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A liquid chromatography–tandem mass spectrometry assay for D-Ala-D-Lac: A key intermediate for vancomycin resistance in vancomycin-resistant enterococci

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ABSTRACT

Vancomycin exerts its antibacterial activity by binding to D-Ala-D-Ala in bacterial cell wall precursors. Vancomycin resistance in vancomycin-resistant enterococci (VRE) is due to an alternative cell wall biosynthesis pathway in which D-Ala-D-Ala is replaced, most commonly by D-Ala-D-Lac. In this study, we extend our recently developed Marfey's derivatization-based liquid chromatography–tandem mass spectrometry (LC–MS/MS) assay for L-Ala, D-Ala, and D-Ala-D-Ala to D-Ala-D-Lac and apply it to the quantitation of these metabolites in VRE. The first step in this effort was the development of an effective washing method for removing medium components from VRE cells. Mar-D-Ala-D-Lac was well resolved chromatographically from Mar-D-Ala-D-Ala, a prerequisite for MS/MS quantitation of D-Ala-D-Ala and D-Ala-D-Lac. Mar-D-Ala-D-Lac gave similar detection parameters, sensitivity, and linearity as Mar-D-Ala-D-Ala. L-Ala, D-Ala, D-Ala-D-Ala, and D-Ala-D-Lac levels in VRE were then determined in the presence of variable vancomycin levels. Exposure to vancomycin resulted in a dramatic reduction of D-Ala-D-Ala, with a response midpoint at approximately 0.06 μg/ml vancomycin and with a broad response profile up to 128 μg/ml vancomycin. In contrast, D-Ala-D-Lac was present in the absence of vancomycin, with its level constant up to 128 μg/ml vancomycin. This method will be useful for the discovery, characterization, and refinement of new agents targeting vancomycin resistance in VRE.

Introduction

Enterococcus species are commensal bacteria of the intestine in humans and animals that can cause problematic infections of the gastrointestinal tract and soft tissues [1,2]. Vancomycin, a glycopeptide antibiotic, is one of the most important antibacterial agents for the treatment of gram-positive bacterial infections resistant to most other antibacterial agents, including vancomycin-sensitive enterococcal and MRSA (methicillin-resistant *Staphylococcus aureus*) infections [3–5]. Vancomycin-resistant enterococci (VRE),[1] first reported during the late 1980s [6], are now widespread and a common cause of nosocomial infections [7]. Given resistance to most alternative classes of antibacterial agents, treatment options for VRE are limited [2,3].

Cell wall biosynthesis is a biochemically unique bacterial pathway that is the target of a number of antibacterial agents. This pathway is complex and has highly conserved elements between bacterial species as well as important differences [8–16]. Vancomycin exerts its antibacterial effect by binding to the D-Ala-D-Ala termini of pentapeptide peptidoglycan precursors [17–21], thereby interfering with the final steps of bacterial cell wall biosynthesis. In the most clinically common resistance mechanisms in VRE, the terminal D-Ala-D-Ala moiety of peptidoglycan precursors is replaced by D-Ala-D-Lac [20,22]. This alternative pathway requires four enzymes to synthesize D-Ala-D-Lac (VanH and VanA) and to degrade D-Ala-D-Ala intermediates before they can be incorporated into nascent peptidoglycan (VanX and VanY) (Fig. 1). Alanine branch metabolites (L-Ala, D-Ala, and D-Ala-D-Ala, and D-Ala-D-Lac) are key intermediates in this process. However, these are small hydrophilic molecules, and there has been a lack of analytical methods for the quantitation of these metabolites. Although methods are available for D-Ala-D-Lac quantitation using radiolabeled precursors, such methods are apparently limited to *in vitro* generated analytes [23,24]. Such methods are inadequate for detailed studies of alanine branch metabolite changes in response to vancomycin in

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[1] *Abbreviations used:* VRE, vancomycin-resistant enterococci; LC–MS/MS, liquid chromatography–tandem mass spectrometry; TFA, trifluoroacetic acid; LC–MS, liquid chromatography–mass spectrometry; QC, quality control; GABA, γ-aminobutyric acid; LLOQ, lower limit of quantification; LOD, limit of detection; MRM, multiple reaction monitoring; VSE, vancomycin-sensitive *Enterococcus faecium*.

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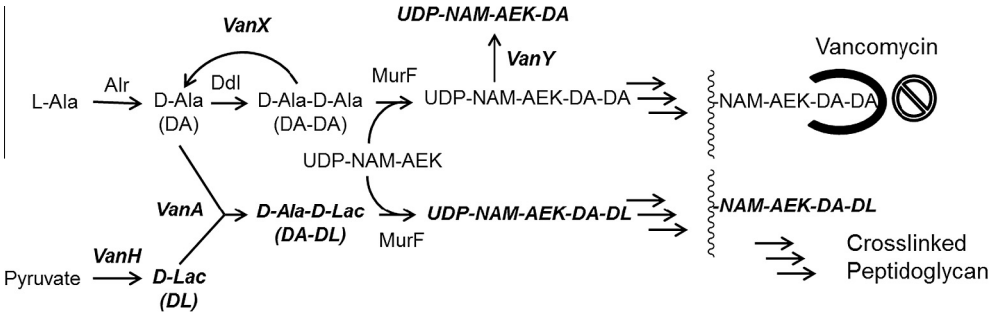

Fig. 1. Cell wall intermediate biosynthesis pathway in VRE. Cell wall biosynthesis intermediates and enzymes specific to the vancomycin resistance pathway are shown in italicized bold text. NAM, *N*-acetylmuramic acid.

VRE, which are at the core of the vancomycin resistance mechanism.

Marfey's reagent is a chiral analog of Sanger's reagent used to derivatize chiral amino acid mixtures prior to separation on achiral medium to determine stereochemical purity [25–27]. In recent studies, we have developed a Marfey's reagent-based liquid chromatography–tandem mass spectrometry (LC–MS/MS) method to quantitate alanine branch intermediates—L-Ala, D-Ala, and D-Ala-D-Ala—in the standard bacterial cell wall biosynthesis pathway [28]. This method has been used to determine the mechanism of antibacterial activity of cycloserine and D-boroAlanine [28,29]. In this study, this method is extended to the detection and quantitation of D-Ala-D-Lac, the key intermediate for most types of vancomycin resistance, and is demonstrated for determining the effect of vancomycin induction on alanine branch metabolites—including D-Ala-D-Lac—in VRE.

Materials and Methods

VRE were a clinical isolate provided by Betty Herndon (University of Missouri–Kansas City School of Medicine). D-Ala, L-Ala, D-Ala-D-Ala, $^{13}C_3$-D-Ala, vancomycin, trifluoroacetic acid (TFA), hemin, and carbonyldiimidazole (CDI) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Boc-D-Ala-OH and D-Lac-OtBu were purchased from Bachem Bioscience (King of Prussia, PA, USA). C18–silica gel was obtained from Sep-Pak Cartridges (Waters, Milford, MA, USA). Marfey's reagent (1-fluoro-2,4-dinitripheny-L-5-alanine amide) was purchased from Novabiochem (a division of EMD Chemicals, Gibbstown, NJ, USA). Other reagents were obtained from standard sources and were reagent grade or better. VRE growth medium—consisting of brain heart infusion (37.5 g/L), NaCl (6.5 g/L), hemin (10 μg/ml), and NAD^+ (10 μg/ml)—was prepared following standard procedures. M9 minimal medium—consisting of Na_2HPO_4 (6 g/L), KH_2PO_4 (3 g/L), NH_4Cl (1 g/L), NaCl (0.5 g/L), $MgSO_4{\cdot}7H_2O$ (1 mM), $FeSO_4{\cdot}7H_2O$ (3 μg/L), vitamin B1 (thiamine, 0.5 mg/L), and glycerol (20%, as carbon source)—was also prepared following standard procedures. LC–MS/MS was performed using electrospray ionization (ESI) in positive mode on an AB-Sciex 2000 QTrap LC–MS/MS mass spectrometer (Framingham, MA, USA) equipped with an Agilent 1100 HPLC (high-performance liquid chromatography) system (Santa Clara, CA, USA). Data acquisition and data processing were performed using the Analyst 1.4.2 software package (Applied Biosystems, Foster City, CA, USA). All chromatographic separations were performed on a Nucleodur 100-3 C8 column (125 × 2.0 mm, Macherey–Nagel, Bethlehem, PA, USA). All centrifuge operations were performed on a Sorvall RT6000 refrigerated centrifuge.

Boc-D-Ala-D-Lac-OtBu

Boc-D-Ala-OH (1.1 eq, 3.8 mmol, 710 mg) was dissolved in anhydrous tetrahydrofuran (THF, 10 ml) and cooled to 0 °C, and then carbonyldiimidazole (1.1 eq, 3.8 mmol, 610 mg) was added. The reaction was allowed to proceed for 30 min to form the acyl imidazole intermediate. D-Lac-OtBu (1 eq, 3.4 mmol, 500 mg) was then added, and the mixture was allowed to warm to room temperature. After 2 days, the reaction was quenched with 2 M acetic acid (10 ml) and extracted three times with methylene chloride (3 × 15 ml) and back-extracted with 1 M HCl (1 × 5 ml), 1 M $NaHCO_3$ (1 × 5 ml), and brine (1 × 5 ml). The organic extract was dried over anhydrous $MgSO_4$ and filtered, and solvent was removed by rotary evaporation. The product was obtained as a light clear oil. Yield: 76%; LC–MS; $[M+H]^+ = 318.2$.

D-Ala-D-Lac

Boc-D-Ala-D-Lac-OtBu (1.6 mmol, 500 mg) was placed in a round-bottom flask and treated with TFA (5 ml) for 1 h. TFA was removed by evaporation using nitrogen gas and then under vacuum (5 μm Hg) for 2 h. The product was a clear oil, which was dissolved in water to yield a concentrated stock solution of D-Ala-D-Lac. LC–MS; $[M+H]^+ = 162.1$.

Preparation of standard solutions and QC samples

Stock solutions (10 mM) of all analytes (D-Ala, L-Ala, D-Ala-D-Ala, and D-Ala-D-Lac) were prepared in water. An equimolar "standard mixture" was prepared by mixing aliquots of 60 μl of 10 mM stock solutions of L-Ala, D-Ala, D-Ala-D-Ala, and D-Ala-D-Lac with 760 μl of H_2O to give 1 ml of 0.6 mM of each. For method validation, quality control (QC) samples of three concentrations of a mixture of all the analytes (400, 40, and 1 pmol) were prepared in VRE extract. Then 20 μM $^{13}C_3$-D-Ala was added to all samples as an internal standard. All solutions were stored at −20 °C until use.

Marfey's derivatization reactions

To a 15-μl aliquot of the standard mixture was added 15 μl of 10 mM Marfey's reagent (in acetone) and then 5 μl of 1 M triethylamine (in water). The contents were mixed well and kept in an incubator at 37 °C for 150 min. The derivatization reaction was quenched and acidified by the addition of 5 μl of 1 M HCl, and the sample was diluted up to 200 μl with 75% H_2O/25% acetonitrile + 0.1% formic acid. This Marfey's derivatized "standard mixture" was used in initial experiments to optimize separation and quantitation parameters. Individual Marfey's adducts of L-Ala, D-Ala, D-Ala-D-Ala, and D-Ala-D-Lac were similarly prepared from 15-μl aliquots of 0.6-mM stocks following the same derivatization procedure.

MS and MS/MS optimization

Salts were observed to interfere with MS detection of Marfey's derivatives. To remove salts prior to MS and MS/MS optimization,