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Impact of peptidoglycan *O*-acetylation on autolytic activities of the *Enterococcus faecalis* *N*-acetylglucosaminidase AtlA and *N*-acetylmuramidase AtlB

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ABSTRACT

Autolysins are potentially lethal enzymes that partially hydrolyze peptidoglycan for incorporation of new precursors and septum cleavage after cell division. Here, we explored the impact of peptidoglycan *O*-acetylation on the enzymatic activities of *Enterococcus faecalis* major autolysins, the *N*-acetylglucosaminidase AtlA and the *N*-acetylmuramidase AtlB. We constructed isogenic strains with various *O*-acetylation levels and used them as substrates to assay *E. faecalis* autolysin activities. Peptidoglycan *O*-acetylation had a marginal inhibitory impact on the activities of these enzymes. In contrast, removal of cell wall glycopolymers increased the AtlB activity (37-fold), suggesting that these polymers negatively control the activity of this enzyme.

1. Introduction

Peptidoglycan is essential for maintaining bacterial cell shape and integrity [1]. This macromolecule is a heteropolymer composed of disaccharide units alternating *N*-acetylglucosamine and *N*-acetylmuramic acid cross-linked by short stem peptides [2,3]. To ensure the insertion of new peptidoglycan precursors and septum cleavage after cell division, peptidoglycan is partially hydrolyzed by potentially lethal enzymes called autolysins. Three autolysins have been characterized in *Enterococcus faecalis*: one *N*-acetylglucosaminidase, AtlA, and two highly similar *N*-acetylmuramidases, AtlB and AtlC [4,5]. AtlA is required for efficient hydrolysis of the septum after cell division. AtlB can act as a surrogate for AtlA and also participates with AtlA in peptidoglycan turnover. No specific contribution of AtlC to peptidoglycan metabolism was detected [5]. To prevent cell lysis during growth, the enzymatic activities of autolysins must be tightly controlled and synchronized with the activity of glycosyltransferases and D,D-transpeptidases, which ensure peptidoglycan polymerization. Peptidoglycan de-*N*-acetylation and *O*-acetylation as well as covalently bound cell wall glycopolymers (CWG), have been suggested to play a role in autolysin control [3]. Recently, several genes encoding peptidoglycan de-*N*-acetylases [6–9] and *O*-acetylases [10–12] have been identified in Gram-positive bacteria. Both peptidoglycan de-*N*-acetylation and *O*-acetylation were found to inhibit peptidoglycan digestion by lysozyme, a eukaryotic *N*-acetylmuramidases enzyme produced in response to bacterial infection. Peptidoglycan de-*N*-acetylation was also demonstrated to inhibit the activity of AcmA, the major *N*-acetylglucosaminidase of *Lactococcus lactis* [8]. Based on mild-base treatment of cell wall preparations, zymogram analyses suggested that *O*-acetylation could modulate autolysin activities in *E. faecalis* [13].

In this study, we compared the enzymatic activity of recombinant AtlA and AtlB on cell walls with variable peptidoglycan *O*-acetylation levels. We also prepared pure peptidoglycan by treating cell walls with hydrofluoric acid. The substrates generated

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were used to assay the activity of recombinant His-tagged AtlA and AtlB in vitro. We found that *O*-acetylation had only a marginal impact on autolytic activities. In contrast, our results suggested that *E. faecalis* cell wall glycopolymers (CWG), including teichoic acids, a polysaccharide capsule and a rhamnopolysaccharide [14] are one of the cell wall properties involved in the negative control of autolytic activities.

2. Materials and methods

2.1. Bacterial strains, and growth condition

E. faecalis JH2-2 and OG1RF are plasmid-free laboratory strains [15,16]. *E. faecalis* JH2-2Δ*oatA* harbors a deletion in the gene encoding the *O*-acetyl transferase OatA (EF0783 at www.tigr.org). *E. faecalis* OG1RF(pOat) is an OG1RF derivative expressing the *oatA* gene from JH2-2. Bacteria were grown at 37 °C in Brain Heart Infusion (BHI) broth or agar (15 g/l) (Difco Laboratories). Expression plasmids pML118 and pEF355 were used to produce recombinant His-tagged mature AtlA [4] and AtlB [5] from *E. faecalis* V583, respectively, as previously described. When required, the growth medium was supplemented with 150 μg/ml of ampicillin, 60 μg/ml of spectinomycin or 200 μg/ml of erythromycin for *Escherichia coli*. Spectinomycin and erythromycin were used at 120 and 30 μg/ml for *E. coli* and *E. faecalis*, respectively.

2.2. Plasmid construction for gene disruption and complementation

Disruption of *oatA* was carried out by allelic exchange as previously described [17] using plasmid pGHHoat. To construct pGHHoat, two fragments of JH2-2 genomic DNA were amplified with primers EF0783_1 (5′AAACCATGGATCAGACAGCAAATAGCAAGAA A3′) and EF0783_2 (5′GGGTAGAACGTCTTGGCTTTCACTCGGTCAGA CCGCGCTTACGA ACAAAGCGTTCC3′), and EF0783_3 (5′TG-ACCGAG TGAAAGCCAAGACGTTCTAC CCACACAACTCACACCAGAGC-AAG3′) and EF0783_4 (5′AAACTCGAGCTACTGTA AAATTTTCTCCG CG3′) using Vent DNA polymerase (New England Biolabs). The two PCR products were fused by the strand overlap extension method [18]. After purification, the final PCR product was cut with XhoI and cloned into the thermosensitive plasmid pGhost9 [19] digested by SmaI and XhoI. The resulting plasmid pGHHoat contains a 1.06 kbp DNA fragment encoding an inactive *oatA* locus due to a 831 bp deletion within the *oatA* open reading frame. To complement the low level of *O*-acetylation in OG1RF, competent OG1RF cells were electroporated with plasmid pOat obtained as follows: the complete open reading frame of *oatA* was PCR-amplified using JH2-2 genomic DNA as a template, Vent DNA polymerase (New England Biolabs) and oligonucleotides EF0783_1 (see above) and EF0783_5 (5′AAAGGATCCCTGTAAAATTTTCTCCGCG3′). The PCR fragment was cut with NcoI and BamHI and cloned into the replicative vector pJEH11 [20] to generate pOat.

2.3. Cell wall purification, hydrofluoric acid treatment, and muropeptide analysis

Bacteria were grown in 500 ml of BHI broth at 37 °C to an OD_{600} of 0.7. Cell walls (*O*-acetylated peptidoglycan with covalently bound glycopolymers) were obtained by extraction of the bacterial pellet with 14 ml of 4% SDS at 100 °C for 30 min followed by pronase and trypsin digestion as previously described [21]. To obtain pure peptidoglycan, cell walls were treated with 48% (m/v) hydrofluoric acid (HF) at 4 °C for 96 h and repeatedly washed with distilled water. This chemical treatment removes cell wall glycopolymers (CWG) covalently bound to peptidoglycan via an acid-labile phosphodiester bond at the C6 group of muramic acid [22]. To allow muropeptides analysis, cell walls were digested with mutanolysin, reduced with $NaBH_4$ and separated by reverse-phase high-performance liquid chromatography (rp-HPLC) as previously described [21].

2.4. NMR analysis of O-acetylated muropeptides

HPLC-purified muropeptides D and G were lyophilized and dissolved in D_2O. NMR spectra were run at 298 K on a Varian Innova spectrometer operating at a proton frequency of 600 MHz. Structural analysis was conducted using standard methods such as correlation spectroscopy, relayed COSY, total correlation spectroscopy (TOCSY), edited gradient selected heteronuclear single-quantum correlation (egHSQC), as well as gradient selected heteronuclear multiple bond correlation (gHMBC). All spectra were processed and plotted using VNMR software (Varian, Palo Alto, USA).

2.5. Determination of AtlA and AtlB specific activities towards purified peptidoglycan

Cell walls or pure peptidoglycan were resuspended at OD_{450} of 0.6 in 50 mM phosphate buffer (pH 7.5 for AtlA or 6.0 for AtlB), and incubated at 37 °C with recombinant purified His-tagged AtlA [4] or AtlB [5] from *E. faecalis* V583. Peptidoglycan hydrolysis was measured by following the decrease in OD_{450}, typically over 2–10 min. The specific activities of AtlA and AtlB were expressed in ΔOD_{450}/min/μmol of enzyme [23]. Specific activities were determined using distinct batches of cell wall preparations.

2.6. Zymographic analyses

Crude extracts were obtained from exponentially growing cells (OD_{600} of 0.7). A cell pellet corresponding to 50 ml of culture was resuspended in 1 ml of Tris 20 mM (pH 7.5), mixed with an equal volume of glass beads (0.17–0.18 mm), and mechanically broken with a Fastprep machine (Qbiogene; 5 cycles of 30 s at maximum speed with 2 min pauses between each round to avoid heating). Proteins in *E. faecalis* JH2-2 crude extracts (20 μg) were separated electrophoretically on a 12% SDS–polyacrylamide gel containing autoclaved cells (OD_{600} of 5). After electrophoresis, the proteins were renatured by incubating the gel at 37 °C for 4 h in 25 mM Bis-Tris–HCl (pH 6.5) buffer containing 0.1% Triton X-100 and then for 24 h in 25 mM Tris (pH 7.5) buffer containing 0.1% Triton X-100. Lytic activities were visualized as clear bands on the opaque SDS–PAGE gel.

3. Results and discussion

3.1. Susceptibility of E. faecalis reference strains JH2-2 and OG1RF to autolytic activities

Zymogram experiments were carried out to compare the autolytic profiles of *E. faecalis* JH2-2 crude extracts on autoclaved cells from two reference strain, JH2-2 and OG1RF. Using JH2-2 cells as a substrate, four bands were detected in crude extracts (Fig. 1, lane 1). Two bands at 72 and 62 kDa correspond to the full length and to a C-terminal fragment of the *N*-acetylglucosaminidase AtlA, respectively [4]. The bands at 50 and 47 kDa correspond to the *N*-acetylmuramidases AtlB and AtlC, respectively [5]. Using OG1RF as a substrate, the autolytic profile revealed an increased intensity of AtlA bands and the appearance of several additional minor bands (Fig. 1, lane 2) absent in extracts from a JH2-2Δ*atlA* mutant [5] (data not shown). This result suggested that OG1RF cells were apparently more susceptible than JH2-2 cells to AtlA present in JH2-2 extracts. In addition, we analyzed *E. faecalis* OG1RF crude