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Modulation of Peptidoglycan Synthesis by Recycled Cell Wall Tetrapeptides

Graphical Abstract

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In Brief

A critical step in peptidoglycan (PG) recycling is the transformation of PG tetrapeptides into tripeptides. Hernández et al. demonstrate that *Vibrio cholerae* accumulates tetrapeptide PG precursors to downregulate PG synthesis in the stationary phase. Tetrapeptide accumulation relies on the substrate preference of L,D-carboxypeptidases for D-alanine versus NCDAAs-modified substrates.

Highlights

- *Vibrio cholerae* releases peptidoglycan (PG) fragments edited with NCDAAs
- Recycling of NCDAAs-muropeptides induces the accumulation of PG-tetrapeptide precursors
- *V. cholerae* recycled tetrapeptides are substrates for PG synthesis
- Recycled PG-tetrapeptides control cell wall synthesis and crosslinking

Article

Modulation of Peptidoglycan Synthesis by Recycled Cell Wall Tetrapeptides

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SUMMARY

The bacterial cell wall is made of peptidoglycan (PG), a polymer that is essential for the maintenance of cell shape and survival. During growth, bacteria remodel their PG, releasing fragments that are predominantly re-internalized and recycled. Here, we show that *Vibrio cholerae* recycles PG fragments modified with non-canonical D-amino acids (NCDAA), which lead to the accumulation of cytosolic PG tetrapeptides. We demonstrate that the accumulation of recycled tetrapeptides has two regulatory consequences for the cell wall: reduction of D,D-cross-linkage and reduction of PG synthesis. We further demonstrate that L,D-carboxypeptidases from five different species show a preferential activity for substrates containing canonical (D-alanine) versus non-canonical (D-methionine) D-amino acids, suggesting that the accumulation of intracellular tetrapeptides in NCDAA-rich environments is widespread. Collectively, this work reveals a regulatory role of NCDAA linking PG recycling and synthesis to promote optimal cell wall assembly and composition in the stationary phase.

INTRODUCTION

The bacterial cell shape-determining peptidoglycan (PG) cell wall provides resistance to cell turgor pressure and protection from environmental threats (Cava and de Pedro, 2014; Vollmer et al., 2008a). PG is a heteropolymer made up of glycan chains consisting of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc). The GlcNAc-MurNAc polymers are cross-linked via short peptides, forming a cell-size mesh known as the murein sacculus (Vollmer et al., 2008a). The composition of these peptides normally includes a terminal D-alanine (D-Ala); however, this amino acid is replaced in some species by non-canonical D-amino acids (NCDAA) when cells enter the stationary growth phase (Alvarez et al., 2018; Cava et al., 2011; Lam et al., 2009).

During growth, the PG sacculus expands through the coordinated action of degradative and synthetic enzymes (Egan et al., 2015; Vollmer et al., 2008b). In *Escherichia coli*, 50% of the murein sacculus is thought to be cleaved at each generation (van Heijenoort, 2011). Although some of the cleaved PG fragments are released into the extracellular medium (Boudreau et al., 2012; Goodell, 1985; Irazoki et al., 2019), most of them are transported back to the cytoplasm for their reutilization, a process referred to as the PG recycling pathway (Park and Uehara, 2008). Cleavage of the sacculus by endopeptidases (EPs) and lytic transglycosylases (LTs) releases monomeric 1,6-anhydro-muropeptides (Vollmer et al., 2008b) that are specifically im-

ported into the cytoplasm by the AmpG permease (Cheng and Park, 2002; Jacobs et al., 1994) to serve as a substrate for the NagZ β -N-acetylglucosaminidase (Cheng et al., 2000; Vötsch and Templin, 2000) and the AmpD amidase (Höltje et al., 1994; Jacobs et al., 1995; Figure 1A). The resulting products of these two enzymes are further cleaved by the L,D-carboxypeptidase LdcA into tripeptides (Templin et al., 1999). The tripeptide is next transformed into uridine diphosphate-N-acetyl-muramyl-tripeptide (UDP-MurNAc-tripeptide) by the muropeptide ligase Mpl (Mengin-Lecreux et al., 1996), thereby connecting PG *de novo* synthesis and recycling (Figure 1A).

Although PG recycling pathways are broadly conserved, this process of salvaging murein components is surprisingly not essential for bacterial growth (Park and Uehara, 2008), at least under standard laboratory conditions. Only the absence of the cytoplasmic LdcA is lethal when *Escherichia coli* enters into the stationary growth phase; Templin et al. (1999) suggested that the incorporation of atypical tetrapeptide PG precursors into the murein sacculus may result in a lethal cross-linkage defect because these muropeptides can only function as acceptors and not as donors in the cross-linking reaction. However, the incorporation of recycled tetrapeptide precursors into PG has not been demonstrated and the consequences of accumulation of these atypical precursors have not been explored.

Here, we show that during PG turnover *Vibrio cholerae* releases NCDAA-modified anhydro-murotetrapeptides, which

Figure 1. Recycling of Non-canonical Murotetrapeptides Leads to Production of Tetrapeptide PG Precursors

(A) Schematic representation of the PG recycling pathway in *E. coli* (EP, endopeptidases; LT, lytic transglycosylases; PBP, penicillin-binding proteins). (B) Extracellular anhydro-murotetrapeptides containing D-Ala (M4N) or D-Met (M4N^{Met}) at the terminal position detected by targeted MS in exponential (Exp) or stationary (Sta) cultures of indicated strains growing in LB + L-Met. (C) UDP-muramyl-peptides (UDP-P3, UDP-P4^{Met}, and UDP-P5) detected by targeted MS in the cytosolic extract of stationary phase cultures of indicated strains growing in LB + L-Met. ND, not detected.

can be re-incorporated via AmpG to be re-used for PG synthesis. Recycled NCDAA-modified tetrapeptides accumulate in the stationary phase due to the preference of the recycling L,D-carboxypeptidase of *V. cholerae* (LdcV) for canonical (D-Ala) versus non-canonical (D-methionine [D-Met]) substrates, a property that is conserved in other L,D-carboxypeptidases. We further investigated the consequences of recycled tetrapeptides in the PG biosynthesis of *V. cholerae* and found that they regulate PG synthesis at two different levels: (1) reducing PG cross-linkage through their incorporation into the murein and (2) decreasing the concentration of UDP-pentapeptide precursor. Therefore, our study underscores a critical role of L,D-carboxypeptidases in the regulatory network controlling PG homeostasis and suggests that NCDAA accumulation may have evolved to circumvent L,D-carboxypeptidase activity as a means of PG architecture regulation.

RESULTS

V. cholerae Recycling of Non-canonical Muropeptides Leads to the Production of Tetrapeptide Precursors

V. cholerae can synthesize different NCDAA (e.g., D-Met) through the expression of the stationary phase-dependent broad-spectrum racemase BsrV (Cava et al., 2011; Lam et al., 2009). Incorporation of NCDAA into the mature PG (primarily through periplasmic L,D-transpeptidases, Ldts) is instrumental for this bacterium to downregulate cell wall biosynthesis to adapt to growth arrest (Alvarez et al., 2018; Cava et al., 2011). Analysis of the turnover muropeptide pool of *V. cholerae* growing in the presence of L-Met revealed the presence of anhydro-murotetrapeptides with D-Met at the terminal position (M4N^{Met}) (Figure 1B). M4N^{Met} was exclusively detected in the stationary phase and never detected in supernatants from a $\Delta bsrV$ strain (Figures 1B and S1A).

Recycled tetrapeptides are normally converted into tripeptides by cytosolic L,D-carboxypeptidases before they are used for the synthesis of the UDP-MurNAc-pentapeptide (UDP-P5) PG precursor (Uehara and Park, 2008; Figure 1A). However, targeted mass spectrometry (MS) analysis of *V. cholerae* stationary phase cytosolic extracts revealed the presence of

UDP-MurNAc-tetrapeptides with D-Met at the terminal position (UDP-P4^{Met}) (Figures 1C and S1B). UDP-tetrapeptides were not detected in the $\Delta ampG$ cytoplasm, indicating that these molecules come from recycled turnover PG fragments (Figure 1C).

Identification and Characterization of the *V. cholerae* L,D-Carboxypeptidase Involved in Peptidoglycan Recycling

The accumulation of UDP-MurNAc-tetrapeptide (UDP-P4) precursors in *E. coli* is observed after deletion of the cytosolic L,D-carboxypeptidase LdcA (Templin et al., 1999). To investigate the biological role of recycled tetrapeptide accumulation in *V. cholerae*, we searched for the Ldc activity in this bacterium. The homolog of LdcA in *V. cholerae* was proposed to be VCA0337 (Templin et al., 1999). However, this protein has been reported as a microcin immunity protein (Nocek et al., 2012; Tikhonov et al., 2010), and we detected no PG hydrolytic activity for VCA0337 *in vitro* (Figures S2A and S2B), suggesting that Ldc in *V. cholerae* is encoded by a different gene.

Inspired by the growth phase-dependent phenotype of $\Delta ldcA$ (Templin et al., 1999), we screened an arrayed mutant library of *V. cholerae* (Cameron et al., 2008) to identify mutants affected in the stationary phase. We found that inactivation of the *vc2153* locus transformed the normal curved rod shape of the wild type into spheres only during the stationary phase (Figure 2A). The altered morphological phenotype of the $\Delta vc2153$ mutant was accompanied by a marked reduction in PG content and a severe reduction in viability that was particularly aggravated under low osmolarity conditions (salt-free LB medium, LB₀) (Figures 2B and 2C). VC2153 is annotated as a VanY D,D-carboxypeptidase-like protein in the NCBI database. However, using protein domain analysis (Marchler-Bauer et al., 2017), we predicted that *vc2153* encodes a putative L,D-carboxypeptidase similar to the *Streptococcus pneumoniae* LdcB. Bacterial PG carboxypeptidases are hydrolytic enzymes that remove the C-terminal amino acid from muropeptides (Vollmer et al., 2008b). While D,D-carboxypeptidases act on pentapeptides (Wright et al., 1992), L,D-carboxypeptidases use tetrapeptides as substrates (Courtin et al., 2006). VC2153 was purified to test

Figure 2. *vc2153* Encodes an L,D-Carboxypeptidase (LdcV) That Contributes to PG Recycling in *V. cholerae*

(A) Phase contrast microscopy of exponential (Exp) or stationary (Sta) phase LB₁₀ cells imaged on agarose pads. (B and C) PG density (content of PG per cell) normalized to the value of the wild type (WT) (B), growth kinetics of strains growing in LB containing 0 (LB₀) or 10 (LB₁₀) g/L of NaCl (C) (left y axis) and number of colony-forming units (CFU) (normalized to WT weight) counted in LB₁₀ cultures at the indicated times (C) (dashed lines, right y axis). (D) Hydrolytic activity of purified VC2153 protein tested on isolated monomeric mucopeptides (M4, mucopeptide; M3, mucopeptide; M5, mucopeptide; N, anhydro form of the mucopeptide). (E) Quantification by untargeted MS of UDP-muramyl-pentapeptide (UDP-P5) and UDP-muramyl-tetrapeptide (UDP-P4) detected in the cytosol of the indicated strains. Data represent means $\pm$ SDs of triplicates; ND, not detected; * $p < 0.05$, unpaired t test.

its carboxypeptidase activity using different monomeric mucopeptides as potential substrates. Purified LdcA from *E. coli* was used as a positive control in these assays. VC2153 fully converted the disaccharide-tetrapeptide substrates (both M4 and its anhydro-derivative, M4N) to disaccharide-tripeptides (M3 and M3N), cleaving the peptide bond between *m*DAP and the terminal D-Ala. VC2153, like LdcA, did not act on pentapeptides (Figure 2D) or cross-linked mucopeptides (e.g., D44) (Figure S2C), although in contrast to a previous report (Leguina et al., 1994), both enzymes acted on high-molecular-weight PG (Figures S2D–S2F), suggesting a potential role for L,D-carboxypeptidases in the modification of the sacculus. These observations demonstrate that VC2153 (renamed LdcV) is an L,D-carboxypeptidase in *V. cholerae*.

To determine whether LdcV is involved in PG recycling, we compared the pool of cytosolic UDP-activated murein precursors in the $\Delta ldcV$ mutant and the wild-type strain, using untargeted ultra-performance liquid chromatography-MS (UPLC-MS). High levels of UDP-P4 (presenting D-Ala at the terminal position) were detected in the $\Delta ldcV$ mutant, but were undetectable in the wild-type strain (Figures 2E and S3), suggesting that LdcV is involved in the *V. cholerae* PG recycling pathway. Furthermore, heterologous expression of *E. coli* *ldcA* under control of the P_{BAD} promoter complemented the growth defect of $\Delta ldcV$ (Figure S2G), indicating that LdcV is the functional homolog of the LdcA of *E. coli*.

L,D-Carboxypeptidases' Preference for Canonical over Non-canonical Recycled Substrates Leads to Tetrapeptide Precursor Accumulation

We previously found that PG editing by NCDAA such as D-Met leads to the accumulation of pentapeptides in the cell wall of some bacteria, likely because these modified mucopeptides are poor substrates for D,D-carboxypeptidases (Cava et al., 2011). We wondered whether NCDAA-modified mucopeptides may also be suboptimal substrates during PG recycling for Ldcs, explaining the accumulation of non-canonical tetrapeptide precursors (UDP-P4^{Met}) detected in *V. cholerae* wild type (Figure 1C).

To assess whether the accumulation of UDP-P4^{Met} could be due to the preference of LdcV for canonical versus non-canonical tetrapeptides (D-Met modified), purified LdcV was incubated with a mixture of canonical M4N and M4 (D-Ala at the fourth position) and non-canonical M4N^{Met} and M4^{Met} (D-Met at the fourth position) mucopeptides as potential substrates. Analyses of the digestion products revealed that while LdcV can hydrolyze both kinds of tetrapeptides (Figure 3A), it exhibits a preference for canonical over D-Met-modified substrates (Figure 3B and S4).

Furthermore, additional L,D-carboxypeptidase *in vitro* assays using LdcV-like (from *Aeromonas hydrophila* and *Proteus mirabilis*) and LdcA-like (from *E. coli* and *Salmonella enterica*) enzymes exhibited a similar preference for canonical rather than NCDAA-modified mucopeptide substrates (Figure 3C). Thus, the

Figure 3. Peptidoglycan Recycling L,D-Carboxypeptidases Exhibit a Preference for Canonical over D-Met-Modified Murotetrapeptides

(A) UPLC chromatograms showing the substrate (in black) and the resultant products (in blue) of LdcV *in vitro* assays (1 h incubation) (M4, murotetrapeptide; M4^{Met}, murotetrapeptide with D-Met at the fourth position; M4N, anhydro-murotetrapeptide; M4N^{Met}, anhydro-murotetrapeptide with D-Met at the fourth position; M3, murotri-peptide; M3N, anhydro-murotri-peptide). (B and C) Dynamics of substrate utilization during *in vitro* reactions of LdcV (B) and purified L,D-carboxypeptidases from *Aeromonas hydrophila* (Ah), *Proteus mirabilis* (Pm), *Salmonella enterica* serovar Typhimurium (St), and *E. coli* (Ec) (C) (MP4 = M4 + M4N; MP4^{Met} = M4^{Met} + M4N^{Met}; MP3 = M3 + M3N).

production of precursor tetrapeptides during the recycling of NCDAA-edited PG seems to be a conserved mechanism.

Tetrapeptide Precursors Downregulate Peptidoglycan Synthesis

To study the regulatory role of recycled tetrapeptides in *V. cholerae*, we used the $\Delta ldcV$ mutant since this strain has increased accumulation compared to the wild type. We took advantage of the survival defect of this mutant to look for genetic determinants involved in the synthesis and function of recycled PG tetrapeptides. Colonies of the $\Delta ldcV$ strain were visibly distinct from wild type and after 3 days produced suppressors, which remained stable upon re-isolation (Figures 4A and S5A). Analyses of the suppressor colonies confirmed that they alleviated $\Delta ldcV$ phenotypes in cell morphology, PG content per cell, and growth in low osmolarity medium (Figures 4B, S5B, and S5C). The suppressor mutations were identified by whole genome sequencing (Figures S5D and S5E). To perform a more comprehensive analysis of suppressors, we carried out a transposon insertion sequencing (Tn-seq)-based screen in LB₀ (Figures 4C and S6). Collectively, more than one-third of the suppressors corresponded to genes associated with PG recycling and turnover.

Double deletion mutants were constructed to validate and extend our suppressor analysis. Combining $\Delta ldcV$ with individual deletions of PG recycling genes *ampG* (permease), *ampD* (amidase), or *mpl* (muropeptide ligase) yielded complete reversion of the $\Delta ldcV$ phenotypic defects, which correlated with the absence of detectable UDP-P4 (Figures 4D–4F, S7A, and S7B). Therefore, these data suggest that the recycled tetrapeptides downregulate PG synthesis through their conversion to UDP precursors by Mpl.

However, the $\Delta ldcV \Delta nagZ$ mutant accumulated UDP-P4 and incompletely suppressed the growth and PG defects of the $\Delta ldcV$ mutant (Figures 4D–4F, S7A, and S7B). UDP-P4 accumulation in the *nagZ ldcV* mutant is likely explained by the catalytic promiscuity of AmpD (Hesek et al., 2009), which can release the tetrapeptide from both the anhydro-muramyl-peptide and M4N (accumulated in the *nagZ* mutant background) (Figures S7C and S7D), thereby promoting Mpl-dependent formation of UDP-P4. Similarly, deletions in PG hydrolytic enzymes only partially alleviated the $\Delta ldcV$ phenotypes and did not prevent UDP-P4 accumulation (Figures 4D–4F). Therefore, these data suggest that in addition to UDP-P4 depletion, there is a second mechanism to suppress $\Delta ldcV$ lethality.

PG hydrolases are also known as autolysins because their uncontrolled activity upon the cessation of PG synthesis can lead to cell lysis (Cavallari et al., 2013; Scheurwater et al., 2008; Shockman et al., 1996). Therefore, we hypothesized that the inactivation of these enzymes could suppress the viability defect of $\Delta ldcV$ by reducing autolysis. To monitor autolysis, we compared the amount of the PG turnover product M4N in the extracellular medium between the PG hydrolase mutants in a $\Delta ampG$ background (to block PG recycling). There were reduced amounts of extracellular M4N in all PG hydrolase mutants (Figure 4G), suggesting that the downregulation of PG synthesis by recycled tetrapeptides is balanced by PG turnover activities to maintain cell wall integrity.

Recycled Tetrapeptides Can Be Incorporated into Peptidoglycan, Leading to Reduced Cross-Linkage

In agreement with a previous report (Templin et al., 1999), our data suggest that recycled tetrapeptides must be transformed into PG precursors to be active. This is further supported by the fact that most Lipid II, the final precursor for PG synthesis (Typas et al., 2011), is in the tetrapeptide form in the *ldcV* mutant (Figures 5A and S8). These data suggest that recycled tetrapeptides can be incorporated into the PG matrix; however, this has not been yet experimentally verified.

To address this question, we designed a strategy to distinguish between the incorporation of tetrapeptides derived from the *de novo* biosynthetic pathway (ending with D-Ala) and those from the recycling pathway. To this end, the growth medium was supplemented with “D-Met-labeled” anhydro-murotetrapeptide (M4N^{Met}), which could be recycled and detected as a D-Met tetrapeptide in the PG (Figures 5B and S9A). To ensure that the detection of D-Met in the PG exclusively relied on the incorporation of recycled tetrapeptide products, these experiments were carried out in a $\Delta ldcV$ derivative (referred to as $\Delta 4$) that was also deficient in endogenous D-Met production (due to the absence of the BsrV racemase) and unable to incorporate

Figure 4. Phenotypes of the $\Delta ldcV$ Mutant Can Be Suppressed by the Inactivation of Genes Involved in PG Recycling and Turnover

(A and B) Colony (A) and cell (B) morphology of WT, $\Delta ldcV$, and a $\Delta ldcV$ suppressor mutant ($\Delta ldcV_{sup}$) growing in LB₁₀.

(C) List of the suppressor candidate genes annotated to be involved in PG homeostasis obtained from sequencing the $\Delta ldcV$ -LB₀ Tn library.

(D–F) Phenotypes of $\Delta ldcV$ suppressor mutants: (D) amount of PG per cell (normalized to the WT levels) of stationary-LB₁₀ cultures; (E) OD₆₀₀ measured after 10 h of incubation in LB₀; and (F) level of cytosolic UDP-P4 of the indicated mutant strains.

(G) Amount of extracellular soluble M4N (normalized to the M4N detected for the $\Delta ampG$ single mutant) detected by untargeted MS in cultures of indicated lytic transglycosylase or endopeptidase mutants.

Data represent means $\pm$ SDs of 2 (E and G) or 3 (D and F) biological replicates; ND, not detected; *p < 0.05, unpaired t test.

exogenous D-Met by L,D-transpeptidation (Cava et al., 2011) (due to the absence of LdtA and LdtB). M4^{Met} was detected in the PG isolated from the M4N^{Met}-treated $\Delta 4$ strain, but not in a control strain ($\Delta 5$), where recycling was disabled via *ampG* inactivation (Figures 5C and S9B). These observations demonstrate that the recycled tetrapeptides are re-used as substrates for PG synthesis.

Accumulation of tetrapeptide precursors in the $\Delta ldcV$ mutants was coupled with a marked reduction in D,D-cross-links (Figures 5D and S10A), which is consistent with the inability of penicillin-binding proteins (PBPs) to use tetrapeptides as donor substrates in D,D-transpeptidation reactions (Vollmer et al., 2008a). Conversely, since L,D-transpeptidases use tetrapeptides instead of pentapeptides as substrates (Magnet et al., 2008; Mainardi et al., 2005; Figure 5E), we speculated that an increase in L,D-cross-links could compensate for the reduction in D,D-cross-links in the $\Delta ldcV$ mutant. Consistent with this idea, we found that an

increased expression of LdtA (the main L,D-transpeptidase of *V. cholerae*) (Cava et al., 2011) improved the fitness of the $\Delta ldcV$ mutant (Figures 5F and S10B), whereas deletion of *ldtA* in the $\Delta ldcV$ background further attenuated its growth (Figure 5G). These results demonstrate that recycled tetrapeptides control PG cross-linking homeostasis through their incorporation into the murein.

Recycled Tetrapeptides Reduce the Abundance of UDP-MurNAc-Pentapeptide

Besides the reduction in PG cross-linkage, we noticed a striking inverse correlation between the accumulation of tetrapeptide (Lipid II-P4 or UDP-P4) and pentapeptide precursors (Figures 5A and 6A). This result is consistent with the reduced PG content of the *ldcV* mutant and suggests that recycled tetrapeptide precursors negatively regulate the *de novo* PG biosynthesis. We hypothesized that the reduction of UDP-P5 may be the result of the

Figure 5. Incorporation of Recycled UDP-P4 into the Cell Wall Leads to a Reduction in D,D-Cross-linking

(A) Detection and quantification of Lipid-II P4 and P5 delipidated forms isolated from WT and $\Delta ldcV$ cultures. ND, not detected.

(B) Schematic representation of the protocol used to demonstrate the incorporation of recycled $M4N^{Met}$ into peptidoglycan. IM, inner membrane; OM, outer membrane.

(C) Exogenous $M4N^{Met}$ was added to cultures of $\Delta ldtA$ $\Delta ldtB$ $\Delta bsrV$ $\Delta ldcV$ ($\Delta 4$) and $\Delta 4$ $\Delta ampG$ ($\Delta 5$). The tandem MS (MS/MS) profile obtained by targeted MS of PG derived from $\Delta 4$ but not $\Delta 5$ revealed the presence of $M4^{Met}$.

(legend continued on next page)

Figure 6. Tetrapeptide Precursors Negatively Regulate the De Novo PG-Biosynthetic Pathway

(A) Correlation between UDP-P5 and UDP-P4 in the indicated strains. The percentage of UDP-P5 left is calculated relative to the reference background (e.g., $\Delta ampG \Delta ldcV$ relative to $\Delta ampG$).

(B) PG density (content per cell) in LB₁₀ of stationary cultures of the $\Delta ldcV$ strain overexpressing the indicated Mur loci from the pBAD vector. PG values are relative to the strain carrying the empty vector (○).

(C) Competition assay in LB₀ of the $\Delta ldcV$ mutant strains overexpressing the indicated Mur loci versus the $\Delta ldcV$ mutant carrying the pBAD-empty vector.

(D) Volcano plot showing the ratio of read counts mapped to individual genes in the $\Delta ldcV$ mutant LB₁₀ transposon library compared with the control WT LB₁₀ library. Genes represented in red and green are significantly underrepresented.

Data represent means $\pm$ SDs of 2 (B) or 3 (A and C) biological replicates; * $p < 0.05$, unpaired t test.

UDP-muramyl substrate (the shared intermediate, i.e., UDP-MurNAc named as NAM in Figure 1A) being diverted from the *de novo* PG synthesis pathway to UDP-P4 synthesis. Therefore, increasing the flow of the *de novo* pathway (i.e., the levels of UDP-P5) (Lovering et al., 2012) should alleviate the PG defect of $\Delta ldcV$. Overexpression of any of the MurA-F enzymes increased the PG density of the $\Delta ldcV$ mutant (2–4 times) and greatly improved its fitness (>1,000 times in the case of MurC overexpression) in competition experiments (Figures 6B, 6C, and S10C). Interestingly, among the Mur enzymes, expression of MurC had the most potent effect on improving $\Delta ldcV$ mutant fitness. MurC mediates the formation of UDP-muramyl-L-Ala, a substrate committed to UDP-P5, regardless of the presence of tetrapeptides. Therefore, our data suggest that competition between the *de novo* synthesis and recycling

pathways for available UDP-muramyl accounts for the reduction in the amount of the UDP-P5 in the $\Delta ldcV$ mutant.

Also consistent with the hypothesis that UDP-P4 negatively regulates pentapeptide levels, a Tn-seq-based screen for *ldcV* genetic interactions revealed a synthetic lethal/sick phenotype with the genes encoding the dominant high-molecular-weight PBP1A (*mrcA*) and its regulatory partner proteins LpoA (Dörr et al., 2014b) and CsiV (Dörr et al., 2014a) (*vc0581* and *vc1887*, respectively) of *V. cholerae* (Figure 6D). Collectively, these observations buttress the idea that Ldc enzymes play a key role in maintaining a proper ratio between UDP-P5 and UDP-P4 to ensure optimal PG synthesis and composition (i.e., cross-linking).

DISCUSSION

The general biological importance of the PG recycling pathway is not well understood, since its inactivation has little impact on bacterial growth under laboratory conditions (Irazoki et al., 2019; Uehara and Park, 2008). Here, our investigations of the *V. cholerae* recycling pathway revealed that in stationary phase this bacterium produces NCDAA-containing PG tetrapeptide precursors in addition to the pentapeptide substrates that feed PG synthesis in most bacteria. Elucidating the genetic determinants and physiological consequences of this apparent anomaly has been instrumental to uncover that L_D-carboxypeptidases exert a critical role in PG homeostasis, modulating PG synthesis and composition.

By using a *V. cholerae* *ldc* mutant, which accumulates high levels of recycled tetrapeptides, we have demonstrated that these molecules control PG homeostasis by (1) decreasing PG cross-linkage through incorporation of activated UDP-P4 into the murein (Figure 5) and (2) downregulating PG synthesis due to the reduction of the canonical UDP-P5 precursor pool (Figure 6). The low levels of tetrapeptide precursors detected in wild-type stationary phase cells (Figure 1C) suggest that accumulation of this molecule has an actual physiological role, wherein it promotes downregulation of PG synthesis in order to adapt the cell envelope of *V. cholerae* to non-growth conditions in the stationary phase. As different Ldcs show distinct biochemical properties (Figure 3C), we anticipate that the levels of tetrapeptide precursors may vary between species, depending on the capacity of their Ldcs to discriminate between canonical and non-canonical substrates. For example, maximum tetrapeptide accumulation will be reached by those species that, despite possessing PG recycling pathways, do not encode L_D-carboxypeptidase orthologs (Park and Uehara, 2008).

Incorporation of recycled tetrapeptides into the murein sacculus has not been demonstrated previously. Distinguishing tetrapeptides that are generated from the activities of periplasmic PBP transpeptidases/carboxypeptidases on pentapeptide substrates from those derived from flipped Lipid II tetrapeptides

(D) Quantification of D_D-cross-linkage (estimated by calculating the D44:M4 ratio and normalized to the WT value) present in the PG of $\Delta ldcV$ suppressor mutants (y axis) and amount of accumulated UDP-P4 detected for those strains (x axis).

(E) Schematic representation of mureopeptide cross-linking mediated by penicillin-binding proteins (PBPs) and L_D-transpeptidases (LDTs).

(F) Growth kinetics of indicated strains grown in LB₀ supplemented with 0.2% arabinose (pB = pBAD₁₈). Error bars represent SD of 3 biological replicates.

(G) Growth kinetics of indicated strains grown in LB₀. Error bars represent SD of 3 biological replicates.

Figure 7. Schematic Representation of the Generation of PG Tetrapeptide Precursors and Their Role in *V. cholerae* Cell Wall Synthesis

(1) Periplasmic BsrV racemase produces NCDAA (e.g., converts L-Met into D-Met). (2) Periplasmic L,D-transpeptidases (e.g., LdtA) incorporate D-Met into the fourth position of the peptide moiety of the muropeptides. (3) Lytic transglycosylases (LT) produce both canonical (i.e., with D-Ala) and non-canonical (e.g., with D-Met) soluble anhydro-muropeptides. (4) Anhydro-muropeptides can be recovered to the cytoplasm through the AmpG permease. (5) L,D-Carboxypeptidase (LdcV) has a preference for canonical recycled muropeptides, which leads to the accumulation of PG tetrapeptides. (6) Recycled tetrapeptides are converted into PG precursors, which downregulate PG synthesis and are further incorporated into the mature murein, thereby decreasing PG cross-linking. (7) Finally, released NCDAA-containing anhydro-muropeptides may also induce *trans*-regulatory effects in the PG synthesis or composition of bacteria inhabiting the same niche.

was challenging. Nevertheless, provision of exogenous traceable D-Met anhydro-muropeptides derivatives to a $\Delta ldcV$ mutant strain incapable of synthesizing NCDAA-modified muropeptides enabled the detection of recycled tetrapeptides in the PG. The use of non-canonical anhydro-murotetrapeptides (e.g., fluorescent derivatives) could facilitate the screening of multiple species for the absence or presence of PG recycling pathways and also to assess Ldc substrate specificity *in vivo*. Furthermore, our findings suggest that using compounds that resemble NCDAA-modified tetrapeptides could be a means to inhibit PG biogenesis; notably, the D-Ser residue in the β -lactam antibiotic nocardicin A is thought to specifically target Ldc enzymes (Metz et al., 1986).

Lipid-II tetrapeptide substrates can only serve as acceptors in PBP-mediated transpeptidase reactions and thus their incorporation into the sacculus results in reduced PG cross-linkage. Consistent with the incorporation of substrates unsuitable for the high-molecular-weight PBPs, we found that LdtA, an L,D-transpeptidase that uses tetrapeptides as donor substrates (Cava et al., 2011), can compensate for the growth defect of the *ldcV* mutant in low osmolarity (Figure 5). A number of bacterial species lack Ldc orthologs, despite apparently encoding other components of the PG recycling pathway (e.g., *Acinetobacter baumannii*; Park and Uehara, 2008). In organisms lacking Ldc homologs, we hypothesize that L,D-transpeptidases (Ldts) may play more prominent roles in maintaining PG homeostasis. Moreover, our data suggest that the inhibition of Ldc (e.g., with nocardicin A) in combination with inhibitors of Ldts (e.g., imipenem) (Mainardi et al., 2007) and/or PBP1a (e.g., β -lactams) may have synergistic effects and thus could represent a potent drug combination for antimicrobial therapy.

Accumulation of recycled tetrapeptides is largely prevented by cytosolic Ldc enzymes. However, NCDAA-edited tetrapepti-

des (e.g., by D-Met) (Figure 1C) can accumulate since these are less preferred substrates for L,D-carboxypeptidation compared to their canonical counterparts (ending with D-Ala) (Figure 3C). The detection of UDP-tetrapeptide precursors modified with D-Met (UDP-P₄^{Met}) in the stationary phase wild-type cultures of *V. cholerae* (Figure 1) suggest that recycling NCDAA-modified muropeptides fine-tunes PG synthesis activity in this bacterium. Thus, production of NCDAA can modulate cell wall synthesis and cross-linking by at least two mechanisms—(1) through periplasmic PG editing (by Ldts) with free NCDAA (Alvarez et al., 2018; Cava et al., 2011; Lam et al., 2009) and (2) incorporation of recycled NCDAA-modified tetrapeptide precursors—that together coordinate cell wall synthesis with growth arrest (Figure 7). It is also possible that in addition to auto-regulatory roles, production of NCDAA induces regulatory changes in the murein of neighboring organisms. In principle, any bacterium that incorporates NCDAA in the fourth position of the muropeptide (e.g., via the activity of Ldts) is a potential source of NCDAA-modified anhydro-muropeptides that could modulate PG synthesis/composition in bacteria inhabiting the same niche (Figure 7).

Finally, extracellular PG fragments are known to be important signals in innate immunity, organ development, and behavior (Arentsen et al., 2017; Humann et al., 2016; Krueger and Opp, 2016; Mukherjee et al., 2019; Troll et al., 2009). Our observation that bacteria can release PG fragments modified with NCDAA suggests that it will be important to consider whether NCDAA-modified PG fragments convey distinct information in interkingdom signaling compared to fragments with canonical DAA. Moreover, as regards microbial ecology, our findings suggest that the release of extracellular non-canonical muropeptides could mediate interspecies modulation of PG synthesis in *trans* if such peptides become substrates for PG recycling in neighboring organisms.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead Contact
  - Materials Availability
  - Data and Code Availability
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - Bacterial strains
  - Growth conditions
  - Construction of plasmids
- **METHOD DETAILS**
  - Cell and colony morphology imaging
  - Peptidoglycan isolation and analysis
  - Protein overexpression and purification
  - *In vitro* protein reactions
  - Whole genome sequencing
  - Transposon insertion sequencing
  - Analysis of soluble muropeptide pools
  - Lipid II extraction and LC-MS analysis
  - M4^{Met} incorporation into the PG by recycling
  - Competition assays
- **QUANTIFICATION AND STATISTICAL ANALYSIS**

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.celrep.2020.107578>.

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AUTHOR CONTRIBUTIONS

S.B.H. and F.C. designed and performed the research. F.C., S.B.H., T.D., and M.K.W. analyzed the data. F.C., S.B.H., T.D., and M.K.W. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Penta-His antibody	QIAGEN	Cat#34660; RRID:AB_2619735
Anti-Mouse IgG	Sigma	Cat#A9044; RRID:AB_258431
Bacterial and Virus Strains		
All bacterial strains are listed in Table S1	This study	Table S1
Chemicals, Peptides, and Recombinant Proteins		
D-Methionine	Sigma	Cat#M9375
L-Methionine	Merck	Cat#105707
Critical Commercial Assays		
Ni-NTA Agarose	QIAGEN	Cat#30210
Bio-Rad Protein Assay Dye Reagent Concentrate	Bio-Rad	Cat#500-0006
GeneJET Genomic DNA Purification Kit	Thermo Fisher Scientific	Cat#K0721
Qubit® dsDNA HS Assay Kit	Thermo Fisher Scientific	Cat#Q32851
Nextera XT DNA Library Preparation Kit	Illumina	Cat#FC-131-1024
MiSeq Reagent Kit v2 (50-cycles)	Illumina	Cat#MS-102-2001
Deposited Data		
Tn-seq experiments_Raw data	This study	https://www.ncbi.nlm.nih.gov BioProject ID: PRJNA623082
WGS suppressor mutants_Raw data	This study	https://www.ncbi.nlm.nih.gov BioProject ID: PRJNA623074
Oligonucleotides		
All oligonucleotides are detailed in Tables S2–S4	This study	Tables S2–S4
Recombinant DNA		
pET28b::vc2153-6xHis	This study	FC281
pET28b::ldcA-6xHis	This study	FC2025
pET22b::vca0337-6xHis	This study	FC2662
pET28b::slt70-6xHis (<i>E. coli</i>)	Espaillat et al., 2016	FC1860
pET28b::ldtA-6xHis	Cava et al., 2011	FC1168
pET28b::AHA1477-6xHis	This study	FC2772
pET28b::PMI1557-6xHis	This study	FC2773
pET28b::STM1800-6xHis	This study	FC2774
pBAD ₃₃ ::ldcA-6xHis	This study	FC2753
pBAD _{18-Km} ::ldtA-6xHis	This study	FC2759
pBAD _{18-Km} ::murA-6xHis	This study	FC2838
pBAD _{18-Km} ::murB-6xHis	This study	FC2839
pBAD _{18-Km} ::murC-6xHis	This study	FC2840
pBAD _{18-Km} ::murE-6xHis	This study	FC2841
pBAD _{18-Km} ::murF-6xHis	This study	FC2842
Software and Algorithms		
GraphPad Prism	GraphPad Software, Inc.	https://www.graphpad.com
Zeiss Zen Blue Software	Zeiss	https://www.zeiss.com/corporate/int/home.html
ImageJ	https://imagej.nih.gov/ij/	https://imagej.nih.gov/ij/
NCBI blast	https://www.ncbi.nlm.nih.gov/BLAST/	https://www.ncbi.nlm.nih.gov/BLAST/
Galaxy server tools	Afgan et al., 2016	https://usegalaxy.org/

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Felipe Cava (felipe.cava@umu.se).

Materials Availability

All bacterial and plasmids generated in this study are available on request from the Lead Contact without restriction.

Data and Code Availability

Raw sequencing data from WGS and Tn-seq experiments are available at Sequence Read Archive (<https://www.ncbi.nlm.nih.gov/BioProject> ID PRJNA623074 and PRJNA623082 respectively). This study did not generate any new code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Bacterial strains

Vibrio cholerae is the experimental model used in this study. All *V. cholerae* strains used in this study are derivatives of the sequenced El Tor clinical isolate N16961 (Heidelberg et al., 2000) and are listed in Table S1. *V. cholerae* deletion mutant strains were constructed using standard allele exchange techniques with derivatives of the suicide plasmid pCVD442 as described previously (Donnenberg and Kaper, 1991). Primers used for constructing pCVD442-derivatives (primers P1-4) and for verifying the deletions (external primers E1-2) are shown in Table S2. Overlapping extension PCR was carried out as described (Nelson and Fitch, 2011). Since the start of *vc2153* overlaps with the terminal 73 nucleotides of the essential locus *vc2152* (Chao et al., 2013), the $\Delta vc2153$ mutant removes *vc2153* nucleotides 171 to 747. Because strains bearing *vc2153* mutations accumulate suppressors, the deletion of *vc2153* was always the final mutation introduced in strains bearing inactivation of more than one gene.

Escherichia coli strains DH5 α and DH5 α λ pir were used as hosts for constructing plasmids. The strains used as template for the amplification reaction of the genes encoding the cytoplasmic L,D-carboxypeptidase of the different bacteria were: *E. coli* K-12 MG1655, *Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966, *Proteus mirabilis* DSM 4479 and *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028.

Growth conditions

Unless otherwise specified, bacteria were grown at 37 °C in LB (Luria Bertani broth) medium. Agar 1.5% (w/v) was used in solid plates. Antibiotics were used at the following concentrations (per ml): streptomycin (Sm), 200 μ g, ampicillin (Ap), 50 μ g, carbenicillin (Cb), 50 μ g, chloramphenicol (Cm), 20 μ g (*E. coli*) and 5 μ g (*V. cholerae*), and kanamycin (Kn), 50 μ g. L- or D-methionine (L/D-Met) were used at a final concentration of 20 mM.

For growth curves, stationary cultures were normalized to an optical density at 600 nm (OD₆₀₀) of 1.5, diluted 1:100 and used for inoculating 96-well plates containing 180 μ L of fresh medium. At least three replicates per strain and condition were carried out. Measurements of the OD₆₀₀ for growth curves were carried at 30°C with shaking using an Eon Biotek microplate spectrophotometer.

Construction of plasmids

Complementation and overexpression plasmids were constructed using the primers indicated in Table S3 by amplifying the gene of interest with its native ribosome binding site. The *ldcA* gene from pET28b::ldcA was inserted into pBAD₃₃ yielding pBAD₃₃::ldcA. Expression of cloned loci from the P_{BAD} promoter was induced by addition of 0.2% arabinose (Guzman et al., 1995). DNA and protein sequence alignments were carried out using the blastn and blastp programs (<https://www.ncbi.nlm.nih.gov/BLAST/>) respectively.

METHOD DETAILS

Cell and colony morphology imaging

Analysis of cell morphology was performed on immobilized bacteria (1% agarose LB pads) by phase-contrast microscopy using a Zeiss Axio Imager.Z2 microscope equipped with a 63x oil immersion objective and a Hamamatsu digital camera controlled by Zeiss Zen Blue software. Pictures of colonies were taken from LB₁₀ plates (10 g L⁻¹ of NaCl) by using a Nikon SMZ1500 Zoom Stereomicroscope and a DS-Fi1 High-Definition Color Camera. Brightness and contrast levels of the images were adjusted using ImageJ software.

Peptidoglycan isolation and analysis

Murein sacculi isolation and muropeptide analysis were performed essentially as described previously (Alvarez et al., 2016; Desmarais et al., 2013; Glauner et al., 1988). Briefly, bacterial pellets of 50 mL (exponential phase OD₆₀₀ ~0.4) or 25 mL (stationary phase OD₆₀₀ ~3) cultures were boiled in 5% SDS. Cell wall material was pelleted and repeatedly washed with water by ultracentrifugation. Clean sacculi were digested with muramidase (100 μ g/ml) and soluble muropeptides were then reduced using 0.5 M sodium borate

pH 9.5 and sodium borohydride at 10 mg ml⁻¹ (final concentration). The pH of the samples was adjusted to 3.5 with phosphoric acid for liquid chromatography.

UPLC analyses were performed on a Waters-UPLC system equipped with an ACQUITY UPLC BEH C18 Column, 130Å, 1.7 μm, 2.1 mm × 150 mm (Water, USA) and detected at Abs. 204 nm. Muropeptides were separated primarily using a linear gradient from buffer A (phosphate buffer 50 mM pH 4.35) to buffer B (phosphate buffer 50 mM pH 4.95 methanol 15% (v/v)) in a 20 min run; a modified method using organic solvents (described below in the LC-MS analysis methods) was used for analysis D-Met muropeptides for a better separation of the peaks and to avoid co-elution of muropeptides. Identity of the peaks was assigned by comparison of the retention times and profiles to other chromatograms in which mass spectrometry data had been collected, and by mass spectrometry when necessary. The relative amount of each muropeptide was calculated by dividing the peak-area of a muropeptide by the total area of the chromatogram. The apparent density of PG (PG content per cell) was assessed by normalizing the total area of the chromatogram to the OD₆₀₀ of the culture used for the PG purification. The degree of cross-linking was calculated as described previously (Glauner et al., 1988) and as a rough estimation of D,D-cross-linkage the ratio between the tetra-tetra dimer (D44) and the tetra monomer (M4) was calculated. All PG analyses were performed using biological triplicates.

Protein overexpression and purification

vc2153 and the genes encoding for the cytoplasmic L,D-carboxypeptidases of *E. coli* (LdcA), *A. hydrophila* (AHA_1477) *P. mirabilis* (PMI1557) and *S. enterica* (STM1800) were cloned into pET28b and vca0337 into pET22b (Novagen) for expression in *E. coli* BL21(DE3) cells (primers used for cloning are listed in Table S4). Expression was induced in LB cultures at exponential phase with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 2 h. Cells were harvested, resuspended in 150 mM Tris HCl pH 7.5, 150 mM NaCl, and stored at -20°C. After thawing on ice, cells were disrupted by passaging through a French press twice. 6 × His-tagged proteins were purified from cleared lysates (30 min, 50,000 rpm) on nickel-nitrilotriacetic acid-agarose columns (QIAGEN), and eluted with 150 mM Tris HCl pH 7.5, 150 mM NaCl, 250 mM imidazole. The eluate was dialyzed for 12 h in 50 mM Tris-HCl pH 7.5, 100 mM NaCl. Purified proteins were visualized by SDS-PAGE and Coomassie Brilliant Blue staining and quantified by Bio-Rad Protein Assay (Bio-Rad).

In vitro protein reactions

For pure muropeptide substrates isolation, *V. cholerae* or *E. coli* peptidoglycan was digested with muramidase (see method above) and used for purification of muropeptides by collection of HPLC-separated muropeptide peaks. For isolation of anhydro- or D-Met-modified muropeptides, *in vitro* reactions were performed using purified Slt70 and LdtA proteins respectively (Cava et al., 2011; Lee et al., 2013). Acetonitrile and formic acid were used as organic solvents for peak separation. Collected peaks were lyophilized, dissolved in water, and stored at -20°C.

In vitro reactions were prepared in a final volume of 50 μL containing as substrates either pure muropeptides (10 μg), intact sacculi or muramidase-digested sacculi; 10 μg of purified protein (50 mM final concentration) in Tris-HCl pH 8 buffer was used. To test the activity of LdcA and LdcV on high molecular weight PG, SDS-free sacculi were incubated with purified proteins and after inactivation by heating at 100°C for 15 min, samples were centrifuged, and the pellet was digested with muramidase. Reactions were performed for 60 min at 37°C, heat inactivated (100°C, 15 min) and centrifuged (15,000 rpm, 10 min) to remove precipitated material. Peptides in the supernatant were monitored by UPLC, identified using standard controls based on their retention time, and confirmed by UPLC-MS analysis.

For studying the substrate preference of Ldcs for different muropeptides, M4, M4^{Met}, M4N and M4N^{Met} were produced and purified as described above. Then a mixture of the four muropeptides (containing approximately equal amounts of each of them) was used as substrate in a reaction of 150 μL final volume in 50mM Tris-HCl pH 8 buffer and 50 μg of purified Ldc protein. After addition of the protein, samples were incubated at 37°C and small aliquotes were removed at several incubation times, boiled to inactivate the protein and centrifuged. Soluble products were injected into the UPLC. The ratio of each muropeptide at each time-point was calculated by dividing its area by the total area calculated for all the muropeptides (substrates and products).

Whole genome sequencing

Genomic DNA was extracted using the GeneJET Genomic DNA Purification Kit and quantified with Qubit dsDNA HS Assay Kit (Thermo Scientific). 1 ng of each DNA was used to generate the genomic libraries following the manufacturer's recommendations (Nextera XT DNA Sample Preparation Kit, Illumina). DNA libraries were then pooled in equimolar proportions and sequenced employing a MiSeq Reagent Kit V2 (Illumina). Paired-end 2x300 bp reads were generated on an Illumina MiSeq instrument. Sequences of the isogenic wild-type strain N16961 and the Δvc2153 mutant were determined in parallel.

The sequences were analyzed using the open, web-based computational platform Galaxy (<https://usegalaxy.org/>) (Afgan et al., 2016) as indicated in Alvarez et al. (2018). Mapping trimmed reads to the reference *V. cholerae* N16961 genome was performed by the BWA-MEM (Li and Durbin, 2009) algorithm. After read alignment, Picard tools were used for the parent strains and each suppressor to mark and remove duplicate sequences mapping to different regions. Subsequently, SNPs (single-nucleotide polymorphisms) and indels (insertions and deletions) were detected by comparison of the results obtained after detecting genetic variants by FreeBayes (Garrison and Marth, 2012) and VarScan (Koboldt et al., 2012). Mapping of the larger deletion in the suppressor mutant Sup₄ was carried out using the Integrative Genome Viewer (IGV) (Thorvaldsdóttir et al., 2013).

Transposon insertion sequencing

V. cholerae transposon insertion libraries, of ~150,000 insertion mutants each, were generated in N16961 and in the Δ vc2153 mutant as described previously (Dörr et al., 2016) using the Himar delivery vector pSC189 (Chiang and Rubin, 2002). Transposon mutants were plated directly onto LB₁₀ (synthetic lethality) or LB₀ (suppressors analysis) agar plates containing Sm and Km. Transposon insertion sequencing was performed as described previously (Chao et al., 2013) using an Illumina MiSeq benchtop sequencer. Data analysis was conducted as described previously (Chao et al., 2013; Pritchard et al., 2014). Visual inspection of transposon insertion profiles was performed with the Sanger Artemis Genome Browser and Annotation tool (Rutherford et al., 2000).

Analysis of soluble muropeptide pools

Sample preparation to determine the level of the different soluble muropeptides was performed following the protocol described previously by Lee et al. (2016) with some modifications. Briefly, bacteria were grown until exponential phase (OD₆₀₀ ~0.7), cooled on ice for 10 min and then, after adjusting the OD₆₀₀ of the cultures (to have the same number of bacteria in each sample), normalized volumes of cells were harvested by centrifugation at 4,000 rpm, 4°C for 20 min. For analysis of the extracellular soluble muropeptides (ESM) normalized volumes of supernatants were collected, boiled for 15 min, centrifuged to remove precipitated material and stored at –20°C. The cell pellets were gently resuspended and washed with ice-cold 0.9% NaCl solution. After pelleting the cells again by centrifugation, they were resuspended in the remaining volume and boiled in water for 15 min. Samples were centrifuged to remove cell debris at 14,000 rpm for 15 min, and soluble fractions (containing intracellular soluble muropeptides, ISM) were transferred to new tubes and stored at –20°C. Both, ESM and ISM samples were filtered with 0.2 µm pore size filters, dried by speed vacuum, resuspended in water and used for LC-MS analyses. Soluble muropeptide analyses were performed on biological triplicates.

Detection and characterization of soluble muropeptides by LC-MS was performed on an UPLC system interfaced with a Xevo G2/XS Q-TOF mass spectrometer (Waters Corp.). Chromatographic separation was achieved as described previously (Alvarez et al., 2016) using an ACQUITY UPLC BEH C18 Column (Waters Corp.) heated at 45°C. 0.1% formic acid in Milli-Q water (Buffer A) 0.1% formic acid in acetonitrile (buffer B) were used as eluents. The gradient of buffer B was set as follows: 0–3 min 5%, 3–6 min 5%–6.8%, 6–7.5 min 6.8%–9%, 7.5–9 min 9%–14%, 9–11 min 14%–20%, 11–12 min hold at 20% with a flow rate of 0.175 mL min^{–1}; 12–12.1 min 20%–90%, 12.1–13.5 min hold at 90%, 13.5–13.6 min 90%–2%, 13.6–16 min hold at 2% with a flow rate of 0.3 mL min^{–1}; and then 16–18 min hold at 2% with a flow rate of 0.25 mL min^{–1}. Chromatograms were recorded at 204 nm. The QTOF-MS instrument was operated in positive ionization mode. Detection of ISM and ESM was in general performed by MS^o to allow the acquisition of precursor and product ion data simultaneously without pre-selection of targeted molecules. For MS^o the following parameters were set for ESI: capillary voltage at 3.0 kV, source temperature to 120°C, desolvation temperature to 350°C, sample cone voltage to 40 V, cone gas flow 100 L h^{–1} and desolvation gas flow 500 L h^{–1}. Detection of D-Met modified tetrapeptides was achieved by targeted MS, by setting the collision energy to scan between 6 eV and 15–40 eV. Mass spectra were acquired at a speed of 0.25 s/scan. The scan was in a range of 100–2000 m/z. Data acquisition and processing was performed using UNIFI software package (Waters Corp.).

The molecular structure of each anhydro-muropeptide and the PG precursor molecules was obtained using ChemSketch (<https://www.acdlabs.com>) to build a compound library in UNIFI. This compound library was used for processing the data, to detect and identify each molecule. Subsequent identification and confirmation of each muropeptide was performed by comparison of the retention-times and mass spectrometric data of experimental samples to purified authentic standards when available. Quantification was done by integrating peak areas from extracted ion chromatograms (EICs) of the corresponding m/z value of each muropeptide.

Lipid II extraction and LC-MS analysis

Lipid extraction was performed according to the protocol described by Qiao et al. (2017) for “large-scale lipid extraction” but using only 500 mL cultures. Strains were grown in LB at 37°C until an OD₆₀₀ of 0.4–0.6 or 2 for exponential or stationary phase samples respectively. After the chloroform/methanol extraction steps, dried recovered interface fractions were resuspended in DMSO. The lipid tail of the extracted Lipid II was removed by ammonium acetate treatment before LC-MS analysis that was performed by targeted MS using the same parameters indicated above for analyzing soluble-muropeptides pools.

M4^{Met} incorporation into the PG by recycling

M4N^{Met} muropeptides were generated by initially obtaining M4N from SDS-free *V. cholerae* sacculi digested with purified *E. coli* His6-tagged Slt70 protein (Höltje et al., 1975; Lee et al., 2013) and then replacing the terminal D-Ala by D-Met via *in vitro* L,D-transpeptidation mediated by purified LdtA protein in the presence of 20 mM D-Met (Cava et al., 2011). Around 15 µg of M4N^{Met} (in water) or the same volume of water as negative control were added to 200 µL of concentrated exponential cultures (containing ~10⁹ bacteria) of Δ ldtA Δ ldtB Δ bsrV Δ ldcV (Δ 4) and Δ ldtA Δ ldtB Δ bsrV Δ ampG Δ ldcV (Δ 5) strains grown in M9 minimal medium with glucose as a carbon source. After incubation of the samples for 45 min at 37°C, bacteria were pelleted and boiled in 2.5% of SDS for sacculi isolation and muramidase digestion. Solubilized muropeptides were reduced and after adjusting the pH, injected into the UPLC/MS for the search of M4^{Met} muropeptides by targeted MS.

Competition assays

In vitro competition indices (CI) were determined from cultures containing the Δ ldcV strain (*lacZ*⁺) carrying the empty pBAD₁₈ vector and the Δ ldcV (*lacZ*[–]) with the specified pBAD₁₈::Mur-construction, mixed in a ratio 5:1. The competition assays were performed by

incubating the cultures in LB₀ (and appropriate antibiotics) supplemented with 0.2% of arabinose for 4h at 37°C. After this time, CFUs were counted by plating serial log dilutions on LB₀ agar containing Sm, Km, 0.2% arabinose and X-gal 40 $\mu\text{g ml}^{-1}$. The CI was defined as the number of white colonies (strains overexpressing Mur proteins)/number of blue colonies (empty vector) counted after the incubation, divided by the white/blue ratio measure in the inoculum. The competition assays were performed in triplicates. In order to ensure protein overexpression from the pBAD promoter, total protein extracts obtained from arabinose induced cultures were prepared and the 6 × His-tagged produced proteins were monitored by western blotting using standard techniques and an anti-His antibody.

QUANTIFICATION AND STATISTICAL ANALYSIS

GraphPad Prism 6 software was used for graphing data and statistical analysis. Details of statistical tests and the number of replicates used for each analysis can be found in the figure legends.