Mechanisms of Incorporation for D-Amino Acid Probes That Target Peptidoglycan Biosynthesis

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Supporting Information

ABSTRACT: Bacteria exhibit a myriad of different morphologies, through the synthesis and modification of their essential peptidoglycan (PG) cell wall. Our discovery of a fluorescent D-amino acid (FDAA)-based PG labeling approach provided a powerful method for observing how these morphological changes occur. Given that PG is unique to bacterial cells and a common target for antibiotics, understanding the precise mechanism(s) for incorporation of (F)DAA-based probes is a crucial determinant in understanding the role of PG synthesis in bacterial cell biology and could provide a valuable tool in the development of new antimicrobials to treat drug-resistant antibacterial infections. Here, we systematically investigated the mechanisms of FDAA probe incorporation into PG using two model organisms Escherichia coli (Gram-negative) and Bacillus subtilis (Gram-positive). Our in vitro and in vivo data unequivocally demonstrate that these bacteria incorporate FDAs using two extracytoplasmic pathways: through activity of their D,D-transpeptidases, and, if present, by their L,D-transpeptidases and not via cytoplasmic incorporation into a D-Ala-D-Ala peptide precursor. Our data also revealed the unprecedented finding that the DAA-drug, D-cycloserine, can be incorporated into peptide stems by each of these transpeptidases, in addition to its known inhibitory activity against D-alanine racemase and D-Ala-D-Ala ligase. These mechanistic findings enabled development of a new, FDAA-based, in vitro labeling approach that reports on subcellular distribution of muropeptides, an especially important attribute to enable the study of bacteria with poorly defined growth modes. An improved understanding of the incorporation mechanisms utilized by DAA-based probes is essential when interpreting results from high resolution experiments and highlights the antimicrobial potential of synthetic DAs.

INTRODUCTION

Of the known bacterial species, nearly all possess a peptidoglycan (PG) cell wall that surrounds the cell. PG is an essential cellular component that maintains the size and shape of the bacterial cell and helps protect the cell from its environment. Given its essential roles in cell growth and division, its relative accessibility when compared to potential cytoplasmic targets, and that it is unique to bacterial cells, some of the most successful antibiotics target PG biosynthesis.1

The PG cell wall is a macromolecule consisting of glycan strands cross-linked by short D-amino acid (DAA)-containing peptides. The PG biosynthetic pathway is promiscuous with...
Figure 1. PG biosynthetic pathways are promiscuous and accept D-amino acid (DAA) based probes. (a) Simplified cartoon representation of the PG synthesis and modification pathways relevant to this work, along with representative PG synthesis inhibitors. (b) Representative DAA-based probes utilized in the experiments described in this manuscript.

Here we have systematically characterized the incorporation mechanisms of common DAA-based probes in two model organisms: Gram-positive *B. subtilis* and Gram-negative *E. coli*. We leveraged chemical genetics, genetics, microscopy, and *in vitro* approaches in combination with labeling experiments that utilized: EDA, a small clickable DAA; HADA, an FDA; along with EDA – DA and DA – EDA, two clickable small DAADs (Figure 1b). Our data suggest that DAADs are incorporated using the cytoplasmic (lipid II) pathway, while single DAA-based probes (e.g., EDA, HADA) are incorporated outside the cytoplasm by the DD-TPase(s) and, if present, the LD-TPase(s). Our chemical genetics experiments revealed a previously unknown mode of action for DCS, specifically, that it is a substrate for both DD-TPases and LD-TPases and that it is readily incorporated into pentapeptide and tetrapeptide stems, respectively (Figure 1b), perhaps providing an important insight for further investigation of novel D-amino acid-based antibacterial agents. Finally, our improved mechanistic understanding helped us design a new, FDAA-based labeling approach that reports on subcellular distribution of muropeptides in bacterial cells.

## RESULTS

Chemical Genetics Approach Reveals Transpeptidase-Mediated Incorporation of DAAs in *Bacillus subtilis*. In order to probe the mechanism of FDAA incorporation in vivo, we first attempted a chemical genetics approach. Since bacterial species usually have multiple LD-TPases and/or commonly essential DD-TPases, deleting them all is not feasible. We first focused on vegetative *B. subtilis* cells and reported a new, FDAA-based labeling approach that reports on subcellular distribution of muropeptides in bacterial cells.

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**Figure 1.** PG biosynthetic pathways are promiscuous and accept D-amino acid (DAA) based probes. (a) Simplified cartoon representation of the PG synthesis and modification pathways relevant to this work, along with representative PG synthesis inhibitors. (b) Representative DAA-based probes utilized in the experiments described in this manuscript.
coli cells (Figure SI1a), it completely removed the signal from vegetative ethanol-fixed pentapeptide-rich B. subtilis ΔdacA cells prelabeled with HADA (Figure SI1b). These results are in agreement with previous observations that vegetative B. subtilis cells incorporate HADA exclusively into their pentapeptides.15

In the absence of L,D-TPase activity, chemical inhibition of extracellular D,D-TPases or inhibition of cytoplasmic D-Ala-D-Ala synthesis and incorporation could provide valuable information on the route of FDAA incorporation. We first confirmed that cells remained viable after such brief drug exposures (Figure SI1c), consistent with a recent report.56 Our initial experiments screening different FDAAs and drugs showed that both β-lactams and D-cycloserine (DCS) had the potential to significantly inhibit the incorporation of FDAAs in live B. subtilis in a drug- and FDAA-dependent manner (Figures 2a and SI1c). For example, the inhibition by DCS was particularly pronounced and comparable to penicillin G, a β-lactam, when the FDAA was TDL, a large red FDAA linked to D-lysine, (∼65–80% inhibition, Figure 2a). A similar effect was observed in E. coli wild-type cells. While D-cycloserine and ampicillin both inhibited HADA incorporation (by ∼30%), meropenem inhibited virtually all HADA incorporation (Figure SI1d).

Given the significant inhibition observed for both DCS and β-lactams—and the well-characterized inhibitory activity of the β-lactams toward the bacterial transpeptidases—we postulated that DCS, a cyclized D-serine analogue with a free amino group (Figure 1b), might behave as a competitive inhibitor of periplasmic L,D- and/or D,D-TPases. In support of this hypothesis, both LdtA_Vc, a representative L,D-TPase from Vibrio cholerae,56 and PBP4_Sa, a soluble version of recombinant PBP4 from Staphylococcus aureus,17 incorporated DCS into

Figure 2. B. subtilis cells do not incorporate (F)DAAs cytoplasmically, but through reactions mediated by D,D-TPases, which can be inhibited by D-cycloserine. (a) A brief pretreatment of live B. subtilis cells with vancomycin, penicillin G (Pen G), and D-cycloserine (DCS), significantly inhibited FDAA incorporation. No inhibition of FDAA incorporation was observed with fosfomycin. (b) LdtA_Vc incorporated DCS into M4, comparable to other DAAs, e.g., D-Met, in vitro. (c) PBP4_Sa incorporated DCS into a synthetic Nα,Nε-Diacetyl-L-Lys-D-Ala-D-Ala tripeptide (3P), in vitro. (d) Live B. subtilis wild-type and B. subtilis Δddl cells, grown in S750 minimal media supplemented with DA−DA, incorporated HADA comparably; HADA incorporation was significantly inhibited by ampicillin pretreatment. (e) DA−EDA was a poor substrate for MurF Bs in vitro, but EDA−DA performed similarly well to the endogenous substrate, DA−DA. Column bar graphs represent mean relative signal ± SD quantified from at least N > 100 cells. Error bars are SEM.
appropriate soluble substrate analogues in vitro.\textsuperscript{13} LdtA\textsubscript{Vc} incorporated DCS into tetrapeptides comparably to D-methionine, a DAA that is naturally produced by Vibrio cholerae cells\textsuperscript{2} (Figure 2b); the product of each reaction was confirmed by MS (Figure SI2a). PBP4\textsubscript{Sa} incorporated DCS into a synthetic N\textsubscript{α},N\textsubscript{ε}-diacetyl-L-Lys-D-Ala-D-Ala tripeptide (3P) albeit to a lower extent when compared to identical experiments utilizing D-methionine (Figure 2c). Moreover, excess DCS directly competed with incorporation of FDAAs into ethanol-fixed cells by LdtA\textsubscript{Vc} in a FDAA-specific manner (Figure SI2b). These data suggest that, in addition to its known cytoplasmic targets (alanine racemase and D-Ala-D-Ala ligase),\textsuperscript{57} DCS, a clinically important antibiotic, has the previously uncharacterized potential to competitively inhibit L,D- and D,D-TPases, and therefore its utilization would not yield clear results probing (F)DAA incorporation mechanisms.

Vegetative B. subtilis Cells Incorporate FDAAs with D,D-TPases and Not Cytoplasmically. Given these difficulties, we turned to a conventional genetics approach for deletion of nonessential (or conditionally essential)}
pathways and interrogation of potential DAA incorporation mechanisms through comparison of FDAA accumulation between otherwise isogenic strains. Because vegetative *B. subtilis* cells lack L,D-Tpase activity (Figure SI1b), knocking out its ability to form DA—DA would also eliminate its ability to incorporate DAAs cytoplasmically and leave D,D-transpeptidation as the only feasible route for DAA incorporation (Figure 1a). Deleting the single and essential DA—DA-ligase gene (*ddl*) in *B. subtilis* provided a strain that was auxotrophic for exogenously provided DA—DA. Under identical growth conditions, wild-type *B. subtilis* and *B. subtilis Δddl* cells accumulated a HADA signal to a comparable extent, while ampicillin treatment inhibited >80% of their initial signal (Figure 2d). This result strongly suggests that FDAAs are not incorporated cytoplasmically in *B. subtilis*.

Our *in vitro* findings with MurF from *Bacillus subtilis* (MurF*)*, the next cytoplasmic enzyme downstream of Ddl, support the conclusion that FDAAs are not incorporated cytoplasmically in *B. subtilis*. These experiments revealed that MurF* accepted EDA—DA, a clickable DDA that eventually generates lipid II tagged in the fourth position of the pentapeptide stem,16 as well as its endogenous substrate, DA—DA both in terms of its affinity and turnover (Figure 2e). However, DA—EDA, a DAAD that generates a pentapeptide tagged in the fifth (i.e., terminal) position, is a significantly poorer (~20-fold) substrate than DA—DA. This observation, along with the enzymatic activity of the endogenous carboxypeptidases (e.g., PBP5), may explain the differential DAAD labeling previously observed in live *B. subtilis* cells; specifically, EDA—DA labeled the PG of *B. subtilis* much more strongly than DA—DA.16 The *in vitro* data also suggest that cytoplasmic DAA incorporation into the terminal position of the pentapeptide stem (e.g., with DA-EDA)—the pentapeptide terminus is the only site labeled by (F)DAAs in vegetative *B. subtilis* cells (Figure SI1b) —is particularly disfavored by MurF in *B. subtilis* (Figure 2e). Furthermore, excess DA—DA did not significantly compete with the FDAA labeling accumulation in live vegetative *B. subtilis* cells, but D-alanine and D-tyrosine, a DAA that is naturally produced by *B. subtilis* cells, (>50-fold decrease) did (Figure SI2c). Thus, we conclude that (F)DAAs are not incorporated cytoplasmically in vegetative *B. subtilis* cells. By the process of genetic elimination and in combination with the Supporting Information, our results strongly suggest that, in vegetative *B. subtilis* cells, FDAAs are incorporated predominantly (if not solely) through the activity of the periplasmic D,D-transpeptidases.

**E. coli** Cells Incorporate FDAAs by L,D-Tpases in a Growth Independent Manner. In contrast to *B. subtilis*, *E. coli* is rich in L,D-Tpase activity.36 We recently reported on a mutant *E. coli* strain in which all the predicted L,D-Tpases were deleted.36 Cells from this strain, *E. coli* BW255113Δ6LDT (hereafter referred to as Δ6LDT), revealed a ∼10-fold lower HADA signal compared to wild-type *E. coli* (Figure 3a). Among the six single L,D-Tpase knockout strains, ΔldtD cells showed the least HADA incorporation (∼50%, Figure S13a). Consistently, double, triple, and quadruple L,D-Tpase mutants showed a significant decrease in labeling compared to wild-type as long as *ldtD* is one of the deleted genes (Figure S13b). These results suggest that the major mode(s) of FDAA incorporation in actively growing *E. coli* is due to the L,D-Tpases, primarily LdtD.41

Early labeling experiments revealed that wild-type *E. coli* cells begin to accumulate FDAA signal at sites of new growth (i.e., septum and side walls) eventually plateauing into uniformly labeled cells over 1–2 generations.15 In contrast, *E. coli* Δ6LDT cells accumulate FDAA signal to a lower extent and preserve the signal persistently at sites of new growth (Figure 3a). This uniform and peripheral accumulation of FDAA signal in wild-type *E. coli* cells suggested a growth-independent, L,D-Tpase-facilitated DAA incorporation.

Consistently, nutrient starved cells, which are kept in phosphate buffered saline (PBS) for 1 h at RT, of wild-type *E. coli* incorporated HADA strongly and uniformly, whereas *E. coli* Δ6LDT cells did not show any significant signal accumulation above background (Figure 3b). A complementation assay with nutrient starved *E. coli* cells suggested that this growth independent FDAA incorporation in buffer was predominantly due to overexpression of L,D-Tpase genes known to generate 3–3 cross-links, *ldtD* and *ldtE*,58 and less so due to L,D-Tpases that anchor Braun’s lipoprotein (*ldtA, ldtB*, or *ldtC*) or *yafK* (Figure S13c). This property was not specific to *E. coli*. Diverse bacteria rich in predicted L,D-Tpases, such as the deltaproteobacterium *Bdellevibrio bacteriovorus*36 and the alphaproteobacterium *Agrobacterium tumefaciens*,72 also incorporated HADA in a growth-independent manner (Figure S1 3d).

Collectively, these results suggested that L,D-Tpase mediated DAA incorporation in *E. coli* can occur independently of cell growth.59 On the other hand, the persistent signal observed in the absence of L,D-Tpase activity (Figure 3a) points to the presence of an additional mode for DAA incorporation in *E. coli*.

**D,D-Tpases Incorporate DAAs into the Pentapeptides of PG in *E. coli* Cells.** Pentapeptides are rare in *E. coli* PG.50 We hypothesized that the relatively faint FDAA signal localized at sites of new growth in cells lacking L,D-Tpases represented probe incorporation into pentapeptides. If so, deleting the gene for one of the major *E. coli* D,D-CPases (*dacA*) in this background should increase the FDAA labeled pentapeptide pools in *E. coli* PG. Indeed, *E. coli* Δ6LDTΔdacA cells showed 2-fold greater incorporation of HADA signal than *E. coli* Δ6LDT (Figure 3c). We observed that HADA accumulation in *E. coli* Δ6LDTΔdacA cells could be eliminated by treatment with a carboxy/endopeptidase (PBP4*,* in *in vitro* (Figure S14a)). The labeling trends observed with these mutants and EDA, a small clickable DAA, were comparable (Figure S14b). These results reveal previously underappreciated DAA incorporation into pentapeptides in *E. coli*.

This pentapeptide labeling, could be achieved via the cytoplasmic route (through Ddl/MurF), an exchange reaction mediated by the D,D-transpeptidases (i.e., the PBP5s), or a combination of the two. Therefore, we deleted *ddlA* and *ddlB* on top of 6 L,D-Tpases generating an *E. coli* Δ6LDTΔddlAB strain that is auxotrophic for DA—DA. This mutant strain lacks both L,D-Tpase activity and the ability to incorporate DAAs cytoplasmically. Similar to the aforementioned case in *B. subtilis*, *E. coli* Δ6LDTΔddlAB cells accumulated HADA (Figure 3d) or EDA (Figure S14c) signals comparably to identically treated *E. coli* Δ6LDT cells. Ampicillin treatment significantly inhibited HADA accumulation in both strains (Figure 3d). Moreover, DCS significantly inhibited (F)DAA signal incorporation even in the absence of Ddl, and in the presence of excess DA—DA, in both *E. coli* and *B. subtilis* (Figure S14c-d), providing further support for the notion that DCS may also function as a DAA-like competitive inhibitor of D,D-Tpases in both organisms. Collectively, these results
suggest that the D,D-TPases, and not the cytoplasmic enzymes, are primarily responsible for incorporation of DAAs into pentapeptides in *E. coli* and in *B. subtilis*.

**Ddl Disfavors the Cytoplasmic Incorporation of DAAs.** For cytoplasmic incorporation, following cellular import, a (F)DAA needs to be tolerated by Ddl and incorporated into a corresponding DAAD. To test our conclusion that (F)DAAs are not incorporated cytoplasmically, we assessed the ability of *E. coli* DdlB (DdlB<sub>Ec</sub>) to ligate D-Ala to EDA, the small clickable DAA, forming DA<sub>−</sub>EDA, in vitro. Under previously published conditions, DdlB<sub>Ec</sub> showed 2-fold higher affinity for EDA than for D-Ala; however, the apparent rate of the reaction with EDA was 10-fold slower than the reaction with D-Ala (Figure 3e). This suggests that cytoplasmic (F)DAA incorporation may be primarily rejected by Ddl, especially since the cytoplasmic steps lying downstream of Ddl seem to be significantly more tolerant. Indeed, MurF from *Pseudomonas aeruginosa* (MurF<sub>Pa</sub>, an enzyme that shows high sequence and kinetic similarity to *E. coli* MurF<sup>61</sup>) incorporated both of the clickable DAADs, EDA<sub>−</sub>DA and DA<sub>−</sub>EDA, with comparable rates and affinities to the native substrate, DA<sub>−</sub>DA (Figure 4a). This tolerance of MurF (and presumably the downstream steps) could explain how small DAADs could be used as common and versatile metabolic PG labeling alternatives to FDAAs and why they can rescue DA<sub>−</sub>DA deficiencies in *E. coli*, *B. subtilis*, and *Chlamydiae*.16,37

Lastly, we wanted to test if *E. coli* L,D-TPases play a role in DAAD incorporation. Cells from *E. coli* Δ6LDT grown for 1–2 generations in the presence of EDA<sub>−</sub>DA incorporated approximately 20% more DAAD signal compared to wild-type *E. coli* cells (Figure 4b). These data provide strong evidence that L,D-TPases do not play a significant role in DAAD incorporation.

From our combined results, we conclude that the incorporation of FDAA and DAAD probes can be used to distinguish between two distinct biosynthetic pathways. FDAA probe incorporation reports on the activity of the extracytoplasmic L,D- and D,D-TPases, whereas DAAD probe incorporation reports on the cytoplasmic (lipid II) pathway, i.e., nascent PG synthesis (Figure 4c).

**FDAAs Report on L,D- and D,D-TPase Activity and Muropeptide Distribution in Fixed Cells.** Our results and previous in vitro work with D-amino acids (DAAs) revealed that both L,D-TPases and D,D-TPases of different bacterial origins can utilize DAAs as acyl-acceptor substrates in the presence of appropriate acyl-donors.17,56,62 Consistently, representative enzymes with known L,D-TPase or D,D-TPase activities could utilize larger fluorescent DAAs (FDAAs) as acyl-acceptor substrates in vitro. A representative

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**Figure 4.** FDAAs are incorporated by periplasmic transpeptidases and DAADs cytoplastically. (a) MurF<sub>Pa</sub> incorporated DAADs as well as the native substrate (DA–DA), in vitro. (b) Live Δ6LDT cells incorporated a greater DA–DA (1 mM for 1 h) signal than *E. coli* wild-type cells. Values in column bar graphs represent the mean relative signal quantified from at least N > 150 cells. (c) Cartoon representation depicting that in *E. coli* DAAs, including FDAAs and DCS, are substrates for periplasmic L,D-TPases, D,D-TPases, or D,D-CPases and that DAADs are substrates for cytoplasmic MurF.
Figure 5. FDAAs are efficiently incorporated into PG precursors. (a) LdtA<sub>Vc</sub> incorporated HADA into the soluble muropeptide disaccharide tetrapeptide (M4) comparable to other DAAs, e.g., D-Met, in vitro. (b) Different high-molecular-weight D,D-TPases from diverse bacteria incorporated HADA and NADA during the in vitro synthesis of nascent PG from lipid-II without significantly changing their total D,D-TPase and D,D-CPase product distribution. The values are the mean ± SD of three independent experiments.

Figure 6. FDAAs report on the abundance and subcellular distribution of muropeptides in ethanol fixed and permeabilized bacterial cells in vitro. (a) Ethanol fixed V. cholerae cells are substrates for LdtA<sub>Vc</sub> and FDAAs, e.g., HADA, in vitro. (b) Ethanol fixed S. aureus cells are substrates for PBP4<sub>Sa</sub> and FDAAs in vitro. (c–d) Ethanol fixed E. coli cells are substrates for LdtA<sub>Vc</sub> or PBP4<sub>Ec</sub> and FDAAs in vitro. (c) FDAAs and LdtA<sub>Vc</sub> can report on PG tetrapeptide abundance of the E. coli Δ6LDT and ΔdacA strains in vitro. (d) FDAAs and PBP4<sub>Ec</sub> can report on PG pentapeptide abundance of the E. coli Δ6LDT and ΔdacA strains in vitro. (e) Sequential PBP4<sub>Ec</sub> (with HADA) and LdtA<sub>Vc</sub> (with BADA) labeling can report on differential subcellular muropeptide distribution of a strain, e.g., in ethanol fixed A. tumefaciens cells, red arrows. Column bar graphs represent mean relative signal quantified from at least N > 100 cells. Error bars are SEM. Scale bars, 2 μm.
L,D-TPase from *Vibrio cholerae* (LdtA\textsubscript{WC}) incorporated HADA into a soluble disaccharide tetrapeptide (Figure 5a) while four recombinant high molecular weight PBPs (E. coli, PBP1\textsubscript{Ae} and PBP1\textsubscript{Bd}; *Haemophilus influenzae*, PBP1\textsubscript{AHi} and *B. subtilis*, PBP1\textsubscript{Bb}) facilitated incorporation of FDAAs during the synthesis of nascent PG from a lipid II precursor (Figure 5b). The FDAA incorporation activities were comparable to naturally produced D-methionine\textsuperscript{2} (Figure 5a) or were at the expense of their native cross-linking/carboxypeptidase activities (Figure 5b). This suggested that FDAAs did not significantly interfere with the native functions of these enzymes and could serve as proxy reporters for the DAA incorporation activities of L,D-TPases or for cross-linking/carboxypeptidase activities of D,D-TPases.

Next, we tested whether these recombinant transpeptidases would incorporate FDAAs into isolated sacci in *vitro*. Early attempts with isolated *V. cholerae* sacci indicated high background/non-specific labeling (Figure S15a). This led us to utilize ethanol-fixed and permeabilized intact cells as transpeptidase substrates, a simple substrate preparation approach that we have previously employed.\textsuperscript{16} Treatment of bacterial cells with ice-cold ethanol inactivates virtually all cellular activity and permeabilizes membranes for downstream applications while keeping the cells intact and well separated for microscopy experiments.\textsuperscript{16} Indeed, recombinant LdtA\textsubscript{WC} incorporated HADA into ethanol-fixed *V. cholerae* cells in *vitro* (Figure 6a). Label incorporation was specific for PG (Figure S15b); parallel experiments with the L-isomer, HALA, resulted in a 5-fold decrease in fluorescence incorporation (Figure S15c). We similarly observed a minor, and *V. cholerae*-specific, HALA incorporation in live *V. cholerae* cells, but not live *E. coli* cells, albeit approximately 20-fold less efficiently than HADA (Figure S15d). LdtA\textsubscript{WC} incorporated HADA into ethanol-fixed cells from a variety of Gram-negative species (Figure S16a). Similarly, a soluble version of recombinant PBP4 from *S. aureus* (PPB4\textsubscript{Sa})\textsuperscript{17} incorporated HADA into ethanol-fixed *Staphylococcus aureus* cells (Figure 6b) and ethanol-fixed *E. coli* cells (Figure S16b). Recombinant PPB4\textsubscript{Sa} also incorporated HALA into fixed *E. coli* cells, although it was incorporated approximately 20-fold less efficiently than HADA (Figure S16b).

The PG of *E. coli* is rich in tetrapeptides,\textsuperscript{58} but the abundance of pentapeptides increases in strains lacking D,D-carboxypeptidases\textsuperscript{27} (e.g., PBP5 encoded by dacA). Consistently, LdtA\textsubscript{WC} incorporated HADA into ethanol-fixed *E. coli* ΔdacA cells comparatively to wild-type cells (Figure 6c), suggesting that the carboxypeptidase activity of DacA may not significantly impact overall tetrapeptide abundance. However, a 7-fold increase in HADA incorporation was observed using ethanol-fixed cells from an *E. coli* strain that lacked all of the six known or predicted genes encoding for L,D-TPases, Δ6LDT (Figure 6c). The enhanced HADA incorporation in the Δ6LDT strain relative to the wild-type strain suggests that LdtA\textsubscript{WC} combined with the FDAAs, can report on the relative abundance of tetrapeptides in PG.

On the other hand, PBP4\textsubscript{Sa} incorporated HADA into fixed cells from an *E. coli* strain that lacked a major D,D-CPase, BW25133ΔdacA, significantly more than the wild-type, strongly suggesting that pentapeptides are the preferred substrate for PBP4\textsubscript{Sa}-mediated HADA incorporation (Figure 6d). Sequential enzymatic labeling of ethanol-fixed cells with differently colored FDAAs was possible. Treatment of ethanol-fixed Δ6LDTΔdacA cells with HADA and PBP4\textsubscript{Sa} followed by BADA, a green, BODIPY-FL containing FDAA that is spectrally distinguishable from HADA, and LdtA\textsubscript{WC} resulted in superimposable labeling patterns (Figure S16c). The same experimental approach with polarly growing *Agrobacterium tumefaciens* cells showed complementary labeling patterns particularly in young cells; a HADA signal (reporting on pentapeptides) appeared enriched at the very tip of the growing pole, followed by a band of BADA signal (reporting on tetrapeptides, Figure 6e). These results show that L,D- and D,D-TPases can incorporate FDAAs into ethanol fixed and permeabilized cells from diverse species in *vitro* while reporting on subcellular tetrapeptide/pentapeptide distribution of a strain. Moreover, these in *vitro* results support the direct role of these periplasmic L,D- and D,D-TPases in incorporating (F)DAAs in different bacteria.

**DISCUSSION**

Bacteria have the evolutionarily preserved ability to produce DAs, and their PG biosynthetic pathways are uniquely promiscuous for utilization of DAAs that provide them with a survival advantage in the wild.\textsuperscript{36,63} The design of multiple classes of DAA-based PG labeling probes that take advantage of this promiscuity are now widely used.\textsuperscript{10,14−18,29} While dissecting the routes for incorporation (and turnover) of these PG probes has been challenging, without this knowledge, the interpretation of the data generated with these probes in a given species is limited. Here, we provide a roadmap to explore the mechanisms by which DAA-based probes are incorporated into the PG by combining genetics, chemical genetics, and *in vitro* approaches for two model organisms, *E. coli* and *B. subtilis*. We provide strong evidence that (F)DAAs, including the DAA drug DCS, are incorporated by extracytoplasmic (Gram-positive) or periplasmic (Gram-negative) L,D-TPases and D,D-TPases in contrast to DAADs, which are cytoplasmically incorporated (Figure 4c).

Prior to our discovery of the FDAAs, previous work with DAADs\textsuperscript{2,17,29,56,62} (e.g., D-methionine) and various DAADs\textsuperscript{5,16,37,49,64} showed that incorporation of any DAA is tolerated by these D-Ala assembly/utilization steps that are common in PG containing bacteria. Previous work strongly suggested DAA probe incorporation was mediated by the bacterial transpeptidases; however, definitive experiments to rule out cytoplasmic incorporation had yet to be reported. The ability to distinguish these pathways has important ramifications with respect to interpretation of results from FDAA labeling experiments. For example, cytoplasmic incorporation of a DAA probe would report on nascent PG synthesis; the probe would be incorporated into the lipid II intermediate that would subsequently be detected upon polymerization into glycan strands. Alternatively, periplasmic incorporation would report on the localized activity of transpeptidases on the bacterial cell surface (Figure 1a). Therefore, any cytoplasmic/periplasmic cross-reactivity of a probe would significantly complicate either of these interpretations. The mechanistic picture is further complicated in experiments that are carried out in the presence of D-cycloserine (DCS). Since DCS is a well-established inhibitor of alanine racemase and D-Ala-D-Ala ligase (Ddl), enzymes that are critical for the cytoplasmic assembly of D-Ala-D-Ala, it is logical to conclude that diminished DAA probe incorporation in the presence of DCS is indicative of a cytoplasmic pathway for probe incorporation. However, our data have revealed that DCS
tetrapeptides and pentapeptides in bacteria with poorly investigated the roles of relative distribution of PG mutant strains. This method will be particularly useful for directly compete with trans peptidase-mediated (F)DAA incorporation. However, our data have revealed that DCS can also be indicative of a cytoplasmic pathway for probe incorporation. This unexpected behavior is likely because DCS is also a DAA and may point to a heretofore unidentified mode of action of DCS on TPase-mediated reactions. Indeed, a D,D-CPase mutation has been shown to confer resistance to DCS in Mycobacterium. Although this multiple target reactivity makes DCS a problematic reagent for investigation of FDAAs in PG of live E. coli and B. subtilis cells through the process of elimination. Ddl knockout strains in B. subtilis and E. coli that also lack L,D-TPase activity showed (F)DAA labeling comparable to the parent strains with native Ddl activity. The observed labeling, in the absence of Ddl and L,D-TPase activity, provides compelling evidence that L,D-TPases incorporate (F)DAAs into pentapeptides in the PG of these two species in vivo. Consistently, β-lactams dramatically reduce in vivo (F)DAA incorporation into pentapeptides in these species. Our in vitro data also suggest that bacteria may have an intrinsic ability to limit utilization of DAAs other than D-Ala by Ddl and MurF. For example, DdBΔ, has a ~10-fold lower specific activity toward EDA relative to D-Ala, suggesting that the cytoplasmic incorporation route may be limited at this point even if (F)DAAs are taken up by the cell. Similarly, MurFΔ, rejects DA–EDA, the terminally tagged DAAD.

The incorporation mechanism for DAADs appears to be simpler. We, and others, have previously shown that DAADs could complement growth in E. coli, B. subtilis, Chlamydia trachomatis, Mycobacterium smegmatis, and even chloroplasts of moss, Physcomitrella patens, when the cytoplasmic DA–DA pools were depleted by DCS and/or by the generation of auxotrophic Ddl mutants. Our data here are in agreement with this finding: DAADs perform similarly to the endogenous substrate (DA–DA) for dipeptide incorporation in vitro.

Collectively, our results imply that (F)DAAs report on the activities of periplasmic D,D-TPases and L,D-TPases, which explains why (F)DAAs label diverse bacteria so effectively. In addition, (F)DAAs do not report on any enzyme activity until the periplasmic biosynthesis of nascent PG is complete (Figure 1a). DAADs, along with a recently reported metabolic glycolabeling strategy, are complementary to FDAAs in that they report on the biosynthesis of nascent PG.
Finally, we note that many clinically relevant antibiotics are DAAs (e.g., DCS, the β-lactams). Our data reveal that DCS, an important antibiotic that targets a D,D-TPase, has been shown to target diverse TPases in evolutionarily diverse bacteria, including E. coli. Like DCS, many β-lactams—widely used and long-standing anti-D,D-TPase drugs—have terminal DAA moieties that confer them improved antibacterial activities relative to their L-amino acid counterparts. For example, D-ampicillin (or ampicillin) is up to 5-fold more potent than L-ampicillin, and a synergy between free DAAs and β-lactams has been shown. It is tantalizing to think that this synergy points to a DAA-dependent D,D-TPase “activation” leading to an enhanced sensitivity toward natural electrophilic β-lactams. If true, and given that (F)DAAs can selectively target bacteria in live animals, this may provide a promising new avenue for DAA-based antibacterial discovery.

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