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Research paper

Cytoplasmic peptidoglycan intermediate levels in *Staphylococcus aureus*☆

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ABSTRACT

Intracellular cytoplasmic peptidoglycan (PG) intermediate levels were determined in *Staphylococcus aureus* during log-phase growth in enriched media. Levels of UDP-linked intermediates were quantitatively determined using ion pairing LC-MS/MS in negative mode, and amine intermediates were quantitatively determined stereospecifically as their Marfey's reagent derivatives in positive mode. Levels of UDP-linked intermediates in *S. aureus* varied from 1.4 μM for UDP-GlcNAc-Enolpyruvyate to 1200 μM for UDP-MurNAc. Levels of amine intermediates (L-Ala, D-Ala, D-Ala-D-Ala, L-Glu, D-Glu, and L-Lys) varied over a range of from 860 μM for D-Ala-D-Ala to 30–260 mM for the others. Total PG was determined from the D-Glu content of isolated PG, and used to estimate the rate of PG synthesis (in terms of cytoplasmic metabolite flux) as 690 μM/min. The total UDP-linked intermediates pool (2490 μM) is therefore sufficient to sustain growth for 3.6 min. Comparison of UDP-linked metabolite levels with published pathway enzyme characteristics demonstrates that enzymes on the UDP-branch range from >80% saturation for MurA, Z, and C, to <5% saturation for MurB. Metabolite levels were compared with literature values for *Escherichia coli*, with the major difference in UDP-intermediates being the level of UDP-MurNAc, which was high in *S. aureus* (1200 μM) and low in *E. coli* (45 μM).

1. Introduction

Bacterial peptidoglycan is a complex material whose synthesis involves a number of steps [1–7]. The cytoplasmic steps of the pathway culminate in the synthesis of a UDP-MurNAc-pentapeptide intermediate (Fig. 1). The peptidoglycan portion of this intermediate is then transferred to a lipid carrier for further elaboration, exported to the outer membrane leaflet, and then assembled into mature cell wall peptidoglycan [1,3,5]. The core cytoplasmic intermediates of this pathway in *Staphylococcus aureus* are a series of UDP-linked intermediates; UDP-GlcNAc (UDP-NAG), UDP-NAG-Enolpyruvate (UDP-EP), UDP-MurNAc (UDP-NAM), UDP-NAM-L-Ala (UDP-Mono), UDP-NAM-L-Ala-D-Glu (UDP-Di), UDP-NAM-L-Ala-γ-D-Glu-L-Lys (UDP-Tri), and UDP-NAM-L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala UDP-Penta). Synthesis of these intermediates in *S. aureus* requires several amine intermediates associated with this pathway; L-Ala, D-Ala, D-Ala-D-Ala, L-Glu, D-Glu, and L-Lys.

The identity of these intermediates, and the enzymes catalyzing their synthesis and their structural and mechanistic details are now generally well established [6]. This pathway is the target for a number of known antibiotics, and is an inviting target for new antibacterial agent development [8,9]. The development of new agents targeting the cytoplasmic steps in this pathway would be facilitated by a deeper understanding of how this pathway functions as an integrated metabolic unit, and how this pathway responds to antibiotics targeting this pathway [10,11]. For these types of studies, it is necessary to be able to quickly and accurately quantify all of the intermediates in this pathway, and the

The abbreviations used are: Alr, Alanine racemase; ATCC, American Type Culture Collection; Ddl, D-Ala-D-Ala ligase; DMHA, N,N-dimethylhexylamine; LC-MS/MS, Liquid chromatography - tandem mass spectrometry; DAP, meso-Diaminopimelic acid; PG, Peptidoglycan; SDS, Sodium dodecylsulphate; t_{double}, Doubling time; UDP-Di, UDP-MurNAc-L-Ala-D-Glu; UDP-EP, UDP-GlcNAc-Enolpyruvate; UDP-GlcA, UDP-Glucuronic Acid; UDP-Mono, UDP-MurNAc-L-Ala; UDP-NAG, UDP-GlcNAc; UDP-NAM, UDP-MurNAc; UDP-Penta, UDP-MurNAc-L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala; UDP-Tri, UDP-MurNAc-L-Ala-γ-D-Glu-L-Lys.

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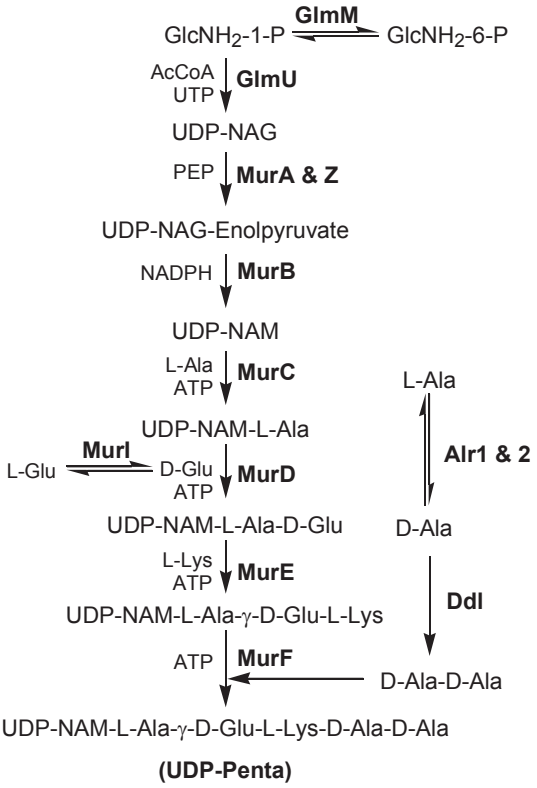

Fig. 1. Cytoplasmic intermediates in the cell wall biosynthesis pathway in *S. aureus*. The enzymes catalyzing the reaction steps are indicated in bold next to the reaction catalyzed. (Note: This a single column figure.)

peptidoglycan product of this pathway.

In this report, recently developed LC-MS/MS-based analytical methods for the quantification of the UDP-linked [12] and amine [13–15] intermediates in this pathway are used to determine their levels in *S. aureus* under log-phase growth conditions. An LC-MS/MS method for PG quantification was also developed to determine total PG biosynthesis. The results of this analysis are the intracellular pool concentrations of all UDP-linked and amine intermediates, and the corresponding amount of PG in the cells and metabolite flow rate (flux) through this pathway. Metabolite level results were analyzed by comparison with the known characteristics of the *S. aureus* enzymes in this pathway, and with prior results for PG metabolite pool level and pathway enzyme characteristic studies in *Escherichia coli*.

2. Materials and methods

2.1. General materials and methods

D-Ala, L-Ala, D-Ala-D-Ala, $^{13}C_3$-D-Ala, Glycine, L-Glutamate, D-Glutamate, L-Lysine, D-Lysine, UDP-glucuronic acid (UDP-GlcA), triethylamine, *N*,*N*-dimethylhexylamine (DMHA), and SDS were purchased from Sigma–Aldrich (St. Louis, MO). C18–silica gel was obtained from Sep-Pak Cartridges from Waters (Milford, MA) and Marfey's reagent (1-fluoro-2,4-dinitripheny-L-5-alanine amide) was purchased from Novabiochem (a division of EMD Chemicals, Gibbstown, NJ). LC-MS/MS was performed on an AB Sciex 3200 QTrap mass spectrometer (Foster City, CA) coupled to a Shimadzu UFLC system (Columbia, MD) using electrospray ionization (ESI) and run with Analyst v.1.4.2 software. Centrifuge operations were performed in a Sorvall RT6000 centrifuge, Beckman Coulter Avanti Tm J-25I centrifuge, or a standard microcentrifuge. M9 minimal medium consisted of Na_2HPO_4 (30 g/L), KH_2PO_4 (15 g/L), NH_4Cl (5 g/L), and NaCl (2.5 g/L). Difco Mueller-Hinton broth BD Biosciences (San Jose, CA) was prepared according to manufacturer instructions. Cell density was measured by absorbance at 600 nm in 1 cm cuvettes in a Biomate 3 thermospectronic spectrophotometer (Waltham, MA). The ATCC #43300 (F-182) strain of methicillin resistant *S. aureus* (MRSA) was obtained from the American Type Culture Collection (Manassas, VA).

2.2. Preparation of the bacterial sample

A 20 mL saturated overnight culture of *S. aureus* was used as inoculum. Sufficient inoculum was added to 200 mL of Mueller-Hinton broth in a baffled flask to provide an OD_{600} of 0.05, and the flask was incubated with good agitation at 37 °C. Bacterial growth was monitored by absorbance at 600 nm (OD_{600}), with an observed doubling time of 45 min. Cultures were grown to an OD_{600} of ~0.8, and cells collected and processed as described below.

2.3. Centrifugation-based metabolite extraction

An actively growing log-phase *S. aureus* culture at 0.8 OD_{600} was rapidly chilled in an ice-water bath. Four samples of 15 mL each were immediately removed into ice-cold 15 mL centrifuge tubes, and the cells were pelleted by centrifugation at 3300 g for 10 min at 4 °C. After supernatant removal, the cells were washed twice by re-suspending in 400 μL ice-cold M9 minimal medium and re-pelleting by centrifugation [15]. Cell pellets were homogenously resuspended in 100 μL minimal medium, and to this suspension was added 400 μL ice-cold methanol/water/formic acid (80:20:0.1 v/v) containing 100 μM $^{13}C_3$-D-Ala and 10 μM UDP-GlcA as internal standards. After 5 min on ice, this mixture was centrifuged and supernatants collected. Extracts were frozen at −80 °C, lyophilized, and redissolved in 200 μL of water/0.1% formic acid.

2.4. Filtration-based metabolite extraction

Filtration has been used as the basis for metabolite level analysis in several other studies [11,16–19], and it was desirable to compare centrifugation and filtration approaches to PG metabolite quantification. The filtration procedure used here was a modification of that described in Meyer et al. [17]. Four samples of 15 mL each were taken from an *S. aureus* culture at 0.8 OD_{600} and immediately filtered through 47 mm diameter 0.2 μm nylon membrane filters (Nalge Company, Rochester, NY). The filter associated bacteria were washed 2x with 5 mL ice-cold 0.9% saline, and the filter attached bacteria transferred to a 15 mL centrifuge tube containing 3 mL of ice-cold methanol/water/formic acid (66:33:1) and 100 μM $^{13}C_3$-D-Ala and 10 μM UDP-GlcA as internal standards. Samples were kept on ice for 5 min with regular vortexing, and then centrifuged at 3300 g and the supernatant collected. Extracts were frozen at −80 °C, lyophilized, and redissolved in 200 μL of water/0.1% formic acid. To determine the effect of chilling on ice on metabolite pool levels, a slightly modified filtration-based approach was tested where the bacterial culture was first chilled on ice to stop metabolism for 30 min prior to filtration.

2.5. UDP-linked intermediate quantification

UDP-linked intermediates were chromatographically separated using *N*,*N*-dimethylhexylamine as an ion-pairing agent on a Nucleodur 100-5 C18 125 × 3 mm column (Macherey–Nagel, Bethlehem, PA), and quantified by MS/MS detection in negative