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# *Escherichia coli* utilizes multiple peptidoglycan recycling permeases with distinct strategies of recycling

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**Bacteria produce a structural layer of peptidoglycan (PG) that enforces cell shape, resists turgor pressure, and protects the cell. As bacteria grow and divide, the existing layer of PG is remodeled and PG fragments are released. Enterics such as *Escherichia coli* go to great lengths to internalize and reutilize PG fragments. *E. coli* is estimated to break down one-third of its cell wall, yet only loses ~0 to 5% of meso-diaminopimelic acid, a PG-specific amino acid, per generation. Two transporters were identified early on to possibly be the primary permease that facilitates PG fragment recycling, i) AmpG and ii) the Opp ATP binding cassette transporter in conjunction with a PG-specific periplasmic binding protein, MppA. The contribution of each transporter to PG recycling has been debated. Here, we have found that AmpG and MppA/Opp are differentially regulated by carbon source and growth phase. In addition, MppA/Opp is uniquely capable of high-affinity scavenging of muropeptides from growth media, demonstrating that AmpG and MppA/Opp allow for different strategies of recycling PG fragments. Altogether, this work clarifies environmental contexts under which *E. coli* utilizes distinct permeases for PG recycling and explores how scavenging by MppA/Opp could be beneficial in mixed communities.**

cell wall | peptidoglycan | peptidoglycan recycling | AmpG | muropeptides

## Significance

Bacteria produce a cell wall that is remodeled as they grow, releasing fragments. Cell wall fragments can trigger inflammation by the immune system of a host. Gastrointestinal bacteria, like *Escherichia coli*, have dedicated pathways to recycle almost all cell wall fragments they produce. *E. coli* contains two known recycling transporters, AmpG and Opp. We explore different growth conditions when these transporters are used, types of fragments recognized, and strategies of how they import. Opp uniquely imports cell wall fragments released by other bacteria. In a mixed community, *E. coli* has the ability to decrease the release of cell wall fragments from neighboring bacteria that cannot recycle. This reduction of released cell wall fragments could modulate recognition by host immune responses.

Bacteria produce a complex cell wall known as peptidoglycan (PG). PG is a large polymer of alternating sugars, GlcNAc and MurNAc (*N*-acetylglucosamine and *N*-acetylmuramic acid, respectively), with cross-linked short peptides extended from MurNAc residues (1). The cell wall is continually remodeled by PG-degrading enzymes during growth, division, and in response to certain environmental conditions (2). Lysozyme, glucosaminidases, and lytic transglycosylases cleave between sugars, with the latter also cyclizing the cleaved terminal MurNAc to form 1,6-anhydro-MurNAc (anhMurNAc) (2). In addition, PG peptides can be cleaved or released by: carboxypeptidases at the C-terminal amino acid, endopeptidases within the peptide, and amidases releasing peptides from MurNAc (2). Remodeling liberates PG fragments, also called muropeptides, which can be either released to the environment or internalized and recycled. Bacteria have adopted strategies to recycle the peptides, sugars, or both portions of muropeptides directly into biosynthesis of PG precursors, a process termed PG recycling (3). The presence or absence of PG recycling in bacteria matches with an organism's lifestyle, as released muropeptides have roles in cell signaling, host communication, host immune stimulation, and adaptive responses (2).

Organisms like *Escherichia coli* are highly dedicated to recycling muropeptides (Fig. 1) and reuse >90% of turned-over PG products (4, 5). This could provide an advantage under nutrient-depleted conditions as an additional source of energy or reduced need for PG biosynthesis. In addition, as a gut endosymbiont, *E. coli* could recycle PG to avoid immune detection of released muropeptides, such as by human host receptors NOD1 and NOD2 (6). *E. coli* contains a PG recycling pathway for direct reutilization of both the peptide and sugar components of muropeptides (Fig. 1). Lytic transglycosylases contribute highly to *E. coli* PG turnover (7), which in conjunction with endopeptidases produce anhMurNAc-peptides and disaccharide muropeptides that can be recycled. Once imported into the cell, either NagZ, a $\beta$-*N*-acetylglucosaminidase (8, 9), or AmpD, an amidase (10, 11), can act first on muropeptides as both have broad substrate preferences. NagZ cleaves between GlcNAc and anhMurNAc sugars, and AmpD cleaves the peptide from anhMurNAc. GlcNAc and anhMurNAc can directly be recycled back into PG synthesis by conversion to GlcNAc-6-phosphate by NagK (GlcNAc recycling) and AnmK and MurQ (anhMurNAc recycling) (3). This is followed by deacetylation of GlcNAc-6-phosphate by NagA to produce glucosamine-6-phosphate (3). Glucosamine-6-phosphate can either be converted into the PG precursor UDP-GlcNAc or be converted to fructose-6-phosphate to enter glycolysis.

The majority of muropeptides contain tetrapeptides since cross-links in the existing cell wall are largely 4-3 cross-links between two tetrapeptides (95 to 97%), with a small amount of 3-3 cross-links between tri- and tetrapeptides (5-3%). In addition, any

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uncrosslinked PG peptides within the cell wall are also trimmed to tetrapeptides by carboxypeptidases. The freed tetramuropeptides need to be further trimmed down to a tripeptide by the L,D-carboxypeptidase, LdcA (12). The tripeptide can then feed into PG biosynthesis via the ligase, Mpl, that creates UDP-MurNAc-tripeptide (13). *E. coli* contains two known permeases, AmpG (14, 15) and MppA/OppBCDF (16, 17), that import peptide-containing muropeptides and will be the focus of this work. Two additional transporters, MurP (18) and NagE (19), are coupled to phosphotransferase systems for import of PG sugars, but will not be explored here. AmpG is a Major Facilitator Superfamily transporter with specific recognition of anhMurNAc-peptides (20). Conversely, OppBCDF is an ATP-binding cassette (ABC) transporter proposed to recognize just PG peptides through a substrate-binding protein MppA (17, 21). However, it is worth noting that MppA was never tested for binding to sugar-linked muropeptides and a recently identified distant homolog of MppA, called YepA, is capable of binding both freed and sugar-linked muropeptides (22). The contribution of AmpG and MppA/OppBCDF to recycling has been debated, with conflicting conclusions over whether MppA/OppBCDF plays a major role (16, 23).

While it is presumed that PG recycling could be a benefit when bacteria grow under nutrient-poor conditions, no evidence of a growth disadvantage in the absence of PG recycling has been identified in rich media (3). The exception is disruption of the encoding gene for LdcA, which does not prevent recycling. Instead, the absence of LdcA causes recycling to become aberrant and in some bacteria even toxic (12, 24, 25). In the absence of LdcA (Fig. 2*A*), tetrapeptides are ligated to UDP-MurNAc by Mpl instead of the LdcA-derived tripeptides. The resulting tetrapeptide PG precursors that are built are unable to be used as donors by D,D transpeptidases which are responsible for the majority of cross-links in the cell wall. Thus, tetrapeptide recycling uses critical PG substrates (UDP-MurNAc, undecaprenyl, etc.) and reduces cross-linking by creating nonideal PG precursors (24). Both of these impacts from tetrapeptide recycling could explain why it is toxic in bacteria like *E. coli*. Increasing expression of any PG biosynthesis gene, MurA-F, has been shown to suppress the toxicity in *E. coli* (26) and *Vibrio cholerae* (24), which would help to correct both biosynthesis and cross-linking issues. In *E. coli*, the toxic recycling mutant (Δ*ldcA*) results in a severe growth defect and stationary phase lysis (12). Thus, the toxic recycling mutant can be used as a genetic tool for studying PG recycling, as disruption of genes upstream or downstream can suppress the toxicity to reduce growth defects and lysis (24). In this work, we utilized the toxic recycling mutant to explore the need for different PG recycling permeases, AmpG and MppA/OppBCDF. This approach suggested that each permease is differentially expressed in response to growth conditions which was supported by transcriptomics. We also find that AmpG and MppA/OppBCDF use different strategies of recycling muropeptides. Altogether, we clarify why *E. coli* utilizes multiple permeases for PG recycling and explore how PG recycling could influence bacterial fitness in mixed cultures.

## Results

**Loss of MppA/OppBCDF Suppresses Δ*ldcA* Toxic PG Recycling.** We found it interesting that the severity of toxicity upon disruption of L,D-carboxypeptidases differs greatly in various bacteria (12, 24, 25, 27). As seen previously in *E. coli* (12), deletion of *ldcA* in our wild-type strain, W3110, causes a growth defect and stationary phase lysis (Fig. 2*B*). To further understand why toxic recycling results in such a severe phenotype in this bacterium, we selected for suppressors that prevented lysis of the Δ*ldcA* mutant. Replicate cultures were serially passaged until lysis was no longer observed. Suppressor populations were whole genome sequenced and possible suppressor mutations identified. We had expected to get suppressors that disrupted Mpl or the major PG recycling permease reported in the literature, AmpG (Fig. 1). We were surprised that all of our populations had become dominated with mutants that disrupted the gene *mppA* (Fig. 2*C*). *mppA* encodes an orphan substrate-binding protein for an ATP-binding cassette (ABC) transporter and has been linked to PG recycling (17) (Fig. 1). MppA is a paralog of the broad-substrate peptide-binding proteins OppA and DppA (oligo-peptide and dipeptide specificity, respectively). MppA has specificity for PG peptides and functions with OppBCDF, the cognate ABC transporter for OppA (17, 21). OppBCDF was previously shown to play only a minor role in PG recycling because mutants with disrupting mutations in *opp* still recycle at similar rates to a wild-type strain (23). However, our suppressor mutations suggested there may be more to the redundancy of AmpG and MppA/OppBCDF.

To test whether the *mppA*-disrupting mutations were responsible for the observed suppression and further dissect the roles of

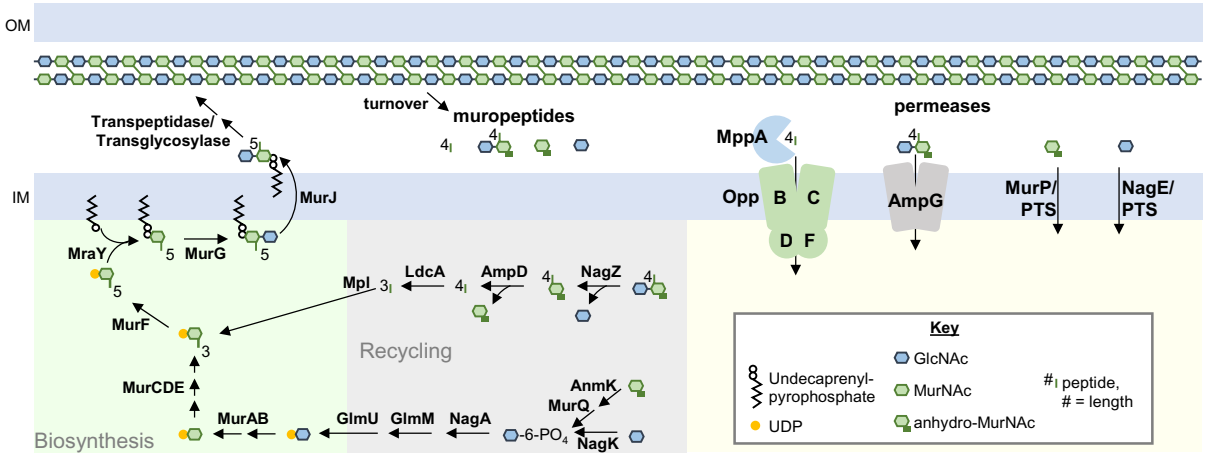
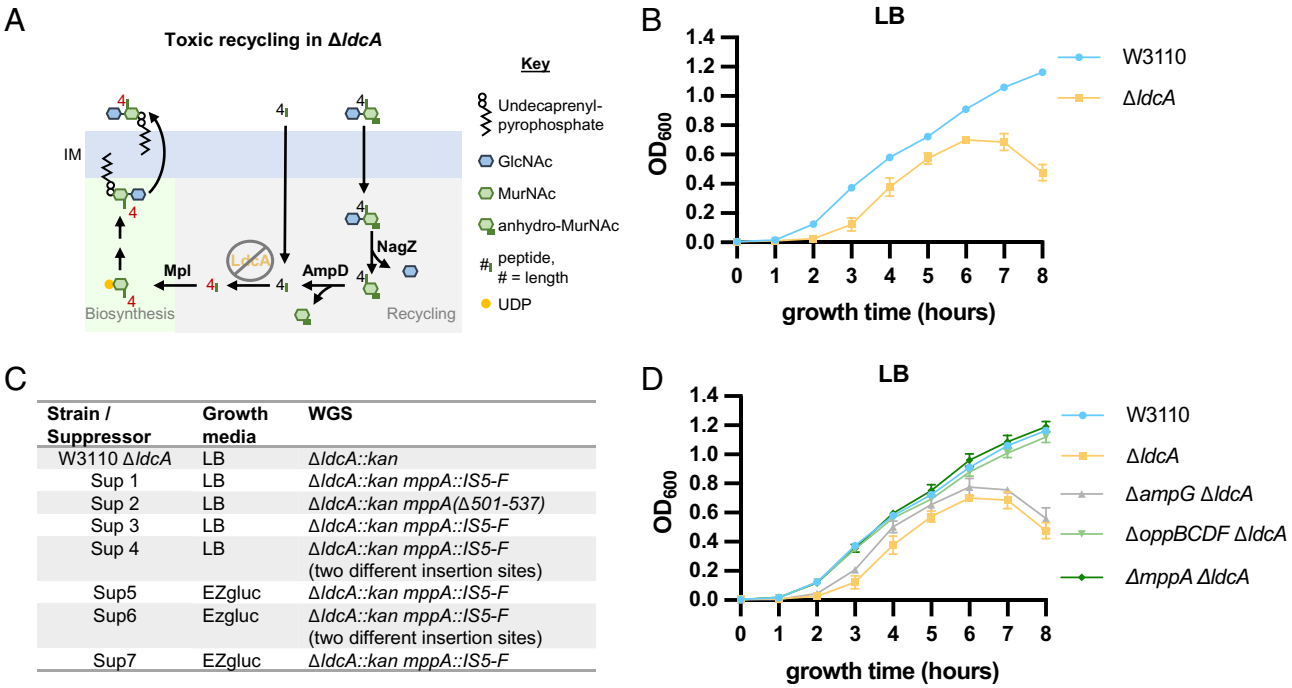



**Fig. 1.** Recycling of muropeptides in *E. coli*. Schematic of muropeptide recycling pathways found in *E. coli*. Muropeptide permeases, the focus of this work, internalize PG turnover products, so they can be either recycled or catabolized. PTS indicates transporters coupled to a phosphotransferase system. In the cytoplasm, muropeptides can be broken down to a tripeptide of m-Dap-D-Glu-L-Ala and the individual sugars anhMurNAc and GlcNAc. These products can either directly feed back into biosynthesis of PG precursors via recycling or be further catabolized.




| Strain / Suppressor | Growth media | WGS |
|---|---|---|
| W3110 Δ*ldcA* | LB | Δ*ldcA::kan* |
| Sup 1 | LB | Δ*ldcA::kan mppA::IS5-F* |
| Sup 2 | LB | Δ*ldcA::kan mppA(Δ501-537)* |
| Sup 3 | LB | Δ*ldcA::kan mppA::IS5-F* |
| Sup 4 | LB | Δ*ldcA::kan mppA::IS5-F* (two different insertion sites) |
| Sup5 | EZgluc | Δ*ldcA::kan mppA::IS5-F* |
| Sup6 | Ezgluc | Δ*ldcA::kan mppA::IS5-F* (two different insertion sites) |
| Sup7 | EZgluc | Δ*ldcA::kan mppA::IS5-F* |

**Fig. 2.** Loss of MppA/OppBCDF suppresses the toxic recycling *ldcA* mutant in LB. (*A*) Model of toxic recycling when LdcA is disrupted. Toxic recycling depletes UDP-MurNAc and other substrates into tetrapeptide lipid II precursors that are unable to act as donors for PBP-catalyzed crosslinks. (*B*) Growth curves showing the impact of toxic peptidoglycan recycling when LdcA is absent. Performed in biological triplicate. (*C*) Suppressors of W3110 Δ*ldcA* stationary phase lysis have disrupted the encoding gene for the MppA periplasmic binding protein. (*D*) Triplicate growth curves testing whether the loss of each transporter suppresses growth defects and/or stationary phase lysis of W3110 Δ*ldcA* mutants. Error bars on growth curves show the SD. When absent, the error bar was smaller than the symbol at that time point.

AmpG and MppA/OppBCDF, we built clean deletion mutants. As previously described (3), individually Δ*ampG*, Δ*mppA*, and Δ*oppBCDF* mutants do not have any obvious growth defects (*SI Appendix*, Fig. S1). To determine how each mutation affected recycling, we next combined them with the toxic recycling mutation in *ldcA*. In support that our *mppA* mutations were responsible for suppression, deletion of *mppA* or *oppBCDF* fully suppressed the growth defect and lysis from toxic recycling (Fig. 2*D*). However, deletion of *ampG* only slightly suppressed the growth defect from toxic recycling and still had severe lysis.

To determine whether any transcriptional regulations complicated our interpretations, we performed transcriptomics on Δ*ampG* and Δ*mppA* mutants (*SI Appendix*, Table S1). We did not expect to find any transcription alterations in our strains, as they do not have the regulator AmpR which senses cytoplasmic muropeptides in other bacteria (15). The Δ*mppA* strains had no significant differences from the wild-type strain. The Δ*ampG* strain only had reduction in transcripts related to sulfate/sulfite uptake and metabolism (*SI Appendix*, Table S1). Altogether, there were no obvious transcriptional changes to explain why Δ*ampG* could not suppress the toxic recycling mutant while Δ*mppA* was able to suppress. If there was a regulatory mechanism that was impacting PG recycling in our mutants, then it would likely be posttranscriptional. Together, these results suggested that MppA/OppBCDF could be critical for PG recycling during growth in LB and as cells transition into stationary phase.

**AmpG and OppBCDF/MppA Expression Is Impacted by Carbon Source and Growth Stage.** Since we had observed that the toxic recycling mutant had severe lysis as cells transitioned to stationary phase, we explored whether recycling permeases were utilized under different growth stages. RNA sequencing was performed on the wild-type strain grown in LB at three phases of growth: mid-log ($OD_{600}$~ 0.7), early stationary ($OD_{600}$ ~ 2), and late stationary ($OD_{600}$~ 4) (Dataset S1). It was found that *ampG* transcripts were expressed at low levels during logarithmic growth (*SI Appendix*, Table S2) and were slightly down-regulated with the transition to stationary phase (–twofold by late stationary phase, Fig. 3*A*). Transcripts for *mppA* were higher and remained steady (Fig. 3*A* and *SI Appendix*, Table S2). In agreement with MppA/OppBCDF playing a major role in stationary phase, *oppBCDF* transcripts increased dramatically (16.3-fold to 22.7-fold) with the transition to stationary phase and leveled out with prolonged stationary phase growth (Fig. 3*A* and *SI Appendix*, Table S2). These results agreed well with the suppression of the toxic recycling mutant and suggested that AmpG and MppA/OppBCDF both contribute to recycling during logarithmic growth in LB. Then, OppBCDF is up-regulated as cells transition to stationary phase increasing the amount of recycling through MppA/OppBCDF.

Peptides serve as the major source of carbon for growth in LB and OppBCDF when coupled to OppA functions in high-affinity import of oligopeptides. We considered the possibility that OppA/BCDF are up-regulated in stationary phase in response to the decrease in the concentration of oligopeptides in the media, which is unintentionally causing a major increase in MppA/OppBCDF recycling of PG fragments. Thus, we retested the toxic recycling mutant combinations when grown in a rich defined medium with glucose as the sole carbon source (EZgluc, Teknova). During logarithmic growth with glucose, loss of either AmpG or MppA/OppBCDF partially suppressed the growth defect of Δ*ldcA* (Fig. 3*B*). Transcriptomics during logarithmic growth in EZgluc indicated that AmpG and Opp were both up-regulated compared to LB (Fig. 3*A* and *SI Appendix*, Table S2) which supported that both were contributing to recycling. As cells transitioned into stationary phase in EZgluc, loss of MppA/OppBCDF suppressed stationary phase lysis, whereas loss of AmpG did not (Fig. 3*B*).

A

| Gene | LB | | EZgluc v LB | EZgluc | |
|---|---|---|---|---|---|
| | Fold Change early stat v log | Fold Change late stat v log | Fold Change both log | Fold Change early stat v log | Fold Change late stat v log |
| ***ampG*** | -1.5 | -2.0 | 4.92 | -8.76 | -15.27 |
| ***mppA*** | 1.3 | 1.3 | -1.62 | 3.37 | 2.58 |
| ***oppB*** | 22.7 | 10.8 | 10.52 | 3 | -1.84 |
| ***oppC*** | 17.9 | 6.9 | 10.6 | 2.17 | -1.53 |
| ***oppD*** | 17.3 | 6.2 | 7.86 | 2.49 | -1.59 |
| ***oppF*** | 16.3 | 5.2 | 8.78 | 2.51 | -1.5 |

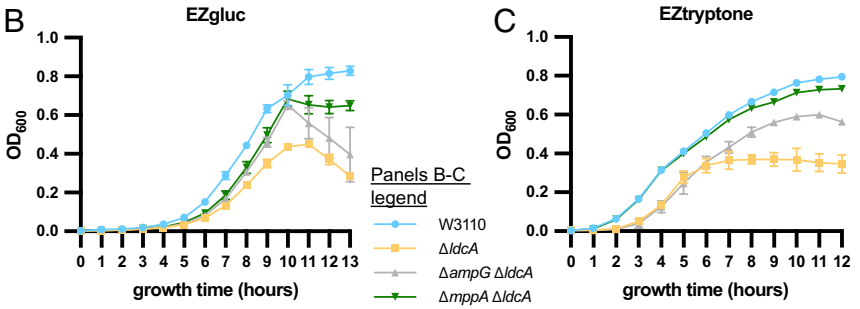



**Fig. 3.** AmpG and OppBCDF/MppA expression is impacted by carbon source and growth phase. (*A*) Weighted fold changes from triplicate RNA-seq in various media or during different growth stages (stat-stationary, log-logarithmic). (*B* and *C*) Triplicate growth curves to test the ability of Δ*ampG* and Δ*mppA* to suppress the toxic recycling Δ*ldcA* mutant when grown with glucose (*B*) and tryptone (*C*) as the carbon source. Error bars on growth curves show the SD. When absent, the error bar was smaller than the symbol at that time point.

Transcriptomics agreed with this finding and showed that AmpG was down-regulated when cells transitioned to stationary phase (~–15-fold by late stationary phase) and OppBCDF were up-regulated during early stationary phase (~twofold to threefold) (Fig. 3*A*).

We also tested suppression of the Δ*ldcA* toxic mutant in EZtryptone as an additional control for the influence of carbon source in PG recycling. The trend in EZtryptone was highly similar to LB, with Δ*mppA* fully suppressing Δ*ldcA* and Δ*ampG* only partially suppressing Δ*ldcA* toxicity (Fig. 3*C*). Altogether, AmpG and MppA/OppBCDF were found to be differentially regulated by carbon source and growth stage. Lrp likely is responsible for both of these responses for *oppBCDF* expression as it is a known regulator of the *opp* operon that senses nutrient richness and growth stage (28, 29). How these conditions impact *ampG* expression is less clear. It is likely that AmpG and MppA/OppBCDF are expressed differentially in response to other physiological cues that have yet to be explored.

**AmpG and OppBCDF/MppA Are Utilized for Different Strategies of PG Recycling.** To assess the ability of each transporter to recycle under different growth conditions, a strain was built lacking all transporter that can contribute to recycling of PG peptides, referred to as the Δpermeases mutant. The Δpermeases mutant is lacking *ampG*, *oppBCDF*, and a third transporter encoding gene, *cadB*. CadB will the focus of a future publication, as we found that it can contribute partially to PG peptide recycling. PG was radiolabeled with tritiated-meso-diaminopimelic acid ($^3$H-Dap), an amino acid incorporated into PG peptides and not into proteins. Labeled cells were then washed, diluted into fresh media containing excess cold m-Dap, and the level of retained $^3$H-Dap monitored per generation. As expected, the Δpermeases mutant lost $^3$H-Dap over time (Fig. 4 *A*–*C*), whereas the wild-type strain retained $^3$H-Dap, indicating that PG was recycled. Similar to recycling rates reported in the literature (4, 5, 15, 23), wild type lost ~3.3% and ~7.2% of $^3$H-Dap per generation in LB and EZgluc, respectively (Fig. 4*A*). The Δpermeases mutant, however, lost ~30.5% and ~36.6% of $^3$H-Dap per generation in LB and EZgluc, respectively (Fig. 4*A*), which was similar to reported loss in null mutants (15).

Next, we expressed a single permease at a time in the Δpermeases mutant from an IPTG-inducible plasmid. Overexpression of AmpG fully restored recycling under both growth conditions to levels similar to wild type (Fig. 4 *B* and *C*), indicating that AmpG is capable of fulfilling the cellular need for PG recycling when expressed. When overexpressing OppBCDF in EZgluc, the strain fully complemented PG recycling (Fig. 4*D*). Strangely, when grown in LB, expression of OppBCDF did not restore recycling during logarithmic growth, but recycling was observed as cells transitioned to stationary phase (Fig. 4*E*). It was also intriguing that when OppBCDF started to recycle in cells grown in LB, the transporter appeared to scavenge $^3$H-Dap that had been released into the growth medium (Fig. 4*E*). If cells had just switched to recycling only periplasmic PG fragments, we would have expected to see $^3$H-Dap loss level off; instead, the cellular $^3$H-Dap rebounded, indicating $^3$H-Dap previously released was now being reincorporated into the cell mass (Fig. 4*E*). The possibility for MppA/OppBCDF to scavenge PG fragments was supported by the published binding affinity of MppA to substrates, $K_D$ ~ 250 nM (21), which is multiple orders of magnitude fold stronger then the reported affinity of AmpG for substrates, $K_{m\ app}$ = 100 μM (20).

The ability of MppA/OppBCDF and AmpG to scavenge muropeptides was also compared. We labeled PG in the Δpermeases strain with $^3$H-Dap and then allowed the strain to release the $^3$H-muropeptides into EZgluc supernatants. Supernatants were collected, filter sterilized, and refreshed for glucose and ammonium. Strains were grown in the refreshed $^3$H-muropeptide media, and the incorporation of the tritiated PG (i.e., scavenging) into the cellular mass was determined. Wild-type W3110 was able to scavenge ~50% of the released $^3$H-muropeptides, whereas the Δpermeases strain could not scavenge $^3$H-muropeptides (Fig. 4*F*). Cells overexpressing only OppBCDF supported scavenging of $^3$H-muropeptides, whereas those expressing only AmpG could not. Altogether, our results suggest that AmpG is fully capable of matching the rate of PG turnover in the periplasm when it is

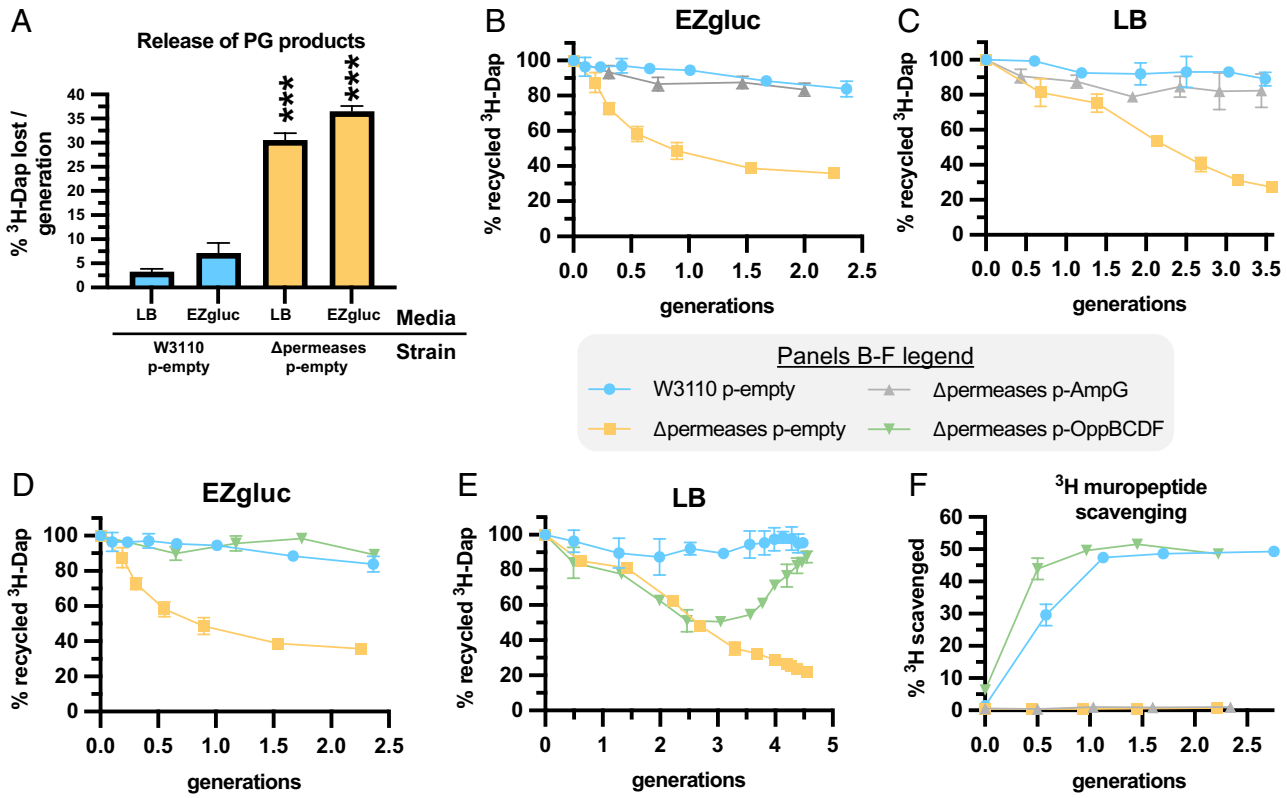



**Fig. 4.** AmpG recycles turned over products, whereas OppBCDF/MppA can also scavenge muropeptides. (*A–F*) Triplicate PG recycling assays utilizing the retention of tritiated $^{3}$H-Dap label in cellular peptidoglycan per generation. (*A*) Calculated loss of $^{3}$H-Dap per doubling. Sole expression of AmpG (*B* and *C*) or OppBCDF (*D* and *E*) to assess the ability of each transporter to recycle during growth on glucose (*B* and *D*) and tryptone (*C* and *E*). (*F*) Triplicate scavenging assays of $^{3}$H-muropeptides released from a strain unable to recycle. Error bars on assays show the SD. When absent, the error bar was smaller than the symbol at that time point.

expressed, whereas the MppA/OppBCDF system functions as a high-affinity scavenger of PG fragments.

**MppA/OppBCDF PG Recycling Is Inhibited by High Oligopeptide Concentrations.** It is important to note that although OppBCDF/MppA-dependent import of muropeptides was decreased during logarithmic growth in LB (Fig. 4*E*), this regulation likely occurred at the posttranscriptional level. This conclusion was drawn because OppBCDF was constitutively expressed from a plasmid and independent of its normal regulators which all bind in the orf of *oppA*. In addition, our transcriptomics have indicated that MppA should be stably expressed under these conditions. To determine whether OppBCDF protein levels were impacted during this stage of growth, we expressed HA-tagged variants of OppD and OppF. Protein levels were immunoblotted during the logarithmic and early stationary phase of growth in both LB and EZgluc media. Compared to an OmpA loading control there was no dramatic change in OppD or OppF protein levels when comparing the same growth stage in the two media (LB/log v EZgluc/log and LB/early stat v EZgluc/early stat, *SI Appendix*, Fig. S2). Importantly in our recycling assays, OppBCDF overexpression was unable to recycle in LB/log, but capable of recycling in EZgluc/log, and protein levels were similar in these conditions (*SI Appendix*, Fig. S2). Together, these suggest that the block of OppBCDF recycling during logarithmic growth in LB was not via a major alteration of protein levels.

Since the carbon source in LB, tryptone, is a rich oligopeptide mixture, we hypothesized that MppA/OppBCDF PG recycling was inhibited by the uptake of oligopeptides by OppBCDF. To assess whether the paralogs of MppA, OppA and DppA, were outcompeting MppA for binding to OppBCDF (*SI Appendix*, Fig. S3*A*), either just *oppA* or both *oppA* and *dppA* were deleted from the Δpermeases strain. Recycling assays in LB were then tested for strains overexpressing OppBCDF in the presence of both paralogs, absence of OppA, and absence of both paralogs. Deletion of *oppA* alone or in combination with *dppA* did not restore PG recycling during logarithmic growth in LB (Fig. 5*A*), indicating that OppA and DppA were not outcompeting MppA.

It was also possible that the oligopeptides in tryptone outcompeted muropeptide binding to MppA. MppA has been demonstrated to have a strong preference for muropeptides (~250 nM affinity) and a 1,000-fold weaker binding to the tripeptide Pro-Phe-Lys (21). PG recycling assays were assessed again in EZgluc in the presence of defined peptides (Pro-Phe-Lys and Ala-Ala-Ala) or tryptone. The OppBCDF expressing strain was recycling in EZgluc until the addition of an excess concentration of oligopeptides (Fig. 5*B* and *SI Appendix*, Fig. S3) that caused an immediate cessation of OppBCDF recycling. After a few generations, cells recovered some or all of the released $^{3}$H-muropeptides (Fig. 5*B* and *SI Appendix*, Fig. S3). A lower dose of Pro-Phe-Lys (1 mM instead of 10 mM) caused only a brief cessation of OppBCDF recycling. Altogether, these results indicated that excessive concentrations of oligopeptides can inhibit MppA recycling of muropeptides.

**MppA/OppBCDF Transports Only Freed PG Peptides.** Because of its homology to OppA and DppA, MppA has only been tested for binding to freed peptides (17, 21). However, it was recently found that a distant homologous ABC transporter found in Alphaproteobacteria, YepA/YejBEF, was capable of transporting

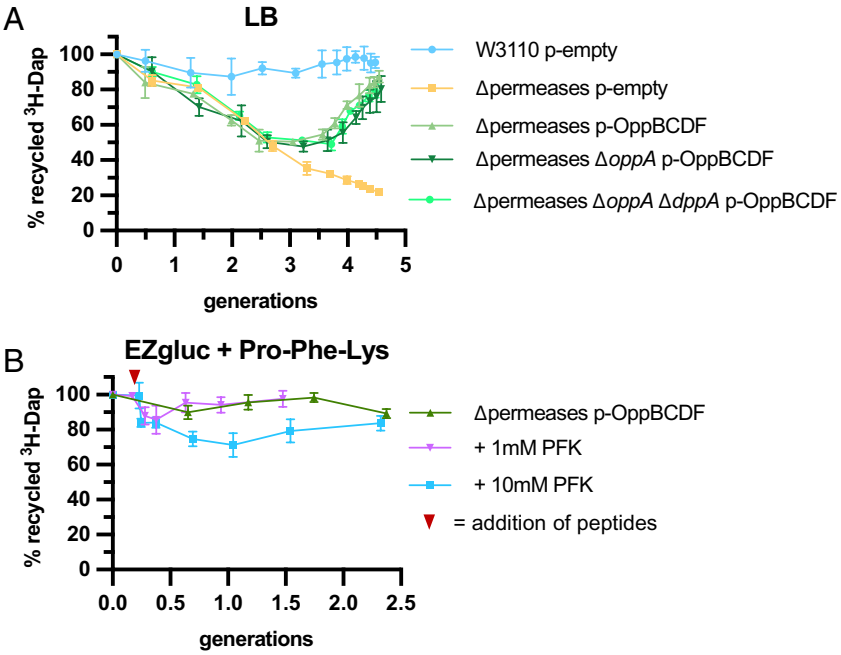



**Fig. 5.** High concentrations of oligopeptides inhibit MppA/OppBCDF PG recycling. (*A*) Triplicate PG recycling assays in LB with OppBCDF overexpression in the presence of OppA and DppA, absence of OppA, or absence of both. (*B*) OppBCDF triplicate recycling assays in EZgluc with the addition of Pro-Phe-Lys (PFK) tripeptide after 1 h timepoint. MppA was previously shown to weakly bind Pro-Phe-Lys with a $K_D$ > 300 μM compared to binding of PG peptides at a $K_D$ ~ 250 nM (21). Error bars on assays show the SD. When absent, the error bar was smaller than the symbol at that time point.

sugar-linked muropeptides (22). *E. coli* MppA could have a more restrictive substrate-binding preference, freed peptides only, or a broad substrate range like YepA. For imported anhMurNAc-linked muropeptides to be recycled, they must first be cleaved by the cytoplasmic amidase AmpD, so we explored the impact of *ampD* deletions on our toxic recycling mutant. If AmpD was only required for recycling of AmpG imported muropeptides and Δ*ampG* was unable to suppress Δ*ldcA*, then Δ*ampD* should also not suppress the Δ*ldcA* toxic recycling mutant (models of hypothetical scenarios in *SI Appendix*, Fig. S4*A*, scenario II.). Alternatively, if AmpD was required for recycling of MppA/OppBCDF imported muropeptides as well, then Δ*ampD* should suppress the toxic recycling mutant (*SI Appendix*, Fig. S4*A*, scenario III.). Surprisingly, Δ*ampD* suppressed toxic recycling in the Δ*ldcA* mutant (*SI Appendix*, Fig. S4 *B* and *C*), supporting that anhMurNAc muropeptides could be the major PG fragment being recycled. However, this result did not preclude the possibility that loss of AmpD instead blocks recycling through a regulatory mechanism (*SI Appendix*, Fig. S4*A*, scenario IV.). We performed transcriptomics on the Δ*ampD* mutant and found no significant changes in gene expression, ruling out that a transcriptional regulation had altered recycling. Together, these initial results suggested either that anhMurNAc was the predominant muropeptide recycled, or that the Δ*ampD* mutation caused an unforeseen posttranscriptional effect that altered PG recycling. If the first interpretation were true, then this would support that MppA/OppBCDF is able to transport anhMurNAc muropeptides similar to YepA/YejBEF.

Therefore, it was critical to directly evaluate whether MppA/OppBCDF was capable or incapable of transporting anhMurNAc muropeptides. To accomplish this, cytoplasmic muropeptides were measured as one permease was expressed. Mutants lacking *ampD* were utilized for this characterization as they accumulate cytoplasmic anhMurNAc-linked muropeptides increasing the sensitivity of the assay. In agreement with published literature (10), the Δ*ampD* mutant accumulates cytoplasmic anhMurNAc with tri-, tetra-, and pentapeptides (Fig. 6). This accumulation requires recycling permeases, as the Δ*ampD* Δpermeases mutant had no cytoplasmic accumulation of muropeptides (Fig. 6). Note that in this assay, *ldcA* is intact, so cytoplasmic tetrapeptides are quickly cleaved into tripeptides. Only AmpG was capable of increasing cytoplasmic accumulation of anhMurNAc muropeptides, while OppBCDF was not capable of transporting this substrate (Fig. 6). These data indicate that MppA/OppBCDF is only able to transport freed peptides.

These results also suggested that Δ*ampD* caused a posttranscriptional block of PG recycling when combined with the Δ*ldcA* mutant in LB. Δ*ampD* mutants accumulate cytoplasmic muropeptides that when unable to be used could serve as a signal to decrease PG recycling. Since AmpG is the only transporter capable of importing anhMurNAc muropeptides, loss of AmpG should relieve regulation due to cytoplasmic accumulation of this product (*SI Appendix*, Fig. S4*A*, scenario V.). When we combined Δ*ampD*, Δ*ampG*, and Δ*ldcA*, the mutant had lysis from toxic PG recycling, indicating that anhMurNAc muropeptides were no longer accumulated in the cytoplasm and that PG recycling was no longer blocked (*SI Appendix*, Fig. S4 *A* and *B*, scenario V). The growth defects observed for the Δ*ampD* Δ*ampG* Δ*ldcA* mutant had to occur from import of freed peptides, supporting that MppA/OppBCDF transports only freed peptides. When anhMurNAc muropeptides accumulate in the periplasm, they can be converted to freed peptides by amidases. The OM-anchored amidase, AmiD, was previously proposed to be the primary periplasmic amidase used for PG recycling (30). Although, cells were still found to generate about 25% of freed tetrapeptides even in the absence of AmpD and AmiD, presumably from cell division amidases (30). To further test whether MppA/OppBCDF are only able to transport freed PG peptides, we deleted *amiD* in the Δ*ampD* Δ*ampG* Δ*ldcA* mutant. We would expect that loss of AmiD in this background would reduce freed-tetrapeptides and suppress toxic recycling growth defects of its parent strain (*SI Appendix*, Fig. S4*A*, scenario VI). Indeed, the Δ*ampD* Δ*ampG* Δ*amiD* Δ*ldcA* mutant combination suppressed growth defects and lysis of its Δ*ampD* Δ*ampG* Δ*ldcA* parent strain (*SI Appendix*, Fig. S4*B*). These results supported previous findings that AmiD contributes to conversion of periplasmic muropeptides and that MppA/OppBCDF are only able to transport freed PG peptides.

**PG Scavenging Could Be Beneficial in Mixed Populations.** We were still intrigued about MppA/OppBCDF's ability to scavenge muropeptides released into the growth medium. This type of

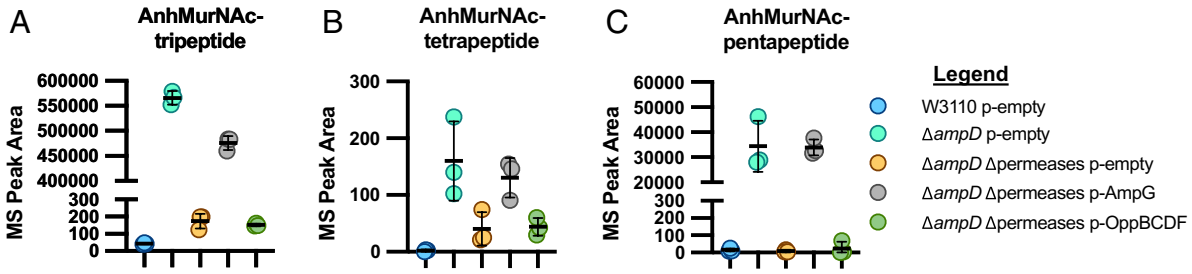



**Fig. 6.** MppA/OppBCDF does not transport AnhMurNAc muropeptides. Triplicate muropeptide analysis of PG products accumulated in the cytoplasm. Loss of AmpD results in cytoplasmic accumulation of anhMurNAc-linked muropeptides containing tri- (*A*), tetra- (*B*), and penta- (*C*) peptides. Overexpression of each transporter in the Δ*ampD* strain can assess whether each transporter is capable of importing anhMurNAc-linked muropeptides. Note that tetramuropeptides are low because LdcA is still present and trims them down to trimuropeptides. Error bars on assays show the SD. When absent, the error bar was smaller than the symbols.

scavenging could allow *E. coli* to uptake PG fragments released by other bacteria that do not recycle. We first retested whether recycling PG provided a growth advantage in LB or EZgluc. Growth competition assays were performed between our wild-type strain with tetracycline resistance and Δpermeases strain with ampicillin resistance. There was no competitive advantage to either strain under the conditions tested (*SI Appendix*, Fig. S5); competitive indices for the wild-type strain compared to the Δpermease strain were all very close to 1, indicating the population stayed at 50% for each strain type.

One environment where PG scavenging could be beneficial is in the gastrointestinal tract as a commensal or pathogen. PG fragments are recognized by human receptors, NOD1 and NOD2, and signal for an inflammatory response (6), which could be detrimental to gut commensals or some pathogenic lifestyles. To assess how prevalent PG recycling and the associated transporters are in gut microbes, we searched for homologs in 60 representative species of the human gut microbiota (Fig. 7). PG peptide recycling (*ampD* and/or *mpl*) is widely spread in Gram-negatives, while PG sugar recycling is prevalent in both Gram-negative (*anmK* and/or *nagK*) and Gram-positive (*amiE* and/or *murQ*) bacteria. Still, many gut bacteria do not recycle PG fragments and Gram-positive members commonly do not recycle the PG peptide. PG peptides can be recognized by the human NOD1 receptor to stimulate inflammation, so how do bacteria prevent inflammation in a community where not all members recycle their PG to an equal degree? It is possible that bacteria with PG scavenging permeases like MppA/OppBCDF, could recycle for their neighbors. These bacteria could reduce localized inflammation by keeping released PG fragments at low levels.

To test whether *E. coli* can scavenge the PG peptides of its neighbors in a mixed community, we performed recycling assays on mixtures of the wild-type and Δpermeases strains prepared at 50:50 and 20:80 ratios and assayed in both LB and EZgluc. The final composition of mixed cultures for assays was determined using different antibiotic resistance markers in each strain (*SI Appendix*, Fig. S6*A*). If the wild-type strain did not recycle PG products released by the Δpermeases mutant, then we would expect the percent $^{3}$H-Dap lost for the mixed communities to be averaged between the rates of the two strains taking into consideration the ratio at which they were mixed. We calculated expected averaged curves and expected averaged rates of $^{3}$H-Dap lost per generation (Fig. 8 *A* and *B* and *SI Appendix*, Fig. S6). Mixed population cultures had higher recycling then the expected averaged values for all conditions tested (Fig. 8 *A* and *B* and *SI Appendix*, Fig. S6). The ratio of our mixtures changed slightly in EZgluc due to a variability in lag times between the strains (*SI Appendix*, Fig. S6*A*), but, even at a 29% wild-type cells and 71% Δpermeases cells mixture, all PG fragments were recycled in the mixed culture (Fig. 8*A* and *SI Appendix*, Fig. S6*C*). Scavenging in a mixed population was not as dramatic during growth in LB as compared to during growth in EZgluc (Fig. 8 *A* and *B* and *SI Appendix*, Fig. S6 *B–E*) but still increased the mixed populations recycling rate above what would be expected if the strains were recycling independently of each other. These results indicate that wild-type cells scavenged $^{3}$H-labeled PG fragments released by the Δpermeases cells.

We next tested whether PG scavenging by our wild-type strain could reduce NOD activation when grown in a mixed culture with the Δpermeases strain. Supernatants were collected from monocultures, 20:80 mixed cultures, and 50:50 mixed cultures of our strains. HEKblue cell lines containing a human NOD1 activation reporter that produced secreted embryonic alkaline phosphatase (SEAP) were used to assess immune stimulation by muropeptides released from bacterial cells. Supernatants collected from monocultures behaved as expected. Wild-type strain's supernatants had no SEAP production and thus no NOD1 activation, whereas the Δpermeases strain's supernatants had robust NOD1 activation (Fig. 8*C*). These results agreed with the wild-type strain recycling nearly all PG fragments versus the Δpermeases strain being unable to recycle. Supernatants from both mixed cultures also had no NOD1 activation (Fig. 8*C*), indicating that the recycler strain (wild-type) had completely scavenged PG muropeptides released from the nonrecycler (Δpermeases) cells and prevented any NOD1 activation. This evidence supports that PG scavenging by *E. coli* MppA/OppBCDF could serve an altruistic behavior modulating inflammation. Further testing would be necessary to determine whether *E. coli* can scavenge muropeptides released by other gut bacteria and whether this is beneficial in a host gastrointestinal tract.

## Discussion

This work highlights the extreme dedication of enterics like *E. coli* to recycling PG turnover products. *E. coli* has four transporters capable of importing the wide array of muropeptides that can be produced during remodeling (Fig. 1). Searching for homologs of these transporters in other gut microbes (Fig. 7) indicated that AmpG is highly prevalent in Gram-negatives, especially in the Proteobacteria and Bacteroidetes phyla. AmpG conservation overlaps with organisms that utilize lytic transglycosylases to produce its substrates, anhMurNAc muropeptides (2). Interestingly, although MppA homologs have only been experimentally explored in proteobacteria, there is evidence of *oppA* duplications in many gut microbes that could encode paralogs similarly specialized for PG import. Of these possible MppA homologs, those of *Enterococcus faecalis* strains are in operons with *murR*, encoding a murein

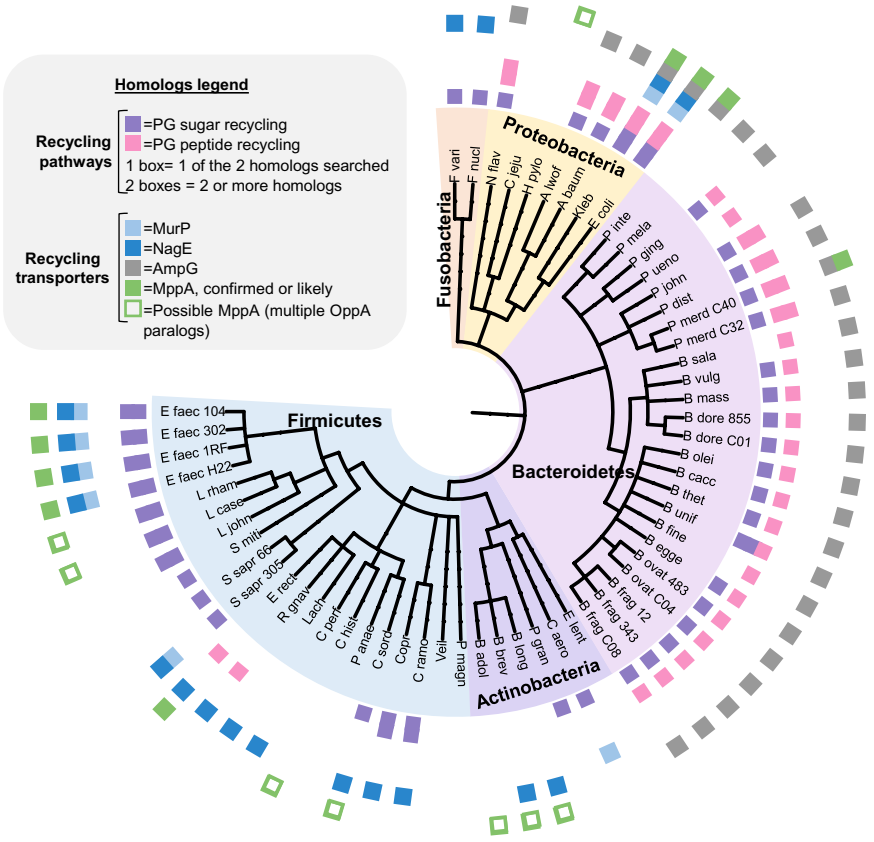



**Fig. 7.** Conservation of PG recycling genes in human gut microbiota. Phylogenetic tree of 60 representative species identified as gut microbes in the human microbiome project, showing distribution of homologs for PG sugar recycling genes (Gram-negative genes *anmK* and/or *nagK*, Gram-positive genes *amiE* and/or *murQ*), PG peptide recycling genes (*mpl* and/or *ampD*), and the four transporters involved in recycling (*murPnagEampG,* and *mppA*). Due to high homology between *mppA* and *oppA, mppA* homologs are split into two groups: i) confirmed/likely (filled box) if experimental evidence, striking similarity to MppA, or regulation by murein regulator, MurR, was present and ii) possible (outlined box) if the organism contained multiple *oppA* paralogs. See Dataset S1 for full details of homologs identified.

regulator. Finally, PG sugar recycling has been found to be critical in several species of the Firmicutes phyla (2) and proteins dedicated to their import are prevalent across Gram positives.

We have also provided evidence that muropeptides are sensed and monitored at several points during recycling. Based on interpretations discussed below of MppA/Opp scavenging, we propose that periplasmic amidases or a partner protein monitor the periplasmic pool of muropeptides. In LB where oligopeptide concentrations were in excess, we observed that the oligopeptides blocked recycling by MppA/Opp for several generations and led to release of muropeptides into the media. However, when oligopeptide concentrations dropped, MppA/Opp scavenged close to 100% of the muropeptides previously

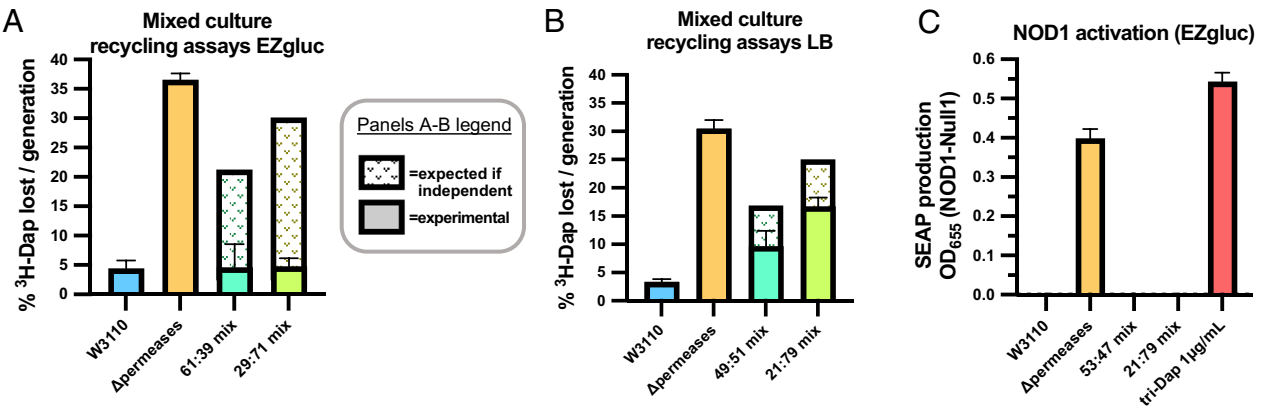



**Fig. 8.** Peptidoglycan recycling could be beneficial in mixed communities. (*A* and *B*) Average and SD of the calculated percent loss of $^{3}$H-Dap per generation in mixed cultures of a strain able to recycle and a mutant lacking all three recycling permeases when grown in EZ-gluc (*A*) and LB (*B*) from triplicate assays. Superimposed expected bars indicate the expected averaged value taking into account the ratio between strains if recycling was performed independently by each strain. (*C*) Triplicate NOD1 activation assays with supernatants from mixed cultures of a strain able to recycle and a mutant lacking all three recycling permeases. The experimentally determined composition of mixed cultures assays is indicated as a ratio of % W3110:% Δpermeases, see *SI Appendix*, Fig. S6*A*. Error bars on assays show the SD.

released. anhMurNAc-linked muropeptides are known to be a major pool of turnover products and yet, OppBCDF is unable to transport anhMurNAc muropeptides. Therefore, periplasmic amidases, possibly AmiD, must contribute to the OppBCDF scavenging observed in LB; periplasmic amidases had to release the peptides from anhMurNAc for them to be transported by MppA/Opp. Since PG scavenging requires tight binding by MppA to capture muropeptides that happen to diffuse into the periplasm, it is even possible that AmiD or another amidase(s) act on muropeptides bound to MppA. This would provide the most direct method of high-affinity capture of muropeptides, coordination of amidases with scavenging, and prevention of peptide loss back to the external environment. How amidases contribute to PG recycling and scavenging will be an exciting topic for future research in our laboratory.

When studying AmpD and AmpG, we also observed evidence that cytoplasmic levels of anhMurNAc muropeptides might be sensed to regulate PG recycling. AmpG was the only *E. coli* permease demonstrated to transport anhMurNAc-peptides, the substrate of AmpD. When AmpD was absent, it suppressed toxic PG recycling possibly via a posttranscriptional regulatory mechanism in response to cytoplasmic accumulation of muropeptides. Loss of AmpG would prevent cytoplasmic accumulation of anhMurNAc-peptides, and indeed deletion of *ampG* restored toxic PG recycling in a Δ*ampD* Δ*ldcA* strain. Other organisms sense these cytoplasmic muropeptides through AmpR (15), but our strains do not contain this regulator, indicating that this could be a different mechanism of regulation. It is possible that cytoplasmic accumulation could negatively impact Mpl recycling or PG precursor biosynthesis. The possible regulation of proteins by anhMurNAc-peptides and what advantage this has on cellular fitness deserve future exploration.

Here, we have also clarified some of the reasons behind redundancy of AmpG and MppA/Opp. AmpG is the only recycling permease produced by *E. coli* that was able to import muropeptides containing both anhMurNAc and the attached peptide. This class of muropeptides are highly prevalent in *E. coli* and are produced through the combined effort of lytic transglycosylases and endopeptidases (2). Sole expression of AmpG was able to almost completely complement wild-type levels of PG recycling, highlighting how prevalent these substrates likely are during growth. These results also insinuate that the affinity of AmpG to its substrate is perfectly matched to the concentration at which they are generated in the periplasm. We found that AmpG is utilized predominantly during active growth and down-regulated as cells transition into stationary phase. In this way, AmpG expression may be fine-tuned to match PG turn-over rates; during active growth when more muropeptides are produced, AmpG may be increased to match the substrate demand. Finally, AmpG was found to not be inhibited by a competing substrate, demonstrating its great specificity for its own substrate. However, there are some conditions where AmpG may not be optimal for PG recycling. The high specificity of AmpG for anhMurNAc muropeptides prevents it from being able to transport freed PG peptides. In addition, AmpG was unable to scavenge muropeptides from the environment.

MppA/Opp was uniquely capable of scavenging muropeptides from the environment likely due to MppA's nanomolar affinity to its substrate (21). OppBCDF were up-regulated as cells transitioned into stationary phase and in EZgluc compared to LB, supporting that high-affinity import of muropeptides is beneficial when nutrients are low. Both of these changes in expression match with the expected regulation of the *opp* operon by Lrp, a leucine sensor that monitors the richness of the media (31). Lrp affects a large number of genes that are up-regulated upon entry into stationary phase (28, 29). MppA-mediated PG recycling has limitations though i) competitive inhibition by excess oligopeptides and ii) only transports freed PG peptides. The limitations of MppA/Opp PG recycling are balanced by the unique ability to scavenge muropeptides. In this way, our work suggests that *E. coli* employs both AmpG and MppA/Opp for PG recycling because their substrates and strategies of recycling are highly distinct.

The most striking implication of MppA/Opp scavenging is its potential to be used for interspecies interactions. We observed that *E. coli* with MppA/Opp could import muropeptide from another *E. coli* strain unable to recycle PG. This supports that *E. coli* could scavenge muropeptides released by neighboring organisms. The breadth of this scavenging ability remains to be seen but likely depends on the amino acid composition of other organisms' PG peptides. *E. coli*'s MppA is likely able to bind best to PG peptides with similar sequences to its own. It is unclear how widely conserved MppA/Opp scavenging is in bacteria due to close homology with OppA (Fig. 7). In addition, a recently identified transporter, YepA, is distantly related to MppA and highly prevalent in Alphaproteobacteria. Interestingly, YepA is also able to transport a broader range of muropeptides, including sugar-linked and those modified with D-Met (22), which could allow for scavenging of a wide array of muropeptides. Scavenging muropeptides from other bacteria could provide additional nutrients, mediate interspecies communication, or help to prevent immune detection in a mixed community. We demonstrated that *E. coli* scavenging from neighbors could reduce NOD1 activation that would lead to inflammation. The implications of MppA scavenging toward host interactions are wide spread requiring further assessment.

## Materials and Methods

**Strains and Growth Conditions.** All strains and primers used in this study are listed in Dataset S1. All LB cultures and agar plates were performed with Difco™ Luria–Bertani Broth or Agar, Miller (BD). EZ defined media were prepared by diluting 10× MOPS buffer from the EZ-rich defined medium kit (Teknova) to their 1× concentration and supplementing with 1.32 mM potassium phosphate dibasic (Teknova), 500 μM thiamine hydrochloride, 0.2 % glucose (w/v), and 0.75 % tryptone (w/v) as appropriate. Where appropriate, media were supplemented with kanamycin (30 μg/mL), tetracycline (5 μg/mL), carbenicillin (100 μg/mL), and isopropyl-$\beta$-$_D$-thiogalactopyranoside (IPTG, 1 mM). The concentration of other supplements is indicated in the respective experiments.

Deletion alleles were obtained from the Keio collection (32) and transduced by P1*vir* transduction into the *E. coli* K-12 W3110 strain. Alleles were confirmed via PCR with primers up and downstream of the appropriate gene. Kan cassettes were removed with pCP20 as described previously (33). To delete the *oppBCDF* operon, DY330 recombineering was utilized as described previously (34), and the deletion allele was P1 transduced into W3110 strains. All additional methods are in *SI Appendix, Supporting Information Text*.

**Data, Materials, and Software Availability.** All study data are included in the article and/or supporting information.

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