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Beyond the wall: can D-amino acids and small molecule inhibitors eliminate infections?

“Recently, the use of small molecules produced by bacteria became a prominent strategy of biofilm dispersal in medicinal microbiology.”

First draft submitted: 15 March 2017; Accepted for publication: 21 March 2017;
Published online: 21 June 2017

Keywords: antibiofilm therapy • antibiotic resistance • biofilms • biofouling • cell-wall
• D-amino acids • dispersal • extracellular matrix • persistent infections • translation

Bacterial multicellular communities called biofilms thrive in a variety of conditions, as they provide significant benefits to the resident bacteria. In clinical settings, biofilms are often associated with persistent infections, and their formation can have deadly outcomes. In a biofilm, the bacteria are effectively sheltered from environmental insults. For example, biofilm cells can be up to 1000 times more resistant to antibiotics than planktonic (free-living) cells [1,2]. The mechanisms supporting this resistance are poorly understood.

Biofilms are tightly held together by a self-produced organic extracellular matrix, as well as by biogenic minerals [1,3]. The extracellular matrix is composed of aggregated proteins, exopolysaccharides and nucleic acids often tightly associated with the bacterial cell envelope [1]. However, once the nutrients available to the community are exhausted, the biofilm state is no longer beneficial for the bacteria – and it is actively dispersed. In the last decade, it was discovered that bacteria often generate small molecules to aid dispersal. Those molecules transcend the diffusion limiting environment and disrupt the complex extracellular matrix encapsulating biofilm cells [4]. Recently, the use of small molecules produced by bacteria became a prominent strategy of biofilm dispersal in medicinal microbiology [4].

Here, we discuss small molecules that interfere with biofilm formation. We will focus on the complex mode of action and applications of D-amino acids (DAAs)

– biofilm inhibitors and dispersal agents which are widely produced by bacteria. We will also discuss how the insights from the study of DAAs can be applied to the study of additional small molecule biofilm inhibitors.

Nearly, all bacteria synthesize a cell wall located outside of the cell membrane. This strong yet elastic network counteracts osmotic pressure, maintains cell shape and serves as a protective barrier against physical, chemical and biological assaults [5,6]. In both Gram-positive and Gram-negative bacteria, the cell wall is composed of a peptidoglycan (PG) polymer – glycan chains (alternating *N*-acetylglucosamine) and *N*-acetylmuramic acid) cross-linked by short peptides. Those are called stem peptides, and normally include the canonical (DAAs), D-Alanine and D-Glutamate (or the amidated form, D-Glutamine). Unlike L-amino acids, DAAs are not used for ribosomal synthesis of proteins. Instead, their main role is as bacterial cell-wall constituents [5,6], though some exceptions have been observed [7]. Incorporation into PG is independent of DAA production; strains that fail to produce DAAs can nonetheless incorporate them into PG at the terminal position of the stem peptide. Diverse Gram-negative and Gram-positive bacteria also produce nonconventional DAAs – other than D-Alanine and D-Glutamate. Once incorporated into the stem peptides of the PG instead of D-Alanine, those DAAs reduce the transpeptidation of PG [6,8].

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In 2010, my colleagues and I have reported that two Gram-positive bacteria – the beneficial *Bacillus subtilis* and the pathogenic *Staphylococcus aureus* – may utilize self-produced non-canonical DAAs to inhibit and disperse established biofilms [9]. It was observed that biofilm interference by DAAs is partially or fully rescued by addition of D-Alanine [9,10]. In agreement with the hypothesis that D-Alanine blocks the incorporation of DAAs into the bacterial envelope, incorporation of ^{14}C -D-Leucine into the cell wall is largely inhibited by applying access to D-Alanine [10]. The specific mechanisms that link cell–wall interference and biofilm formation remain to be determined. Intriguingly, macro-analysis of *B. subtilis* biofilms indicated that DAAs induced a malfunction of the anchoring of the extracellular matrix to the cell wall [10,11]. DAAs can also directly interfere with protein translation, leading to production of defective proteins. In some cases, aminoacyl tRNA synthase, an enzyme responsible for charging of tRNA with L-amino acids may instead load tRNA with DAAs, resulting into the formation of a highly toxic DAA–tRNA complex. It was first reported in *Escherichia coli* and *B. subtilis* that tRNA molecules are charged with D-tyrosine in the presence of tyrosyl tRNA synthetase [12], followed by similar reports on D-Valine, D-Aspartate and D-Tryptophan. Noncanonical DAAs were suggested to have a global role in inhibition of protein translation [13], although, this hypothesis was never tested directly, and a later study found no change in the overall protein levels in treated biofilm cells [10].

“The specific role proposed for D-Tyr-tRNA deacylase (DTD) in translational quality control of biofilm development makes this enzyme a compelling therapeutic target for biofilm infections.”

The proofreading activity of D-Tyr-tRNA deacylase (DTD) is responsible for hydrolyzing DAA–tRNA complex. It cleaves the bond formed between DAA and tRNA, freeing the tRNA to reconnect to the correct L-amino acid. In *B. subtilis*, the presence of a defective *dtd* allele sensitized the cells to the application of DAAs [10,13], which would be consistent with a general toxic effect of tRNA–DAA on translation. However, the biofilm inhibition clearly occurred at sub-toxic concentrations even in the presence of a defective *dtd* allele [10]. In addition, and in contrast to the expectations that the addition of D-Alanine will further impair translation and thus biofilm growth, its application actually rescued biofilm formation [9,10]. Furthermore, DAAs induced a translational response identical to a response induced by a canonical cell–wall stress [10]. Overall, those findings suggest an additional, unknown link between

protein synthesis, cell–wall stress and the assembly of macrostructures. The specific role proposed for DTD in translational quality control of biofilm development makes this enzyme a compelling therapeutic target for biofilm infections [7].

DAAs interfere with biofilm formation & promote antibiotic sensitivity of biofilm

Initially, DAAs and their effects on biofilm were studied in *B. subtilis*, a Gram-positive root associated bacterium [1]. Since then, DAAs became an example of successful translational medicine approach – demonstrating how basic principles uncovered in a model organism can be translated into therapeutic strategies relevant to pathogens. The first pathogen model to be evaluated for DAAs sensitivity was the Gram-positive pathogen *S. aureus* [14,15]. Biofilms formed by *S. aureus* play a critical role in many device-related infections, infective endocarditis, urinary tract infections and acute septic arthritis. *In vitro*, *S. aureus* biofilms were found to be inhibited and dispersed by various DAAs. Later, local delivery of sub-molar concentrations of DAAs from biodegradable polyurethanes (PUR) scaffolds inhibited biofilm formation by clinical isolates of *S. aureus* both *in vitro* and *in vivo*. An equimolar mixture of D-Met:D-Pro:D-Trp shifted the dose–response curve toward lower doses compared with the individual DAAs and exhibited minimal cytotoxicity at concentrations that are effective at dispersing biofilms. Furthermore, the addition of DAAs enhanced the activity of several antibiotic classes against biofilms of genetically distinct isolates of methicillin-resistant *S. aureus*. The greatest synergy was observed in the combination of DAAs and rifampin. Interestingly, the authors report similar trends for the Gram-negative pathogen multidrug-resistant *Pseudomonas aeruginosa* [16], with a clear potentiation toward ciprofloxacin. DAAs may have augmented the activity of antimicrobials against biofilms by promoting biofilm dispersal. More recently, another Gram-positive pathogen emerged as a potential candidate for DAAs treatments: *E. faecalis* can cause life-threatening infections such as endocarditis, bacteremia, urinary tract infection and meningitis [17], and is especially problematic in hospitals where antibiotic resistance is prevalent. In this clinically relevant pathogen, DAAs were recently demonstrated to inhibit and disperse biofilms while restoring antibiotic sensitivity to the biofilm cells [18,19]. There is also an emerging evidence for the potential role of DAAs in regulating biofilm formation of Gram-negative pathogens. While never tested for pathogenic *E. coli* strains involved in acute device related infections, DAAs directly inhibit adhesion to surfaces of a lab strain of *E. coli* [20]. In addition, the virulence of a prominent plant pathogen, *Xanthomonas citri* [21], is compromised in the presence of D-Leucine.

Recent technological and chemical breakthroughs are bridging the gap between the laboratory findings and the clinical applications for DAAs. For example, the development of noncytotoxic PUR scaffolds that allow a slow release of DAAs [15]. In a more recent study, a promising nanodevice for biofilm infections was introduced [22]. Under near infrared radiation (NIR), this nanodevice can release free DAAs, (D-Tyr) and ROS ($\cdot\text{OH}$) in a specific spatiotemporal pattern, for combined biofilm dispersion and bacterial eradication.

“...a critical mass of labs and investigators must assess and validate new developments.”

The dispersing properties of DAAs were also demonstrated in industrial settings. On industrial occasions where biofilms are an issue, the term microbial fouling or biofouling is used. In industry, biofouling causes serious problems leading to energy and product losses [23]. DAAs were shown to combat biofouling in various settings. For example, D-Tyr triggered *P. aeruginosa* biofilm dispersal from membrane filters [24]. For *Desulfovibrio vulgaris*, a Gram-negative sulfur-reducing bacterium forming biofilms on carbon steel coupons, D-Tyr and D-Met effectively inhibited biofilm formation [25,26]. DAAs also inhibited biofilm formation in industrial water by direct interactions with exopolymeric substances [27]. In addition, DAAs enhanced the efficacy of THPS, a broad-spectrum biocide, in mitigation of *D. vulgaris* biofilms [27]. Most recently, DAAs were shown to augment the action of two additional industrial biocides against a biofilm consortium of sulfate reducing bacteria. The application of a DAAs cocktail that was previously optimized for *B. subtilis* [9] together with THPS reduced by a three to four order of magnitudes the biofilm cell counts compared with THPS alone, depending on the consortia composition [28]. Strikingly, just as in the original study [9], a mix of DAAs was extremely efficient in dispersing biofilm consortia, while the individual DAAs were less potent [28].

Biofilms are an optimal ‘test-tube’ for the development of novel therapeutics

The role of DAAs in biofilm inhibition and dispersal was originally described for *B. subtilis* and *S. aureus* [9,14]. Then, a handful of conflicting publications emerged, generating a discussion within the field [11,13]. Variation in results and conclusions at an early stage is not a unique to the study of DAAs. Another example to a difficult journey to consensus is establishing quorum-sensing as a target for biofilm interference in *P. aeruginosa* [29]. In 1998, Davies and colleagues suggested a role of the *P. aeruginosa las* quorum-sensing in biofilm forma-

tion [30]. In this first study, *lasI* mutants deficient in the synthesis of 3-oxododecanoyl-HSL formed biofilms that were flat, densely packed and homogenous relative to the highly structured, heterogeneous biofilms of the wild-type parent PAO1. Then, some conflicting reports emerged. Of note it was reported that a *lasI* mutant of PAO1 formed flat homogenous biofilms that were indistinguishable from the wild-type under their experimental conditions [29]. With time, the powerful premise of quorum-sensing inhibitors was established [29]. Just as in case of DAAs, the discrepancies in initial observations often reflect the limited methodology used to analyze the influence of small molecules on biofilm biology [29] and the inadequacy of relying on biofilm formation *per se* (e.g., ‘all or none’ analysis). Higher resolution single cell imaging techniques, as well as systemic transcriptional and translational profiling of the biofilm cells [10] are necessary for detection of functional differences in biofilms grown under laboratory conditions. Furthermore, it is clear that establishing a consistent and robust framework relying on multiple independent criteria for biofilm inhibition is essential, and several experimental conditions (e.g., growth media, temperature, inoculum condition) should be used [11]. If only a single condition is tested and the kinetics of biofilm development is not taken under consideration, false-negative results are likely. Similarly, the challenges of optimizing effective subtoxic concentrations may generate an understandable tendency to increase working concentrations toward nonspecific toxic concentrations [13]. Last and most importantly, a critical mass of labs and investigators must assess and validate new developments. While initial observations may vary, different groups utilizing new methodologies and consistent experimental framework will eventually achieve a ‘quorum’. It is now clear that the role of DAAs as biofilm interfering agents, both in medicinal and industrial microbiology, has met this important criterion.

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No writing assistance was utilized in the production of this manuscript.

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