Characterization of DalS, an ATP-binding Cassette Transporter for D-Alanine, and Its Role in Pathogenesis in *Salmonella enterica*#§

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**Background:** Bacterial pathogens must acquire nutrients for survival during host infection.

**Results:** DalSTUV is an ABC transporter for D-alanine and contributes to virulence in vivo.

**Conclusion:** Nutrient exchange during the host-pathogen interaction can direct disease outcome.

**Significance:** This is the first report of an ABC transporter for D-alanine.

Expansion into new host niches requires bacterial pathogens to adapt to changes in nutrient availability and to evade an arsenal of host defenses. Horizontal acquisition of *Salmonella* Pathogenicity Island (SPI)-2 permitted the expansion of *Salmonella enterica* serovar Typhimurium into the intracellular environment of host cells by allowing it to deliver bacterial effector proteins across the phagosome membrane. This is facilitated by the SsrA-SsrB two-component regulatory system and a type III secretion system encoded within SPI-2. SPI-2 acquisition was followed by evolution of existing regulatory DNA, creating an expanded SsrB regulon involved in intracellular fitness and host infection. Here, we identified an SsrB-regulated operon comprising an ABC transporter in *Salmonella*. Biochemical and structural studies determined that the periplasmic solute-binding component, STM1633/DaLS, transports D-alanine and that DaLS is required for intracellular survival of the bacteria and for fitness in an animal host. This work exemplifies the role of nutrient exchange at the host-pathogen interface as a critical determinant of disease outcome.

Horizontal gene transfer (HGT)4 is a major driver of bacterial evolution, allowing rapid gains in function through acquisition of virulence machinery and other fitness factors. Evolution of *Salmonella enterica* as a mammalian pathogen was driven by two major HGT events. Acquisition of *Salmonella* pathogenicity island (SPI)-1 conferred the ability to invade intestinal epithelial cells and SPI-2 allowed for intracellular proliferation and systemic dissemination. SPI-2 encodes a type III secretion system that delivers virulence proteins called effectors into the host cell where they modify host cell biology. Common targets include the host cell cytoskeleton, endosome trafficking and immune signaling cascades including NF-κB (1–3). Expression of SPI-2 is driven by the two-component regulatory system SsrA-SsrB (4) with additional inputs from the ancestral regulatory networks of OmpR (5), SlyA (6), PhoP (7), and H-NS (8) to fine-tune virulence gene expression in response to environmental cues.

Host cells detect and respond to microbial infection through recognition of pathogen-associated molecular patterns (PAMPs) including lipopolysaccharide, flagellin, and peptidoglycan. Peptidoglycan, a critical and ubiquitous component of bacterial cell walls, is composed of glycan chains of alternating N-acetylmuramic acid and N-acetylmuramic acid residues linked by short peptides (9). Newly made peptides typically contain L-alanine, D-glutamic acid, *meso*-diaminopimelic acid, and two terminal D-alanine residues, and these peptides are cross-linked and trimmed during peptidoglycan synthesis and maturation.

During infection of host cells *Salmonella* resides in an intracellular vacuole called the *Salmonella*-containing vacuole (SCV). Nutrient and small molecule exchange across the SCV provides an opportunity for host-pathogen interaction however very little is known about this. Bacteria commonly employ ATP-binding Cassette (ABC) transporters for nutrient exchange with the environment. ABC importers consist of four components: a periplasmic-binding protein that binds ligand with high affinity, two transmembrane proteins that span the inner membrane through which the ligand is shuttled, and a

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*The abbreviations used are: HGT, horizontal gene transfer; SPI, *Salmonella* Pathogenicity Island; ABC, ATP-binding cassette; SCV, *Salmonella*-containing vacuole; DaLS, D-alanine transporter in *Salmonella*; SOE, splicing by overlapping extension.*
D-Alanine ABC Transporter Required for Salmonella Virulence

Cytoplasmic ATPase dimer that energizes the transport process (10). ABC importers vary in their specificity and can bind a wide variety of ligands, including cations, amino acids, polysaccharides, and polypeptides.

Here we have structurally and biochemically characterized the periplasmic-binding protein component of a novel D-alanine ABC transporter encoded within the STM1633-STM1636 operon in Salmonella enterica serovar Typhimurium. The soluble-binding component (STM1633) was named DalS (D-alanine transporter in Salmonella) to reflect its binding specificity, with the other linked components of the system named according to standard nomenclature for ABC transport systems. We found that dalS was co-regulated with the SPI-2 virulence locus through direct activation by SsrB, suggesting a role for this system during intracellular infection. Accordingly, we demonstrate that a dalS deletion mutant has a fitness defect in vivo. We identified D-alanine as the ligand for DalS and verified this specificity by determining the crystal structure to 1.9 Å in complex with D-alanine. This work provides the first example of an ABC transporter for D-alanine and highlights the importance of this amino acid for Salmonella fitness during infection.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—All strains are isogenic derivatives of Salmonella enterica serovar Typhimurium SL1344 (S. Typhimurium). Unless otherwise indicated, bacteria were grown in LB at 37 °C, 225 rpm. For expression of SsrB-regulated genes, a low phosphate, low magnesium minimal medium with an acidic pH (LPM pH 5.8) was used as described (11). For nutrient drop-out studies, casamino acids were replaced with individual amino acids added separately at the same concentration as that found in casamino acids. The medium with an acidic pH (LPM pH 5.8) was used as described (11).

To generate dalS containing a C-terminal 2-HA tag, the entire open reading frame for dalS including 1 kb of the upstream promoter region were PCR amplified from S. Typhimurium genomic DNA using primers SEO027 and SEO037. The resulting PCR fragment was cloned into pWSK29 as a SalI/XbaI fragment and verified by sequencing. Constructs were transformed into Escherichia coli BL21 (DE3) (12). The C-terminal DNA binding domain plus linker region of SsrB (SsrBc) (bases 420–644) was cloned in pET3a using primers SsrBc-6HIS and SsrBc-HISF and SsrBc-HISR as a Ndel/BamHI fragment to generate C-terminal 6×His tag (SsrBc-6HIS). Constructs were confirmed by sequencing and transformed into E. coli BL21 (DE3). Protein Purification—E. coli BL21 (DE3) carrying pDalS-6HIS, pDalSM146A-6HIS or pDalSM146T-6HIS was grown in LB at 37 °C to an A600 of 0.5 and then induced with 0.5 mM IPTG (Bioshop) and grown at 22 °C for an additional 3 h. Cells were centrifuged at 4000 × g for 20 min at 4 °C and resuspended in 20 ml of cold lysis buffer containing 20 mM Tris, pH 7.5, 0.5 mM NaCl. Cells were lysed by sonication (Misonix Sonicator, Ultrasonic Processor S-400) at 40% amplitude with 3 pulses of 30 s in 1 min intervals. Whole cell lysates were centrifuged at 10,000 × g for 20 min at 4 °C. The supernatant was added to Ni-NTA beads (Qiagen) following equilibration with TBS (40 mM Tris, pH 7.5, 0.5 mM NaCl). The column was washed with 50 ml of TBS containing increasing concentrations of imidazole (10 mM, 20 mM, 40 mM), and then protein was eluted in TBS containing 80 mM imidazole and run on SDS-PAGE for purity determination. Pure protein aliquots were pooled and concentrated using 3K Amicon Ultra Centrifugal filters (Millipore, UFC800324) and stored at −80 °C.

Cloning and Mutant Construction—Primers used for cloning and mutant construction are listed in supplemental Table S1. An in-frame, unmarked deletion of dalS (ΔdalS) was constructed using homologous recombination from a suicide plasmid. The entire dalS open reading frame plus 700 bp flanking either side of the gene was amplified by PCR using primers SEO068 and SEO069. PCR product was cloned into pBLUESCRIPT as a KpnI/SacI fragment. Primers SEO070 and SEO071 were used for inverse PCR of pBLUESCRIPT (DalS). Product was then self ligation to generate the deletion allele of dalS which was subsequently subcloned into the mobilizable plasmid pRE112 and transformed into DH5α βpir. Transformants were conjugated with wild type S. Typhimurium and merodiploids selected based on resistance to both streptomycin and chloramphenicol. Merodiploids were grown for 6 h without antibiotics and then counter selected on agar plates containing 5% (w/v) sucrose. Clones having lost chloramphenicol resistance were PCR screened for deletion of dalS using primers SEO068 and SEO069.

Construction of the dalS chromosomal β-galactosidase transcriptional reporter, 1 kb of DNA immediately upstream of dalS was PCR amplified using primers SEO007 and SEO008 and cloned into pIVET5 as a XhoI/MfeI fragment and confirmed by sequencing. pPdalS-β-galZ was transformed into DH5α βpir and conjugated into wild type and ΔssrB S. Typhimurium. Merodiploids were selected based on resistance to both streptomycin and ampicillin.

To generate dalS containing a C-terminal 2-HA tag, the entire open reading frame for dalS including 1 kb of the upstream promoter region were PCR amplified from S. Typhimurium genomic DNA using primers SEO027 and SEO037. The resulting PCR fragment was cloned into pWSK29 as a SalI/XbaI fragment and verified by sequencing.

The expression construct for purification of DalS-6HIS was generated by PCR amplification with primers SEO172 and SEO173. Point mutants were constructed using splicing by overlapping extension (SOE) PCR (13). Initial fragments were amplified from S. Typhimurium chromosomal DNA using primer pairs; SEO172, BRT36 and BRT37, SEO173 for the M146T mutant and SEO172, BRT38 and BRT39, SEO173 for the M146A mutant. Products were purified, combined, and a second PCR was performed using primers SEO172 and SEO173. PCR products were cloned into pET3a as Ndel/BamHI fragments and confirmed by sequencing. Constructs were transformed into Escherichia coli BL21 (DE3) (13). The C-terminal DNA binding domain plus linker region of SsrB (SsrBc) (bases 420–644) was cloned in pET3a using primers SsrBc-6HIS and SsrBc-HISR as a Ndel/BamHI fragment to generate C-terminal 6×His tag (SsrBc-6HIS). Constructs were confirmed by sequencing and transformed into E. coli BL21 (DE3).

Protein Purification—E. coli BL21 (DE3) carrying pDalS-6HIS, pDalSM146A-6HIS or pDalSM146T-6HIS was grown in LB at 37 °C to an A600 of 0.5 and then induced with 0.5 mM IPTG (Bioshop) and grown at 22 °C for an additional 3 h. Cells were centrifuged at 10,000 × g for 13 min at 4 °C, washed in PBS, and then resuspended in 20 ml of cold lysis buffer containing 20 mM Tris, pH 7.5, 0.5 mM NaCl. Cells were lysed by sonication (Misonix Sonicator, Ultrasonic Processor S-400) at 40% amplitude with 3 pulses of 30 s in 1 min intervals. Whole cell lysates were centrifuged at 10,000 × g for 20 min at 4 °C. The supernatant was added to Ni-NTA beads (Qiagen) following equilibration with TBS (40 mM Tris, pH 7.5, 0.5 mM NaCl). The column was washed with 50 ml of TBS containing increasing concentrations of imidazole (10 mM, 20 mM, 40 mM), and then protein was eluted in TBS containing 80 mM imidazole and run on SDS-PAGE for purity determination. Pure protein aliquots were pooled and concentrated using 3K Amicon Ultra Centrifugal filters (Millipore, UFC800324) and stored at −80 °C.

E. coli BL21 (DE3) carrying pSSrBc-6HIS was inoculated 1:50 into LB and grown at 37 °C with aeration to A600 of 0.8. Cultures were induced with 1 mM IPTG and grown at 16 °C for 24 h. Pellets were collected by centrifugation at 4000 rpm, 4 °C for 10 min and washed with PBS. Pellets were resuspended in Nickel Buffer A (20 mM Tris, pH 8.5, 500 mM KCl, 10% glycerol, 0.1% LDAD, 10 mM imidazole with protease inhibitors). Bacteria were lysed by three passages through a French Press (French Press Cell Disruptor, Thermo) and clarified by centrifugation at 20,000 rpm, 4 °C for 40 min. Supernatant was filter sterilized and applied to a nickel column (Amersham Biosciences Hi-Trap Ni2+ -NTA) pre-equilibrated with Nickel Buffer A. Pro-
tein was washed with 10 ml Nickel Buffer A, then 10 ml each of 5, 10, and 15% Nickel Buffer B (20 mM Tris, pH 8.5, 500 mM KCl, 10% glycerol, 0.1% LDAO, 300 mM imidazole). Protein was eluted with 10 ml 100% Nickel Buffer B into 5 ml of Nickel Buffer B to limit precipitation. Purified protein was then dialyzed overnight with storage buffer (20 mM Tris, pH 8.5, 150 mM NaCl, then centrifuged to isolate soluble protein. Glycerol was added to 20%, and protein was stored at −80 °C.

**Genetic Analysis**—Genome sequences for *S. Typhimurium* LT2, SL1344, DT104, *S. enterica* Gallinarum 287/91, *S. enterica* Hadar, *S. enterica* Infantis, *S. enterica* Typhi Ty2, and CT18, *S. enterica* Paratyphi A and *S. enterica* Choleraesuis were obtained from the Wellcome Trust Sanger Institute Pathogen Sequencing Unit. Promoter regions were aligned using MAFFT (v6.707b). Identification of the SsrB regulatory motif was determined previously (4).

β-Galactosidase Assays—Wild type and ΔssrB Salmonella containing chromosomally encoded P<sub>ΔssrB</sub>-lacZ were inoculated 1:100 into LPM, pH 5.8 and grown at 37 °C with aeration. At the indicated time points the A<sub>600 nm</sub> was determined, and 200 μl of culture was collected for transcriptional reporter activity as described previously (14). Relative light units (RLU) of the ΔssrB promoter activity were quantified using 96-well black microtiter plates (Corning) using a top-reading plate luminometer (Envision, PerkinElmer). For each time point, RLU was normalized to the A<sub>600 nm</sub>.

**Immunoblotting**—Bacteria were subcultured 1:100 into LB or LPM pH 5.8 and grown to A<sub>600 nm</sub> ~0.6. Cells were centrifuged, and pellets resuspended in SDS-PAGE sample buffer (100 mM Tris-HCl (pH 6.8), 20% (v/v) glycerol, 4% (w/v) SDS, 0.002% (w/v) bromophenol blue, and 200 mM DTT) in a volume adjusted to the optical density of the parent culture as described previously (11). 5 μl from each sample was run on 12% SDS-PAGE and analyzed by immunoblotting using the following antibodies: mouse anti-HA (1:2000, Covance), mouse anti-DnaK (1:5000, Stressgen), rabbit anti-SeeB (1:2000) (11), rabbit anti-SseC (1:5000, gift from Michael Hensel), rabbit anti-YidC (1:2000, gift from Eric Brown), mouse anti-MBP (1:2000, New England Biolabs), rabbit anti-RssB (1:2000, gift from Susan Gottesman) and rabbit anti-RpoS (1:2000, gift from Susan Gottesman), goat anti-mouse HRP (1:5000, Sigma) and goat anti-rabbit HRP (1:5000, Sigma). Conjugated HRP was detected using chemiluminescence (Western Lightning Plus-ECL, PerkinElmer).

Electrophoretic Mobility Shift Assays—The dals promoter region from position −252 to −91 relative to the translational start site was PCR amplified using primers SEO161 and SEO162 to generate a 5′ biotin-tagged fragment and SEO163 and SEO162 to generate the identical fragment lacking the biotin tag. PCR products were purified from native PAGE using Qiagen Gel Extraction kit. 50 μl Tris-HCl pH 7.5, 50 ng/μl poly dI:dC (Sigma, P4929), and 0.5 μM of 5′ biotin DNA were mixed and purified SsrBc-6HIS was added to each reaction mixture and sample incubated at 37 °C for 20 min. Controls contained a 100-fold excess of unlabeled DNA or biotin labeled nonspecific rrsH amplified using primers rrsHF and rrsHR. Samples were run on 8% native PAGE and transferred to a Biodyne B-positive nylon membrane (Pierce, 77016). Membranes were dried, and DNA crosslinked for 15 min using a transilluminator. Membranes developed using a chemiluminescence-based kit (Chemiluminescence Nucleic Acid Detection Module, Pierce) according to the manufacturer’s directions.

**Cell Culture**—RAW264.7 cell culture lines were grown at 37 °C, 5% CO<sub>2</sub> in DMEM/10%FBS (Invitrogen) unless otherwise indicated. Overnight cultures of wild type, ΔssrB, Δdals, and Δdals (pdalS-2HA) were opsonised in DMEM/20% human serum at 37 °C for 30 min. Bacteria were diluted in DMEM/10% FBS and added to −5 × 10<sup>5</sup> RAW 264.7 macrophages at multiplicity of infection of 1000 in tissue culture plate wells. Infected cells were centrifuged at 500 × g for 5 min and then incubated for 30 min to allow for uptake. Infected cells were washed three times with PBS and 100 μg/ml gentamicin (Bio-shop) was added to each well and incubated for 1.5 h. Cells were washed as before and either lysed or cultured for an additional 18 h in medium containing 10 μg/ml gentamicin. At the indicated times macrophages were lysed in 250 μl of lysis buffer (1% Triton X-100, 0.1% SDS). Lysates were serial diluted and plated for CFU determination. Fold replication was determined as CFU (20 h)/CFU (2 h). Data were normalized to fold replication of wild type for each experiment.

**Competitive Infection of Animals**—All animal protocols were performed in accordance with the Canadian Council on the Use of Laboratory Animals and approved by the McMaster University Animal Ethics Committee. Female C57BL/6 mice (Charles River) were orally infected with −1 × 10<sup>6</sup> *Salmonella* in 0.1 ml Heps pH 8.0, 0.9% NaCl containing an equal number of chloramphenicol-resistant wild type bacteria and an unmarked mutant under study. At 3 days post, mice were sacrificed by cervical dislocation and spleen, liver and cecum were harvested. Tissues were homogenized in a Mixer Mill (5min, 30 Hz) (Retisch), serial diluted and plated on LB agar containing streptomycin to determine total CFU counts. Colonies were replica plated onto LB agar containing both streptomycin and chloramphenicol (10 μg/ml) to determine the ratio of chloramphenicol resistant to sensitive colonies. Competitive index was calculated as (mutant/wild type<sub>output</sub>/(mutant/wild type<sub>input</sub>). Statistical analysis was performed using a one sample t test. For survival studies, female C57BL/6 mice were orally infected with −1 × 10<sup>6</sup> CFU of either wild type or Δdals S. Typhimurium and endpoints were determined based on body condition scoring and loss of > 20% body weight.

**Fluorescence Thermal Shift Assay**—The fluorescence thermal shift assay was performed essentially as described previously (15). Amino acids were purchased from Sigma unless otherwise indicated. The assay was performed with a 480 Lightcycler (Roche) (498 nm excitation, 610 nm emission). Each reaction contained 10 μM protein, 5× concentration of SyPro Orange (Invitrogen) and 1 mM of the indicated amino acid dissolved in 100 mM HEPES, 150 mM NaCl, pH 7.5 in a 96-well plate (Roche). Samples were initially equilibrated at 25 °C for 10 min and a melting curve program was run on the LightCycler with a 1 °C temperature increase every 30 s up to 99 °C. Software calculated the first derivative values (−dΔT<sub>m</sub>/dt) from raw fluorescence data to determine T<sub>m</sub>. A ΔT<sub>m</sub> of ≥ 2 °C upon addition of amino acid was considered positive binding as described (15).
Protein Crystallization—Selenomethionine-derivatized DalS was expressed in B834 (DE3) cells using M9 SeMET high-yield growth media kit (Mediclon). The protein was purified using nickel affinity chromatography and buffer exchanged to 20 mM Tris, pH 7.5, and 100 mM NaCl. Protein crystals were grown using the hanging drop vapor diffusion method. Purified DalS-6His (concentrated to 4 mg/ml) was mixed at a 2:1 ratio with a precipitant of 10% (w/v) PEG-3000, Na/potassium phosphate pH 6.2. The mixture was equilibrated against 800 µl of 1.65 M (NH₄)₂SO₄, and crystals formed after 2 days at 4 °C. To form a complex with glycine, purified DalS was mixed at a 2:1 ratio with a precipitant containing 10% (w/v) PEG-3000 and CHES pH 9.5. Glycine was added to the drop to a final concentration of 5 mM, and the drop was equilibrated over 800 µl of 1.5 M (NH₄)₂SO₄. Crystals formed overnight at 4 °C. Crystals of sufficient size were flash frozen with liquid nitrogen. Anomalous data were collected under cryogenic conditions (100 K) at beam line X29 at the National Synchrotron Light Source at Brookhaven National Labs and processed using HKL2000 (16). Phenix-AutoSol (17) was used to locate 7 heavy atom sites and also for phasing and density modification. Iterative rounds of model building and refinement were carried out using Coot (18) and Phenix-Refine (17). The final Rwork and Rfree values for crystals grown in the presence of glycine were 19% and 20.3%, respectively. The final Rwork and Rfree values for crystals grown in the absence of glycine were 19% and 21.6% respectively. All final models were analyzed using PROCHECK which indicated 93.5% residues lie in favorable regions, 5.9% lie in allowed regions and 0.5% lie in generously allowed regions. Residues 1–24 were not included in any of the final models due to disorder. Structural images were generated using PyMol (The PyMOL Molecular Graphics System, Version 1.2, Schrödinger, LLC).

Transport Assay—Wild type and ΔdalS were grown in 5 ml of LPM pH 5.8 for 3 h at 37 °C with shaking. Cells were centrifuged and resuspended in PBS and grown at 37 °C for 30 min. 5 µl of [3H]alanine (60 Ci/mmol, 1 mCi/ml; ART 0179; American Radiolabeled Chemicals Inc., Missouri) or [3H]glycine (60 Ci/mmol, 1 mCi/ml, Perkin Elmer) were added to the cultures. Every 30 s, 200 µl aliquots were spotted onto 0.2 µm nitrocellulose filters under vacuum. Filters were washed with 4 ml of ice-cold PBS and added to a Fast Turn Cap Mini Poly-Q scintillation vial (Beckman Coulter) containing 4 ml of EcoScint A scintillation fluid (LS-273, National Diagnostics). Filter samples were left to saturate in the vials for 30 min, before reading on the Beckman Coulter LS 6500 scintillation counter.

RESULTS

Co-regulation of dalS with SPI-2—The dalSTUV operon (Fig. 1A), which is conserved across all Salmonella lineages, was co-regulated with the SPI-2 virulence machinery in S. Typhimurium in transcriptional profiling experiments, where dalS showed an ~10-fold decrease in mRNA levels in an srbB deletion mutant (4). In accord with these data, another group showed that dalS/STM1633 clustered into an SsrB-controlled regulatory network enriched in virulence genes including genes in SPI-2 and with srfN (19), the latter of which we previously showed was required for in vivo fitness (20). To confirm the microarray results, we constructed a chromosomally encoded lacZ transcriptional reporter (P_dalS-lacZ) in both wild type (wt) and ΔsrbB backgrounds and measured β-galactosidase activity over time in SPI-2 inducing minimal media (LPM pH 5.8) (11). In agreement with the microarray data, the activity of the dalS promoter increased over time, and this expression was diminished in ΔsrbB (Fig. 1B). DalS protein analyzed by Western blot in wt and ΔsrbB cells showed that DalS levels were increased in minimal medium compared with LB and that SsrB was required for full expression of DalS in minimal medium (Fig. 1C).

The dalS promoter region was bound by SsrB in chromatin immunoprecipitation (ChIP)-on-chip experiments (4). To confirm that SsrB directly binds to the dalS promoter, an electrophoretic mobility shift assay was performed using a purified DNA fragment spanning nucleotide position −252 to −92 that incorporated the SsrB binding peak. Incubation of this DNA fragment with increasing concentrations of purified SsrBc (5) resulted in a retardation of band migration (Fig. 1D) that was inhibited in the presence of unlabeled competitor DNA confirming that SsrB binds to the dalS promoter. Consistent with this finding, an SsrB binding motif was identified at position −185 to −168 relative to the dalS translational start site (Fig. 1E). Together these results indicate that SsrB has direct transcriptional input into the expression of dalS.

DalS Is a Virulence Factor Required for Intracellular Survival—Given the well-documented role of SsrB in S. enterica pathogenesis we hypothesized that DalSTUV was required for full intracellular virulence. We constructed an unmarked, in-frame deletion of dalS (ΔdalS), which had no effect on growth of the bacteria under any in vitro condition examined (supplemental Fig. S1A). C57BL/6 mice orally infected with wt S. Typhimurium had a mean survival time of 5 days (Fig. 2A) whereas mice infected with ΔdalS had a mean survival time that was delayed by 1 day (6 days; p = 0.03). To determine whether the dalS mutant had a reduced ability to replicate in peripheral tissues we performed a competitive infection of C57BL/6 mice with a mixed inoculum of wt and ΔdalS. After 3 days of infection, the bacterial load in the spleen, liver, and cecum was determined and expressed as a competitive index (21). The dalS mutant was consistently recovered in lower amounts compared with wt, with competitive index values of 0.72 (spleen), 0.66 (liver), and 0.76 (cecum) (Fig. 2B).

To examine whether ΔdalS was deficient for intracellular replication, macrophage-like RAW264.7 cells were infected with wt or ΔdalS and the ability of the bacteria to survive and replicate over 20 h was determined by standard gentamicin protection assays. Although ΔdalS was able to replicate in macrophages, it did not reach the same level of replication as wt, a defect that was recovered by complementation (Fig. 2C). In mice, complementation of the ΔdalS mutant also restored the replication defect seen in earlier competitive infections (data not shown). These results indicate that DalS contributes to intracellular survival and to the competitive fitness of Salmonella during animal infections.

DalS Is an ABC Importer for d-Alanine—Each protein encoded in the dalSTUV operon has strong similarity to components of ATP-binding cassette (ABC) transporters (supplemental Fig. S2). DalS was identified as the periplasmic binding
protein, DalT and DalV are homologous to the membrane spanning transport channel and DalU was identified as the cytoplasmic ATPase. Specifically, the dalSTUV operon was related to a family of polar amino acid (PAA) importers. Alignments for DalU showed particularly strong conservation of the Walker A, Walker B boxes, and the ABC signature motif (supplemental Fig. S2).

The membrane-spanning permeases DalT and DalV were predicted to have 4 to 5 membrane spanning domains (supplemental Fig. S3). Consistent with the prediction that DalS is the periplasmic component of this ABC transporter, cellular fractionation experiments showed localization of DalS-2HA exclusively to the periplasmic fraction (supplemental Fig. S2).

To identify the substrate(s) of DalS, we purified DalS and used this protein in a fluorescence thermal shift (FTS) assay previously developed to identify the amino acid specificity of solute-binding proteins in the bacterial ABC transport family (15). Amino acids were added to DalS-6HIS in the presence of SyPro Orange and fluorescence measurements were acquired during thermal shift. In the FTS assay, we identified glycine and D-alanine as specific ligands for DalS, whereas the glycine derivative betaine, L-alanine, or other amino acids did not register as positive in this screen (Fig. 3).

To confirm that DalSTUV functions to transport the ligands identified by FTS, we studied the accumulation of [3H]glycine and D-[3H]alanine by wild type and ΔdalS cells. Cells were grown in minimal medium to exponential phase then transferred to PBS for a 30 min starvation period prior to addition of ligand. Cells were collected at 30-s time intervals following ligand addition and intracellular radioactivity was measured. In
these experiments, cells lacking DalS consistently had lower levels of intracellular D-[3H]alanine compared with wild type cells (Fig. 3B), whereas the uptake of [3H]glycine was unaffected by DalS (Fig. 3C). These data suggested that D-alanine was the biological substrate, which we set out to verify using structural information.

To structurally characterize this D-alanine periplasmic-binding protein, we crystallized DalS and solved the structure to 1.9 Å resolution (supplemental Table S2). DalS crystallized with a monomer in the asymmetric unit and behaved as a monomer in solution during size exclusion chromatography (data not shown). Based on the structure, charged side chains Arg-102 and Glu-191, and a hydroxyl belonging to Tyr-148 additionally confer hydrophobic interactions. Although Met-146 does not directly interact with D-alanine, it likely contributes to ligand specificity due to steric hindrance (Fig. 4D). To test this hypothesis we made point mutants with similar (M146T) and smaller side chains (M146A). M146T was found to additionally accommodate methionine as a binding ligand (Fig. 4E) whereas the DalS variant with the M146A mutation was highly promiscuous and allowed for the accommodation of seven new binding ligands (Fig. 4E). Although the binding profile for glycine was altered in both mutants, the binding of D-alanine was unaffected. This finding highlights Met-146 as a critical residue in conferring ligand specificity. DalS demonstrated significant structural similarity despite sharing only moderate sequence similarity (14% identity, 57% similarity) to the previously characterized histidine-binding periplasmic-binding protein, HisJ (22) and to the lysine-arginine-ornithine-binding protein LAO from Salmonella (23) with RMSD values within the binding pocket of 0.429 and 0.470 Å, respectively. Interestingly, the amino acids responsible for direct ligand binding are conserved as well (Fig. 4, C and D). In addition to orthologs in the Salmonella genus, similarity searches identified a putative periplasmic-binding protein and extracellular binding protein from Dickeya dadantii and D. zaeae, respectively, in which all ligand-binding interacting residues were conserved (data not shown). These data provide strong evidence that DalSTUV comprise the components of an ABC transporter of this specificity.

FIGURE 2. Deletion of dalS attenuates S. Typhimurium virulence in vivo. A, survival of C57BL/6 mice orally infected with wild type or ΔdalS (n = 8); B, C57BL/6 mice orally infected with a mixed inoculum of wild type and ΔdalS. Competitive index values were determined after 3 days of infection. Each data point represents an individual animal, and horizontal bars are the geometric means. C, contribution of DalS to Salmonella intracellular replication in macrophages was assessed by gentamicin protection assays in RAW264.7 cells. Macrophages were infected with the indicated strains, and the change in intracellular bacteria numbers was determined between 2 and 20 h post-infection and normalized to wild type. ΔassB was used as a negative control (n = 5). (*, p < 0.05; ***, p < 0.001).
**DISCUSSION**

In this work we identified and characterized the first example of an ABC transporter for \( \alpha \)-alanine. We found that DalS was co-regulated with the SPI-2 encoded virulence machinery through direct SsrB-dependent activation, allowing its expression to be modified during intracellular infection. Accordingly, cell culture and animal infections demonstrated that DalS contributed to a quantifiable fitness gain for \( S. \) Typhimurium only during host infection whereas its presence for growth \( \text{in vitro} \) was completely dispensable. Biochemical and structural characterization identified DalSTUV as an ABC importer for \( \alpha \)-alanine where DalS is localized to the periplasm to serve as the periplasmic binding component in this system.

Peptidoglycan is a key pathogen-associated molecular pattern (PAMP) recognized by the host innate immune system. Given its substrate binding profile, we thought that DalS might play a role in peptidoglycan structure but this was found not to be the case. \( \alpha \)-Alanine is the terminal subunit of the peptidoglycan stem pentapeptide and is released during the processes of peptide cross-linking by transpeptidases and trimming of tetrapeptides to tripeptides by \( \text{DD-carboxypeptidase} \). It is possible that DalSTUV plays a unique role in the intracellular transport of polar amino acids. How-ever data from our structural analysis, led us to initial predictions that DalS was involved in transporting polar amino acids. Consequently, the expression of DalS mutants would be sensitized to wall-active drugs, which they were not (data not shown). In addition, recycling of murein cell wall in \( S. \) Typhimurium occurs by re-uptake of two major intact cell wall peptides (\( L-\text{Ala-D-Gly-\( \gamma \)-meso-diaminopimelic acid} \) and \( L-\text{Ala-D-Gly-\( \gamma \)-meso-diaminopimelic acid} \)) by the oligopeptide permease, \( Omp \), as opposed to periplasmic hydrolysis followed by reutilization of the constituent amino acids (28). Importantly, \( Omp \) mutants were growth-defective, showing a clear physiological role for peptide recycling, whereas the \( dalS \) mutants were not affected in growth in any \( \text{in vitro} \) media tested. Alternatively, given the limited distribution of DalS to the \( S. \) Typhimurium genus, it is possible that DalSTUV plays a unique role in the intracellular lifestyle of this organism during infection, possibly through modulation of the host innate immune system, production of a secondary metabolite, or satisfying a within-host nutritional requirement that will require further work to address.

The high conservation of amino acids within the DalS binding pocket in comparison with HisJ and LAO, which we confirmed with our structural analysis, led us to initial predictions that DalS was involved in transporting polar amino acids. However data from our ligand screen did not support this hypothesis but rather showed an interaction with \( \alpha \)-alanine and glycine. A second crystal structure of STM1633 deposited in the Protein Data Bank (PDB 3R39) contains two monomers within the asymmetric unit, one bound to \( \alpha \)-alanine and the other to glycine. Despite extensive dialysis even in a large excess of glycine, DalS was reproducibly purified in the presence of bound \( \alpha \)-alanine, making determinations of binding affinity difficult (data not shown). These data suggested that glycine might be a weaker or non-biological ligand. Future work will deter-
As an intracellular pathogen, Salmonella must rapidly adapt to changing conditions as it transitions between environmental and host lifestyles. Up-regulation of existing nutrient importers through regulatory evolution, which is the case for DalS through its incorporation into the SsrB regulon, confers a fitness advantage to the bacteria that promotes infection of a host. This work highlights the importance of nutrient exchange across the host-pathogen interface as a critical determinant of disease outcome.

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