Twin-arginine translocation (Tat) mutants in Salmonella enterica serovar Typhimurium have increased susceptibility to cell wall targeting antibiotics

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ABSTRACT

The twin-arginine translocation (Tat) system is a protein secretion system that is conserved in bacteria, archaea and plants. In Gram-negative bacteria, it is required for the export of folded proteins from the cytoplasm to the periplasm. There are 30 experimentally verified Tat substrates in Salmonella, including hydrogenase subunits, enzymes required for anaerobic respiration and enzymes involved in peptidoglycan remodeling during cell division. Multiple studies have demonstrated the susceptibility of tat mutants to antimicrobial compounds such as SDS and bile; however, in this work, we use growth curves and viable plate counts to demonstrate that cell wall targeting antibiotics (penicillins, carbapenems, cephalosporins and fosfomycin) have increased killing against a Δtat strain. Further, we demonstrate that this increased killing is primarily due to defects in translocation of critical Tat substrates: MepK, AmiA, AmiC and SufI. Finally, we show that a ΔhyaAB ΔhybABC ΔhydBC strain has an altered Δg that impacts proper secretion of critical Tat substrates in aerobic growth conditions.

Keywords: stress response; tat; ampicillin

INTRODUCTION

Salmonella enterica serovar Typhimurium (S. Typhimurium) is a Gram-negative pathogen that causes an array of diseases in a variety of hosts. In humans, S. Typhimurium causes self-limiting gastroenteritis in otherwise healthy individuals; however, in immunocompromised or otherwise susceptible hosts, S. Typhimurium can cause life-threatening system infection (Mastroeni and Grant 2011). Systemic salmonellosis has historically been treated with ampicillin and ciprofloxacin; however, the emergence of resistant strains has led to the use of cephalosporins in recent years (Gal-Mor, Boyle and Grassl 2014). Penicillins and cephalosporins work by irreversibly binding the enzyme transpeptidase, preventing the synthesis of peptidoglycan during cell division. Use and overuse of these...
antibiotics have led to an increase of multidrug-resistant isolates of Salmonella species (Mather et al. 2013; Krueger et al. 2014; Nair, Venkitanarayanan and Kollanoor Johny 2018), hastening the need to develop new and alternative treatment methods. The twin-arginine translocation (Tat) system is a protein secretion system present in the cytoplasmic membrane of many bacteria and archaea. In Gram-negative organisms, the Tat translocon transports substrate proteins from the cytoplasm to the periplasmic space where the protein can undergo further translocon transports substrate proteins from the cytoplasm to the periplasmic space where the protein can undergo further.

Table 1. Salmonella enterica serovar Typhimurium Tat substrates. Adapted from Craig et al. (2013) and Sargent, Berks and Palmer (2010).

| Molybdopterin-independent Tat substrates | Protein | Functional knockout | Substrate function |
|----------------------------------------|---------|---------------------|--------------------|
| AslA (STM0084)                         | ΔaslA   |                      | Acid-inducible sulfatase |
| MigS (PSL046)                          | ΔmigS   |                      | Carbonic anhydrase     |
| CueO                                   | ΔcueO   |                      | Multicopper oxidase    |
| FhuD                                   | ΔfhuD   |                      | Fe²⁺ hydroxamate siderophore transport component |
| HyaA                                   | ΔhyaA   |                      | Hydrogenase 1 small subunit |
| HybA                                   | ΔhybABC |                      | Hydrogenase 2 subunit   |
| HypO                                   | ΔhypABC |                      | Hydrogenase 2 small subunit |
| HydA                                   | ΔhydBC  |                      | Hydrogenase 3 small subunit |
| MoeG                                   | ΔmoeG   |                      | Glucan biosynthesis    |
| WcaM                                   | ΔwcaM   |                      | Colanic acid biosynthesis |
| NrfC                                   | ΔnrfA   |                      | Nitrite reductase       |
| MepK (YcbK)                            | ΔmepK   |                      | 3–3 DAP murein endopeptidase |
| AmiAl                                  | ΔamiAl  |                      | N-Acetylmuramoyl-1-alanine amidase |
| AmiC                                   | ΔamiC   |                      | N-Acetylmuramoyl-1-alanine amidase |
| SufI (FtsP)                            | ΔsufI   |                      | Cell division protein  |

| Molybdopterin-dependent Tat substrates | Protein | Functional knockout | Substrate function |
|---------------------------------------|---------|---------------------|--------------------|
| STM0611                                | ΔmoaDE  |                      | Putative oxidoreductase |
| DmsA                                   | ΔmoaDE  |                      | Dimethyl sulfoxide reductase, subunit A |
| DmsA1                                  | ΔmoaDE  |                      | Dimethyl sulfoxide reductase, subunit B |
| DmsA2                                  | ΔmoaDE  |                      | Dimethyl sulfoxide reductase, subunit C |
| TtrA                                   | ΔmoaDE  |                      | Tetrahionate reductase, subunit A |
| TtrB                                   | ΔmoaDE  |                      | Tetrahionate reductase, subunit B |
| YnfE                                   | ΔmoaDE  |                      | Putative anaerobic reductase |
| YnfE                                   | ΔmoaDE  |                      | Putative anaerobic reductase |
| FdxG                                   | ΔmoaDE  |                      | Formate dehydrogenase N, alpha subunit |
| PhsA                                   | ΔmoaDE  |                      | Thiosulfate reductase |
| NapC                                   | ΔmoaDE  |                      | Quinol dehydrogenase |
| NapA                                   | ΔmoaDE  |                      | Nitrate reductase, large subunit |
| YedY                                   | ΔmoaDE  |                      | Sulfite oxidase |
| TorA                                   | ΔmoaDE  |                      | TMAO reductase |
| FdoG                                   | ΔmoaDE  |                      | Formate dehydrogenase O, alpha subunit |

The total number of Tat substrates varies by species. Salmonella Typhimurium has 30 proteins that are substrates of Tat, either predicted with bioinformatics or experimentally confirmed (Craig et al. 2013). These Tat substrates include enzymes necessary for anaerobic respiration, hydrogenases and cell wall amidases, among others (Table 1). Indeed, the Tat system has been shown to be critical for the virulence of S. Typhimurium and other pathogenic species (Caldelari et al. 2006; Lavander et al. 2006; Reynolds et al. 2011; Craig et al. 2013; Fujimoto et al. 2018).
while not being essential for in vitro growth. In S. Typhimurium, this virulence defect is due to the combined loss of three genes: amiA, amiC and suf (Craig et al. 2013; Fujimoto et al. 2018). AmiA and AmiC are N-acetylmuramyl-L-alanine amidases that remove cross-links in peptidoglycan during cell division (Heidrich et al. 2001; Bernhardt and De Boer 2003), while SufL (ftsP) is important for stabilization of the divisome during stress conditions (Heidrich et al. 2001; Samaluru, Saisree and Reddy 2007; Tarry et al. 2009). Interestingly, in Escherichia coli, overproduction of a third amidase not secreted via Tat, Ami8, is able to compensate for the deletion of taitC (Ize et al. 2003). Overproduction of AmiA or AmiC does not provide the same benefit for E. coli (Ize et al. 2003). Indeed, overproduction of Tat substrates in a Tat+ background is actually detrimental to the cell as it prevents efficient translocation of Tat substrates (DeLisa et al. 2004). MepK (formerly YcbK) is an endopeptidase that cleaves mDAP–mDAP cross-links (Chodisetti and Reddy 2019) and deletion of mepK also causes a virulence defect in mice (Craig et al. 2013). The combined data from E. coli and S. Typhimurium demonstrate a critical role of the Tat system in maintenance of the Gram-negative cell envelope. Strains deleted for genes encoding the Tat apparatus experience several growth phenotypes, including elongated cells and septal defects during division (Heidrich et al. 2001; Samaluru, Saisree and Reddy 2007).

In this study, we demonstrate that the deletion of tatABC is significantly more susceptible to low levels of cell wall targetting antibiotics as compared with wild type. Further, we show that susceptibility is primarily due to loss of specific substrates MepK, AmiA, AmiC and SufL, all play critical roles in maintenance of peptidoglycan. Our data also suggest an important role for hydrogenses in aerobic growth, as a ΔhyyAΔΔhyyBΔΔhydBC strain has an altered Δϕ that likely leads to inefficient translocation of critical Tat substrates.

**MATERIALS AND METHODS**

**Media, reagents and enzymatic assays**

Luria–Bertani (LB) medium was used in all experiments for growth of bacteria and SOC was used for the recovery of transformants (Maloy et al. 1996). Bacterial strains were routinely grown at 37 °C except for strains containing the temperature-sensitive plasmids, pCP20 or pKD46, which were grown at 30 °C. Antibiotics were used at the following concentrations for selection purposes: 50 μg/mL ampicillin (Amp); 20 μg/mL chloramphenicol (Cm); and 50 μg/mL kanamycin (Km). Enzymes were purchased from New England BioLabs and were used according to the manufacturer’s recommendations. Primers and gBlocks gene fragments were purchased from Integrated DNA Technologies (San Diego, CA, USA). Antibiotics were purchased from MilliporeSigma (St. Louis, MO, USA).

**Growth curves and viable plate counts**

Growth curves were performed in 96-well plates. Briefly, overnight cultures were subcultured 1:100, grown for 6 h and then subcultured 1:100 again into medium for the growth curve. Four independent replicates were grown in LB with appropriate antibiotics and concentrations. Growth curves were performed in a temperature-controlled BioTek Cytation 3 plate reader at 37 °C with 200 r.p.m. of agitation. OD600 readings were taken every 15 min. The median for each time point was plotted with standard deviation. Column 1 is always the uninoculated, LB only negative control. After 14 h, serial dilutions were performed in LB and 10 μL spot plated on non-selective LB agar to determine viable cells/mL.

**Measurement of Δϕ with flow cytometry**

BaLight Bacterial Membrane Potential Kit purchased from ThermoFisher Scientific (Waltham, MA, USA) was used for solutions of DiOC3(3) carboxyfluorescein dye (3,3′-diethyloxacabocyanine iodide) and CCCP (carbonyl cyanide 3-chlorophenylhydrazone). Stationary cultures were diluted 1:100 in 1× PBS. DiOC3(3) was added to final concentration of 30 μM. A depolarized control was running with the addition of CCCP with the final concentration of 5 μM. Samples were incubated for 50 min at 37 °C, then data were collected on a BioRad 35e cell sorter. Mean fluorescence intensity (MFI) was calculated with a derived parameter utilizing the following formula for each event: red fluorescence – green fluorescence + 500, where 500 is a constant used to ensure that all ratiometric values are positive. Then, the geometric mean is generated for each independent run based on 10 000 events.

**Fluorescent microscopy**

pAT5 (pBR322:::TorA–GFP) was transformed into strains via electroporation. A single colony from an LB with ampicillin plate was suspended in water to create a bacterial smear on a glass slide; coverslip was mounted with 2% agarose. Slide was then visualized on Nikon Eclipse Ci-L microscope with Nikon DS-Fi3 color camera and NIS-Elements software using 100× objective and an EGFP filter.

**Strain and plasmid construction**

Bacterial strains and plasmids are described in Table 1. All S. enterica serovar Typhimurium strains used in this study are isogenic derivatives of the strain ATCC 14028 (American Type Culture Collection) and were constructed using P22 HT105/1 int-201 (P22)-mediated transduction (Maloy et al. 1996). Deletion of various genes and concomitant insertion of an antibiotic resistance cassette was carried out using Lambda Red-mediated recombination as described (Datsenko and Wanner 2000). In all cases, the appropriate insertion of the antibiotic resistance marker was checked by P22 linkage to known markers and/or polymerase chain reaction (PCR) analysis. The constructs resulting from this procedure were moved into a clean wild-type background (14028) by P22 transduction. In some strains, the antibiotic resistance cassettes were removed using the temperature-sensitive plasmid pCP20 carrying the FLP recombinase (Cherepanov and Wackernagel 1995). Salmonella enterica serovar Enteritidis (S. Enteritidis; ATCC 13076) and Salmonella enterica serovar Heidelberg (S. Heidelberg; ATCC 8326) were purchased from the American Type Culture Collection (Manassas, VA, USA). The ΔtatABC::Km allele initially generated in ATCC 14028 was moved into ATCC 8326 and ATCC 13076 via modified P22 transduction as previously reported (Edwards, Helm and Maloy 1999) with the modification of pretreatment of recipient cells at 45 °C for 25 min. Constructs containing the OmpA signal sequence were designed and ordered as gBlocks fragments from Integrated DNA Technologies. Sequences are included in the Supporting Information. Oligos were used to amplify the fragments and clone into pBAD33 with appropriate restriction enzymes. Plasmids constructed in this work were verified by sequencing analysis at the Arizona State University Genomics Facility (Tempe, AZ, USA). Primers used for cloning and deletions are described in Table S2 (Supporting Information).
RESULTS AND DISCUSSION

Low concentrations of ampicillin readily kill a ΔtatABC strain

With the effects of bile, SDS and other compounds that disrupt the cell envelope on tat mutants (Ize et al. 2003; Pradel et al. 2009; Reynolds et al. 2011), we predicted that peptidoglycan targeting antibiotics would also have a stronger effect on a Δtat strain as compared with wild-type S. Typhimurium. We determined the effects of ampicillin and other cell wall targeting antibiotics using growth curves. The data (Fig. 1) show that deletion of tatABC causes S. Typhimurium to be more susceptible to low concentrations of ampicillin. Even 0.78 μg/mL of ampicillin, which has no impact on the final OD₆₀₀ of wild-type S. Typhimurium, caused a 5-fold decrease in final OD₆₀₀ of the ΔtatABC strain. Several previous studies have noted the elongated cell phenotype associated with deletion of tat genes and some Tat substrates (Heidrich et al. 2001; Stanley et al. 2001; Craig et al. 2013). We predicted the elongated cells and chains formed by cell division defects associated with ΔtatABC could be artificially elevating the OD₆₀₀ of our cultures; thus, we did serial dilutions and spot plating to determine the viable cells/mL after the 14-h growth curve. The major benefit of this dual approach is that low concentrations of ampicillin do indeed kill the ΔtatABC strain readily; however, we saw spontaneous resistant mutants develop at low ampicillin concentrations. These manifest as a recovery in growth late in the curve with large error bars. The raw data show that in these cases, one of the four replicates grew to an optical density far higher than the rest. Thus, using growth curves allows us to verify that strains have remained ampicillin sensitive and using viable plate counts gives an accurate read of cell viability, unaltered by the elongated cell phenotype associated with ΔtatABC. While we are reporting only viable plate counts in most cases, these were indeed taken from the end of 14-h growth curves.

Viable plate counts show that ampicillin causes a 1 000 000-fold decrease in viability of ΔtatABC as compared with wild type (Fig. 2B). Other peptidoglycan targeting antibiotics have similar effects. Ceftriaxone (1 000 000-fold), meropenem (10 000-fold) and fosfomycin (10 000-fold) all have a much more dramatic effect on the viability of the ΔtatABC strain as compared with wild type. To demonstrate that this phenotype is specific to antibiotics targeting the production of peptidoglycan, we included chloramphenicol as a control. The data show that there is no significant difference between ΔtatABC and wild-type S. Typhimurium in chloramphenicol sensitivity. Since these antibiotics all work by preventing peptidoglycan synthesis, we used ampicillin as a proxy to study the effect of cell wall targeting antibiotics on the Tat system.

The ampicillin effect on ΔtatABC deletions is not serovar specific

To determine whether the effect of ampicillin on ΔtatABC is specific to S. Typhimurium, we purchased S. Enteritidis and S. Heidelberg from the ATCC. The ΔtatABC allele was moved from S. Typhimurium into S. Enteritidis and S. Heidelberg via modified P22 transduction and growth curves were performed to determine the effects of ampicillin. The data (Fig. 3) show that deletion of tatABC has a similar phenotype in S. Enteritidis and S. Heidelberg. While the concentrations of ampicillin that give a ΔtatABC phenotype in S. Enteritidis (1.56 μg/mL) and S. Heidelberg (3.125 μg/mL) are higher than S. Typhimurium (0.78 μg/mL), the general effect of ampicillin on the ΔtatABC strains is otherwise similar. Both serovars demonstrate ~1000-fold increased susceptibility of the ΔtatABC strains as compared with their wild-type counterparts. While the 1000-fold effect on serovars Heidelberg and Enteritidis is less dramatic than the 10 000-fold impact on Typhimurium, the general trend holds. It is difficult to speculate as to why the phenotype is less severe in these...
serovars; nonetheless, these data suggest that the phenotype would be applicable to other \textit{S. enterica} serovars not tested.

The ampicillin effect on \( \Delta \text{tatABC} \) is primarily due to loss of MepK, AmiA, AmiC and SufI

The Tat system has 30 substrates in \textit{Salmonella} that have been identified via bioinformatics or experimentally (Craig et al. 2013); for the full list of Tat substrates, see Table 1. We predicted that growth defect of the \( \Delta \text{tatABC} \) mutant in ampicillin was due to loss of specific substrates important in biogenesis and maintenance of the cell wall: AmiA, AmiC, MepK and SufI. AmiA and AmiC are amidases that remove cross-links in peptidoglycan during cell division (Heidrich et al. 2001; Bernhardt and De Boer 2003), while SufI is involved in stabilizing divisome (Heidrich et al. 2001; Tarry et al. 2009) during replication. MepK is a murein endopeptidase that cleaves 3–3 DAP cross-links in E. coli (Chodisetti and Reddy 2019).

We tested functional deletions of all known Tat substrates using growth curves and viable plate counts in LB alone and LB with 0.78 \( \mu \text{g/mL} \) of ampicillin. We individually deleted genes encoding each of the molybdopterin-independent Tat substrates, while a deletion of \( \text{moeA} \) knocks out biosynthesis of the molybdopterin cofactor required by half of the Tat substrates that are involved in anaerobic respiratory pathways (Table 1). Thus, \( \Delta \text{moeA} \) is used as a single test to determine whether molybdopterin-requiring Tat substrates have an ampicillin phenotype. The data (Fig. 4) demonstrate that several Tat substrates have modest viability phenotypes in ampicillin as compared with wild-type 14028. A deletion of \( \text{cueO} \) shows a slight decrease in overall growth in 0.78 \( \mu \text{g/mL} \) of ampicillin, though the difference compared with 14028 wild type is not statistically significant. Figure 4 shows that any difference in growth and viability in \( \Delta \text{moeA} \) as compared with wild-type 14028 is statistically insignificant; therefore, none of the molybdopterin-requiring Tat substrates are impacted by the presence of ampicillin. A deletion of \( \text{frdA} \) had a notable growth defect in plain LB and a very modest difference compared with wild-type 14028 when treated with ampicillin; however, given the lack of difference between the LB control and the LB with ampicillin treatment, we did not follow up further. A single deletion of \( \text{mig5} \) and a triple deletion of hydrogenase subunits (\( \Delta \text{hydABC} \), \( \Delta \text{hydABC} \), \( \Delta \text{hydABC} \)) had significant phenotypes in ampicillin. These will be discussed further in another section.

Deletions of AmiA, AmiC, SufI and MepK cause more dramatic, significant phenotypes (Fig. 4). Given the apparent redundancy of AmiA and AmiC functions, we made an \( \text{amiA amiC} \) double-deletion strain and predicted that this double deletion would have a more substantial ampicillin phenotype. The data demonstrate that the \( \Delta \text{amiA} \Delta \text{amiC} \) strain is \( \sim 10 \)-fold more attenuated than the single-deletion constructs and the difference between the single \( \text{amiA} \) and \( \text{amiC} \) deletions and the \( \text{amiA amiC} \) double deletion is significant (Fig. 4). Data from Craig et al. (2013) demonstrate the importance of AmiA, AmiC and SufI, but the authors only briefly note the significant 3-fold effect of the \( \text{ycbK} \) (mepK) deletion. Given the apparent importance of AmiA, AmiC, SufI and MepK, we constructed a \( \Delta \text{amiA} \Delta \text{amiC} \Delta \text{sufI} \Delta \text{mepK} \) quadruple deletion strain. Indeed, the viable plate counts show that the quadruple deletion is statistically equivalent to the \( \Delta \text{tatABC} \) strain (Fig. 4).

There seems to be no reason for AmiA, AmiC and SufI to be exported specifically via Tat, as they are not predicted to have a required cofactor that is absent in the periplasm like other Tat substrates. Other work has demonstrated some flexibility in translocation of AmiA, AmiC and SufI using the Sec pathway even with their native Tat signal sequences (Tullman-Ercek et al. 2007). Additionally, E. coli AmiB is secreted via the Sec pathway rather than Tat and overproduction of AmiB compensates for a tat deletion (Heidrich et al. 2001). We overexpressed native AmiA, AmiC and SufI via \( p\text{BAD33} \) and none of the substrates complemented the \( \Delta \text{tatABC} \) deletion (Fig. S1, Supporting Information). This result was expected given that with no functioning Tat system AmiA, AmiC and SufI would have to be exported via Sec. While there may be some flexibility in the pathway used for localization of these three substrates (Tullman-Ercek et al. 2007), any export of Tat substrates via Sec is clearly not efficient. Further, the data show that overproduction of AmiC and AmiA cause 10-fold and 100-fold decreases in viability of 14028, respectively, as compared with the \( p\text{BAD33} \) empty vector control (Fig. S1, Supporting Information). Overproduction of SufI in the 14028 background has no impact on viability. This fits with previous work demonstrating that overproduction of Tat substrates can block efficient translocation via the Tat system (DeLisa et al. 2004) and we likely see that reflected in stunted growth in ampicillin when AmiA and AmiC are overproduced.

\[ \text{AmiA, AmiC and SufI exported via Sec complement } \Delta \text{tatABC} \]

Critical Tat substrates MepK, AmiA, AmiC and SufI were engineered to have the OmpA signal sequence for the Sec pathway (OmpA\(_{1-21}\)) replacing the native Tat signal sequence. The resulting constructs (OmpA\(_{1-21}\)-AmiC\(_{22-416}\); OmpA\(_{1-21}\)-AmiA\(_{5-296}\); OmpA\(_{1-21}\)-SufI\(_{28-471}\); OmpA\(_{1-21}\)-MepK\(_{11-183}\)) were cloned into pBAD33 for expression in the \( \Delta \text{tatABC} \) background. For short-hand purposes, we have named these constructs SecSP (Sec Signal Peptide)-substrate. The data show that overproduction of SecSP-AmiA, SecSP-AmiC and SecSP-SufI each compensate for the \( \Delta \text{tatABC} \) deletion (Fig. 5). Each construct increased growth of the \( \Delta \text{tatABC} \) strain in the presence of ampicillin by \( \sim 1000 \)-fold.
MepK, AmiA, AmiC and SufI account for most of the \(\Delta tatABC\) phenotype in ampicillin. Mig5 also plays a significant role. Strains were grown in LB with indicated concentrations of ampicillin and OD\(_{600}\) monitored over 14 h. After 14-h growth curves in indicated concentration of ampicillin, strains were diluted and plated on non-selective LB to determine viable plate counts. Strains used were ATCC 14028, JRE 140, JS 1189 through JS 2004, JRE 507 and JRE 509. Significance was determined using unpaired \(t\)-tests: \*\(P < 0.05\); \*\*\(P < 0.01\); \*\*\*\(P < 0.001\).

AmiA, AmiC and SufI exported via Sec complement the \(\Delta tatABC\) phenotype in ampicillin. Strains were grown in LB with 0.2% arabinose and indicated concentrations of ampicillin and OD\(_{600}\) monitored over 14 h. After 14-h growth curves in indicated concentration of ampicillin, strains were diluted and plated on non-selective LB to determine viable plate counts. Strains used were JRE 458, JRE 460, JRE 462, JRE 464 and JRE 530. Significance was determined using unpaired \(t\)-tests: \*\(P < 0.05\); \*\*\(P < 0.01\); \*\*\*\(P < 0.001\).

Figure 4. MepK, AmiA, AmiC and SufI account for most of the \(\Delta tatABC\) phenotype in ampicillin. Mig5 also plays a significant role. Strains were grown in LB with indicated concentrations of ampicillin and OD\(_{600}\) monitored over 14 h. After 14-h growth curves in indicated concentration of ampicillin, strains were diluted and plated on non-selective LB to determine viable plate counts. Strains used were ATCC 14028, JRE 140, JS 1189 through JS 2004, JRE 507 and JRE 509. Significance was determined using unpaired \(t\)-tests: \*\(P < 0.05\); \*\*\(P < 0.01\); \*\*\*\(P < 0.001\).

Figure 5. AmiA, AmiC and SufI exported via Sec complement the \(\Delta tatABC\) phenotype in ampicillin. Strains were grown in LB with 0.2% arabinose and indicated concentrations of ampicillin and OD\(_{600}\) monitored over 14 h. After 14-h growth curves in indicated concentration of ampicillin, strains were diluted and plated on non-selective LB to determine viable plate counts. Strains used were ATCC 14028, JRE 140, JS 1189 through JS 2004, JRE 507 and JRE 509. Significance was determined using unpaired \(t\)-tests: \*\(P < 0.05\); \*\*\(P < 0.01\); \*\*\*\(P < 0.001\).

Figure 6. Hydrogenase deletion strain has altered \(\Delta\). Each diamond represents the MFI of 10,000 events from a single biological replicate. Strains used were JRE 104 (ATCC 14028), JRE 140 (\(\Delta tatABC\)), JS 2001 (\(\Delta mig5\)), JRE 507 (\(\Delta amiA\) \(\Delta amiC\) \(\Delta sufI\) \(\Delta mepK\)) and JRE 509 (\(\Delta hydBC\) \(\Delta hybABC\) \(\Delta hyaAB\)). Significance was determined using unpaired \(t\)-tests: \*\(P < 0.05\); \*\*\(P < 0.01\); \*\*\*\(P < 0.001\).

be exported via Tat after folding. Indeed, previous work has demonstrated that CueO with a Sec signal sequence is non-functional and it must be translocated from the cytoplasm via Tat in a partially folded state (Stolle, Hou and Brüser 2016).

It is interesting that overproduction and export via Sec of SecSP–AmiA, SecSP–AmiC or SecSP–SufI all compensate for the deletion of \(\Delta tatABC\) given the very different roles of AmiA/AmiC and SufI in the cell. Previous studies have shown that \(\beta\)-lactam antibiotics not only irreversibly bind PBPs but also induce a futile cycle that leads to disruption of cell wall synthesis machinery (Cho, Uehara and Bernhardt 2014). Given these data, it is...
likely that deletion of Tat exacerbates the futile cycle induced by β-lactams and overproduction of even one key peptidoglycan-modifying enzyme relieves it. It is worth noting that fosfomycin disrupts formation of peptidoglycan by acting on MurA, a UDP-N-acetylgalactosamine enolpyruvyl transferase that is essential in E. coli (Brown et al. 1995). Though fosfomycin and β-lactams target peptidoglycan via different mechanisms, they have a similar effect on the tatABC deletion (Fig. 2B). It is possible that fosfomycin also induces a similar futile cycle as noted with β-lactams, but that has not been shown.

Oddly, while we were able to transform pSecSP–AmiA, pSecSP–AmiC and pSecSP–SufI into the ΔtatABC background, we were unable to get transformants in the wild-type background after repeated attempts. Previous studies show that pBAD33 promoter is uninduced in LB without arabinose (Guzman et al. 1995) and we performed growth curves to verify it. Our data show that pSecSP–AmiA compensates for loss of Tat even when arabinose is not added to the media (Fig. S2A, Supporting Information). Thus, it seems that the constitutive level of expression of these engineered constructs is detrimental to the cell, changing conformation and fluorescing red. The relative Δσ is established by determining the red:green MFI. If the ratio decreases, it means there is a decrease in the efficiency of dye import. The red:green MFI was determined by flow cytometry. The data from five independent runs of 10,000 events each demonstrate that wild-type 14028 has a red:green MFI of ~700 units, which indicates the normal Δσ level and accumulation of dye in the cell (Fig. 6). Wild-type 14028 was treated with CCCP to quench the reaction and indicate a disrupted Δσ. When 14028 is treated with CCCP, the MFI drops to ~200. The ΔtatABC, ΔhyABABC ΔhydBC and Δmig5 strains show disrupted Δσ with red:green MFI values lower than that of wild-type 14028 (Fig. 6). The ΔamiA ΔamiC ΔsufI ΔmepK quadruple deletion strain also had a red:green MFI lower than that of wild-type 14028, indicating that the loss of these critical substrates has a dramatic effect on the health of the cell, including on the Δσ. Additionally, a representative histogram from one of the five independent flow cytometry runs shows the shift in red:green ratio in the deletion strains as compared with wild type (Fig. S3, Supporting Information). The combined Δσ data show an important role for hydrogenases in aerobic growth of S. Typhimurium.

These data are mirrored in Craig et al. (2013) where a hydrogenase triple mutant was attenuated for virulence 5-fold. Here, the authors clearly demonstrate that Tat and Tat substrates AmiA, AmiC and SufI are required for virulence in the mouse model of infection, and further show that a strain deficient in anaerobic respiration (ΔmoaDE ΔnfrA ΔfrdA) is not attenuated. Additionally, deletions of aerobic respiratory components are
significantly attenuated. Thus, Craig et al. show that conditions inside the animal are aerobic. This is important because the authors also demonstrate a ΔhyaAB ΔhybABC ΔhydB phenotype in the mouse, suggesting that these hydrogenases are necessary for full virulence in aerobic conditions. Indeed, the translocation of Tat substrates is driven by PMF; thus, disruption of PMF likely leads to inefficient translocation of Tat substrates. The combined data from Craig et al. and our study suggest an important role for these hydrogenases in the maintenance of PMF during aerobic growth. The ΔhyaAB ΔhybABC ΔhydB phenotype in both the animal model (Craig et al. 2013) and our ampicillin model is likely caused by poor translocation of Tat substrates AmiA, AmiC, SufF and MepK due to disrupted PMF. To show that the ΔhyaAB ΔhybABC ΔhydB and Δmig5 strains are deficient in translocation of Tat substrates, we used a TorA–GFP plasmid previously described (Craig et al. 2013). In this construct, GFP is cloned into pBR322 and engineered to carry the signal sequence from the Tat substrate TorA. The data demonstrate (Fig. 7) that GFP is periplasmic in the wild-type 14028 background and cytoplasmic in the ΔtatABC background, as expected. The ΔhyaAB ΔhybABC ΔhydB and Δmig5 strains show clear GFP localization to the cytoplasm, similar to the ΔtatABC background. This confirms that the ΔhyaAB ΔhybABC ΔhydB and Δmig5 strains are deficient in translocation of Tat substrates to the periplasm due to the changes in Δhya of these strains.

Together, these data demonstrate the importance of Tat system in the Salmonella response to peptidoglycan targeting antibiotics. Our results indicate that MepK, AmiA, AmiC and SufF are critical periplasmic proteins and loss of these enzymes leaves the bacterium exceptionally sensitive to antibiotics that target peptidoglycan synthesis. Further, deletions of hydrogenase subunits and the Mig5 carbonyl anhydrase alter the Δψ of the cell and impact delivery of MepK, AmiA, AmiC and SufF to the periplasm, leaving the cell sensitive to cell wall targeting antibiotics. We have also demonstrated the importance of these hydrogenases and Mig5 in contributing to the PMF of Salmonella. There are notable differences between the Tat systems of E. coli and Salmonella, including the translocation of Mig5, which is not present in E. coli. The Tat system is an attractive target for identification of new antimicrobial compounds. Our data show that drugs targeting the Tat system or the hydrogenases that power it could be used in combination with β-lactams to increase microbial killing capacity and could potentially extend the spectrum of existing β-lactams.

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SUPPLEMENTARY DATA

Supplementary data are available at FEMSMC online.

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Conflict of Interest. None declared.

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