Role of N-Acetylglucosaminidase and N-Acetylmuramidase Activities in Enterococcus faecalis Peptidoglycan Metabolism

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Identification of the full complement of peptidoglycan hydrolases detected by zymogram in Enterococcus faecalis extracts led to the characterization of two novel hydrolases that we named AtlB and AtlC. Both enzymes have a similar modular organization comprising a central catalytic domain fused to two LysM peptidoglycan-binding modules. AtlB and AtlC displayed N-acetylmuramidase activity, as demonstrated by tandem mass spectrometry analyses of peptidoglycan fragments generated by the purified enzymes. The genes encoding AtlB and AtlC were identified for a limited number of prototypic enzymes, and the specificity of the enzymes is most frequently inferred from sequence comparisons. Single gene inactivation is rarely associated with a loss of function, this fact.

Peptidoglycan is the major component of the bacterial cell wall, which determines cell shape and confers resistance to internal osmotic pressure. The peptidoglycan network consists of glycan strands made of alternating N-acetylmuramic acid (GlcNAc)2 and N-acetylmuramyl (MurNAc) residues that are cross-linked by short peptides of various compositions. In Enterococcus faecalis, the e-amino group of L-Lys in the L-Ala-γ-D-Gln-L-Lys-D-Ala-D-Ala pentapeptide stem is substituted by N-[14] acetylmuramyl; BHI, brain heart infusion; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; rp-HPLC, reverse-phase high performance liquid chromatography.

The abbreviations used are: GlcNAc, N-acetylglucosamine; MurNAc, N-acetylmuramyl; BHI, brain heart infusion; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; rp-HPLC, reverse-phase high performance liquid chromatography.
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vitro an E. faecalis multimodular N-acetylglycosaminidase (AtlA) that requires a LysM peptidoglycan-binding domain for full activity (16). To gain insight into the physiological role of the peptidoglycan hydrolases, we have identified in this study all the autolytic activities detectable by zymogram in E. faecalis JH2-2. We report deletion of the corresponding genes in various combinations and extensive analysis of the impact of the deletions on cell morphology and peptidoglycan turnover. None of the detectable activities were essential for viability, although two enzymes, AtlA and AtlB, appeared to account almost completely for septum digestion and peptidoglycan turnover.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions—Details of plasmid and strain constructions are described in the supplemental Experimental Procedures. The bacteria were routinely grown at 37°C in brain heart infusion (BHI) broth or agar (Difco) unless otherwise stated.

Detection of Peptidoglycan Hydrolyses by Zymogram—Proteins were separated by SDS-PAGE using gels containing 0.2% (w/v) autoclaved E. faecalis JH2-2 cells (17). After electrophoresis, the proteins were renatured by incubating the gel in 25 mM Tris-HCl (pH 6.5) containing 0.1% Triton at 37°C.

Partial Purification of EF0355 (AtlB)—The hyperlytic mutant 78E9, obtained by random mutagenesis of E. faecalis JH2-2,3 was grown in 500 ml of BHI until the A{sub 600} reached 0.7. Cells were centrifuged and used to inoculate 6 liters of a synthetic medium containing (per liter): 6 g of Na{sub 2}HPO{sub 4}, 3 g of KH{sub 2}PO{sub 4}, 0.5 g of NaCl, 1 g of NH{sub 4}Cl, 1 mM MgSO{sub 4}, 0.1 mM CaCl{sub 2}, 0.1% glucose, 20 mg of each amino acid, 100 mg of thiamine, 20 mg of pyridoxine, 20 mg of pantothenic acid, 20 mg of biotin, 2 mg of nicotinic acid, 2 mg of riboflavin, and 2 mg of folic acid. Cells were removed by centrifugation (8,000 × g for 10 min at 4°C), and 4.2 kg of ammonium sulfate was gradually added to the supernatant under stirring at 4°C. Proteins were collected by centrifugation (20,000 × g for 30 min at 4°C) and dissolved in 33.5 ml of 50 mM Tris-HCl (pH 8.5). The autolytic activity was partially purified by two anion exchange chromatographies (HiTrap Q-Sepharose fast flow 5 ml and Mono Q HRS/5 columns; Amersham Biosciences) using NaCl gradients in 50 mM Tris-HCl (pH 8.5). Autolytic activity in the fractions was determined spectrophotometrically by following the decrease in A{sub 450} produced by the hydrolysis of E. faecalis JH2-2 peptidoglycan, as described previously (16).

N-terminal and MALDI-TOF Sequencing—For N-terminal sequencing, proteins were transferred onto polyvinylidene difluoride membranes and sequenced using a PerkinElmer Life Sciences Procise 494 HT protein sequencer as described elsewhere (18). For MALDI-TOF sequencing, proteins were separated by SDSPAGE, and trypsic digestions were performed as follows: gel plugs were washed in 50% CH{sub 3}CN in 50 mM NH{sub 4}CO{sub 3} and desiccated under vacuum. The digestion was performed with 0.5 μg of modified trypsin (Promega, sequencing grade) in 25 μl of 50 mM ammonium bicarbonate (pH 8.0) for 18 h at 37°C. A 0.5-μl aliquot was spotted directly onto the stainless steel MALDI plate. The sample was dried at room temperature before addition of 0.5 μl of 3 mg/ml α-cyano-4-hydroxycinnamic acid matrix in 50% acetonitrile and 0.1% trifluoroacetic acid. Mass spectra were acquired on a Voyager DE-STR+ time-of-flight mass spectrometer (Applied Biosystems) equipped with a nitrogen laser emitting at 337 nm. For acquisition, the accelerating voltage used was 20 kV. Peptide spectra were recorded in the positive reflector mode and with a delayed extraction of 130 ns and a 62% grid voltage. The spectra were calibrated using an external calibration as described by Cencic et al. (19).

Production and Purification of Histidine-tagged AtlB and AtlC—E. coli BL21(DE3) harboring recombinant plasmids were grown at 37°C in BHI broth containing 100 μg/ml ampicillin. When the cultures reached an absorbance at 600 nm of 0.7, production of the recombinant proteins was induced by addition of 0.5 mM isopropyl β-D-thiogalactopyranoside, and incubation was continued for 4 h. The cells were harvested and resuspended in 50 mM Tris-HCl (pH 8.0) containing 300 mM NaCl, and crude lysates were obtained by sonication (six times for 30 s, 20% output, Bronson Sonifier 450). Inclusion bodies containing AtlB and AtlC were collected using centrifugation (10,000 × g for 10 min at 4°C) and washed three times in 10 mM Tris-HCl (pH 8.0) containing 150 mM NaCl, 10 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 0.5% Triton X-100. The inclusion bodies were dissolved in buffer A (100 mM sodium phosphate buffer, 10 mM Tris-HCl (pH 8.0), 8 M urea) and incubated for 1 h at room temperature with Ni{sup 2+}-nitrilotriacetate-agarose resin (Qiagen). The resin was extensively washed with buffer A adjusted to pH 7.0 and eluted with buffer A adjusted to pH 4.5. Purified AtlB and AtlC were dialyzed against 25 mM sodium phosphate buffer (pH 7.5), containing 50 mM NaCl and 40% glycerol. Proteins were stored at −80°C.

Cell Wall Purification and Peptidoglycan Structural Analysis—Peptidoglycan was purified from exponentially growing cells as described previously (16), freeze-dried, and resuspended in distilled water at a concentration of 20 mg/ml. Peptidoglycan (5 mg) was digested overnight with 300 μl of purified AtlB or AtlC at 37°C in 500 μl of 25 mM sodium phosphate buffer (pH 6.5) containing 50 mM NaCl. Soluble disaccharide peptides were recovered in the supernatant following ultracentrifugation (100,000 × g for 30 min at 20°C) and reduced with sodium borohydride as described previously (16).

The reduced muropeptides were separated by reverse-phase high performance liquid chromatography (RP-HPLC) on a C{sub 18} column (3 μm, 4.6 × 250 mm; Interchrom, Montluçon, France) at a flow rate of 0.5 ml/min with a 0–20% gradient applied between 10 and 90 min (buffer A, 0.05% trifluoroacetic acid in water; buffer B, 0.035% trifluoroacetic acid in acetonitrile). Mass spectral data were collected with an electrospray time-of-flight mass spectrometer operating in the positive mode (Qstar Pulsar I; Applied Biosystems). The data were acquired with a capillary voltage of 5,200 V and a declustering potential of 20 V. The mass scan range was from m/z 350 to 1,500, and the scan cycle was 1 s. Tandem mass spectrometry was carried out as described previously (20).

Flow Cytometry Analysis—Strains were grown overnight with shaking at 180 rpm at 37°C. Bacteria were diluted (1:100) into fresh broth and incubated with shaking until the A{sub 600}
reached 0.2. Bacteria were diluted (1:100) in phosphate-buffered saline and analyzed by flow cytometry using a Beckman Coulter EPICS flow cytometer, as described elsewhere (21). Light scatter data were obtained with logarithmic amplifiers for 20,000 events.

Incorporation of [14C]GlcNAc by Exponentially Growing E. faecalis Strains—One ml of an exponentially growing culture (A600 = 0.3) was used to inoculate 5 ml of pre-warmed Luria Bertani medium containing [14C]GlcNAc (7.3 μM final; 2.1 GBq/mmol; Amersham Biosciences). Aliquots (400 μl) were withdrawn; an equal volume of 0.1% SDS was added, and bacteria were centrifuged (15,000 × g for 5 min at room temperature). The pellet was resuspended in 150 μl of water, and radioactivity was determined by liquid scintillation counting in 4 ml of ACS II scintillation mixture (Amersham Biosciences).

Determination of Radioactivity Released in the Culture Medium—Exponentially growing E. faecalis were labeled with [14C]GlcNAc in the conditions described above, collected when the A600 reached 0.25, washed by centrifugation, and resuspended at the same cell density in Luria Bertani medium containing 1 mM GlcNAc. Aliquots were withdrawn; bacteria were centrifuged, and the amount of radioactivity released by the bacteria was determined by liquid scintillation counting of the supernatant.

RESULTS

Autolytic Activities of E. faecalis JH2-2—Four protein bands displaying autolytic activities (72, 62, 50, and 47 kDa) were detected in crude extracts of the wild-type strain JH2-2 (Fig. 1A, lane 1). The profile obtained for the secreted proteins was similar to that of crude extracts, except that the 47-kDa band was absent and the 72-kDa band was slightly more intense (Fig. 1B, lane 1). Analyses of strains with deletion of the atlA gene (Fig. 1, A and B, lane 2) indicated that the 72- and 62-kDa bands correspond to two forms of the major N-acetylmuramidase, as described previously (16). The remaining 50- and 47-kDa bands were not detected in our previous study in which we used Micrococcus lysodeikticus instead of E. faecalis cells as the substrate.

Identification of the Gene Encoding the 50-kDa Autolysin (AtlB) by Reverse Genetics—The 50-kDa secreted protein was purified from strain 78E9, a hyperlytic mutant of E. faecalis JH2-2 that overproduces this autolysin (Fig. 1, lane 3). The mutant 78E9 was grown in a synthetic medium, and proteins present in the culture supernatant were concentrated by ammonium sulfate precipitation. The 50-kDa autolysin was partially purified by two successive anion exchange chromatographies (Fig. 2, A and B). The second chromatography increased the specific activity only 2-fold (Fig. 2C), but this step was crucial for protein identification because it allowed the separation of several polypeptides with molecular masses close to 50 kDa, which were poorly resolved by SDS-PAGE. The N-terminal sequence of the polypeptide at 50 kDa present in the protein fraction enriched in autolytic activity (Fig. 3A, arrow) was determined by Edman degradation. The sequence, AKGDQGVD, matched the predicted N-terminal sequence of three putative secreted proteins encoded by the E. faecalis V583 genome after cleavage of their signal peptides (EF0355, EF1992, and EF2802). As depicted in Fig. 3B, EF0355 and EF2802 are nearly identical (97.5% identity), whereas EF1992 is more distant (~69% identity). MALDI-TOF tandem-mass spectrometry of tryptic fragments was used to determine which of these three candidate genes encoded the 50-kDa protein band with autolytic activity. Three [M + H]+ ions at m/z 875.46, 1,424.74, and 2,459.22 matched the calculated mass of tryptic fragments of EF0355 (Fig. 3, B and C). Because digestion of EF1992 and EF2802 is not predicted to produce fragments of the same mass, this observation indicates that EF0355 was present in the 50-kDa protein band. Conversely, none of the fragments specific for EF1992 and EF2802 were detected suggesting that these proteins were not present in the 50-kDa protein band.

Inactivation of the Gene Encoding EF0355 (AtlB)—The 50-kDa protein band with autolytic activity disappeared following deletion of the gene encoding EF0355 by allelic exchange (Fig. 1, lane 4). Complementation of the deletion with a plasmid encoding EF0355 with an additional histidine tag restored the parental autolytic profile except for a slightly reduced electrophoretic mobility because of the tag (Fig. 1, lane 6). These

FIGURE 1. Autolytic activities of E. faecalis JH2-2 and isogenic mutants. A, detection of cell-associated autolytic activities. Crude extracts from over-night cultures (20 μg of proteins) were separated electrophoretically on a 12% SDS-polyacrylamide gel containing 0.2% (w/v) of E. faecalis JH2-2 auto-claved cells. Protein bands with autolytic activities were detected after renatu-ration. WT, wild-type JH2-2; ΔA, ΔatlA mutant; 78E9, mutant overproducing AtlB; ΔB, ΔatlB mutant; ΔC, ΔatlC mutant; ΔBΔC, ΔatlB mutant complemented with a plasmid encoding histidine-tagged AtlB; ΔBΔCΔatlB double mutant; ΔBC+, ΔatlB double mutant complemented with a plasmid encoding AtlC; ΔAB, ΔatlAB double mutant. B, detection of secreted autolytic activities. Culture supernatants (20 μl) were analyzed as described in A.
results indicate that EF0355 encodes an autolysin, which we named AtlB. These results also confirmed that a single autolysin is present in the 50-kDa protein band, as inferred from MALDI-TOF sequencing (see above). Deletion of *altB* alone (Fig. 1, lane 4) or in combination with *atlA* (lane 9) revealed that *E. faecalis* JH2-2 produced a third autolysin of 47 kDa that we named AtlC.

**Inactivation of the Gene Encoding AtlC**—Because the calculated mass of the secreted EF1992 (42,967 Da, Fig. 3B) was close to 47 kDa, the corresponding gene was inactivated in *E. faecalis* JH2-2. The deletion suppressed production of the 47-kDa autolysin (Fig. 1, lane 5) indicating that EF1992 encodes AtlC. Complementation analysis was performed in a double *atlBC* mutant as the 47-kDa activity could be clearly detected in the absence of AtlB (50 kDa) (Fig. 1). The triple *ΔatlABC* mutant did not display any autolytic activity (data not shown). Together, these results indicate that three enzymes, AtlA, AtlB, and AtlC, account for all the autolytic activities detected by zymogram using *E. faecalis* peptidoglycan as the substrate.

**Both AtlB and AtlC Display N-Acetylmuramidase Activity**—We have previously shown that AtlA is an N-acetylmuraminidase (16). To identify the peptidoglycan bond cleaved by AtlB and AtlC, the autolysins were produced in *E. coli* as recombinant proteins lacking the signal peptide and harboring a C-terminal histidine tag (Fig. 4A, inset). The peptidoglycan of *E. faecalis* JH2-2 was digested with the purified proteins. The resulting disaccharide peptides were reduced by sodium borohydride and separated by rp-HPLC (Fig. 4 and data not shown). Mass spectrometry analysis of the main monomer generated by AtlB (Fig. 4A, arrow) gave an [M + H]⁺ ion at m/z 1,110.6 that matched the calculated mass of a reduced disaccharide pentapeptide substituted by an L-Ala-L-Ala side chain (Fig. 4B). Thus, AtlB cleaved the glycan strands. To distinguish between N-acetylglucosaminidase and N-acetylmuramidase activity, the sugar harboring the reducing end (GlcNAc or MurNAc) was identified by tandem mass spectrometry (Fig. 4C). The fragmentation pattern of the ion at m/z 1,110.6 showed that GlcNAc was not reduced as shown by [M + H]⁺ ions at m/z 204.1 (GlcNAc) and 907.5 (loss of GlcNAc) and was therefore typical of a disaccharide pentapeptide substituted by an i-Ala-i-Ala side chain JH2-2 (AA) (Fig. 4B). Thus, AtlB cleaved the glycan strands. To distinguish between N-acetylglucosaminidase and N-acetylmuramidase activity, the sugar harboring the reducing end (GlcNAc or MurNAc) was identified by tandem mass spectrometry (Fig. 4C). The fragmentation pattern of the ion at m/z 1,110.6 showed that GlcNAc was not reduced as shown by [M + H]⁺ ions at m/z 204.1 (GlcNAc) and 907.5 (loss of GlcNAc) and was therefore typical of a disaccharide pentapeptide substituted by an i-Ala-i-Ala side chain (20). The same approach revealed that AtlC is also an N-acetylmuramidase (data not shown).

**Role of AtlA, AtlB, and AtlC in Cell Separation**— Cultures of enterococci, including *E. faecalis* JH2-2 (22), contain mostly diplococci with rare short chains (typically 4–8 cells). The impact of deletion of *atlA*, *atlB*, and *atlC* on the distribution of these different forms was analyzed by flow cytometry (Fig. 5).
The wild-type distribution of forward scattered light gave a sharp peak characteristic of a homogeneous population mostly consisting of diplococci both in the exponential and stationary growth phases. Analysis of exponentially growing cells of the ΔatlA mutant revealed a broad peak that was shifted toward higher forward scattered light intensities. Formation of long...
chains by the ΔatlA mutant indicated that AtlA was required for digestion of the septum after cell division. Because the difference between the ΔatlA mutant and the parental strain disappeared after 24 h of incubation, the septum was digested independently from AtlA in the stationary phase. Deletion of atlB and atlC, alone or in combination, had no impact on cell morphology. Combination of the ΔatlA and ΔatlB mutations increased bacterial chain length both in exponentially growing cells and after 24 h of incubation. Thus, AtlB contributed to digestion of the septum in the absence of AtlA.

To further investigate the contribution of E. faecalis autolysins to cell separation, we analyzed the dispersion of chains by purified AtlA and AtlB (Fig. 6). Complementation of the ΔatlA mutation was observed with both autolysins. AtlA appeared more active than AtlB in this assay because higher enzyme concentrations of AtlB (~10-fold) were required for chain dispersion. These results indicate that externally added autolysins are active in the digestion of the septum and confirm that AtlA is the major enzyme responsible for cell separation after division.

**Contribution of AtlA, AtlB, and AtlC to Peptidoglycan Metabolism**—Incorporation and release of [14C]GlcNAc was analyzed according to the procedures previously developed for Bacillus subtilis (23). Comparison of the parental strain JH2-2 and the triple ΔatlABC mutant (Fig. 7A) indicated that the three autolysins were not required for a wild-type rate of incorporation of GlcNAc into peptidoglycan under the experimental conditions tested. To evaluate the release of peptidoglycan fragments, bacteria labeled with [14C]GlcNAc
were washed and resuspended in pre-warmed medium containing a 140-fold molar excess of unlabeled GlcNAc (Fig. 7B). After four generations of exponential growth, only 15% of the radioactivity was released in the culture medium by the parental strain JH2-2. Under similar conditions, 80% of the radioactivity was previously found to be released in three generations by B. subtilis (23). These results indicate that peptidoglycan turnover is much lower in E. faecalis than in B. subtilis.

During the stationary phase, radioactivity was released at a slow and constant rate by JH2-2 to reach 83% at 48 h. Release of radioactivity was not associated with cell lysis because A600 did not decrease during this time period. Thus, release of radioactivity should be attributed to a partial hydrolysis that did not compromise cell wall integrity.

Individual deletion of atlA, atlB, and atlC had little or no impact on the release of radioactivity during the exponential phase of growth (10, 14, and 17% versus 15%, respectively, for JH2-2). During the stationary phase, the release of radioac-

**FIGURE 5.** Flow cytometry analysis of E. faecalis mutants harboring single or multiple deletions of the atlA, atlB, and atlC genes. A, distribution of forward scattered light intensities in cells harvested in early exponential phase (A600 = 0.2; red) or in stationary phase after 24 (green) or 96 h (blue) of incubation. Light scatter data collected for 20,000 events are shown for JH2-2 (WT) and relevant mutants with the ΔatlA (ΔA), ΔatlBC (ΔBC), and ΔatlAB (ΔAB) deletions. FS, forward scattered light. B, mean values and standard deviations were calculated for the complete set of mutants.

**FIGURE 6.** Separation of bacterial chains by purified AtlA and AtlB. A, ΔatlA mutant was grown in exponential phase (A600 = 0.2), collected by centrifugation, and bacterial chains were resuspended in phosphate-buffered saline. Purified AtlA or AtlB was added, and cell size distribution was determined by flow cytometry after 15 min of incubation at 37 °C. FS log, relative log of forward scattered light collected for 20,000 events. 100% corresponds to the control without autolysin. For the sake of comparison, the dotted line indicates the relative forward scattered light observed for wild-type JH2-2 (WT).

**FIGURE 7.** Contribution of AtlA, AtlB, and AtlC to peptidoglycan synthesis and turnover. A, incorporation of [14C]GlcNAc by E. faecalis JH2-2 (diamonds) and the triple mutant ΔatlABC (circles). Exponentially growing cells were used to inoculate a pre-warmed Luria Bertani medium containing [14C]GlcNAc (time 0) and incorporation of radioactivity (filled symbols) and growth profiles (empty symbols) were recorded after various times of incubation at 37 °C. B, release of radiolabeled peptidoglycan fragments into the culture medium. Exponentially growing cells labeled with [14C]GlcNAc were washed and transferred to nonradioactive Luria Bertani medium. The radioactivity released in the culture supernatant was measured (solid lines) for JH2-2 (diamonds) and the ΔatlA (×), ΔatlB (circles), ΔatlC (+), and ΔatlAB (triangles) mutants. A representative growth profile is shown (dashed line).
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tivity was diminished for the \( \Delta atlasA \) (26%) and \( \Delta atlasB \) (29%) mutants in comparison to JH2-2 (83%) or to the \( \Delta atlasC \) mutant (95%). Combination of the \( \Delta atlasA \) and \( B \) mutations suppressed the release of radioactivity, except in the late stationary phase (9% between 24 and 48 h). Together, these results indicate that the hydrolytic activities of AtlA and AtlB account for production of virtually all peptidoglycan fragments that are released by E. faecalis in the culture medium during both the exponential and stationary phases of growth. AtlA and AtlB appear to compensate for each other only during the exponential phase of growth.

DISCUSSION

Genome sequencing revealed that the complement of genes encoding putative peptidoglycan hydrolases is complex in most bacteria because it typically includes 10–30 candidate genes. Redundancy is often observed for the same cleavage specificity, and the relative contribution of the different enzymes to peptidoglycan metabolism remains largely unknown except in the model organisms E. coli and B. subtilis (10, 24). Sequence comparisons with known autolysins (data not shown) revealed that the E. faecalis genome encodes 20 putative peptidoglycan hydrolases, including 6 glucosaminidases, 7 muramidases, 2 amidases, 2 endopeptidases, and 3 proteins related to lytic transglycosylases. Among these putative enzymes, AtlA was previously shown to be an N-acetylmuramidase (16). In this study, gene inactivation (Fig. 1) and reverse genetics (Figs. 2 and 3) led to the identification of two additional autolysins (AtlB and AtlC). Both enzymes were produced in E. coli, and their N-acetylmuramidase (lysozyme) activity was demonstrated by tandem mass spectrometry analysis of the peptidoglycan digestion products (Fig. 4). Construction of a triple mutant (\( \Delta atlasABC \)) revealed that AtlA, AtlB, and AtlC were not essential for growth and accounted for all the autolytic activities detectable by zymogram in E. faecalis JH2-2 (Fig. 1). The absence of any autolytic band in the zymogram of the triple mutant (data not shown) implied that serial deletions of \( atla \), \( atlb \), and \( atlc \) did not result in the activation of any autolysin that could have been cryptic in the wild-type strain.

The impact of the \( atla \), \( atlb \), and \( atlc \) mutations on cell morphology was analyzed by flow cytometry (Fig. 5). This method was recently developed to quantify the cellular dimensions of E. coli PBP mutants (25). Formation of bacterial chains by the \( \Delta atlasA \) mutant during the exponential phase of growth indicated that AtlA is the major autolysin responsible for septum hydrolysis following cell division. During the stationary phase, the distribution of cell length returned to normal in 24 h in the \( \Delta atlasA \) mutant but not in the \( \Delta atlasAB \) mutant, indicating that AtlB cleaved the septum in the absence of AtlA. Purified AtlA and AtlB were able to digest the septum, although higher concentrations of the latter protein were required (Fig. 6) suggesting that AtlB is less efficient than AtlA for septum cleavage. Both AtlA and AtlB harbor a domain organization typical for autolysins, i.e. a catalytic domain fused to a cell wall binding domain (24). Although both AtlA and AtlB harbor a LysM peptidoglycan domain, these domains are composed of a different number of LysM repeats (six for AtlA and two for AtlB) and display a very different pl (10.1 for AtlA and 5.5 for AtlB). The distinct properties of the AtlA and AtlB LysM domains may account for the fact that AtlA is specifically targeted to the septum. Preferential cleavage of the septum by AtlA could also depend upon local differences in the chemical composition of peptidoglycan, which could affect the catalytic activity of the autolysin.

Cell separation was observed in the absence of AtlA and AtlB upon prolonged incubation (96 h), suggesting that the septum was slowly cleaved. This additional activity cannot be attributed to AtlC because deletion of \( atlc \) did not result in any detectable modification of cell morphology. This conclusion is supported by deletions of the \( atla \), \( atlb \), and \( atlc \) genes in all possible combinations (Fig. 5B).

In E. coli and B. subtilis, peptidoglycan hydrolases release fragments from the wall during growth, a process termed peptidoglycan turnover (26, 27). Here we show that peptidoglycan turnover is much lower in E. faecalis than in B. subtilis because only 15% of radio-labeled GlcNAc was released in four generations (Fig. 7). As inferred from our zymogram analyses (Fig. 1) and from assays using purified peptidoglycan (Fig. 4) (16), the autolytic activities of AtlA, AtlB, and AtlC are readily detectable in vitro. This is in contrast with the yet unexplained, particularly low, turnover rate in E. faecalis. This paradox implies that autolysins, which are potentially lethal enzymes, are tightly controlled during cell growth by mechanisms that remain to be discovered. The activity of AtlA and AtlB fully accounted for this low turnover during exponential growth. This observation implies that peptidoglycan synthesis in the double \( \Delta atlasAB \) mutant was not associated with any significant release of peptidoglycan fragments and raises the question previously mentioned (27) of whether autolysins are essential for enlargement of the cell.

AtlA and AtlB also accounted for the release of peptidoglycan fragments in the culture medium during the stationary phase (Fig. 7B). The activity of these enzymes was efficiently controlled because cell lysis was not detected. The peptidoglycan fragments released in the medium are likely to originate, at least in part, from digestion of the septum by AtlA and AtlB as documented by flow cytometry (Fig. 6).

In contrast to \( atla \), the \( atlb \) and \( atlc \) genes are located in putative prophages, designated 01 and 04, respectively (28, 29). The location of \( atlb \) and \( atlc \) suggests that the autolysins participate in a lytic cycle, although the functionality of these genetic elements as phages is unknown. We have shown that AtlB and AtlC are produced by JH2-2 (Fig. 1) in the absence of cell lysis or production of phage progeny. AtlB contains a canonical signal peptide of 22 residues, and N-terminal sequencing revealed cleavage at the predicted site (Fig. 3). Thus, AtlB appears to be secreted independently from any phage-encoded function, despite the presence of a putative holin gene upstream from \( atlb \). An impact of AtlB production on cell morphology has been detected in a \( \Delta atlasA \) background revealing that AtlB could act as a surrogate for AtlA. Together, these results suggest that AtlB, and perhaps
AtIC, may have been hijacked by *E. faecalis* to contribute to peptidoglycan metabolism.

**Acknowledgments**—We thank Anne-Lise Bravetti for the construction of the ΔatlAB mutant, Bruno Fantin for support with flow cytometry, Céline Henry (INRA, Jouy-en-Josas) for MALDI-TOF and N-terminal sequencing, and Alain Rince (Université de Caen) for the gift of the *E. faecalis* JH2-2 78E9 mutant. We are indebted to Laurent Gutmann, Jean-Luc Mainardi, and Emma Pilling for constructive comments on the manuscript.

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doi: 10.1074/jbc.M802323200 originally published online May 19, 2008

Access the most updated version of this article at doi: 10.1074/jbc.M802323200

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