis in [%] unit against the total concentration of the titrated partner on a log10 scale on the x-axis²⁷. (a) To determine K_d , increasing concentrations of LiaS (blue circles), LiaS in the presence of 0.2 mM ATP (light blue triangles) or adaptive mutant LiaS^{T120A} (magenta diamonds) were added to 84 nM of fluorescently labeled Peptide 3. (b) To determine K_d , we added increasing concentrations of *Efm* LiaR to 20 nM of fluorescently labeled LiaS (blue circles), LiaS in the presence of 0.2 mM ATP (light blue triangles) or in the presence of 2 mM AMP-PNP (light green squares).