ABSTRACT  During evolution, enzymes can undergo shifts in preferred substrates or in catalytic activities. An intriguing question is how enzyme function changes following horizontal gene transfer, especially for bacterial genes that have moved to animal genomes. Some insects have acquired genes that encode enzymes for the biosynthesis of bacterial cell wall components and that appear to function to support or control their obligate endosymbiotic bacteria. In aphids, the bacterial endosymbiont Buchnera aphidicola provides essential amino acids for aphid hosts but lacks most genes for remodeling of the bacterial cell wall. The aphid genome has acquired seven genes with putative functions in cell wall metabolism that are primarily expressed in the aphid cells harboring Buchnera. In analyses of aphid homogenates, we detected peptidoglycan (PGN) muropeptides indicative of the reactions of PGN hydrolases encoded by horizontally acquired aphid genes but not by Buchnera genes. We produced one such host enzyme, ApLdcA, and characterized its activity with both cell wall derived and synthetic PGN. Both ApLdcA and the homologous enzyme in Escherichia coli, which functions as an L,D-carboxypeptidase in the cytoplasmic PGN recycling pathway, exhibit turnover of PGN substrates containing stem pentapeptides and cross-linkages via L,D-endopeptidase activity, consistent with a potential role in cell wall remodeling. Our results suggest that ApLdcA derives its functions from the promiscuous activities of an ancestral LdcA enzyme, whose acquisition by the aphid genome may have enabled hosts to influence Buchnera cell wall metabolism as a means to control symbiont growth and division.

IMPORTANCE  Most enzymes are capable of performing biologically irrelevant side reactions. During evolution, promiscuous enzyme activities may acquire new biological roles, especially after horizontal gene transfer to new organisms. Pea aphids harbor obligate bacterial symbionts called Buchnera and encode horizontally acquired bacterial genes with putative roles in cell wall metabolism. Though Buchnera lacks cell wall endopeptidase genes, we found evidence of endopeptidase activity among peptidoglycan muropeptides purified from aphids. We characterized a multifunctional, aphid-encoded enzyme, ApLdcA, which displays L,D-endopeptidase activities considered promiscuous for the Escherichia coli homolog, for which these activities do not contribute to its native role in peptidoglycan recycling. These results exemplify the roles of enzyme promiscuity and horizontal gene transfer in enzyme evolution and demonstrate how aphids influence symbiont cell wall metabolism.

KEYWORDS Buchnera, carboxypeptidase, cell wall, endopeptidase, enzyme promiscuity, horizontal gene transfer, multifunctional, pea aphid, peptidoglycan, symbiosis

Editor Joerg Graf, University of Connecticut
Copyright © 2021 Smith et al. This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International license.
Address correspondence to Thomas E. Smith, smit4227@gmail.com.
Received 7 September 2021
Accepted 19 October 2021
Published 21 December 2021
The near-universal ability of enzymes to perform promiscuous reactions is increasingly recognized as a starting point in the evolution of novel functions (1). Promiscuous activities become functional when, in the context of a new environment and/or mutation(s), they contribute to fitness, often via complementation of metabolic inadequacies (2–4). Host-associated bacteria that have experienced extreme genome reduction often lack essential genes or whole pathways (5), such that multifunctional enzymes derived from ancestrally promiscuous enzymes have been suggested as a likely means of compensation (6, 7). This idea is supported by examples in multiple bacterial lineages, including the mammalian pathogen Chlamydia (8, 9) and insect-associated Wolbachia (10) and Buchnera aphidicola (11) symbionts.

Alternatively, host genomes may acquire genes via horizontal-gene transfer (HGT) to supplement symbiont shortcomings. While HGT is relatively rare in eukaryotes, it occurs most often from host-associated bacteria (12) and has proven instrumental in eukaryotic evolution (13–16), most notably in the context of mitochondrial and plastid evolution (17, 18). Among insect symbioses, horizontally transferred genes (HTGs) appear to provide hosts with novel functions and, in some cases, may improve their ability to regulate or benefit from symbionts (12, 19). Recently, the compensatory nature of several mealybug HTGs has been revealed—the insect genome encodes enzymes of the peptidoglycan (PGN) synthesis pathway that symbionts lack (20), and these proteins localize within symbionts and participate in cell wall construction (21).

Aphids require Buchnera symbionts to provide essential amino acids that are missing from their exclusive diet of phloem sap. Interestingly, the pea aphid (Acyrthosiphon pisum) genome contains eight HTGs with putative functions in PGN metabolism (Fig. 1) (22, 23). Seven of these aphid HTGs appear important for symbiosis based on their increased expression in bacteriocytes (23), the specialized host cells where Buchnera reside, relative to other host tissues. In addition, RNAi knockdown of HTG expression reduces Buchnera abundance (24), and HTG expression is correlated with aphid genotypes displaying high symbiont abundances (25). Considering the close coordination of PGN metabolism with cell growth and division machinery in bacteria (26), these observations suggest that host PGN enzymes may play a role in regulating Buchnera proliferation.

Of the seven aphid HTGs implicated in symbiosis, all but one putatively function in cell wall remodeling. This gene, ldcA, encodes a homolog of the L,D-carboxypeptidase (ApLdcA) involved in PGN recycling (27), a cytoplasmic process that is absent in Buchnera but present in free-living bacteria like Escherichia coli, a close relative of Buchnera (Fig. 1) (28). While E. coli LdcA (EcLdcA) is known to utilize only solubilized PGN fragments (muropeptides), LdcA homologs from some intracellular pathogens are exported to the periplasm and display a shift in substrate tolerance, modifying the polymeric cell wall in addition to soluble muropeptides (29, 30). Furthermore, LdcA homologs exhibit multifunctionality, demonstrating endopeptidase activities in addition to their carboxypeptidase function (29–31). We hypothesized that EcLdcA might display a similar shift in activity that could enable aphids to control or support its symbionts. Specifically, endopeptidases are essential for E. coli because they are required to make space for the insertion of nascent PGN strands into the cell wall (32)—an endopeptidase may be required by Buchnera but encoded by the host. If ApLdcA is an endopeptidase, this novel function may derive from a promiscuous enzyme activity present in the ancestral enzyme.

In the present work, we investigated the hypothesis that ApLdcA displays key differences from a free-living bacterial homolog, EcLdcA. First, we provide evidence that PGN hydrolases, including an endopeptidase, are active in the aphid-Buchnera system, producing muropeptides that can be isolated from the aphid hemolymph indicative of their physiological relevance. Second, we demonstrate that ApLdcA retains its L,D-carboxypeptidase function toward soluble muropeptides and also exhibits L,D-endopeptidase activity against both stem pentapeptides and cross-linked peptidoglycan. This feature likely derives from ancestral enzyme promiscuity, since the closely related EcLdcA is also capable, albeit to a lesser extent, of L,D-endopeptidase activity. Taken
together, these results reveal potential host adaptations that have capitalized on the catalytic and substrate promiscuity of LdcA in order to target symbiont PGN.

RESULTS
Complex PGN can be isolated from whole aphids. We first sought to understand the role that host HTGs may play in Buchnera PGN metabolism by characterizing Buchnera’s cell wall. Cell wall PGN is comprised of repeating units of β-1,4-linked N-acetylgalactosamine (GlcNAc), N-acetylmuramic acid (MurNAc), L-alanine, γ-D-glutamate, meso-diaminopimelic acid (Dap), and D-alanine. Cell wall digestion with muramidase produces muropeptides, many of which are suitable substrates for known LdcA enzyme activities, while AmiD and RlpA act on the polymeric cell wall. Enzyme reactions appear above reaction arrows, while enzyme names are shown below. Enzymes shown in green indicate those for which aphid-encoded homologs exist. Organism abbreviations, shown in parentheses, denote the species for which the reaction has been demonstrated for the enzyme homolog: Eco, E. coli (27, 67); Nar, Novosphingobium aromaticivorans (31); Ftu, Franciscella tularensis (29); Ngo, Neisseria gonorrhoeae (30); and Pae, Pseudomonas aeruginosa (68). Organism abbreviations shown in orange designate enzymes capable of utilizing both muropeptides and cell wall as substrates.
homogenate to high-performance liquid chromatography (HPLC) and screened fractions for muropeptides using nano ultrahigh performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) and a novel proteomics-based approach for the automated identification of muropeptides (37).

To compare *Buchnera* PGN with that of its closest free-living relative, we also analyzed *E. coli* PGN derived from digestion of the isolated sacculus with the muramidase mutanolysin. The *E. coli* cell wall exhibits a range of chemical modifications, including GlcNAc-anhydro-MurNAc (G-aM) disaccharides at the termini of PGN strands, stem peptide cross-linkages that give the cell wall its mesh-like architecture, and a low level of substitution of stem peptide D-Ala residues for noncanonical L- or D-amino acids (NCLAAs and NCDAAs, respectively). We detected a similarly complex assortment of muropeptides from aphids with both GlcNAc-MurNAc (GM) and G-aM glycans, variable stem peptide lengths and sequences, and diverse cross-linked compounds, including those derived from three strands of peptidoglycan (Fig. 2; see also Fig. S1A and B in the supplemental material). We analyzed fractions from two distinct homogenate treatments—both were sonicated to lyse *Buchnera* cells, while one was additionally treated with hen egg-white lysozyme (HEWL) to digest the cell wall. Untreated and lysozyme-treated samples had largely similar PGN profiles and sonication alone is insufficient to shear glycan chains into small units (38), suggesting that soluble muropeptides are produced in aphids in the absence of exogenously added lysozyme.

The composition of muropeptides in aphid homogenate implicates several host and/or symbiont PGN enzymes in their origin. Muropeptides containing terminal G-aM (including G-aM itself) are products of lytic transglycosylases such as AprRP (Fig. 1) or *Buchnera*’s MltA and MltE enzymes, nonhydrolytic enzymes that fragment PGN chains (35, 39). On the other hand, GM-substituted muropeptides could be produced by hydrolytic muramidases, like the two endogenous invertebrate (i-type) lysozymes (NCBI gene IDs 100160909 and 100168424) that are more highly expressed in bacteriocytes relative to other host tissues (40). We detected muropeptides containing tripeptide and tetrapeptide stems, which are likely derived from carboxypeptidase and/or endopeptidase activities (Fig. 1). Since the *Buchnera* genome encodes no recognizable

FIG 2 Composition of *Buchnera*-derived cell wall fragments purified from *A. pisum*. Aphid homogenate was successively filtered to components of ≤10 kDa and subjected to LC-MS analysis. Muropeptide compounds were identified from MS/MS spectra and extracted-ion chromatogram (XIC) peak areas determined using Byonic and Byologic softwares, respectively (Protein Metrics). Areas were baseline subtracted and normalized by sample, such that the data shown is the percentage of total PGN represented by each compound. Untreated mutanolysin-derived *E. coli* muropeptides are shown for comparison (purple). Two treatments were used without replicates: aphid homogenate was sonicated to lyse *Buchnera* cells (light gray) or additionally treated with lysozyme to digest *Buchnera* cell walls (dark gray). The table describes the compound structure: PGN compounds vary by stem peptide sequence and glycan (GM = GlcNAc-MurNAc, GaM = GlcNAc-anhydro-MurNAc). For distinct compounds that are equivalent in mass (differing either in stem peptide sequence or cross-linkage type), we were unable to quantify each compound abundance independently, because the two compounds could not be chromatographically resolved—such structural isomers were integrated together, and their sequences are reported with variable residues shown separated by backslashes within parentheses, such that the same relative position within parentheses refers to the sequence of one isomer.

![Composition of Buchnera-derived cell wall fragments purified from A. pisum. Aphid homogenate was successively filtered to components of ≤10 kDa and subjected to LC-MS analysis. Muropeptide compounds were identified from MS/MS spectra and extracted-ion chromatogram (XIC) peak areas determined using Byonic and Byologic softwares, respectively (Protein Metrics). Areas were baseline subtracted and normalized by sample, such that the data shown is the percentage of total PGN represented by each compound. Untreated mutanolysin-derived E. coli muropeptides are shown for comparison (purple). Two treatments were used without replicates: aphid homogenate was sonicated to lyse Buchnera cells (light gray) or additionally treated with lysozyme to digest Buchnera cell walls (dark gray). The table describes the compound structure: PGN compounds vary by stem peptide cross-linkages that give the cell wall its mesh-like architecture, and a low level of substitution of stem peptide D-Ala residues for noncanonical L- or D-amino acids (NCLAAs and NCDAAs, respectively). We detected a similarly complex assortment of muropeptides from aphids with both GlcNAc-MurNAc (GM) and G-aM glycans, variable stem peptide lengths and sequences, and diverse cross-linked compounds, including those derived from three strands of peptidoglycan (Fig. 2; see also Fig. S1A and B in the supplemental material). We analyzed fractions from two distinct homogenate treatments—both were sonicated to lyse Buchnera cells, while one was additionally treated with hen egg-white lysozyme (HEWL) to digest the cell wall. Untreated and lysozyme-treated samples had largely similar PGN profiles and sonication alone is insufficient to shear glycan chains into small units (38), suggesting that soluble muropeptides are produced in aphids in the absence of exogenously added lysozyme.](mbio.asm.org/content/12/6/e02636-21/f2)
L-D-carboxypeptidases or endopeptidases and LdcA homologs from intracellular pathogens collectively exhibit both of these functions (29, 30), it is possible that ApLdcA could be responsible for producing these stem peptides in Buchnera.

Aphid PGN also includes cross-linked PGN compounds containing 4,3- and 3,3-cross-linkages (Fig. 1). The 4,3-cross-linkages, which predominate in E. coli, are likely formed by the D,D-transpeptidases PBP1B and PBP3 encoded by Buchnera during cell wall synthesis, but 3,3-cross-linkages typically require the L,D-transpeptidases YnhG and YcbB (41), which Buchnera spp. lack. Because some cross-linked muropeptides are equivalent in mass and yet display distinct cross-linkage types (i.e., 4,3-cross-linked tetra-tripeptide and 3,3-cross-linked tri-tetrapeptide), these could not be definitively distinguished by their MS/MS fragmentation patterns, as described by Bern et al. (37). However, the presence of tri-tripeptide cross-linked stems, which contain only 3,3-cross-linkages, indicates that both 4,3- and 3,3-cross-linkage types are represented among PGNs derived from aphid homogenate (Fig. 2). In addition, while E. coli PGN is devoid of cross-linked stem peptides lacking any glycan substituents, we observed glycancanless cross-linked stem peptides among muropeptides from aphid homogenate that are likely products of ApAmiD or Buchnera’s AmiB amidase (Fig. 2).

Stem peptides may include noncanonical residues in place of one or both terminal D-Ala residues. NCDAAs are introduced to stem peptides either during lipid II synthesis via racemase enzymes or during cell wall synthesis via L,D-transpeptidases (42) and play a role in regulating PGN composition (42–44). NCLAAs found within stem peptides derive from covalent attachment of outer membrane proteins, such as murein lipoprotein (Lpp) in E. coli (45) and can be detected following proteolytic digest (46). Though our approach is incapable of discerning amino-acid stereochemistry, we observed that 33.1% of all E. coli detected muropeptides contain atypical amino acids, which are present in both non-cross-linked (see Fig. S1A) or cross-linked stem peptides (see Fig. S1B and Table S1). Some noncanonical stem peptides reach proportions similar to that of canonical tri- or pentapeptides. Among these, AE-mDap-K and AE-mDap-KR stem peptides are derived from Lpp. In contrast, only 10.7% of aphid muropeptides contain noncanonical amino acids, and a much lower diversity of stem sequences is represented. Despite this, we detected AE-mDap-K and AE-mDap-KR stems in aphids (see Fig. S1A and B). This observation is unexpected for two reasons: (i) the Buchnera genome lacks the gene encoding Lpp, as well as any homologs of the three L,D-transpeptidases, in E. coli that cross-links Lpp to PGN (ldtA to ldtC) (41), and (ii) unlike the E. coli sacculus, aphid PGN samples were not treated with proteases. Most Gram-negative bacteria lack Lpp homologs—in these species, the cell wall is covalently linked to different outer membrane proteins (46), suggesting the same may be true for Buchnera. While the source of AE-mDap-KR muropeptides in aphid homogenate is unclear, these molecules are abundant and are likely part of a specific and significant process in Buchnera.

Soluble muropeptides purified from aphid homogenate are derived from the Buchnera cell wall and reflect the collective enzymatic activities of both (i) cell wall remodeling and (ii) any downstream processing of soluble remodeling products. Our results show that endopeptidase and L,D-carboxypeptidase activities are involved in one or both of these processes. Furthermore, we found that Buchnera PGN is essentially as complex as that of E. coli, notwithstanding the limited set of PGN enzymes. This level of complexity also suggests that Buchnera muropeptides are not exhaustively degraded by symbiont or host PGN enzymes, indicating that these enzymes may play a more specific role in sculpting the Buchnera cell wall architecture.

Characterization of LdcA activities using a multisubstrate, automated assay. Next, we investigated the reactions of ApLdcA and EcLdcA in vitro. We expressed the ldcA genes in E. coli and purified recombinant ApLdcA and EcLdcA proteins by immobilized metal-affinity chromatography (IMAC) (see Fig. S1C and D). We then treated muropeptides derived from mutanolysin digestion of E. coli cell walls with the recombinant proteins (Fig. 3A). These compounds are similar to the substrates normally encountered by EcLdcA in the cytoplasm, consisting of at most a single glycan substituent per stem peptide. In
addition, the complexity of *E. coli* PGN in terms of the abundance and diversity of cell wall modifications allows for simultaneous evaluation of a wide range of potential enzyme substrates. Treated muropeptides were reduced with sodium borohydride and desalted by HPLC before applying the same proteomics-based approach used above to identify individual muropeptide compounds and evaluate LdcA activity toward each potential substrate (Fig. 3B). The same quantity of an identical preparation of *E. coli* saccus was used for each reaction, such that, for a given compound, relative differences in abundance between treatments are essentially quantitative (see Table S1).

Relative to untreated *E. coli* muropeptides, we observed reduced tetrapeptide abundance for both *EcLdcA* and *ApLdcA* with concurrent increases in the amount of tripeptides, demonstrating that the L,D-carboxypeptidase activity of these enzymes can be readily detected by our approach as a decrease in substrate and an accumulation of product (Fig. 3B; see also Table S1). Interestingly, we observed a decrease in pentapeptide abundance for *ApLdcA* and an increase for *EcLdcA*. The former suggests that *ApLdcA* acts as an L,D-endopeptidase that converts pentapeptides directly to tripeptides, an activity previously reported for LdcA homologs from *Novosphingobium aromaticivorans* and *Francisella tularensis* (29, 31). An explanation for the latter observation is described in the following section. We also detected a lower abundance of several noncanonical tetrapeptide monomers for both enzymes (see Fig. S1E). When these are taken into account, *ApLdcA* generally shows less turnover of non-cross-linked muropeptides than does *EcLdcA*, likely indicating a reduced preference for these substrates relative to *EcLdcA*.

Unlike the cell wall fragments that *EcLdcA* might encounter during PGN recycling, mutanolsin-derived muropeptides contain cross-linked stem peptides that would normally be hydrolyzed by endopeptidases prior to being imported into the cytoplasm. Though *EcLdcA* is not known to hydrolyze cross-linkages, we observed a decrease in the abundance of nearly all cross-linked compounds for both *EcLdcA* and *ApLdcA*-treated...
muropeptides, including both 4,3- and 3,3-cross-linked stem peptides (Fig. 3B). Among cross-linked compounds with two glycan substituents (one on each stem peptide), we observed greater turnover of substrates containing G-aM over GM glycans (2 G-aM and 1 GM). Cross-linked compounds containing only one glycan (on either stem peptide), likely resulting from partial amidase activity, are generally less affected by either LdcA enzyme than di-substituted compounds, though G-aM-containing muropeptides are still preferred over those with GM (Fig. 3B). We found the same trends among some noncanonical cross-linked stem peptides (see Fig. S1G). In general, ApLdcA treatment decreased the abundance of cross-linked muropeptides to a greater extent than EcLdcA (Fig. 3B; see also Fig. S1F and G and Table S1).

Collectively, these results demonstrate that ApLdcA can act as an L,D-endopeptidase on pentapeptide substrates and that both EcLdcA and ApLdcA are capable of hydrolyzing PGN cross-linkages, an activity previously not reported for EcLdcA (47).

| Glycan | Tetrapeptide (Ala-Glu-mDap=Ala) | Pentapeptide (Ala-Glu-mDap=Ala-Ala) | Tetra-tripeptide (4,3) (Ala-Glu-mDap-Ala=mDap-Glu-Ala) |
|--------|-----------------------------|----------------------------------|--------------------------------|
| mM     | ApLdcA 100 | EcLdcA 100 | ApLdcA 75 | EcLdcA 38 | ApLdcA 8/35 | EcLdcA 7/22 |
| aM     | ApLdcA 100 | EcLdcA 100 | ApLdcA 84 | EcLdcA 41 | ApLdcA 41/80 | EcLdcA 18/26 |
| G-mM   | ApLdcA 41/80 | EcLdcA 18/56 | ApLdcA *0/12 | EcLdcA *0/3 | ApLdcA 18/66 | EcLdcA 13/29 |
| G-aM   | ApLdcA 43/89 | EcLdcA 8/25 | ApLdcA *0/5 | EcLdcA *0/0 | ApLdcA NA | EcLdcA NA |
| GMG-mM | ApLdcA 37/58 | EcLdcA 14/51 | ApLdcA *0/12 | EcLdcA *0/3 | ApLdcA NA | EcLdcA NA |
| GMG-aM | ApLdcA 100 | EcLdcA 100 | ApLdcA NA | EcLdcA NA | ApLdcA NA | EcLdcA NA |

*Product formation was determined after 2 h (single value) or at 2 and 24 h (values separated by “/”, respectively). Values preceded by an asterisk (*) represent the percentage of N-acetyl muramyl-L-Ala amidase product observed. For glycans, mM indicates that the MurNAc C-1 hydroxyl is replaced by β-OCH₃, and aM indicates 1,6-anhydro-MurNAc. NA indicates where substrates were not available.

We next sought to validate the results of our proteomic analysis of LdcA activity and to characterize the transformations for each LdcA enzyme with authentic synthetic muropeptide samples. The activities of the LdcA enzymes were assayed with each of 11 authentic PGN substrates produced by multistep chemical syntheses developed previously in our laboratory (Table 1; see also Fig. S2) (48, 49). Three different types of peptide were used to assess each activity observed above for LdcA—tetrapeptide for the L,D-carboxypeptidase activity, pentapeptide for the L,D-endopeptidase activity, and 4,3-cross-linked tetra-tripeptide for cross-linkage endopeptidase activity (Fig. 1). Substrate glycans also varied in length and composition (Table 1). Reactions were monitored by UPLC-MS, with products identified by an analysis of retention times, high-resolution mass measurements, and MS/MS spectra (Fig. 4 and 5; see also Fig. S3 to S6). Comparisons of reaction products to authentic synthetic standards were made whenever possible (Fig. 4 and 5; see also Fig. S3 to S6), and negative controls were included for most synthetic endopeptidase substrates (see Fig. S7 at https://figshare.com/articles/figure/Figure_S7_Negative_controls/16823347). In this section, we refer to specific substrates and products with a lowercase “s” and “p,” respectively, preceding a number referring to the structures shown in Fig. S2 to S6.

All four tetrapeptide substrates, including three non-cross-linked muropeptides (s1, s3, and s5) and both stem peptides of a glycan-linked dimer (s8), were hydrolyzed completely to the corresponding tripeptide products by both EcLdcA and ApLdcA (Table 1, Fig. 4A to L; see also Fig. S3 to S5). Pentapeptide substrates (s2, s4, s6, s7, and s9), though not as rapidly consumed as tetrapeptide substrates, were also converted to tripeptide products by both EcLdcA and ApLdcA, with ApLdcA demonstrating higher turnover ability than EcLdcA with each substrate (Table 1 and Fig. 4M to X; see also...
FIG 4  Single-substrate assays demonstrating the L,D-carboxypeptidase and L,D-endopeptidase activities of LdcA enzymes on stem tetrapeptides (A to L) and pentapeptides (M to X), respectively. LC-MS traces are shown for each reaction. Chemical structures of substrates (preceded by an "s") are shown in Fig. S2. MS data are shown in Fig. S3 to S5.
Fig. S3 to S5). This disparity appears even more pronounced with disaccharide-pentapeptides (s6 and s7; Table 1; see also Fig. 4S to U) and glycan-linked pentapeptide dimers (s9; Table 1 and Fig. 4V to X; see also Fig. S5) than with monosaccharide pentapeptides (s2 and s4; Table 1 and Fig. 4M to R; see also Fig. S3). No products were

**FIG 5** Single-substrate assays demonstrating the L,D-endopeptidase activity of LdcA enzymes on cross-linked muropeptides. (A) Two hydrolyzable bonds in s11 indicated with black and gray arrows correspond to routes “a” and “b,” respectively. (B to G) LC-MS traces of LdcA reactions with s11. (H to J) Mass spectra of s11 reaction products p2, p13, and p14. (K and L) Collision-induced dissociation (CID) MS/MS spectra of p13 and p13’ confirm hydrolysis of s11 by route “a.” The pink bar indicates the loss of Ala from the N terminus (71 Da), while the blue bar indicates the loss of Ala from the C terminus (89 Da). (M to Q) Reactions of LdcA with s12 (M) and the resulting substrate and product LC-MS traces (N to Q). (R to U) CID MS/MS spectra of detected route “a” products (p16 and p17) and potential route “b” products (p16’ and p17’). Further LC-MS/MS data are shown in Fig. S6.
detected in 24-h control reactions of pentapeptide substrates with either heat-inactivated enzymes or bovine serum albumin (BSA; see Fig. S7 at https://figshare.com/articles/figure/Figure_S7_Negative_controls/16823347), indicating that noncatalytic degradation does not occur under the conditions employed. It is possible, however, that trace amounts of unidentified peptidoglycan-degrading enzymes were copurified with the recombinant LdcA enzymes, such that products accumulated only after long incubation times. Tandem MS fragmentation patterns of tetra- and pentapeptide substrates and products are shown in Fig. S5.

We confirmed that LdcA exhibits cross-linkage endopeptidase activity and found that LdcA carries out a reaction that is not performed by any known endopeptidase. For 4,3-cross-linked tetra-tripeptide substrates (s10 and s11), there are two potential hydrolysable bonds (Fig. 5; see also Fig. S6), both of which yield tripeptide and tetrapeptide products. While the tripeptide product from either reaction is identical, the tetrapeptide products, though equivalent in mass, differ by the position of D-Ala on either the Dap side chain (Fig. 5A; see also Fig. S6, route “a”) or the main chain (Fig. 5A; see also Fig. S6, route “b”). The two products are readily differentiated from their MS/MS fragmentation patterns, revealing that both EcLdcA and ApLdcA proceed through route “a” (Fig. 5K and L; see also Fig. S6), reinforcing the specificity of LdcA for cleavage of L,D-amide bonds. This specificity distinguishes LdcA endopeptidase activity from that of the D,L-endopeptidase activity of *Pseudomonas aeruginosa* penicillin-binding protein 4, which we previously showed proceeds over the same cross-linked substrate by route “b” (49). ApLdcA was more active than EcLdcA toward both cross-linked substrates (s10 and s11; Table 1 and Fig. 5B to G; see also Fig. S6), suggesting some differences in glycan preference. No substrate degradation or product formation was observed when 4,3-cross-linked substrates were treated with heat-inactivated enzymes or BSA (see Fig. S7 at https://figshare.com/articles/figure/Figure_S7_Negative_controls/16823347).

In our automated analysis, we observed a decreased abundance of 3,3-cross-linked tripeptide compounds following LdcA treatment (Fig. 3B), but did not have synthetic 3,3-cross-linked substrates readily available to confirm this reaction. To address this remaining question, we purified a mixture of cross-linked substrates from mutanolysin-derived *E. coli* muropeptides (s12), containing 4,3-cross-linked tetra-tripeptide (s12a), 3,3-cross-linked tri-tetrapeptide (s12b), and 4,3-cross-linked tetra-tetrapeptide (s12c) in a ratio of ~1:1:5, respectively. Figure 5M illustrates the reaction of LdcA with these three substrates. Reaction mixtures showed complete turnover of the 3,3-cross-linked s12b and partial turnover of 4,3-cross-linked compounds s12a and s12c, demonstrating that both ApLdcA and EcLdcA are more active against 3,3-cross-linkages than 4,3-cross-linkages (Fig. 5N to Q; see also Fig. S6). MepK is the only other enzyme known to display L,D-endopeptidase activity toward 3,3-cross-linkages (50). MepK is also capable of cleaving 4,3-cross-linkages, but via D,D-endopeptidase activity (Fig. 5M, route “b”) (50). Reaction of the 4,3-cross-linked substrates with LdcA was evident by the accumulation of route “a”-type tetrapeptide (p16) and pentapeptide (p17) products (Fig. 5N to Q). We confirmed that p16 and p17 are not the mass-equivalent route “b” products (p16’ and p17’) by comparison of their MS/MS fragmentation patterns (Fig. 5R to U). While p16 represents a reaction end product, p17 can be further converted to p16 by the L,D-carboxypeptidase activity of LdcA. Reactions with ApLdcA showed accumulation of p16 only, while EcLdcA treatment produced more p17 than p16 (Fig. 5N to Q; see also Fig. S6), suggesting that the conversion of p17 to p16 proceeds more slowly for EcLdcA than ApLdcA. This result may explain why the proportion of stem pentapeptides in mutanolysin-derived *E. coli* PGN decreased after treatment with ApLdcA but increased for EcLdcA (Fig. 3B).

Taken together, these results confirm the majority of our conclusions from the proteomic analysis of LdcA treatment on *E. coli* PGN (Fig. 3), demonstrating that: (i) EcLdcA and ApLdcA act as L,D-carboxypeptidases with un-cross-linked stem tetrapeptides and as L,D-endopeptidases with both 4,3- and 3,3-stem peptide cross-linkages, and (ii) ApLdcA exhibits increased turnover of endopeptidase substrates relative to EcLdcA (Table 1). The LdcA homolog from *N. gonorrhoeae* is also capable of hydrolyzing 3,3-
cross-linkages in vitro; although the exact bond cleavage was not determined in that case, this finding suggests that the endopeptidase activity of this enzyme exhibits the same specificity for L,D-amide bonds that we observed for EcLdcA and ApLdcA (30). In addition, whereas our automated analysis successfully identified ApLdcA as an L,D-endopeptidase with regard to un-cross-linked stem pentapeptides, single substrate analysis revealed that EcLdcA, too, exhibits this activity (Table 1).

**DISCUSSION**

Despite its role in PGN recycling, a cytoplasmic process involving soluble muropeptides, there is growing evidence that LdcA is an efficient starting point for the evolution of novel enzyme activities that modify the cell wall (29, 30). In aphids, the use of LdcA in their symbiotic association with Buchnera implies a derived ability of ApLdcA to target symbiont cell walls. We found by biochemical characterization that both EcLdcA and ApLdcA exhibit L,D-carboxypeptidase and L,D-endopeptidase activities. The endopeptidase activities of EcLdcA might be considered promiscuous, since the cytoplasmic EcLdcA does not encounter cross-linked stem peptides in nature. In contrast, ApLdcA is likely to encounter cross-linked Buchnera PGN because the enzyme is produced by the host, outside of symbiont cells. Whether ApLdcA is transported into the Buchnera periplasm, where it directly remolds the cell wall, or is present in the bacteriocyte cytoplasm and acts on soluble muropeptides released during Buchnera cell wall remodeling is unknown. There is evidence of host protein transport to Buchnera for at least one host HTG, RlpA4 (51), although this enzyme contains a eukaryotic signal peptide that is absent from ApLdcA (23). Because the endopeptidase activities of EcLdcA are shared by ApLdcA but are likely physiologically relevant in aphids, our results suggest an evolutionary link between the inherent enzyme promiscuity of EcLdcA and the higher turnover of endopeptidase substrates by some extant LdcA enzymes, including ApLdcA.

Catalytically promiscuous enzyme reactions vary greatly in magnitude among related proteins, sometimes approaching a level of efficiency similar to that of their primary functions (52). In addition, many models of protein evolution emphasize enzymatic tradeoffs between promiscuous and primary activities (1). Nonetheless, ApLdcA and some other multifunctional LdcA enzymes retain a high level of L,D-carboxypeptidase activity (Fig. 3) (29–31). Thus, enzyme function is not easily distinguished from promiscuity without a demonstrable role in biology. We provide evidence that each catalytic activity of ApLdcA plays a role in the aphid-Buchnera symbiosis, implying selection for multifunctionality. Some of the muropeptides identified from aphid homogenate are likely the products of Buchnera cell wall digestion by ApLdcA, implying its functionality in vivo (Fig. 2). Specifically, tripeptide monomers can only be formed from tetrapeptides or by the cleavage of stem peptide cross-linkages via L,D-carboxypeptidase or endopeptidase activities, respectively, both of which can be catalyzed by ApLdcA and are not encoded elsewhere in the Buchnera or host genomes. Though additional enzyme functions cannot be ruled out for the other PGN-modifying enzymes that are retained in the Buchnera genome, our data indicate that these roles can be fulfilled by the catalytic functions of ApLdcA.

Several LdcA enzymes from bacteria have now been biochemically characterized and, in conjunction with our own results, some patterns emerge that hint at the evolutionary origin of enhanced endopeptidase functions within the LdcA family. The A. pism LdcA gene is thought to have been acquired by an ancient aphid ancestor from Wolbachia-like bacteria (22), which are frequently associated with arthropod hosts as intracellular pathogens or facultative symbionts (53). Given that all other LdcA enzymes known to target cell wall and display endopeptidase activity are derived from intracellular bacteria, we hypothesize that the ldcA gene originally acquired by ancestral aphids already exhibited these abilities and provided an immediate advantage to the insect host, possibly enabling aphids to establish the high degree of control over Buchnera that exists today. An analysis of crosswise promiscuity within the LdcA family
that includes both extant and ancestrally reconstructed enzymes of free-living, intracellular, and aphid origins could be used to test this idea.

Besides ApLdcA, aphids harbor six other HTGs with putative roles in PGN remodeling and symbiosis: amiD, encoding an amidase, and rlpA1 to rlpA5, all encoding lytic transglycosylases (Fig. 1). Together with ApLdcA, they possess each of the necessary enzyme functions required for PGN remodeling in free-living bacteria (54). Based on their high levels of bacteriocyte-specific expression (23), their importance for both aphid and symbiont growth (24), and their correlation with high symbiont abundance among distinct aphid genotypes (25), these genes appear to contribute to Buchnera proliferation. HTGs could be involved in releasing cell wall fragments that mediate host-microbe interactions (55) or in degrading those fragments as a means for hosts to curb their own immune response to indigenous microbiota (56, 57). However, because aphids lack PGRPs (58), it seems unlikely that Buchnera cell wall fragments affect aphid hosts at all. Though it is possible that some other aphid signaling pathway has been coopted for recognition of Gram-negative PGN, we propose an alternative hypothesis in which host control of PGN metabolism enables aphids to regulate symbiont PGN metabolism and regulate their growth and/or cell division.

Multifunctionality is apparently common among individual PGN hydrolase domains (29, 30, 59–61), suggesting that multifunctionality in the other aphid HTGs or in Buchnera’s own PGN remodeling enzymes may exist. For example, Buchnera contains AmiB and the typically nonenzymatic NlpD. In E. coli, NlpD is the designated activator of AmiC, while EnvC, missing in Buchnera, activates AmiA and AmiB (62). In Waddlia chondrophila and Chlamydia pneumoniae, NlpD acts as a bifunctional d,L-carboxypeptidase and d,D-endopeptidase, independent of any amidase (59, 60). In addition, E. coli PBP1B exhibits d,L-carboxypeptidase activity under acidic conditions and in the presence of its activator, LpoB (61)—both of these proteins are encoded by Buchnera. Thus, in addition to ApLdcA, either Buchnera NlpD or PBP1B could contribute to the production of trimmed stem peptides among muropeptides from aphid homogenate (Fig. 2).

In another example of multifunctionality, Buchnera lacks Alr and DadX, each capable of producing d-Ala for lipid II biosynthesis via alanine-racemase activity, but contains GlyA, to which the alanine-racemase activity of C. pneumoniae has been attributed (63). Our results support the idea that aphid PGN hydrolases are involved in Buchnera PGN metabolism, but further interrogation of these pathways is required to understand how host enzymes contribute. To add another layer of complexity, Buchnera symbionts of different aphid species vary greatly in PGN gene repertoire (64, 65), which could translate to substantial differences in cell wall and/or enzyme chemistry depending on their metabolic needs.

In conclusion, aphids encode each of the three enzyme functions required for PGN remodeling. While both EcLdcA and ApLdcA behave as endopeptidases, ApLdcA exhibits enhanced endopeptidase activity toward pentapeptides and cross-linked dimers, revealing adaptations in this enzyme specific to the aphid–Buchnera system. Host acquisition and retention of these HTGs throughout aphid evolution reflect their importance in symbiosis. Potentially, their acquisition by aphid ancestors was instrumental in establishing control over symbionts that has since evolved further in extant aphids.

**MATERIALS AND METHODS**

Purification of PGN. Buchnera muropeptides were purified from homogenates of 7-day-old fourth-instar A. pisum nymphs by successive filtration. The filtrate was subjected to HPLC, and the collected fractions were subjected to proteomic analysis. Whole E. coli DH5α murein sacculus was purified following the methods of Desmarais et al. (66). More details are provided in the supplemental Materials and Methods (see Text S1).

**Protein production and purification.** The A. pisum and E. coli ldcA genes were amplified by PCR using primers in Table S2, then cloned into the pET-28b plasmid. E. coli Rosetta (DE3) was transformed with plasmid and induced with isopropyl β-D-1-thiogalactopyranoside (IPTG). Proteins were purified from cell pellets by (i) lysis with HEWL and high-pressure homogenization, (ii) binding to Ni-NiA IMAC resin, and (iii) elution with imidazole.

**Proteomics-based muropeptide analysis.** E. coli sacculus was digested with mutanolysin (Sigma-Aldrich) and the resulting muropeptides treated with sodium borohydride and desalted using HPLC.
Muropeptides were incubated with LdcA enzyme and then desalted again by HPLC. Treated samples were subjected to nano LC-MS/MS analysis following the methods of Bern et al. Individual PGN compounds were identified and quantified from MS and MS/MS spectra using Byonic and Byologic software, respectively (Protein Metrics). All MS data and associated Byonic and Byologic files are available for download via the mass spectrometry database MassIVE (MSV000087634). Data transformation, statistical comparison, and plotting were accomplished using custom R scripts, available along with raw and transformed data at GitHub (https://github.com/smit4227/ApLdcA_proteomics).

**Single-substrate enzyme assays.** Synthetic PGN substrates (s1 to s11) used in this study were synthesized using previously reported methods (48, 49). The muropeptide mixture s12 was purified from mutanolysin-derived *E. coli* muropeptides by HPLC. Reactions of LdcA enzymes with synthetic PGNs were stopped at different time points (2, 8, and 24 h) and analyzed by UPLC-MS. Reactions of LdcA enzymes with s12 were carried out under the same conditions, except that reaction mixtures were reduced with sodium borohydride after treatment.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**TEXT S1**, DOCX file, 0.1 MB.
**FIG S1**, TIF file, 2.5 MB.
**FIG S2**, TIF file, 0.7 MB.
**FIG S3**, TIF file, 1.2 MB.
**FIG S4**, TIF file, 1.1 MB.
**FIG S5**, TIF file, 1.4 MB.
**FIG S6**, TIF file, 1.3 MB.
**TABLE S1**, DOCX file, 0.1 MB.
**TABLE S2**, DOCX file, 0.1 MB.

**ACKNOWLEDGMENTS**

This study was supported by NIH awards F32GM126706 (to T.E.S.), R35GM131738 (to N.A.M.), and R35GM131685 (to S.M.) and NSF award 1551092 (to N.A.M.). The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

We thank the DNA Sequencing and Proteomics Facilities at the University of Texas at Austin for Sanger sequencing and mass spectrometry services, the lab of Andreas Matouschek for use of lab equipment, the lab of David Taylor for assistance with protein purification by FPLC, and Marshall Bern and James Moore for providing academic licenses for Protein Metrics softwares Byonic and Byologic.

T.E.S., N.A.M., and S.M. conceptualized the research idea. T.E.S., M.L., and S.M. developed the methodology. T.E.S. and M.L. performed all experimental investigations. M.L., D.H., and M.D.P. provided the resources used in this study in the form of synthesis of PGN substrates (M.L. and D.H.) and LC-MS instrumentation and operation (M.D.P.). T.E.S. performed all other formal analyses. N.A.M. and S.M. supervised all research activities. T.E.S. and M.L. prepared figures and tables for data visualization. T.E.S. wrote the original draft of the manuscript with contributions from M.L. and M.D.P., while review and editing was completed by T.E.S., M.L., M.D.P., S.M., and N.A.M.

**REFERENCES**

1. Glasner ME, Truong DP, Morse BC. 2020. How enzyme promiscuity and horizontal gene transfer contribute to metabolic innovation. FEBS J 287:1323–1342. https://doi.org/10.1111/febs.15185.
2. Kim J, Kershner JP, Novikov Y, Shoemaker RK, Copley SD. 2010. Three serumopid pathways in *Escherichia coli* can bypass a block in pyridoxal-5′-phosphate synthesis. Mol Syst Biol 6:436. https://doi.org/10.1038/msb2010.88.
3. Juárez-Vázquez AL, Edirisinghe JN, Verduzzo-Castro EA, Michalska K, Wu C, Noda-García L, Babnigg G, Endres M, Medina-Ruíz S, Santoyo-Flores J, Carrillo-Tripp M, Ton-That H, Joachimiak A, Henry CS, Barona-Gómez F. 2017. Evolution of substrate specificity in a retained enzyme driven by gene loss. Elife 6:e22679. https://doi.org/10.7554/ elife.22679.
4. Pontrelli S, Fricke RC, Teoh ST, Laviña WA, Putri SP, Fitz-Gibbon S, Chung M, Pellegrini M, Fukusaki E, Liao JC. 2018. Metabolic repair through emergence of new pathways in *Escherichia coli*. Nat Chem Biol 14:1005–1009. https://doi.org/10.1038/s41589-018-0149-6.
5. McCutcheon JP, Moran NA. 2011. Extreme genome reduction in symbiotic bacteria. Nat Rev Microbiol 10:13–26. https://doi.org/10.1038/nrmicro2670.
6. Kelkar YD, Ochman H. 2013. Genome reduction promotes increase in protein functional complexity in bacteria. Genetics 193:303–307. https://doi.org/10.1534/genetics.112.145656.
7. Newton MS, Arcus VL, Gerth ML, Patrick WM. 2018. Enzyme evolution: innovation is easy, optimization is complicated. Curr Opin Struct Biol 48:110–116. https://doi.org/10.1016/j.sbi.2017.11.007.
8. Adams NE, Thiaville JJ, Proestos J, Juárez-Vázquez AL, McCoy AJ, Barona-Gómez F, Iwata-Reuyl D, de Crécy-Lagard V, Maurelli AT. 2014. Promiscuous and adaptable enzymes fill “holes” in the tetrahydrofolate pathway in *Chlamydia* species. mBio 5:e01378-14–e01314. https://doi.org/10.1128/mBio.01378-14.
29. Zellner B, Mengin-Lecreulx D, Tully B, Gunning WT, Booth R, Huntley JF. 2018. Peptidoglycan in obli-
ner bacteria and the nexus to antibiotic resistance. Chem Rev 118: 5952–5984. https://doi.org/10.1021/acs.chemrev.8b00292.

30. Lenz JD, Hackett KT, Dillard JP. 2017. A single dual-function enzyme controls the production of inflammatory NODs against peptidoglycan fragments by Neisseria gonorrhoeae. mbio 8:e01464-17. https://doi.org/10.1128/mbio.01464-17.

31. Das D, Hervé M, Elsliger M-A, Kadam RU, Grant JC, Chiu H-J, Knuth MW, Klock HE, Miller MD, Godzik A, Lesaay SA, Deacon AM, Mengin-Lecreulx D, Wilson IA. 2013. Structure and function of a novel \( \omega \)-carboxypeptidase A involved in peptidoglycan recycling. J Bacteriol 195:5355–5366. https://doi.org/10.1128/JB.00900-13.

32. Singh SK, Sašée R, Amrutha RN, Reddy M. 2012. Three redundant murein endopeptidases catalyze an essential cleavage step in peptidoglycan synthesis of Escherichia coli K12. Mol Microbiol 86:1036–1051. https://doi.org/10.1111/j.1365-2958.2012.07470.x.

33. Benedetti SD, Fisher JB, Mobashery S. 2021. Bacterial cell wall: morphology and biochemistry. p 167–204. In Goldman E, Green LH (ed), Practical handbook of microbiology, 4th ed. Taylor & Francis, New York, NY.

34. Johnson JW, Fisher JB, Mobashery S. 2013. Cell wall recycling of the Gram-nega-

35. Adams KL, Palmer JD. 2003. Evolution of mitochondrial gene content: the diversi-

36. Turner RO, Vollmer W, Foster SI. 2014. Different walls for rods and balls: the diversity of peptidoglycan. Mol Microbiol 91:862–874. https://doi.org/10.1111/j.1365-2958.2012.07470.x.

37. Bern M, Beniston R, Mesnage S. 2017. Towards an automated analysis of bacterial peptidoglycan structure. Anal Bioanal Chem 409:551–560. https://doi.org/10.1007/s00216-016-9857-5.

38. Verwer RW, Beauchey EH, Keck W, Stoub AM, Poldermans JEM. 1980. Orien-
ted fragmentation of \( \omega \)-carboxypeptidase on biosynthetically modi-

39. Dik DA, Marous DR, Fisher JF, Mobashery S. 2017. Lytic transglycosylases: concinnity in concision of the bacterial cell wall. Crit Rev Biochem Mol Bio 52:503–542. https://doi.org/10.1080/10409238.2017.1373705.

40. Hansen AK, Moran NA. 2011. Aphid genome expression reveals host–symbiont cooperation in the production of amino acids. Proc Natl Acad Sci U S A 108:2849–2854. https://doi.org/10.1073/pnas.103465108.
52. Khersonsky O, Tawfik DS. 2010. Enzyme promiscuity: a mechanistic and evolutionary perspective. Annu Rev Biochem 79:471–505. https://doi.org/10.1146/annurev.biochem-030409-143718.

53. Werren JH, Baldo L, Clark ME. 2008. Wolbachia: master manipulators of invertebrate biology. Nat Rev Microbiol 6:741–751. https://doi.org/10.1038/nrmicro1969.

54. Vollmer W, Joris B, Charlier P, Foster S. 2008. Bacterial peptidoglycan (murein) hydrolases. FEMS Microbiol Rev 32:259–286. https://doi.org/10.1111/j.1574-6976.2007.00099.x.

55. Irazoki O, Hernandez SB, Cava F. 2019. Peptidoglycan muropeptides: release, perception, and functions as signaling molecules. Front Microbiol 10:500. https://doi.org/10.3389/fmicb.2019.00500.

56. Maire J, Vincent-Monégat C, Balmand S, Vallier A, Hervé M, Masson F, Parisot N, Vigneron A, Anselme C, Perrin J, Orlans J, Rahioui I, Da Silva P, Fauvarque M-O, Mengin-Lecreulx D, Zaidman-Rémy A, Heddi A. 2019. Weevil pgrp-lb prevents endosymbiont TCT dissemination and chronic host systemic immune activation. Proc Natl Acad Sci U S A 116:5623–5632. https://doi.org/10.1073/pnas.1821806116.

57. Troha K, Nagy P, Pivovar A, Lazzaro BP, Hartley PS, Buchon N. 2019. Nephrocytes remove microbiota-derived peptidoglycan from systemic circulation to maintain immune homeostasis. Immunity 51:625–637. https://doi.org/10.1016/j.immuni.2019.08.020.

58. Gerard NM, Altincicek B, Anselme C, Atamian H, Barribau SM, de Vos M, Duncan EJ, Evans JD, Gabaldón T, Ghanim M, Heddi A, Kaloshian I, Latorre A, Moya A, Nakabachi A, Parker BJ, Pérez-Brocal V, Pignatelli M, Rahbé Y, Ramsey JS, Spragg CJ, Tamames J, Tamarit D, Tamborindegy C, Vincent-Monegat C, Vilcinskas A. 2010. Immunity and other defenses in pea aphids, Acyrthosiphon pisum. Genome Biol 11:R21. https://doi.org/10.1186/gb-2010-11-2-r21.

59. Frandi A, Jacquier N, Théraulaz L, Greub G, Viollier PH. 2014. FtsZ-independent septal recruitment and function of cell wall remodelling enzymes in chlamydiyal pathogens. Nat Commun 5:4200. https://doi.org/10.1038/ncomms5200.

60. Klöckner A, Otten C, Deroaux A, Vollmer W, Bühl H, De Benedetti S, Münch D, Josten M, Möllerken K, Sahli H-G, Henrichfreise B. 2014. AmiA is a penicillin target enzyme with dual activity in the intracellular pathogen Chlamydia pneumoniae. Nat Commun 5:4201. https://doi.org/10.1038/ncomms5201.

61. Egan AJF, Biboy J, van’t Veer I, Breukink E, Vollmer W. 2015. Activities and regulation of peptidoglycan synthases. Philos Trans R Soc B 370:20150031. https://doi.org/10.1098/rstb.2015.0031.

62. Uehara T, Parzych KR, Dinh T, Bernhardt TG. 2010. Daughter cell separation is controlled by cytokinin-ring-activated cell wall hydrolysis. EMBO J 29:1412–1422. https://doi.org/10.1038/emboj.2010.36.

63. De Benedetti S, Bühl H, Gaballah A, Klöckner A, Otten C, Schneider T, Sahli H-G, Henrichfreise B. 2014. Characterization of serine hydroxymethyltransferase GlyA as a potential source of α-alanine in Chlamydia pneumoniae. Front Cell Infect Microbiol 4:19. https://doi.org/10.3389/fcimb.2014.00019.

64. Chong RA, Park H, Moran NA. 2019. Genome evolution of the obligate endosymbiont Buchnera aphidicola. Mol Biol Evol 36:1481–1489. https://doi.org/10.1093/molbev/msz082.

65. Atwal S, Chuenklin S, Bonder EM, Flores J, Gillespie JJ, Driscoll TP, Salje J. 2021. Discovery of a diverse set of bacteria that build their cell walls without the canonical peptidoglycan polymerase aPBP. mBio 12:e01342-21. https://doi.org/10.1128/mBio.01342-21.

66. Desmarais SM, Cava F, de Pedro MA, Huang KC. 2014. Isolation and preparation of bacterial cell walls for compositional analysis by ultra performance liquid chromatography. J Vis Exp 2014:e51183. https://doi.org/10.3791/51183.

67. Jorgenson MA, Chen Y, Yahashiri A, Popham DL, Weiss DS. 2014. The bacterial septal ring protein RlpA is a lytic transglycosylase that contributes to rod shape and daughter cell separation in Pseudomonas aeruginosa. Mol Microbiol 93:113–128. https://doi.org/10.1111/mmi.12643.