Modulation of Peptidoglycan Synthesis by Recycled Cell Wall Tetrapeptides

Highlights

- *Vibrio cholerae* releases peptidoglycan (PG) fragments edited with NCDAAs
- Recycling of NCDAA-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

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-ala versus NCDAA-modified substrates.

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Modulation of Peptidoglycan Synthesis by Recycled Cell Wall Tetrapeptides

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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 reinternalized and recycled. Here, we show that Vibrio cholerae recycles PG fragments modified with non-canonical ω-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 ω,ω-cross-linkage and reduction of PG synthesis. We further demonstrate that L,ω-carboxypeptidases from five different species show a preferential activity for substrates containing canonical (ω-alanine) versus non-canonical (ω-methionine) ω-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 ω-alanine (ω-Ala); however, this amino acid is replaced in some species by non-canonical ω-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 imported 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-acetylg glucosaminidase (Cheng et al., 2000; Vötsch and Templin, 2000) and the AmpD amidase (Hötting et al., 1994; Jacobs et al., 1995; Figure 1A). The resulting products of these two enzymes are further cleaved by the L,ω-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-muro-tetrapeptides, 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 (M4NMet) 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-P4Met, 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 NCDAAs-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 NCDAAs 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 NCDAAs (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 NCDAAs 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 D-Met revealed the presence of anhydro-murotetrapeptides with D-Met at the terminal position (M4NMet) (Figure 1B). M4NMet was exclusively detected in the stationary phase and never detected in supernatants from a ΔbsrV 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 (Iuehara 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-P4Met) (Figures 1C and S1B). UDP-tetrapeptides were not detected in the ΔampG 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 ΔldcA (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 Δvc2153 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 L,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 (Völlmer et al., 2008b). While L,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...
its carboxypeptidase activity using different monomeric muropeptides 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 mDAP and the terminal D-Ala. VC2153, like LdcA, did not act on pentapeptides (Figure 2D) or cross-linked muropeptides (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 ΔldcV 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 ΔldcV 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 PBAD promoter complemented the growth defect of ΔldcV (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 muropeptides are poor substrates for D,D-carboxypeptidases (Cava et al., 2011). We wondered whether NCDAA-modified muropeptides may also be suboptimal substrates during PG recycling for LdcS, explaining the accumulation of non-canonical tetrapeptide precursors (UDP-P4Met) detected in *V. cholerae* wild type (Figure 1C).

To assess whether the accumulation of UDP-P4Met could be due to the preference of LdcV for canonical versus non-canonical tetrapeptide (D-Met modified), purified LdcV was incubated with a mixture of analogue M4N and M4 (D-Ala at the fourth position) and non-canonical M4NMet and M4Met (D-Met at the fourth position) muropeptides 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 rather than NCDAA-modified murotetrapeptide substrates (Figure 3B). 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 murotetrapeptide substrates (Figure 3C). Thus, the
transposon insertion sequencing (Tn-seq)-based screen in LB0

more comprehensive analysis of suppressors, we carried out a

(MP4 = M4 + M4N; MP4Met = M4Met + M4NMet; MP3 = M3 + M3N).

Salmonella enterica serovar Typhimurium

and purified L,D-carboxypeptidases from

position; M3, murotripeptide; M3N, anhydro-murotripeptide).

Analyses of the suppressor colonies confirmed that they allevi-

and turnover.

pressors corresponded to genes associated with PG recycling

NCDAA-edited PG seems to be a conserved mechanism.

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; M4Met, murotetrapeptide with D-Met at the fourth position; M4N, anhydro-murotetrapeptide; M4NMet, anhydro-murotetrapeptide with D-Met at the fourth position; M3, murotripeptide; M3N, anhydro-muramitripeptide).

(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 Typhimium (St), and E. coli (Ec) (C) (MP4 = M4 + M4N; MP4Met = M4Met + M4NMet; MP3 = M3 + M3N).

production of precursor tetrapeptides during the recycling of NCDAAnedited 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 ΔldcV 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 ΔldcV 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 ΔldcV phenotypes in cell morphology, PG content per cell, and growth in low osmolality 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 LB2 (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 ΔldcV with individual deletions of PG recycling genes ampG (permease), ampD (amidase), or mpl (muropeptide ligase) yielded complete reversion of the ΔldcV 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 ΔldcV ΔnagZ mutant accumulated UDP-P4 and incompletely suppressed the growth and PG defects of the ΔldcV mutant (Figures 4D–4F, S7A, and S7B). UDP-P4 accumulation in the nagZ ΔldcV 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 ΔldcV 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 ΔldcV 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; Scheuerwater et al., 2008; Shockman et al., 1996). Therefore, we hypothesized that the inactivation of these enzymes could suppress the viability defect of ΔldcV 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 ΔampG 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 S5A 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 (M4NMet), which could be recycled and detected as a D-Met tetrapeptide in the PG (Figures S5B 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 ΔldcV derivative (referred to as Δ4) that was also deficient in endogenous D-Met production (due to the absence of the BsrV racemase) and unable to incorporate...
exogenous D-Met by L,D-transpeptidation (Cava et al., 2011) (due to the absence of LdtA and LdtB). M4Met was detected in the PG isolated from the M4NMet-treated D4 strain, but not in a control strain (D5), 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 Δdcv 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 Δdcv 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 Δdcv mutant (Figures 5F and S10B), whereas deletion of ldtA in the Δdcv 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 ldtC mutant and suggests that recycled tetrapeptides control PG cross-linking homeostasis through their incorporation into the murein.
Figure 5. Incorporation of Recycled UDP-P4 into the Cell Wall Leads to a Reduction in d,dl-Cross-linking

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

(B) Schematic representation of the protocol used to demonstrate the incorporation of recycled M4NMet into peptidoglycan. IM, inner membrane; OM, outer membrane.

(C) Exogenous M4NMet was added to cultures of ΔldtA ΔldtB ΔbarV ΔdcV (Δ4) and Δ4 ΔampG (Δ5). The tandem MS (MS/MS) profile obtained by targeted MS of PG derived from Δ4 but not Δ5 revealed the presence of M4NMet.

(legend continued on next page)
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 ΔldcV. Overexpression of any of the MurA-F enzymes increased the PG density of the ΔldcV 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 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 ΔldcV 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 (Dorr et al., 2014b) and CssV (Dorr 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 tetrapeptides 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 3) 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...
was challenging. Nevertheless, provision of exogenous traceable D-Met anhydro-muropeptides derivatives to a ΔldcV mutant strain incapable of synthesizing NCDA-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 NCDA-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, NCDA-edited tetrapeptides (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-P4Met) in the stationary phase wild-type cultures of V. cholerae (Figure 1) suggest that recycling NCDA-modified muropeptides fine-tunes PG synthesis activity in this bacterium. Thus, production of NCDA can modulate cell wall synthesis and cross-linking by at least two mechanisms—(1) through periplasmic PG editing (by Ldts) with free NCDA (Alvarez et al., 2018; Cava et al., 2011; Lam et al., 2009) and (2) incorporation of recycled NCDA-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 NCDA induces regulatory changes in the murein of neighboring organisms. In principle, any bacterium that incorporates NCDA in the fourth position of the muropeptide (e.g., via the activity of Ldts) is a potential source of NCDA-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 NCDA suggests that it will be important to consider whether NCDA-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 modulate interspecies modulation of PG synthesis in trans if such peptides become substrates for PG recycling in neighboring organisms.
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SUPPLEMENTAL INFORMATION

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

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AUTHOR CONTRIBUTIONS


DECLARATION OF INTERESTS

The authors declare no competing interests.


## STAR METHODS

### KEY RESOURCES TABLE

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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 Δ*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 DH5α and DH5α Δpir 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 μg, ampicillin (Ap), 50 μg, carbenicillin (Cb), 50 μg, chloramphenicol (Cm), 20 μg (E. coli) and 5 μg (V. cholerae), and kanamycin (Km), 50 μg. 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 μL of fresh medium. At least three replicates per strain and condition were carried out. Measures 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 PBAD 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 Stereoscope 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; Desmaraïs et al., 2013; Glauner et al., 1988]). Briefly, bacterial pellets of 50 mL (exponential phase OD₆₀₀ ~0.4) or 25ml (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 μg/ml) and soluble muropeptides were then reduced using 0.5 M sodium borate
and stored at 4°C. Purified proteins were visualized by SDS-PAGE and Coomassie Brilliant Blue staining and quantified using QIAFAST (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 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 muramidase-digested sacculi 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 (PM11557) 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 passing 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, M4Met, M4N and M4NMet 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 aliquots 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 Δvc2153 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 LB2 (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 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 MS2 to allow the acquisition of precursor and product ion data simultaneously without pre-selection of targeted molecules. For MS3 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.

M4Met incorporation into the PG by recycling

M4Met 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 M4Met (in water) or the same volume of water as negative control were added to 200 μL of concentrated exponential cultures (containing ~10⁷ bacteria) of ΔldtA ΔldtB ΔbsrV ΔldcV (Δ4) and ΔldtA ΔldtB ΔbsrV ΔampG ΔldcV (Δ5) 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 sacculli isolation and muramidase digestion. Solubilized muropeptides were reduced and after adjusting the pH, injected into the UPLC/MS for the search of M4Met muropeptides by targeted MS.

Competition assays

In vitro competition indices (CI) were determined from cultures containing the ΔldcV strain (lacZ+) carrying the empty pBAD18 vector and the ΔldcV (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 μg 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.