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An Anhydro-*N*-Acetylmuramyl-L-Alanine Amidase with Broad Specificity Tethered to the Outer Membrane of *Escherichia coli*[▽]

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From its amino acid sequence homology with AmpD, we recognized YbjR, now renamed AmiD, as a possible second 1,6-anhydro-*N*-acetylmuramic acid (anhMurNAc)-L-alanine amidase in *Escherichia coli*. We have now confirmed that AmiD is an anhMurNAc-L-Ala amidase and demonstrated that AmpD and AmiD are the only enzymes present in *E. coli* that are able to cleave the anhMurNAc-L-Ala bond. The activity was present only in the outer membrane fraction obtained from an *ampD* mutant. In contrast to AmpD, which is specific for the anhMurNAc-L-alanine bond, AmiD also cleaved the bond between MurNAc and L-alanine in both muropeptides and murein sacculi. Unlike the periplasmic murein amidases, AmiD did not participate in cell separation. *ampG* mutants, which are unable to import GlcNAc-anhMurNAc-peptides into the cytoplasm, released mainly peptides into the medium due to AmiD activity, whereas an *ampG amiD* double mutant released a large amount of intact GlcNAc-anhMurNAc-peptides into the medium.

During growth, 40 to 50% of the *Escherichia coli* murein (peptidoglycan) is broken down and reused each generation. This is sufficient to provide 25% of the murein in the daughter cells. During the past 12 years, nine enzymes have been found to be specifically involved in the recycling process. The principal murein degradation product produced by lytic transglycosidases is *N*-acetylglucosaminyl-1,6-anhydro-*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-D-alanine (GlcNAc-anhMurNAc-L-Ala-D-Glu-Dap-D-Ala). It is imported via AmpG, a permease specific for GlcNAc-anhMurNAc and GlcNAc-anhMurNAc-peptides (anhydromuropeptides) (2). Once in the cytoplasm, anhydromuropeptides are further degraded into GlcNAc, anhMurNAc, murein tripeptide, and D-Ala by an anhMurNAc-L-Ala amidase, AmpD (13, 15); a β-*N*-acetylglucosaminidase, NagZ (1, 37); and an L,D-carboxypeptidase, LdcA (31). Murein tripeptide is directly ligated to UDP-MurNAc by Mpl, a UDP-MurNAc:L-Ala-γ-D-Glu-Dap ligase (21), thereby returning it to the pathway for synthesis of murein. GlcNAc and anhMurNAc are also made available for reuse by conversion to GlcNAc-6-phosphate, which can enter the glycolysis pathway or the pathway to generate UDP-GlcNAc, the main precursor of murein and lipopolysaccharide (28, 34–36). The GlcNAc derived from the murein sacculus is phosphorylated by NagK, a specific GlcNAc kinase, thus facilitating its utilization (34). The anhMurNAc is phosphorylated by an anhMurNAc kinase, AnmK, to generate MurNAc-6-phosphate (36), which is then converted to GlcNAc-6-phosphate by a MurNAc-6-phosphate etherase, MurQ (16, 35), thus allowing its utilization.

In cells lacking an enzyme involved in murein recycling, the substrate of the enzyme will accumulate in the cytoplasm unless another pathway exists to metabolize it or to export it to the medium. For example, anhMurNAc-tripeptide accumulates in cells lacking the AmpD anhMurNAc-L-Ala amidase (14), and GlcNAc-anhMurNAc accumulates in a mutant lacking the NagZ β-*N*-acetylglucosaminidase (1). However, only a small amount of murein tripeptide accumulates in an *mpl* mutant (21), leading us to suspect that an enzyme is present that degrades murein tripeptide and to identify MpaA, which cleaves the γ-D-Glu-Dap bond in the murein tripeptide (33). As expected, in an *mpl mpaA* double mutant, a very large amount of murein tripeptide accumulates (33). Surprisingly, in a mutant lacking NagK (GlcNAc kinase), GlcNAc does not accumulate, though in a *nagA* (GlcNAc-P deacetylase) mutant, both GlcNAc and GlcNAc phosphate accumulate, suggesting that another pathway to metabolize or to export free GlcNAc may be activated in a *nagK* mutant (34). In an *anmK* mutant, anhMurNAc accumulates in the medium instead of the cytoplasm, indicating that *E. coli* has an efflux pathway for anhMurNAc (36). As a final example of this phenomenon, in a *murQ* etherase mutant, the substrate, MurNAc phosphate, accumulates in the cytoplasm (35).

Surprisingly, in an *ampD nagZ* double mutant, in addition to the expected GlcNAc-anhMurNAc-tripeptide, a significant amount of the disaccharide, GlcNAc-anhMurNAc, accumulates (1), suggesting that another AmpD-like enzyme may exist in *E. coli.* This led us to identify and characterize the AmpD-like lipoprotein, AmiD, described here.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. TP71 [F⁻ *lysA opp araD139 rpsL150 deoC1 ptsF25 ftbB5301 rbsR relA1* Δ(*argF-lac*)] (14) was used as a parent strain. TP78B (*nagB*::Kan *nagZ*::Cm *ampD* mutant of TP71) (1) and TP72 (*ampG*::Kan mutant of TP71) (14) were constructed earlier. The *ampD* mutation in TP78B, which was derived from TP73 (14) and therefore was assumed to be Δ*ampDE*, was found to be a 1-bp deletion of the 509th nucleotide of the *ampD* open reading frame. This resulted in replacement of the C-terminal 14 amino acids of AmpD with an altered sequence of 29 amino acids. TP78BD (Δ*amiD* mutant of TP78B) and TP72D (Δ*amiD* and *ampG*::Kan mutant of TP71) were constructed as described below. The expression vector, pJG1, and a plasmid carrying the *amiD* (*ybjR*) open reading frame, pOCUS2-ybjR, were obtained from the *E. coli* Genome Project at the University of Wisconsin—Madison (http://www.genome

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.wisc.edu/index.htm). Plasmids pJG1AmiD, containing the *amiD* gene under the control of the T7 promoter, and pTrcAmiD, expressing AmiD from the P_{trc} promoter, were constructed as described below. Cultures were grown in LB broth at 37°C with aeration unless otherwise noted. To label murein, cells were grown in M9 minimal medium (29) containing 0.1% Casamino Acids, 1 mM $MgCl_2$, 1 μg of thiamine/ml, 200 μg of lysine/ml, and either 0.2% glucose or 0.6% glycerol as a carbon source, as indicated.

Construction of the *amiD* mutant. *amiD* was deleted as described previously (4), using primers 5′-*GCGTCAGCGGTACGGCGGTGAAAACGCGTCGATG AGAAGA***TGTAGGCTGGAGCTGCTTCG** and 5′-*CTGGCTACGCGACCACG GAAAACTGCCGCGCTAATCCTGCCC***ATGAATATCCTCCTTAGT** (italicized sequences are homologous to the *amiD*-flanking sequence, and boldfaced sequences are homologous to the pKD3 vector sequence flanking the Cm^r gene) and pKD3 as the template to synthesize the PCR product used for one-step inactivation. The Cm^r gene replacement was confirmed by PCR with primers 5′-CTGATGGTTAGCGTCAG and 5′-AAACTGCCGCGCTAATC. To construct TP78BD, the *amiD*::Cm mutation was first transduced into TP73 (*ampD* mutant of TP71), and the Cm^r cassette was removed by the FLP recombinase present in plasmid pCP20 as described elsewhere (4). This was followed by the transduction of *nagZ*::Cm (1) and *nagB*::Km (28) into the strain, to yield TP78BD. TP72D was constructed by the transduction of *amiD*::Cm into TP72. TP71amiAC (*amiA*::Cm *amiC*::Km mutant of TP71) was constructed by transduction of *amiA*::Cm and *amiC*::Km mutations (11). TP71amiACD (Δ*amiD amiA*::Cm *amiC*::Km mutant of TP71) was constructed by transduction of *amiD*::Cm and removal of the Cm^r cassette by the FLP recombinase, followed by transduction of the *amiA*::Cm and *amiC*::Km mutations (11). All transductions were performed with T4gt7 phage (22).

Measurement of murein breakdown products labeled with [6-^{3}H]GlcN or [^{3}H]Dap. Cells were labeled for 4 to 5 generations in the glycerol Casamino Acid minimal medium containing 1 μCi of [6-^{3}H]glucosamine ([6-^{3}H]GlcN) (21.6 Ci/mol; Perkin-Elmer, Waltham, MA)/ml as described previously (28) or in the glucose Casamino Acid minimal medium containing 1 μCi of [^{3}H]Dap (20 Ci/mmol; Moravek Biochemicals, Inc., Brea, CA)/ml as described previously (14). To label cells exclusively in their amino sugars with [6-^{3}H]GlcN, a *nagB* mutation was introduced into the strain. When cells were labeled with [^{3}H]Dap, *lysA* mutants were used to prevent the conversion of Dap to lysine. The amounts of radioactivity in the murein degradation products present in the cytoplasm of labeled cells were determined by analysis of hot-water extracts of cells by high-performance liquid chromatography (HPLC) as described below. When required, the spent medium was analyzed by HPLC.

HPLC analysis. The amounts of GlcNAc-anhMurNAc tripeptide and GlcNAc-anhMurNAc in hot-water extracts from TP78B and TP78BD were measured by HPLC as described previously (1, 33). All other samples were separated by a Waters Symmetry C_{18} reverse-phase column (4.6 by 150 mm; particle size, 5 μm; Waters Co., Milford, MA) employing a Waters 1525 binary HPLC pump, a Waters 2487 dual-wavelength absorbance detector, and Waters Breeze software (Waters Co., Milford, MA). Compounds were eluted at a flow rate of 0.5 ml/min with isocratic elution with solvent A for 10 min, followed by a linear gradient of 0 to 20% solvent B over a period of 70 min. Two solvent systems were used as indicated: either 0.1% formic acid (solvent A) and 0.1% formic acid in acetonitrile (solvent B) or 0.05% trifluoroacetic acid (solvent A) and 0.035% trifluoroacetic acid in acetonitrile (solvent B).

Preparation of substrates. The radioactive intermediates in the murein recycling pathway were purified by HPLC from hot-water extracts of mutants as described previously (36). [^{3}H]GlcNAc-[^{3}H]anhMurNAc was purified from *nagZ* mutant cells labeled with [6-^{3}H]GlcN. The [^{3}H]GlcNAc-anhMurNAc-tripeptide was from a *nagZ ampD* double mutant labeled with either [6-^{3}H]GlcN or [^{3}H]Dap. The [^{3}H]Dap-labeled anhMurNAc-tripeptide was prepared from the [^{3}H]Dap-labeled GlcNAc-anhMurNAc-tripeptide by treatment with ExoII, a β-*N*-acetylglucosaminidase from *Vibrio furnissii* purified as described elsewhere (3), followed by purification using HPLC (36).

anhMurNAc-L-Ala amidase assay. The radioactive GlcNAc-anhMurNAc-tripeptide was incubated with cellular fractions or purified AmiD at 37°C. Following heat inactivation, the substrate and the radioactive product were separated by HPLC. When the enzyme solution contained 1 mM EDTA, 2 mM $ZnCl_2$ was added to the reaction mixture.

Cellular fractionation of the periplasmic, membrane, and cytoplasmic proteins by using osmolysis. During fractionation steps, phenylmethylsulfonyl fluoride (PMSF) and EDTA were added to inhibit proteases, because otherwise AmiD might be detached from the membrane by cellular proteases. This actually occurred in the case of MltB, a lipoprotein that was originally reported to be a soluble lytic transglycosylase, Slt35 (5, 6). TP78B cells from 40 ml of culture in log phase were chilled, harvested, and resuspended in 4 ml of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) containing 20% sucrose, 10 μg/ml of lysozyme (freshly made, 10 mg/ml dissolved in water), and 1 mM PMSF (freshly made, 100 mM dissolved in dimethyl sulfoxide). The suspension was placed on ice for 30 min and centrifuged (at 3,000 × *g* for 5 min at 4°C). The supernatant contained the periplasmic fraction. The pellet was suspended in 4 ml of TE buffer and centrifuged (at 3,000 × *g* for 5 min at 4°C). Two milliliters of the supernatant was ultracentrifuged (at 200,000 × *g* for 60 min at 4°C) using a 100.3 rotor (Beckman Coulter, Fullerton, CA), and the pellet (membrane fraction) was resuspended in 0.2 ml of TE buffer. The supernatant was the cytoplasmic fraction.

Membrane fractionation by sucrose gradient centrifugation. Inner and outer membranes were separated as described previously (23, 27) with the following modification: TP78B/pTrcAmiD cells were grown at 37°C in 1 liter of LB broth containing 100 μg of ampicillin/ml and 0.2% glucose. When the culture reached late log phase, 50 μM isopropyl-β-D-thiogalactoside (IPTG) was added to half of the culture (500 ml) to induce AmiD. After an hour, cells in the induced and uninduced cultures were chilled, harvested, washed, resuspended in 5 ml of TE buffer containing 20% sucrose, and frozen. Cells thawed in the presence of 200 μg of PMSF/ml were broken by a French press at 15,000 lb/in^2 twice. Unbroken cells were removed by low-speed centrifugation. The suspension was centrifuged (at 200,000 × *g* for 2 h at 4°C), and the pellet (membrane fraction) was resuspended in 0.5 ml of TE buffer containing 20% sucrose. The membrane fraction was loaded on top of a 12-ml sucrose density gradient (30 to 60% [wt/wt] sucrose in TE buffer) formed by Gradient Master (Biocomp Instruments Inc., Brunswick, Canada), and then the tube was centrifuged at 200,000 × *g* for 42 h at 4°C in an SW41 rotor (Beckman Coulter, Fullerton, CA). Fractions of 1 ml were collected with a piston gradient fractionator (Biocomp Instruments Inc., Brunswick, Canada). Aliquots (20 μl) of the fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with staining with Coomassie brilliant blue (CBB) (29), and 0.1-μl aliquots were used for the amidase assay.

Construction of plasmids overexpressing AmiD. A pJG1AmiD plasmid was constructed by ligation of a SapI-digested 828-bp fragment of pOCUS2-ybjR into the SapI site of the pJG1 vector. The 872-bp HindIII-XbaI fragment of pJG1AmiD was cloned into the HindIII-XbaI-digested vector pTrc99a, generating pTrcAmiD. To express AmiD in the cytoplasm, a plasmid with AmiD containing hexahistidine instead of the signal sequence was constructed. The *amiD* DNA fragment amplified from pTrcAmiD by PCR using 5′-GAGGAAT TCGCAGGCGAAAAAGGCATTG and 5′-CGCAAGCTTTAATCCTGCCC was digested with EcoRI and HindIII and ligated into the EcoRI and HindIII sites of the pET28a vector (Promega, Madison, WI), generating plasmid pET28a-AmiD. The DNA approaches were based on methods described previously (29), and the XL1-Blue strain (Stratagene, La Jolla, CA) was used as a host for DNA cloning.

Overexpression and purification of AmiD. All purification steps were performed at 4°C or on ice unless otherwise noted. BL21(DE3) cells (Stratagene, La Jolla, CA) carrying plasmid pJG1AmiD were grown in 2 liters of LB broth supplemented with 100 μg ampicillin/ml and 0.2% glucose, and at late-log phase 1 mM IPTG was added to the culture. After 3 h of induction, cells were chilled, harvested, washed, resuspended in 20 ml of 50 mM Tris-HCl (pH 8.0) containing 0.3 M NaCl, and broken by sonication. Following low-speed centrifugation, the supernatant was centrifuged at 100,000 × *g* for 60 min. The pellet was washed with 50 mM Tris-HCl (pH 8.0) containing 1 M NaCl and ultracentrifuged twice. About 80% of the protein in the pellet was AmiD, as judged by SDS-PAGE and CBB staining. The pellet was resuspended by stirring overnight at 4°C in 10 ml of buffer containing 50 mM Tris-HCl (pH 8.0), 1 M NaCl, 2% Triton X-100, and 1 mM PMSF; then it was ultracentrifuged. In this step, while about one-half of the AmiD was solubilized together with other membrane proteins, the pellet fraction contained the other half of AmiD, which now represented 90% of the protein in the fraction. The pellet was resuspended again in the same buffer overnight and ultracentrifuged. In a final purification step, the sample was dialyzed against 20 mM Tris-HCl (pH 8.0) containing 1% Triton X-100 and was then loaded onto a MonoQ HR 5/5 column (Amersham Pharmacia, Uppsala, Sweden), followed by the elution of AmiD with a linear gradient of 0 to 0.5 M NaCl. AmiD was eluted at around 100 mM NaCl in 20 mM Tris-HCl (pH 8.0) containing 1% Triton X-100. Purified AmiD was stable at −80°C for at least 2 years.

His-tagged AmiD was overexpressed in BL21(DE3)/pET28a-AmiD cells with 1 mM IPTG for 3 h and purified from the cell extract with a HiTrap chelating HP column (1 ml) (Amersham Pharmacia, Uppsala, Sweden) by following the manufacturer's instructions.

Digestion of murein by AmiD. *E. coli* sacculi were purified from log-phase cells by the method described by Glauner (8). Two hundred fifty micrograms of sacculi (murein) was incubated with 2.5 μg of purified AmiD in 50 μl of 10 mM HEPES-NaOH (pH 7.0) at 37°C overnight. Following centrifugation (at 14,000 × *g*

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AmpD  MLLEQGWLVGARRVPSPHYDCRPDDETPTLLVVHNISLPPGEFGGPWIDALFTGTIDPQA 60
YbjR  -MRRFFWLVAAALLLA---GCAGEKGIVEKEGYQLDTRRQAQAAYPRIKVLVIHYTADDF 56
       :   *** *  : :    *  :          :  :    :   * *  *        :

AmpD  HPFFAEIAHLRVSAHCLI-----RRDGE--IVQYVPFDKRAWHAGVSQYQGRERCNDFSI 113
YbjR  DSSLATLTDKQVSSHYLVPAVPPRYNGKPRIWQLVPEQELAWHAGISAWRGATRLNDTSI 116
         :* ::  :**:* *:     * :*:  * * ** :: *****:* ::*  * ** **

AmpD  GIELE---------GTDTLA-YTDAQYQQLAAVTRALIDCYPDIAKNMTGHCDIAPDRKTD 164
YbjR  GIELENRGWQKSAGVKYFAPFEPAQIQALIPLAKDIIARYHIKPENVVAHADIAPQRKDD 176
      *****         *   :* :  ** * *  ::: :*  *    :*:  * ****:** *

AmpD  PGPAFDWARF----------------------------------------RVLVSKETT-- 183
YbjR  PGPLFPWQQLAQQGIGAWPDAQRVNFYLAGRAPHTPVDTASLLELLARYGYDVKPDMTPR 236
      *** * * ::                                          *  : *

AmpD  ------------------------------------------
YbjR  EQRRVIMAFQMHFRPTLYNGEADAETQAIAEALLEKYGQD 276
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FIG. 1. Alignment of the amino acid sequences of the AmpD amidase and YbjR (AmiD) from *Escherichia coli* by the T-Coffee program (25). Identical amino acids and conserved substitutions are indicated by asterisks and colons, respectively. Dashes indicate a gap in the sequence. Amino acid numbers for both proteins are given on the right. The residues of the zinc-binding triad His^{34}-His^{154}-Asp^{164} of AmpD are shaded, and the lipobox sequence ($L^{14}AGC^{17}$) of YbjR is underlined.

for 10 min at room temperature), the soluble components were separated by HPLC with the trifluoroacetic acid solvent system. The products were detected by their UV absorbances at 206 nm. The three main fractions were recovered, lyophilized, and identified by mass spectrometry with a Voyager DE Pro matrix-assisted laser desorption ionization—time-of-flight (MALDI-TOF) mass spectrometer (Applied Biosystems, Foster City, CA) at the Tufts Core Facility.

RESULTS

Identification of YbjR as a possible anhMurNAc-L-Ala amidase. Since the accumulation of GlcNAc-anhMurNAc in the *ampD nagZ* double mutant suggested that *E. coli* had a second anhMurNAc-L-Ala amidase in its cytoplasm, we searched for a protein similar to AmpD in the *E. coli* genome by NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). A protein called YbjR (b0867) was identified. As shown in Fig. 1, YbjR is a 276-amino-acid protein that includes a 123-amino-acid sequence (amino acids 61 to 183) with 40% identity to amino acid sequence 65 to 171 of AmpD. Two of the three components of the zinc-binding triad essential for AmpD activity (7, 19) appear to be conserved in YbjR. We have given YbjR the name AmiD to distinguish it from the three periplasmic murein amidases (11).

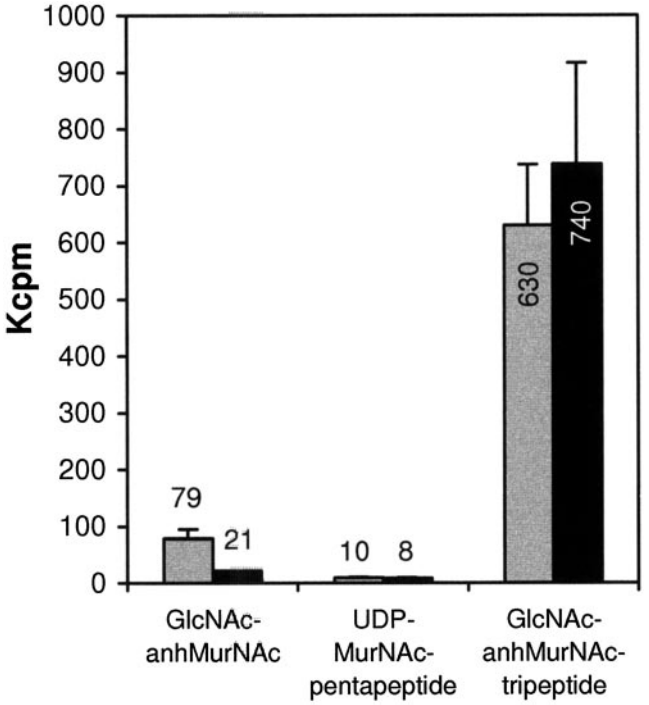

FIG. 2. Effect of AmiD on the GlcNAc-anhMurNAc content of *ampD nagZ* cells. Shaded bars, the $amiD^+$ strain TP78B (*nagB nagZ ampD*); solid bars, the *amiD* deletion mutant TP78BD (*nagB nagZ ampD amiD*). Cells were labeled with [6-^{3}H]GlcN in 4 ml of M9 glycerol minimal medium, and hot-water extracts were analyzed by HPLC. The amounts of radioactivity in the fractions corresponding to GlcNAc-anhMurNAc, UDP-MurNAc-pentapeptide, and GlcNAc-anhMurNAc-tripepide were measured and adjusted to equal cell populations. Values are averages from two separate experiments.

Effect of an *amiD* mutation on the level of GlcNAc-anhMurNAc in the cytoplasm of the *ampD nagZ* mutant. To examine whether AmiD plays a role of the accumulation of GlcNAc-anhMurNAc disaccharide in a *nagZ ampD* mutant, a strain lacking NagZ, AmpD, and AmiD was examined. Note that the *amiD*-null mutation itself did not cause any defect in growth or cell shape under the conditions tested. Hot-water extracts of cells growing at mid-log phase in the presence of 1 μCi of [6-^{3}H]GlcN/ml were analyzed by HPLC. As shown in Fig. 2, TP78BD (*nagB nagZ ampD amiD*) contained one-fourth as much GlcNAc-anhMurNAc as TP78B (*nagB nagZ ampD*), indicating that the formation of GlcNAc-anhMurNAc in cells lacking AmpD is mainly due to AmiD. However, TP78BD still contained a small amount of GlcNAc-anhMurNAc, raising the possibility that *E. coli* may have a third anhMurNAc-L-Ala amidase. To test whether the *ampD amiD* double mutant has an activity to

TABLE 1. anhMurNAc-L-Ala amidase activity in various cell fractions

Strain	Relevant mutation(s)	Fraction[a]	% Cleaved[b]
TP78B	*ampD*	Cell extract	22
		Periplasm	0
		Cytoplasm	0
		Membrane	54
TP78BD	*ampD amiD*	Cell extract	0

[a] Fractions were separated from TP78B (*ampD nagZ* mutant of TP71) cells as described in Materials and Methods.

[b] The fractions separated from cells equivalent to 0.9 ml culture at late-log phase were incubated with 5,000 cpm of [^{3}H]Dap-labeled GlcNAc-anhMurNAc-tripeptide at 37°C overnight in 50 mM HEPES-NaOH (pH 7.0)–1 mM $ZnCl_2$, followed by HPLC analysis using the trifluoroacetic acid solvent system. The amidase activity is shown as the percentage of tripeptide released from GlcNAc-anhMurNAc-tripeptide.

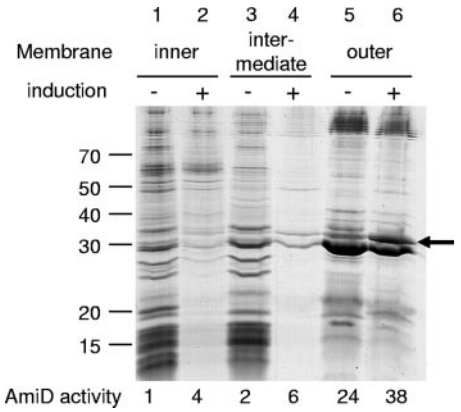

FIG. 3. Subcellular location of AmiD. Membranes of TP71/pTrcAmiD cells before (lanes 1, 3, and 5) and after (lanes 2, 4, and 6) induction of AmiD for 1 h following the addition of 50 μM IPTG were separated by sucrose gradient centrifugation into inner membrane (lanes 1 and 2), intermediate membrane (lanes 3 and 4), and outer membrane (lanes 5 and 6) fractions. The samples were fractionated by SDS-PAGE and stained with CBB. Arrow indicates AmiD protein. Each value below the gel is the percentage of [^{3}H]GlcN-labeled GlcNAc-anhMurNAc-tripeptide (1,000 cpm) cleaved by 1/200 of the fraction loaded on the SDS-PAGE gel and was determined by incubation with the substrate at 37°C for 30 min, followed by heat inactivation and thin-layer chromatographic analysis.

cleave anhydromuropeptides, whole-cell extracts consisting of cytoplasm, periplasm, and envelopes from TP78B and TP78BD were incubated overnight at 37°C with [^{3}H]Dap-labeled GlcNAc-anhMurNAc-tripeptide, followed by analysis using HPLC. As shown in Table 1, the whole-cell extract from TP78BD totally lacked activity to release the tripeptide from the substrate, while that from TP78B cleaved 22% of the substrate. These results indicate that AmiD is the only anhMurNAc-L-Ala amidase in the *ampD* mutant.

Cellular location of AmiD. Surprisingly, the N terminus of AmiD contains a typical, though relatively short, signal sequence followed by a lipobox motif (L_{14}AGC) (Fig. 1) (39), suggesting that AmiD is a lipoprotein. In fact, it has been verified that YbjR, i.e., AmiD, is a lipoprotein (17). This conflicted with our expectation that a second anhMurNAc-L-Ala amidase existed in the cytoplasm. Thus, the location of AmiD was examined by measuring the anhMurNAc-L-Ala amidase activity in cellular fractions of Δ*ampD* cells in which AmiD was the only active anhMurNAc-L-Ala amidase. As shown in Table 1, the amidase activity was present only in the membrane fraction.

It is known that the amino acid next to the cysteine that carries diacylglycerol determines whether a lipoprotein resides in the inner or outer membrane (30, 32). Since the amino acid following the lone cysteine in AmiD is alanine, AmiD was expected to be transferred to the outer membrane. To establish this, the inner and outer membranes of *ampD* cells overexpressing AmiD were separated by sucrose gradient centrifugation. The majority of the overproduced protein was present in the outer membrane fraction (Fig. 3), and most AmiD amidase activity was present in the outer membrane, indicating that AmiD is indeed located in the outer membrane.

Inhibition of growth by overexpression of AmiD. When AmiD was overexpressed from the pTrcAmiD plasmid by the addition of 100 μM IPTG to growing cells of a wild-type strain,

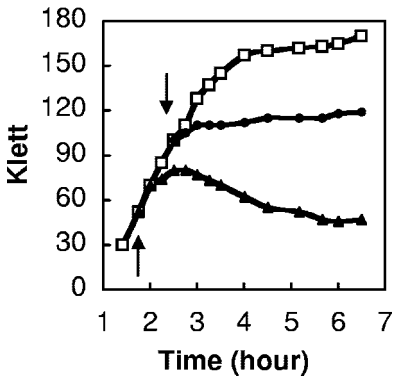

FIG. 4. Overexpression of AmiD inhibits growth. TP71 carrying pTrcAmiD was grown at 37°C in LB broth containing 100 μg ampicillin/ml and 0.2% glucose. IPTG (100 μM) was added at a turbidity of 52 Klett units (filled triangles) or 100 Klett units (filled circles), as indicated by the arrows. Open squares, control culture.

TP71, growth stopped in 90 min, and then the turbidity decreased gradually (Fig. 4). Cells in late-log phase ceased growth soon after addition of IPTG (Fig. 4). In contrast, TP71 carrying a control plasmid, pTrc99a, showed no growth defect in the absence or presence of 100 μM IPTG (data not shown).

AmiD is not involved in cell separation. The periplasmic murein amidases, AmiA, AmiB, and AmiC, have been shown to be involved in cell separation (11, 38). Mutants of each of these amidases form short chains of cells, and the length of chains and the frequency of chain formation increase when two or more *ami* genes are deleted (11). However, the *amiD*-null mutation neither caused any morphological defect, such as chain formation, nor exacerbated the phenotype of an *amiA* mutant, an *amiC* mutant, or an *amiA amiC* double mutant (data not shown). These results indicate that AmiD is not involved in cell separation.

Characterization of AmiD activity. Since AmpD is highly specific for the anhMurNAc-L-Ala bond (15), one would predict that AmiD might be equally specific because of the homology between them. To test this, AmiD was overproduced and purified from the membrane fraction. Two hundred fifty

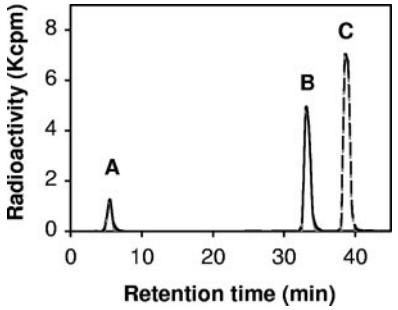

FIG. 5. HPLC analysis of GlcNAc-anhMurNAc-L-Ala-D-Glu-Dap before (dashed line) and after (solid line) digestion with AmiD. A mixture of 11 kcpm of [^{3}H]GlcNAc-[^{3}H]anhMurNAc-tripeptide and 2.5 kcpm of GlcNAc-anhMurNAc-L-Ala-D-Glu-[^{3}H]Dap was incubated with 0.25 μg of purified AmiD at 37°C for 30 min and separated by HPLC with the formic acid solvent system. Fraction A, L-Ala-D-Glu-Dap; fraction B, GlcNAc-anhMurNAc; fraction C, GlcNAc-anhMurNAc-L-Ala-D-Glu-Dap.

TABLE 2. AmiD prefers a substrate containing GlcNAc-anhMurNAc[a]

Substrate	Amt of AmiD (ng)	% Cleaved
GlcNAc-anhMurNAc-tripeptide	5	83
anhMurNAc-tripeptide	5	2
	25	21
	500	94
UDP-MurNAc-pentapeptide	500	0

[a] [^{3}H]Dap-labeled substrates were incubated with purified AmiD in 10 μl of 10 mM Tris-HCl buffer (pH 7.5) for 30 min at 37°C. Breakdown products were separated by HPLC.

nanograms of purified AmiD completely cleaved 13.5 kcpm of [^{3}H]GlcNAc-[^{3}H]anhMurNAc-L-Ala-D-Glu-[^{3}H]Dap in 30 min at 37°C to release the tripeptide L-Ala-D-Glu-[^{3}H]Dap and [^{3}H]GlcNAc-[^{3}H]anhMurNAc (Fig. 5).

To examine whether AmiD, like AmpD, requires Zn^{2+} for its activity, the effect of a specific Zn^{2+} chelator, 1,10-phenanthroline, was studied. Preincubation of AmiD with 5 mM 1,10-phenanthroline inhibited AmiD activity by >98%. Treatment of AmiD with 1 mM 1,10-phenanthroline decreased AmiD activity fivefold, and addition of 5 mM $ZnCl_2$ to the reaction mixture largely restored AmiD activity. We conclude that AmiD requires Zn^{2+} for its activity. AmiD was fully active at pH 6 and pH 7, less active at pH 4.5 and pH 9, and inactive at pH 10.5 (data not shown). As shown in Table 2, anhMurNAc-tripeptide was a poor substrate for AmiD, requiring almost 100-fold more enzyme than was required for the digestion of GlcNAc-anhMurNAc-tripeptide. UDP-MurNAc-pentapeptide was not cleaved by 500 ng of AmiD (Table 2). Thus, AmiD prefers a substrate containing GlcNAc-anhMurNAc over a substrate containing only anhMurNAc, while AmpD cleaves both substrates at about the same rate (15).

Surprisingly, we have found significant differences in substrate specificities between AmiD and AmpD. When muropeptides from mutanolysin-digested murein were incubated with AmiD at 37°C overnight and the digest was analyzed by HPLC following reduction with sodium borohydride, all peaks corresponding to muropeptides were absent (Fig. 6), indicating that AmiD cleaved MurNAc peptide bonds as well as anhMurNAc-peptide bonds. When murein digested by AmiD was analyzed by HPLC, free murein tripeptide, tetrapeptide, and tetra-tetra-cross-linked peptide were the principal products identified (Fig. 7). These results demonstrate that AmiD cleaves the MurNAc-L-Ala bond in muropeptides and intact murein.

We have constructed a plasmid that expresses in the cytoplasm AmiD whose signal sequence is replaced with a hexahistidine tag. The purified His-tagged AmiD was active on murein and GlcNAc-anhMurNAc-tripeptide (data not shown), indicating that AmiD folds properly in the cytoplasm.

Does AmiD play a significant role in the metabolism of the murein sacculus or the anhydromuropeptides released during growth? We have shown that AmiD is present in the outer membrane and cleaves the bond between *N*-acetylmuramic acid and L-Ala in intact sacculi, in muropeptides, and in anhydromuropeptides. The question arises whether AmiD has a demonstrable effect in growing cells. In order to address this question, we compared the effect of an *amiD* deletion in our standard strain, TP71, and in TP72, a strain lacking the AmpG permease. Cultures were grown in the presence of [^{3}H]Dap for about 5 generations. Comparison of the spent medium of the *ampG* strain with that of an *ampG amiD* strain by HPLC revealed that GlcNAc-anhMurNAc-tripeptide and GlcNAc-anhMurNAc-tetrapeptide accumulated in the medium when AmiD was absent from the *ampG* strain (Fig. 8). In contrast, when AmiD was present, the anhydromuropeptides were cleaved by AmiD amidase and free tetrapeptide accumulated in the medium. We were unable to measure the amount of free tripeptide in the spent medium, because the HPLC method did not resolve the relatively small amount of tripeptide from the huge amount of radioactive Dap present. The effect of AmiD in the *ampG*-positive cells was small: the amount of free tetrapeptide in the medium was reduced only twofold by the *amiD* mutation (Fig. 8). This indicates that the anhydromuropeptides are swept into the cytoplasm rapidly through the AmpG permease.

DISCUSSION

We have identified AmiD (YbjR) as a second anhMurNAc-L-Ala amidase in *E. coli* and demonstrated that the GlcNAc-anhMurNAc accumulating in an *ampD nagZ* mutant was produced mainly by degradation of GlcNAc-anhMurNAc-peptides in the periplasm by AmiD, followed by uptake of GlcNAc-anhMurNAc via the AmpG permease.

The small amount of GlcNAc-anhMurNAc generated by cells lacking AmiD, AmpD, and NagZ had led us to speculate that *E. coli* has another anhMurNAc-L-Ala amidase. In fact, *E. coli* has three periplasmic murein amidases, AmiA, AmiB, and AmiC, which cleave the MurNAc-L-Ala bonds of peptidoglycan (11). AmiA, AmiB, and AmiC are similar to each other but not to either AmiD or AmpD. Since the cell extract from the *amiD ampD* double mutant completely lacked the activity to cleave the anhMurNAc-L-Ala bond (Table 1), AmiD and AmpD were the only enzymes present in *E. coli* that cleave the anhMurNAc-L-Ala bond. Hence, AmiA, AmiB, and AmiC must not cleave the anhMurNAc-L-Ala bond under the condi-

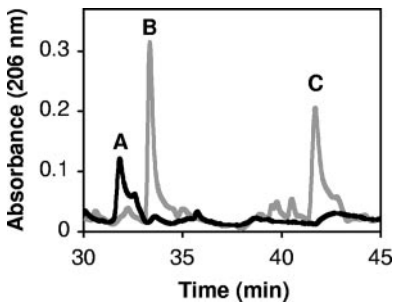

FIG. 6. Cleavage of muropeptides by AmiD. Purified sacculi were first digested with 10 U of mutanolysin (Sigma, St. Louis, MO) at 37°C overnight in 10 mM morpholineethanesulfonic acid-NaOH (pH 6.0), followed by incubation with (black line) or without (gray line) 5 μg of AmiD in 50 mM HEPES-NaOH (pH 7.0) overnight. The samples were reduced with sodium borohydride and separated by HPLC with the trifluoroacetic acid solvent system. Based on the elution time, peak A corresponds to cross-linked tetra-tetrapeptide; peak B, GlcNAc-MurNAc tetrapeptide; peak C, cross-linked GlcNAc-MurNAc tetrapeptide-tetrapeptide-MurNAc-GlcNAc.

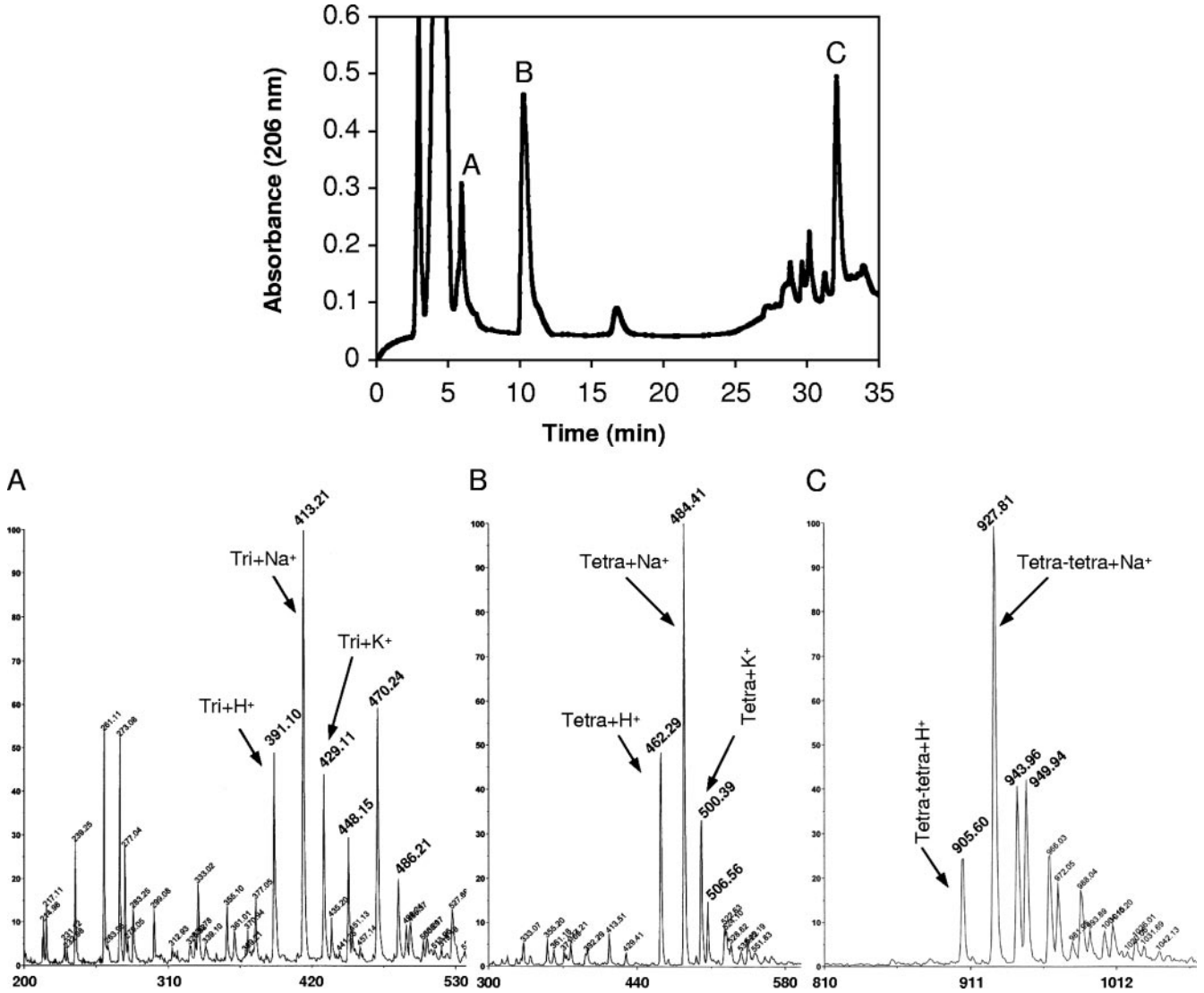

FIG. 7. Release of peptides from murein by AmiD. A 0.2-mg portion of purified murein digested by purified AmiD was separated by HPLC with the trifluoroacetic acid solvent system as described in Materials and Methods. Compounds in the three main peaks (A, B, and C) were collected, lyophilized, and analyzed by MALDI-TOF mass spectrometry. Fraction A, L-Ala-D-Glu-Dap (*m*/*z* 391) tripeptide; fraction B, L-Ala-D-Glu-Dap-D-Ala (*m*/*z* 462) tetrapeptide; fraction C, tetra-tetrapeptide (*m*/*z* 905).

tions tested. The expression levels of AmiA, AmiB, and AmiC amidases in the *ampD amiD* mutant should be similar to those of its parent cells, because the double mutant did not produce any chained cells, which *amiA*, *amiB*, or *amiC* mutants produce (11). Probably the 1,6-anhydro bond in the MurNAc moiety interferes with the activities of these amidases. Since major peptidoglycan glycosyl hydrolases of *E. coli* are lytic transglycosylases that form GlcNAc-anhMurNAc-peptides (12), the GlcNAc-anhMurNAc accumulating in an *ampD amiD nagZ* mutant must be derived from the cleavage of MurNAc-L-Ala bonds of intact murein by the periplasmic amidases, followed by the action of lytic transglycosylases, such as Slt70 (12).

Like AmpD, purified AmiD cleaved the anhMurNAc-L-Ala bond and required Zn^{2+} for its activity. Unlike AmpD, AmiD preferred a substrate containing GlcNAc-anhMurNAc to a substrate containing only anhMurNAc. Interestingly, AmiD also cleaved MurNAc-L-Ala bonds in murein. The crystal structure of AmiD from *E. coli* is available at the RCSB Protein Data Bank (http://www.rcsb.org/pdb/), though it is not published. The AmiD structure (PDB IDs, 2BGX and 2BH7) contains a zinc ion bound to a triad (His^{50}-His^{166}-Asp^{176}) of AmiD, two residues of which were aligned with the zinc triad of AmpD shown in Fig. 1. The broad substrate specificity of AmiD indicates that the active site of AmiD must be much more accessible than that of AmpD. However, both zinc ions of the active sites of the crystal structure of AmiD and the nuclear magnetic resonance structure of AmpD (19) appear to be surface exposed. Hence, more crystallographic and biochemical studies are required to explain the difference in the substrate specificities of AmpD and AmiD. The amino acid alignment of AmpD and AmiD (Fig. 1) also shows that AmiD has an extra C-terminal tail consisting of about 100 amino

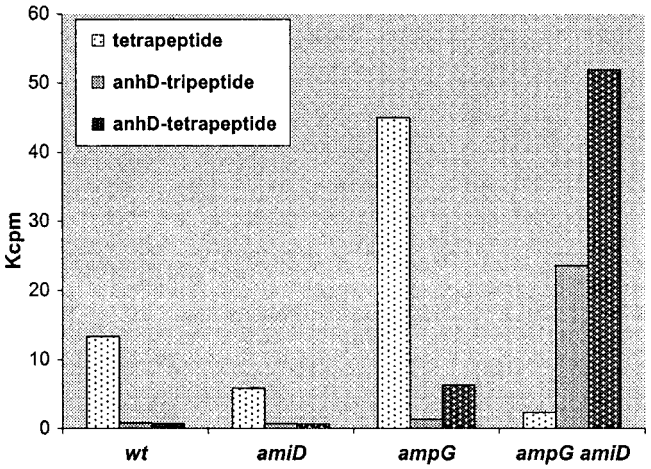

FIG. 8. Amounts of Dap-containing compounds in the spent medium from cultures growing for about 5 generations in 2 ml of M9 minimal medium containing 1 μCi of [^{3}H]Dap/ml. The medium was lyophilized and analyzed by HPLC to measure the amount of each compound. wt, wild type; anhD, GlcNAc-anhMurNAc.

acids, which weakly resembles a peptidoglycan-binding sequence. In the crystal structure of AmiD, it appears to form a domain distant from the zinc ion of the active center.

We have also shown that AmiD exists in the outer membrane. Considering the facts that the signal sequence of AmiD has the features of an outer membrane lipoprotein and that AmiD lacking its signal sequence forms a soluble, active enzyme in the cytoplasm, it was not unexpected that AmiD proved to be tethered to the outer membrane. Unpublished experiments mentioned in reference 17 have also shown that YbjR (AmiD) is a lipoprotein.

AmiD was shown to be active in growing cells by the demonstration that *nagZ* cells lacking both AmpD and AmiD contained more GlcNAc-anhMurNAc-tripeptide than *nagZ* cells lacking only AmpD (Fig. 2). Overexpression of AmiD inhibited cell growth, followed by a slow loss of turbidity which may be the result of AmiD hydrolytic activity on peptidoglycan. However, another reason could be that the *amiD* gene has tandem rare arginine codons, AGA_AGA, as its second and third codons. When overexpressed, an mRNA with a pair of the rare codons near the initiation codon is known to interfere with protein synthesis and hence to inhibit cell growth (26, 40).

Though AmiD has the same activity on murein as the periplasmic amidases involved in cell separation, AmiD did not participate in cell separation. This may be because AmiD is attached to the outer membrane, which may prevent the movement of AmiD to the septum.

It is known that during growth *E. coli* releases the murein peptides L-Ala-D-Glu-Dap-D-Ala, L-Ala-D-Glu-Dap, and Dap-D-Ala to the medium (10). We also observed the release of the tetrapeptide to the medium (Fig. 8). When AmpG permease is absent, the anhydromuropeptides derived from murein accumulate in the periplasm rather than being returned to the cytoplasm for recycling. Under these circumstances, AmiD is able to cleave most of the anhydromuropeptides in the periplasm. Though one might predict that the accumulation of the murein breakdown products in the periplasm would induce AmiD expression, the AmiD activity in the *ampG* mutant was the same as that in its parent strain (data not shown). Surprisingly, in *ampG* cells lacking AmiD, huge amounts of the uncleaved anhydromuropeptides, with molecular weights of 850 (GlcNAc-anhMurNAc-tripeptide) and 921 (GlcNAc-anhMurNAc-tetrapeptide), were released into the medium. This suggests that even compounds that exceed the "exclusion limit" of 600 Da (24) can cross the outer membrane if the concentration gradient is large. However, the release of GlcNAc-anhMurNAc-peptides from *Bordetella pertussis* (9), *Neisseria gonorrhoeae* (20), and *Vibrio fischeri* (18) in amounts sufficient to destroy ciliated epithelial cells illustrates that significant release occurs even in bacteria possessing orthologs of AmpG. Peptidoglycan degradation products also induce innate immune responses (3a). The peptidoglycan recycling pathway starting with the import of the breakdown products via AmpG permease into the cytoplasm could be a primary bacterial strategy to avoid innate immune responses in these environments. AmiD may function as a secondary bacterial strategy to avoid innate immune responses in these environments by degrading the bioactive compounds in the periplasm.

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REFERENCES

1. **Cheng, Q., H. Li, K. Merdek, and J. Park.** 2000. Molecular characterization of the β-*N*-acetylglucosaminidase of *Escherichia coli* and its role in cell wall recycling. J. Bacteriol. **182:**4836–4840.
2. **Cheng, Q., and J. Park.** 2002. Substrate specificity of the AmpG permease required for recycling of cell wall anhydro-muropeptides. J. Bacteriol. **184:**6434–6436.
3. **Chitlaru, E., and S. Roseman.** 1996. Molecular cloning and characterization of a novel β-*N*-acetyl-D-glucosaminidase from *Vibrio furnissii*. J. Biol. Chem. **271:**33433–33439.

3a. **Cloud-Hansen, K. A., S. B. Peterson, E. V. Stabb, W. E. Goldman, M. J. McFall-Ngai, and J. Handelsman.** 2006. Breaching the great wall: peptidoglycan and microbial interactions. Nat. Rev. Microbiol. **4:**710–716.

4. **Datsenko, K., and B. Wanner.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA **97:**6640–6645.
5. **Ehlert, K., J. Höltje, and M. Templin.** 1995. Cloning and expression of a murein hydrolase lipoprotein from *Escherichia coli*. Mol. Microbiol. **16:**761–768.
6. **Engel, H., A. Smink, L. van Wijngaarden, and W. Keck.** 1992. Murein-metabolizing enzymes from *Escherichia coli*: existence of a second lytic transglycosylase. J. Bacteriol. **174:**6394–6403.
7. **Généreux, C., D. Dehareng, B. Devreese, J. Van Beeumen, J. Frère, and B. Joris.** 2004. Mutational analysis of the catalytic centre of the *Citrobacter freundii* AmpD *N*-acetylmuramyl-L-alanine amidase. Biochem. J. **377:**111–120.
8. **Glauner, B.** 1988. Separation and quantification of muropeptides with high-performance liquid chromatography. Anal. Biochem. **172:**451–464.
9. **Goldman, W. E., D. G. Klapper, and J. B. Baseman.** 1982. Detection, isolation, and analysis of a released *Bordetella pertussis* product toxic to cultured tracheal cells. Infect. Immun. **36:**782–794.
10. **Goodell, E., and U. Schwarz.** 1985. Release of cell wall peptides into culture medium by exponentially growing *Escherichia coli*. J. Bacteriol. **162:**391–397.
11. **Heidrich, C., M. Templin, A. Ursinus, M. Merdanovic, J. Berger, H. Schwarz, M. de Pedro, and J. Höltje.** 2001. Involvement of *N*-acetylmuramyl-L-alanine amidases in cell separation and antibiotic-induced autolysis of *Escherichia coli*. Mol. Microbiol. **41:**167–178.
12. **Höltje, J.** 1998. Growth of the stress-bearing and shape-maintaining murein sacculus of *Escherichia coli*. Microbiol. Mol. Biol. Rev. **62:**181–203.
13. **Höltje, J., U. Kopp, A. Ursinus, and B. Wiedemann.** 1994. The negative regulator of β-lactamase induction AmpD is a *N*-acetyl-anhydromuramyl-L-alanine amidase. FEMS Microbiol. Lett. **122:**159–164.
14. **Jacobs, C., L. Huang, E. Bartowsky, S. Normark, and J. Park.** 1994. Bacterial cell wall recycling provides cytosolic muropeptides as effectors for β-lactamase induction. EMBO J. **13:**4684–4694.

15. **Jacobs, C., B. Joris, M. Jamin, K. Klarsov, J. Van Beeumen, D. Mengin-Lecreulx, J. van Heijenoort, J. Park, S. Normark, and J. Frère.** 1995. AmpD, essential for both β-lactamase regulation and cell wall recycling, is a novel cytosolic *N*-acetylmuramyl-L-alanine amidase. Mol. Microbiol. **15:**553–559.
16. **Jaeger, T., M. Arsic, and C. Mayer.** 2005. Scission of the lactyl ether bond of *N*-acetylmuramic acid by *Escherichia coli* "etherase." J. Biol. Chem. **280:** 30100–30106.
17. **Juncker, A., H. Willenbrock, G. Von Heijne, S. Brunak, H. Nielsen, and A. Krogh.** 2003. Prediction of lipoprotein signal peptides in Gram-negative bacteria. Protein Sci. **12:**1652–1662.
18. **Koropatnick, T. A., J. T. Engle, M. A. Apicella, E. V. Stabb, W. E. Goldman, and M. J. McFall-Ngai.** 2004. Microbial factor-mediated development in a host-bacterial mutualism. Science **306:**1186–1188.
19. **Liepinsh, E., C. Généreux, D. Dehareng, B. Joris, and G. Otting.** 2003. NMR structure of *Citrobacter freundii* AmpD, comparison with bacteriophage T7 lysozyme and homology with PGRP domains. J. Mol. Biol. **327:**833–842.
20. **Melly, M. A., Z. A. McGee, and R. S. Rosenthal.** 1984. Ability of monomeric peptidoglycan fragments from *Neisseria gonorrhoeae* to damage human fallopian-tube mucosa. J. Infect. Dis. **149:**378–386.
21. **Mengin-Lecreulx, D., J. van Heijenoort, and J. Park.** 1996. Identification of the *mpl* gene encoding UDP-*N*-acetylmuramate: L-alanyl-γ-D-glutamyl-*meso*-diaminopimelate ligase in *Escherichia coli* and its role in recycling of cell wall peptidoglycan. J. Bacteriol. **178:**5347–5352.
22. **Miller, J. H.** 1992. A short course in bacterial genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
23. **Nikaido, H.** 1994. Isolation of outer membranes. Methods Enzymol. **235:** 225–234.
24. **Nikaido, H.** 1996. Outer membrane, p. 29–47. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed., vol. 2. American Society for Microbiology, Washington, DC.
25. **Notredame, C., D. Higgins, and J. Heringa.** 2000. T-Coffee: a novel method for fast and accurate multiple sequence alignment. J. Mol. Biol. **302:**205–217.
26. **Olivares-Trejo, J., J. Bueno-Martínez, G. Guarneros, and J. Hernández-Sánchez.** 2003. The pair of arginine codons AGA AGG close to the initiation codon of the lambda *int* gene inhibits cell growth and protein synthesis by accumulating peptidyl-tRNA^{Arg4}. Mol. Microbiol. **49:**1043–1049.
27. **Osborn, M., J. Gander, E. Parisi, and J. Carson.** 1972. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. Isolation and characterization of cytoplasmic and outer membrane. J. Biol. Chem. **247:** 3962–3972.
28. **Park, J.** 2001. Identification of a dedicated recycling pathway for anhydro-*N*-acetylmuramic acid and *N*-acetylglucosamine derived from *Escherichia coli* cell wall murein. J. Bacteriol. **183:**3842–3847.
29. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
30. **Seydel, A., P. Gounon, and A. Pugsley.** 1999. Testing the '+2 rule' for lipoprotein sorting in the *Escherichia coli* cell envelope with a new genetic selection. Mol. Microbiol. **34:**810–821.
31. **Templin, M., A. Ursinus, and J. Höltje.** 1999. A defect in cell wall recycling triggers autolysis during the stationary growth phase of *Escherichia coli*. EMBO J. **18:**4108–4117.
32. **Tokuda, H., and S. Matsuyama.** 2004. Sorting of lipoproteins to the outer membrane in *E. coli*. Biochim. Biophys. Acta **1693:**5–13.
33. **Uehara, T., and J. Park.** 2003. Identification of MpaA, an amidase in *Escherichia coli* that hydrolyzes the γ-D-glutamyl-*meso*-diaminopimelate bond in murein peptides. J. Bacteriol. **185:**679–682.
34. **Uehara, T., and J. Park.** 2004. The *N*-acetyl-D-glucosamine kinase of *Escherichia coli* and its role in murein recycling. J. Bacteriol. **186:**7273–7279.
35. **Uehara, T., K. Suefuji, T. Jaeger, C. Mayer, and J. Park.** 2006. MurQ etherase is required by *Escherichia coli* in order to metabolize anhydro-*N*-acetylmuramic acid obtained either from the environment or from its own cell wall. J. Bacteriol. **188:**1660–1662.
36. **Uehara, T., K. Suefuji, N. Valbuena, B. Meehan, M. Donegan, and J. Park.** 2005. Recycling of the anhydro-*N*-acetylmuramic acid derived from cell wall murein involves a two-step conversion to *N*-acetylglucosamine-phosphate. J. Bacteriol. **187:**3643–3649.
37. **Vötsch, W., and M. Templin.** 2000. Characterization of a β-*N*-acetylglucosaminidase of *Escherichia coli* and elucidation of its role in muropeptide recycling and β-lactamase induction. J. Biol. Chem. **275:**39032–39038.
38. **Wolf-Watz, H., and S. Normark.** 1976. Evidence for a role of *N*-acetylmuramyl-L-alanine amidase in septum separation in *Escherichia coli*. J. Bacteriol. **128:**580–586.
39. **Wu, H.** 1996. Biosynthesis of lipoprotein, p. 1005–1014. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed., vol. 2. American Society for Microbiology, Washington, DC.
40. **Zahn, K.** 1996. Overexpression of an mRNA dependent on rare codons inhibits protein synthesis and cell growth. J. Bacteriol. **178:**2926–2933.