Advances in Bacterial D-Amino Acid Biosynthesis and Regulation: Postprint

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Abstract

L/D-type amino acids are ubiquitous in bacteria. Unlike L-amino acids (L-AAs), D-amino acids (D-AAs) do not participate in protein synthesis but are involved in the synthesis of cell wall peptidoglycan, directly affecting the shape, quantity, and strength of bacterial cell walls. D-AAs play important roles in bacterial characterization, drug bacteriostatic activity, target identification, and other aspects. Currently, some research progress has been made on the mechanism of exogenously added D-AAs participating in peptidoglycan synthesis, and their fluorescent derivatives have been applied to bacterial visualization for specifically probing cell wall formation/remodeling, bacterial growth, and cell morphology. However, the mechanism by which D-AAs affect bacterial growth and its stress resistance remains unclear. This article reviews the current research status of D-AAs, focusing on the biosynthesis of D-AAs in bacteria and their mechanisms of involvement in cell wall synthesis, the regulation of bacteria by atypical D-AAs and their applications in bacterial visualization, and provides prospects for future research directions on D-AAs.

Full Text

Preamble

Research Progress in the Biosynthesis and Regulation of D-Amino Acids in Bacteria

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Abstract

L- and D-type amino acids are ubiquitous components in bacteria. Unlike L-amino acids (L-AAs), D-amino acids (D-AAs) do not participate in protein synthesis but are instead involved in cell wall peptidoglycan (PG) biosynthesis, directly influencing bacterial cell wall shape, quantity, and strength. D-AAs play crucial roles in bacterial characterization, drug bacteriostasis, and target identification. While significant progress has been made in understanding the mechanisms by which exogenous D-AAs participate in PG synthesis, and fluorescent derivatives have been applied for bacterial visualization to specifically probe cell wall formation/remodeling, bacterial growth, and cell morphology, the mechanisms through which D-AAs affect bacterial growth and stress resistance remain unclear. This review summarizes current research on D-AAs, focusing on their biosynthetic pathways in bacteria, mechanisms of participation in cell wall synthesis, regulatory effects of non-canonical D-AAs on bacteria, and applications in bacterial visualization. Finally, future research directions for D-AAs are discussed.

Keywords: Peptidoglycan, D-amino acid, amino acid isomerase, amino acid aminotransferase

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1. Introduction

With the exception of glycine, the 19 amino acids that constitute proteins are all α-amino acids that exist in nature as two stereoisomeric forms: L-amino acids (L-AAs) and D-amino acids (D-AAs). Living organisms have effectively selected L-AAs as the basic building blocks for ribosomal polypeptide synthesis and as key metabolic intermediaries, making them far more abundant in nature than D-AAs. Nevertheless, research into the potential functions of D-AAs has never ceased [1]. D-AAs are widely distributed in microbial, plant, and animal cells, and find applications in the food, pharmaceutical, and cosmetics industries [2]. Many bacterial culture media have been found to contain D-AAs, such as *Vibrio cholerae* and *Bacillus subtilis* [3-6], which participate in microbial metabolism [7-10] and regulate various physiological processes including cell wall biosynthesis, biofilm integrity, and spore germination [4, 11-13].

D-AAs primarily participate in bacterial cell wall PG formation. The cell wall is a mesh-like polymer located outside the cell membrane that plays a vital role in maintaining cell integrity and shape stability. PG is an essential and specific component of the cell wall that provides anchoring sites for other cell

wall components [14]. PG polymer chains are primarily formed by cross-linking N-acetylglucosamine (GlcNAc)-N-acetylmuramic acid (MurNAc)-pentapeptide units [15]. The pentapeptide composition differs between Gram-positive (G^+) and Gram-negative (G^-) bacteria: G^+ bacteria have L-Ala-D-Glu/Gln-L-Lys-D-Ala-D-Ala, while G^- bacteria have L-Ala-D-Glu/Gln-m-DAP-D-Ala-D-Ala, with the main difference being the third amino acid—L-Lys in G^+ bacteria and meso-diaminopimelic acid (m-DAP), a non-chiral carboxyl derivative of Lys, in G^- bacteria [Figure 1: see original paper]. The cross-linking patterns between adjacent peptide chains also vary among different bacteria. In G^+ bacteria, adjacent peptide chains are directly cross-linked or connected via peptide bridges, forming position 2 (D-Glu/Gln)-4 (D-Ala) or 3 (L-Lys)-4 (D-Ala) linkages. In G^- bacteria, abundant 3 (m-DAP)-4 (D-Ala) linkages or less common 3 (m-DAP)-3 (m-DAP) linkages are formed through cross-linking [16-18]. PG assembly and maturation require transglycosylation, transpeptidation (TP), and carboxypeptidation (CP) processes [15]. Notably, the composition of PG peptide chains changes when D-AAs are added exogenously to the culture medium. Below, we provide a detailed review of D-AA research and discuss future development prospects.

2. Biosynthesis of D-AAs in Bacteria

Bacteria synthesize D-AAs through two primary pathways. The first involves amino acid isomerases that catalyze the interconversion between L- and D-stereoisomers in a reversible reaction [Figure 2a: see original paper]. Amino acid isomerases are classified as either epimerases (catalyzing amino acids with two or more chiral centers) or racemases (catalyzing amino acids with only one chiral center). The second pathway involves the stereospecific conversion of the corresponding α-keto acid to D-AAs via D-amino acid aminotransferase (D-AAT), which is also reversible [1] [Figure 2b: see original paper].

2.1 Amino Acid Racemases

Although multiple racemases for D-AA synthesis have been identified in bacteria, most exhibit specificity for particular amino acids. Based on their requirement for the cofactor pyridoxal-5-phosphate (PLP), amino acid racemases are divided into two classes: PLP-dependent and PLP-independent racemases [20]. Among reported racemases, alanine racemase [21], serine racemase [22], lysine racemase [23], and broad-spectrum racemase [24] are PLP-dependent, while proline racemase [25], aspartate racemase [13, 16], and glutamate racemase [26, 27] are PLP-independent. The following sections categorize these enzymes based on whether their synthesized amino acids participate in conventional PG synthesis.

2.1.1 Alanine, Aspartate, and Glutamate Racemases Alanine racemase participates in PG synthesis and amino acid metabolism while inhibiting spore formation. In the absence of exogenous D-Ala sources, deficiency in alanine racemase is typically lethal. Some G^- bacteria encode two alanine racemases:

constitutive Alr and inducible DadX, which function in D-Ala biosynthesis and D-Ala catabolism, respectively [28]. During metabolism, L-Ala promotes spore formation, whereas D-Ala inhibits spore germination. Aspartate racemase exists only in a few bacteria, such as lactic acid bacteria, and the D-Asp it synthesizes is primarily used for peptide bridge formation during peptide chain cross-linking. Glutamate racemase exhibits high specificity for Glu racemization and is essential for D-Glu synthesis in *Lactobacillus*, *Pediococcus*, and *Staphylococcus* species [27]. Recently, two glutamate racemases, YrpC and RacE, have been identified in *Bacillus subtilis*, with YrpC providing D-Glu for PG synthesis and RacE activity being associated with poly-γ-D-Glu synthesis [29, 30].

2.1.2 Other Racemases Serine racemase has been identified in some G^+ bacteria, such as *Enterococcus faecalis*, and is the only known racemase with a transmembrane domain. Studies have shown that serine racemase also possesses alanine racemase activity [20]. Two racemases catalyze the stereoisomeric conversion of Lys and Arg: lysine racemase and broad-spectrum racemase, with lysine racemase acting preferentially when both are present [31]. Currently, proline racemase has only been found in a few eukaryotes and pathogenic bacteria, including *Clostridium Prazmowski*, *Clostridium difficile*, and *Thermococcus litoralis*.

In addition to substrate-specific racemases, broad-spectrum racemase (Bsr) can accept multiple amino acids as substrates for racemization. To date, characterized Bsr family racemases have only been found in a few G^- bacteria. In *V. cholerae*, BsrV can reversibly racemize ten natural chiral amino acids, including non-β-branched aliphatic amino acids (Ala, Leu, Met, Ser, Cys, Gln, and Asn) and positively charged amino acids (His, Lys, and Arg) [31]. In G^+ bacteria such as *B. subtilis*, Espaillat et al. [32] detected D-AAs in culture medium but found no orthologous genes similar to *bsrV* in the genome, suggesting the existence of other genes with Bsr-like functions [3]. Recently, Kawakami et al. [32-34] identified a novel G^+ broad-spectrum amino acid racemase (PH0138) in the hyperthermophilic archaeon *Pyrococcus horikoshii* OT-3, with preliminary analysis via spectrophotometric assays and ultra-performance liquid chromatography (UPLC).

2.2 Epimerases and Amino Acid Aminotransferases

Compared to racemases, epimerases are relatively rare in bacteria. Mutaguchi et al. [35] demonstrated that γ-aminobutyrate aminotransferase catalyzes the racemization of nonpolar amino acids, enabling efficient epimerization-mediated interconversion between L/D-Ile. D-AAT is a PLP-dependent enzyme that catalyzes the reversible transamination between various D-AAs and α-keto acids [36]. Except for Lys and Thr, all other α-amino acids can be synthesized by aminotransferases. Fotheringham et al. [37] discovered the *dat* gene encoding D-AAT in *Bacillus* species, which can catalyze D-Glu synthesis.

3. D-AAs in Peptidoglycan Synthesis

3.1 D-AAs Commonly Found in PG

D-AAs are indispensable components of bacterial cell wall PG, influencing cell wall shape, quantity, and strength [3]. The D-AAs involved in PG synthesis primarily include D-Ala, D-Glu/Gln, and D-Asp. Two D-Ala molecules are ligated by Ddl (D-Ala-D-Ala ligase) to form D-Ala-D-Ala, which then connects to the third position of the short peptide chain to form a monosaccharide-pentapeptide unit [Figure 1: see original paper]. D-Asp/Asn mainly serve as components of peptide bridges during peptide chain cross-linking in some bacteria.

3.2 Non-Canonical D-AAs Participate in PG Synthesis

D-AAs detected in bacterial culture media that are not components of typical PG are termed non-canonical D-amino acids (NCDAAs). Interestingly, when NCDAAs are added to culture media, they can be detected in bacterial PG [15, 19, 38]. Felipe et al. [5] demonstrated that exogenously added NCDAAs can participate in PG synthesis even when the bacteria cannot synthesize these D-AAs themselves. Similarly, Gly has been shown to replace Ala at positions 1, 4, or 5 in bacterial PG peptide chains [39]. D-Met, D-Trp, or D-Phe can substitute for D-Ala at position 4 in *E. coli* PG, whereas exogenous addition of L-isomers does not affect PG structure or participate in PG synthesis. Further studies revealed that high concentrations of exogenous D-AAs inhibit bacterial growth, while low concentrations have no significant effect on growth rate or morphology but can affect PG synthesis and cross-linking, leading to reduced PG content per cell. Two possible mechanisms have been proposed: (1) D-AAs compete with L,D-transpeptidase substrates, mimicking natural substrates and interfering with transpeptidase activity; and (2) D-AAs inhibit at least one high-molecular-weight penicillin-binding protein that catalyzes PG synthesis and cross-linking [40].

3.3 Mechanisms of NCDAAs Participation in PG Synthesis

PG biosynthesis requires coordinated action of multiple enzymes. The early stage involves the Mur family enzymes for disaccharide-pentapeptide synthesis, while the later stage requires penicillin-binding proteins (PBPs) for glycan chain synthesis and modification [Figure 3: see original paper]. Disaccharide-pentapeptide units are cross-linked to adjacent glycan chains by transpeptidases [41]. Most proteins involved in these enzymatic reactions are PBPs, which are classified into three classes (A, B, and C) based on their conserved functional domains [42]. Lupoli et al. [19] demonstrated through in vitro experiments using single amino acids as substrates that *E. coli* PBP1A can incorporate D-Phe, D-Trp, and D-Tyr into PG via TP, replacing D-Ala. Lebar et al. [43] further showed that *B. subtilis* PBP1 catalyzes the incorporation of NCDAAs and D-aminocarboxamides into nascent PG, whereas *E. coli* PBP1A only exchanges NCDAAs. In *V. cholerae*, NCDAA incorporation involves two enzymes:

Ddl in the cytoplasm, which catalyzes D-Ala-D-Ala synthesis but with low substrate specificity, leading to D-Ala-NCDAA dipeptide formation; and Ldt in the periplasm, which can replace NCDAAs onto peptide chains [5]. Fura et al. [15] found that NCDAA replacement is related to transpeptidation reactions and hypothesized that increased D,D-carboxypeptidase (PBP5) activity in *B. subtilis* leads to enhanced PBP transpeptidase activity, consequently increasing NCDAA replacement rates.

Two main mechanisms enable NCDAAs to participate in PG synthesis: (1) L,D-transpeptidases in the periplasm, such as LdtA and LdtB, directly replace NCDAAs at positions 4/5 of cross-linked peptide chains; and (2) D-AAs are transported into the cytoplasm via ABC transporters, where Ddl catalyzes D-Ala-NCDAA dipeptide formation, followed by MurF-mediated synthesis of new peptide chains that are flipped to the outer surface of the cytoplasmic membrane and incorporated into the macromolecular sacculus through transglycosylation and transpeptidation [Figure 4: see original paper].

Caparrós et al. [40] investigated how functional groups interfere with incorporation reactions by modifying D-Trp (as N-acetyl-D-Trp and D-Trp methyl ester) to determine the roles of amino and carboxyl groups. Their results showed that D-Trp methyl ester could be incorporated into PG, whereas the N-acetyl derivative could not, indicating that a free α-D-amino group is required while a free carboxyl group is non-essential. Cava et al. [5] verified through in vitro experiments that *V. cholerae* can synthesize NCDAAs in vivo during stationary phase and promote PG remodeling, with only 10% of disaccharide-pentapeptide monomers undergoing replacement compared to 96% of dimers, suggesting that NCDAAs are more readily replaced in dimers. This report not only demonstrated two distinct processes mediating NCDAA participation in PG synthesis—cytoplasmic disaccharide-pentapeptide synthesis and periplasmic peptide chain cross-linking—but also revealed that multiple bacteria can incorporate NCDAAs into cell wall synthesis.

4. Regulatory Effects of NCDAAs on Bacteria

NCDAA incorporation into PG affects bacterial cell wall shape, quantity, strength, and biofilm formation. Recent studies indicate that NCDAAs can directly regulate periplasmic transpeptidase activity [3], act as signaling molecules to disassemble biofilms under specific conditions [12], and inhibit spore germination [44].

4.1 Regulatory Effects of NCDAAs on Bacterial Cell Walls

Caparrós et al. [40] reported that D-AA supplementation in culture media can alter PG synthesis and structure. Addition of D-Met to culture media reduced PG content per cell and decreased the proportion of lipoprotein-bound peptide chains by 27%. Compared to normally growing PG, modified peptide chains accounted for 45% of total PG, with D-Met rapidly incorporating into peptide

chains to form new, significantly stable unconventional PG polymers within approximately 30 minutes. Lam et al. [3] found that D-Leu and D-Met synthesized by BsrV racemase during stationary phase could induce rod-shaped *V. cholerae* to become spherical while reducing cell wall PG content by about 50%. The PG glycan chain length in *bsrV* mutant strains was approximately 80% of that in wild-type strains, with pentapeptides reduced by 50% and trimeric oligopeptides increased by 40%, indicating negative regulation of cell wall PG synthesis. This mechanism helps *V. cholerae* cope with environmental stress, as wild-type strains demonstrated higher survival rates than mutant strains in stress resistance experiments, suggesting that BsrV controls cell wall integrity during stationary phase by reducing PG synthesis and modifying cell walls to adapt to stationary-phase conditions.

Cava et al. [5] further discovered that both Ldts (LdtA/LdtB; LdtA catalyzes DAP-DAP cross-links between peptide chains in some G^- bacteria, while LdtB catalyzes covalent binding between DAP and lipoprotein [Lpp]) and *bsrV* mutant strains exhibited PG content approximately 1.7 times higher than wild-type strains, though *ldt* mutants showed significantly higher sensitivity to low osmotic pressure than wild-type strains. Through experiments on PG content and sensitivity in mutants, it was demonstrated that Ldts can incorporate NCDAAs produced by BsrV into PG, controlling PG quantity and strength during stationary phase and thereby regulating PG content per cell while maintaining resistance to low osmotic pressure. Recently, Alvarez et al. [45] found that D-Met toxicity in *dacA1* (D,D-carboxypeptidase) mutants results from accumulated D-Met-containing pentapeptides that inhibit PG transpeptidase activity (i.e., PBPs), thereby damaging the cell wall and causing impaired growth and morphological changes.

4.2 Regulatory Effects of NCDAAs on Bacterial Biofilms

Tong et al. [46] found that D-Cys, D-Asp, and D-Glu, as well as their mixtures, significantly enhanced the antibacterial activity of nisin against *Streptococcus mutans* (S. mutans), inhibiting its growth and biofilm formation. The proposed mechanism is that pores formed by nisin treatment facilitate NCDAA penetration into cells, affecting normal metabolism. Aliashkevich et al. [11] suggested that D-AAs such as D-Tyr, D-Pro, and D-Phe do not prevent bacterial adhesion but rather inhibit colony growth by affecting protein components of extracellular polymeric substances (EPS), thereby influencing biofilm formation without significantly impacting exopolysaccharide production and localization.

Kolodkin-Gal et al. [12] reported that D-Tyr affects biofilm formation by inhibiting TasA fiber anchoring to the cell wall. Fluorescent labeling of TasA fiber fusion localization genes in S. mutans revealed that TasA fibers anchor to film cells not treated with D-Tyr. In contrast, D-Tyr-treated cells consisted mostly of unmodified cells and fiber aggregates not anchored to cells, indicating that D-Tyr impairs TasA fiber anchoring and consequently affects biofilms. D-Tyr also significantly reduces surface attachment capacity in E. coli and B. subtilis,

causing cell detachment. By inducing changes in extracellular proteins, D-Tyr leads to altered biofilm structure and composition, inhibiting biofilm formation and further disassembling existing biofilms. Different D-Tyr concentrations may involve different mechanisms that could be species-specific [47].

5. Applications of NCDAAs in PG Synthesis

The phenomenon of NCDAA participation in PG synthesis enables the detection of cell wall dynamic processes through exogenous additives. Fluorescent D-amino acids (FDAAs) are fluorescent derivatives that can efficiently label PG in situ across multiple bacterial species, specifically probing cell wall formation/remodeling, bacterial growth, and cell morphology. Fura et al. [15] added FDAAs to B. subtilis culture medium and monitored changes in PBP TP activity through fluorescence observation. Kuru et al. [48] used time-lapse pulsing of FDAAs to elucidate dynamic changes in cell walls of "predator and prey"bacteria at different stages, visualizing bacterial invasion dynamics where the invading predator penetrates the prey bacterium' s outer membrane, degrades certain cell wall components to form pores, reseals the pores after entry, and modifies PG. With continued in-depth research on FDAAs, their emission wavelengths now span the entire visible spectrum, enabling visualization of PG synthesis in both G^- and G^+ bacteria [49].

Vancomycin, a large hydrophilic molecule (MW 1485.7) and clinically important antibiotic, has fluorescent derivatives that are specific to G^+ bacteria but ineffective against G^- bacteria because their outer membrane acts as a permeability barrier [3, 50-52]. Vancomycin conjugated with a fluorescent marker (FLUOS) forms a fluorescent derivative (Van-FL) that can bind to the terminal D-Ala-D-Ala of newly synthesized PG polypeptide chains, serving as a sensitive tool for tracking PG synthesis and probing PG topological structure with important application prospects for bacterial cell wall research. Daniel et al. [47] used fluorescence microscopy to observe Van-FL-labeled bacteria and detect nascent wall synthesis, finding that in B. subtilis, newly formed or division sites stained most intensely and that nascent PG synthesis follows a helical pattern. Lam et al. [3] used Van-FL to label cell wall precursor lipid II to monitor cell wall synthesis after adding D-AA mixtures to culture medium, observing that Van-FL staining in bacterial side walls decreased significantly 30 minutes after D-AA mixture addition, with differential nascent PG synthesis leading to varying staining intensities at different stages.

6. Future Perspectives

The mechanisms of D-AA participation in cell wall synthesis and related applications have attracted increasing attention. The bacteriostatic function of D-AAs makes them a research focus for biological sterilization, and their combination with nisin could support disease treatment. The functional roles of D-AAs and their relationship with bacterial ecology remain important topics for future re-

search. Although amino acids are nutritional substances for humans, further investigation into their safety and practicality as drugs is needed. Additionally, the mechanisms by which D-AAs enter cells to participate in metabolism remain unclear, and the relevant enzymes involved require further study. Continuous improvement in D-AA detection methods and expanding technologies provides technical support for future scientific research and lays a foundation for multidisciplinary cross-fertilization and in-depth investigation.

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Note: Figure translations are in progress. See original paper for figures.