D-Amino Acids Do Not Inhibit *Pseudomonas Aeruginosa* Biofilm Formation

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Objective: *Pseudomonas aeruginosa*, a known biofilm-forming organism, is an opportunistic pathogen that plays an important role in chronic otitis media, tracheitis, cholesteatoma, chronic wounds, and implant infections. Eradication of biofilm infections has been a challenge because the biofilm phenotype provides bacteria with a protective environment from the immune system and antibiotics; thus, there has been great interest in adjunctive molecules that may inhibit biofilm formation or cause biofilm dispersal. There are reports that D-amino acids may inhibit biofilms. In this study, we test the ability of various D-amino acids to inhibit *P. aeruginosa* biofilm formation in vitro.

Study Design: We evaluated the effect of D-alanine (10 mM), D-leucine (10 mM), D-methionine (10 mM), D-tryptophan (10 mM), and D-tyrosine (10 uM and 1 mM) on biofilm formation in two commonly studied laboratory strains of *P. aeruginosa*: PAO1 and PA14.

Methods: Biofilms were grown in 24-well and 96-well tissue culture plates, documented photographically and stained with 0.1% crystal violet and solubilized in 33% glacial acetic acid for quantification.

Results: In strains PAO1 and PA14, the addition of D-amino acids did not result in an inhibitory effect on biofilm growth in 24-well plates. Repeating the study in 96-well plates confirmed our findings that D-amino acids do not inhibit biofilm formation of *P. aeruginosa*.

Conclusion: We conclude that D-amino acids only slow the production of biofilms rather than completely prevent biofilm formation; therefore, D-amino acids represent a poor option for potential clinically therapeutic interventions.

Key Words: D-amino acids, P. aeruginosa, biofilms, chronic biofilm infections.

Level of Evidence: N/A.

INTRODUCTION

Chronic biofilm infections play an important role in otitis media,[1] cholesteatoma,[2] sinusitis,[3] osteoradionecrosis,[4] tracheitis,[5] and tonsillitis,[6] making these infections difficult to eradicate. In nature, bacteria have been observed to exist in both planktonic or biofilm forms (large, complex, multicellular communities encased in a dynamic extrapolysaccharide matrix composed of extracellular DNA, polysaccharides, and proteins).[7–11] Bacteria within biofilms are highly tolerant to antibiotics and host defenses, making them a significant problem in healthcare today. Healthcare-associated infections are responsible for 1.7 to 2 million infections per year in the

United States.[12,13] According to a report by the National Institutes for Health (Bethesda, MD), biofilms account for over 80% of infections in the body,[14,15] and it is estimated that 60% to 70% of nosocomial infections in the United States specifically are associated with implanted devices.[16] The economic burden of healthcare-associated infections is considerable; the overall direct costs to hospitals in the United States range from $28 billion to $45 billion.[13] Biofilms are thus an important area of study given their impact on morbidity, mortality, and annual healthcare expenditure.

Perhaps the biggest challenge that bacteria within biofilms present is their ability to evade antibiotics and the host immune defenses. This unique ability of biofilms has been attributed to stationary phase physiology, horizontal exchange of antibacterial resistance genes, tolerance, adaptive resistance, and efflux pumps.[7,17] Resistance to host immune defenses still is being investigated; recent studies have suggested that biofilms trigger impaired neutrophil activity and use neutrophils to enhance initial biofilm development.[18,19] These factors make biofilms a particularly unique therapeutic challenge. For this reason, there has been interest in investigating molecules and surfaces that can disperse or inhibit biofilm formation and eradicate persister cells.

Recently, Kolodkin-Gal et al. reported that D-amino acids inhibited and disassembled biofilms formed by *Bacillus subtilis*.[20] However, this group later discovered that the strain had a mutation in the gene *dtd*, which prevents misincorporation of D-amino acids into

proteins.[21] When that gene was repaired, *B. subtilis* biofilms were not susceptible to inhibition or dispersion by D-amino acids.[21] Since then, there have been several other reports that D-amino acids inhibit biofilm formation in various bacterial species, including *S. aureus*, *S. epidermidis*, and other pathogens. However, numerous inconsistencies exist between these studies.[20–25]

In addition to *B. subtilis*, Kolodkin-Gal et al. reported that D-amino acids inhibit biofilm formation by *P. aeruginosa*.[20] *P. aeruginosa* is an important pathogen in opportunistic infections and cystic fibrosis and has become a major cause of nosocomial infections worldwide (10% of nosocomial infections in the European Union).[26] The ability of *P. aeruginosa* to cause these infections, colonization of biomedical implants, and infection in chronic wounds is dependent on biofilm formation by this pathogen. Therefore, we re-examined the ability of D-amino acids (leucine, methionine, alanine, tryptophan, and tyrosine) to inhibit biofilm formation in two commonly studied wild type strains of *P. aeruginosa*: PAO1 and PA14.

MATERIALS AND METHODS

Bacterial Strains

Bacterial strains used in this study were PAO1 and PA14. Our PAO1 strain was obtained from Colin Manoil's laboratory, UW Genome Sciences, at the University of Washington (Seattle, WA); PA14 was obtained from Stephen Lory's laboratory, department of Microbiology and Immunobiology, at Harvard University (Boston, MA). Both strains were stored at -80°C in 20% glycerol and plated onto Luria-Bertani (LB) agar to obtain single, isolated colonies, which were then inoculated into LB broth for planktonic growth to log phase. Planktonic cultures were grown at 37°C, with shaking at 225 rotations per minute (rpm) for 16 to 18 hours (New Brunswick Scientific C-24 Classic Benchtop Incubator Shaker, Edison, NJ). The absorbance of an overnight culture of PAO1 and PA14 grown in LB broth (described above) was measured at an optical density of 600 nanometer (nm) using a 1:10 dilution (Barnstead Thermolyne Turner Spectrophotometer Model 340, Dubuque, IA). All cultures were adjusted with sterile LB to a uniform OD of 0.3 at 600 nm.

24-Well Plate Qualitative Biofilm Assay

Eight D-amino acid conditions and eight L-amino acid conditions were tested: 10 mM D/L-leucine, 10 mM D/L-methionine, 10 mM D/L-alanine, 10 mM D/L-tryptophan, 1 mM D/L-tyrosine, 10 uM D/L-tyrosine, D/L-amino acid mix 1 (mixture of 10 mM Leu, Met, Ala, Trp, and 10 uM Tyr), and D/L-amino acid mix 2 (mixture of 10 mM Leu, Met, Ala, Trp, and 1 mM Tyr). The amino acids and concentrations tested were chosen based on previously published studies.[20] Planktonic cultures were diluted 1:1000 in sterile M63 minimal media supplemented with MgCl$_2$ (1 mM), D-glucose (0.2%), casamino acids (0.5%),[20] and the D- or L-amino acid condition described above. One milliliter of this mixture was then distributed into each well of a sterile, untreated, 24-well polystyrene cell culture plate (CellStar 24W suspension multiwall plate, Greiner Bio One, Austria) and incubated at 30°C for 24, 48, and 72 hours (Fisher Isotemp Incubator Model 255D, Waltham, MA). PA14 biofilm formation in D/L-leucine, -methionine, -mix1, -mix2, and tryptophan were incubated for 7 days. PAO1 biofilm formation in D/L-mix1 and -mix2 was also incubated for 7 days. Prior to incubation, each plate was sealed with a gas-permeable membrane to allow for a high, uniform rate of gas exchange in all wells while maintaining a contaminant-free environment within each well (Breathe Easy Gas Permeable Sealing Membrane for Microtiter Plates, Diversified Biotech, Dedham, MA). At each time point (24, 48, 72, and 168 hours), the plates received a gentle wash in tap water. The remaining adherent biofilms were dried for at least 48 hours and then stained with 0.1% crystal violet and rinsed. Quantitative measures of remaining crystal violet were not performed in this part of the study (in 24-well plates) because of the variability of adherence of these large biofilms. Alternatively, this measure was performed in 96-well plates (see next section, 96-Well Plate Quantitative Biofilm Assay).

96-Well Plate Quantitative Biofilm Assay

The bacterial strains were prepared in the same manner as in the 24-well plate biofilm assay section. After diluting the bacterial cultures in 1:1000 M63 media supplemented with the appropriate single amino acid or amino acid mixture, 100 µL of this dilution was distributed into each well of a 96-well plate (Corning® 96 Well Clear Flat Bottom Polyvinyl Chloride (PVC) not Treated Microplate Corning Inc., Corning NY) with 16 replicates for each bacterial strain/amino acid condition to control for well-to-well variation. An additional condition was tested; biofilms were grown in the presence of 20× minimal inhibitory concentration (MIC) ciprofloxacin as a comparison. Ciprofloxacin will inhibit biofilm growth by inhibiting bacterial growth and proliferation. A gas-permeable membrane was applied to each plate and incubated for 6, 12, 18, 24, 36, 48, 72, and 96 hours at 30°C. To assess biofilm formation, each plate was washed twice by submerging and gently shaking the plate back and forth in tap water. The plates were then dried for at least 48 hours. After drying, 125 microliters of 0.1% crystal violet was added to each well and incubated for 10 minutes at room temperature to stain for adherent biofilm. After 10 minutes incubation, the plates were washed in a tap water basin four times by complete submersion of the plate with gentle shaking. The plates were then dried for at least 48 hours. When the plates were dry, the crystal violet was extracted by solubilization in 200 µL of 33% glacial acetic acid for 15 minutes on an orbital shaker at 140 rpm (Lab Line Junior Orbit Shaker, Melrose Park, IL). The amount of crystal violet eluted was then measured using a microplate reader (Å595 nm) (Biotek Synergy HT, Winooski, VT).

RESULTS

We began by examining biofilm formation by two strains of *P. aeruginosa*: PAO1 and PA14. The strains were grown in LB media, diluted into M63 minimal media, and added to 24-well plates. A diffusion membrane was placed over the wells, and the plates were incubated at 30°C for up to 7 days. Amino acids were added to designated wells prior to incubation. Biofilm formation was assayed visually, and photographs were taken.

PAO1

In the absence of amino acids, there was increased biofilm formation with increased incubation time as seen in both the dark field and crystal violet stains (Fig. 1A). Addition of L- versus D-amino acids (alanine, leucine, tyrosine, methionine, or tryptophan) did not have an inhibitory effect on formation of biofilms at 24, 48, and 72 hours (Fig. 1B). Mixes of L-amino acids also had no effect, whereas mixes of D-amino acids delayed biofilm formation at 24, 48, and 72 hours. However, this effect dissipated by 168 hours, indicating that D-amino acids do not prevent biofilm formation (Fig. 1C).
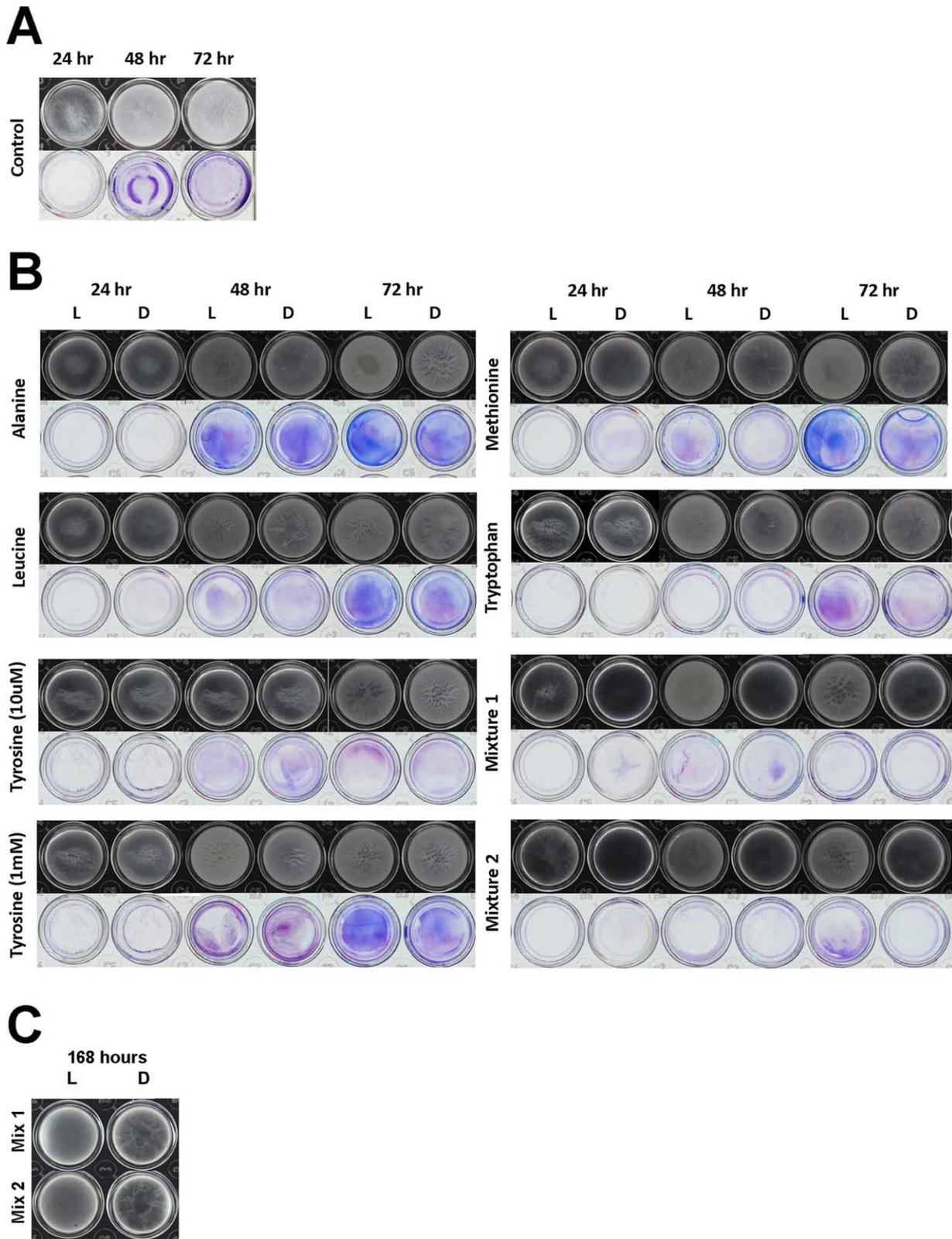
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Fig. 1. Effect of D-amino acids on biofilm formation by PA01 in 24-well plates. Side-by-side comparison of biofilms grown in the absence or presence of D- and L-amino acids at 24, 48, 72, and 168 hours. The amino acid added is listed to the left of each panel. Mixture 1 contains 10 mM concentration of alanine, leucine, methionine, tryptophan, and tyrosine at 10 uM. Mixture 2 contains the same, but the tyrosine is at 1 mM. (A) Biofilm growth in the absence of amino acid. (B) Biofilm grown in the presence of amino acids at 24, 48, and 72 hours. (C) Biofilm growth in the presence of amino acids at 168 hours.

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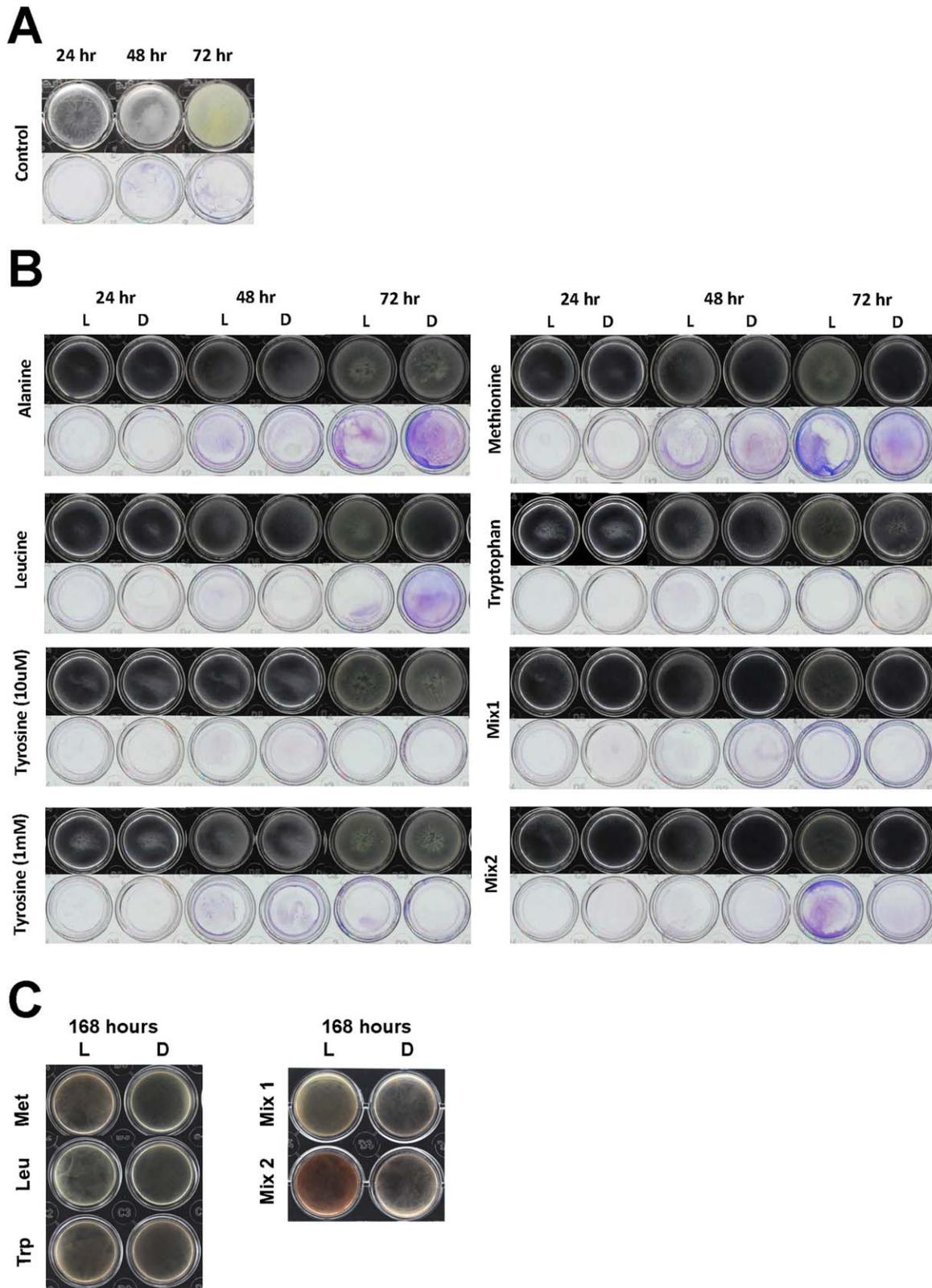
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Fig. 2. Effect of D-amino acids on biofilm formation by PA14 in 24-well plates. Side-by-side comparison of biofilms grown in the absence or presence of D- and L-amino acids at 24, 48, 72, and 168 hours. The amino acid added is listed to the left of each panel. Mixture 1 contains 10 mM concentration of alanine, leucine, methionine, tryptophan, and tyrosine at 10 uM. Mixture 2 contains the same, but the tyrosine is at 1 mM. (A) Biofilm growth in the absence of amino acid. (B) Biofilm grown in the presence of amino acids at 24, 48, and 72 hours. (C) Biofilm growth in the presence of amino acids at 168 hours.
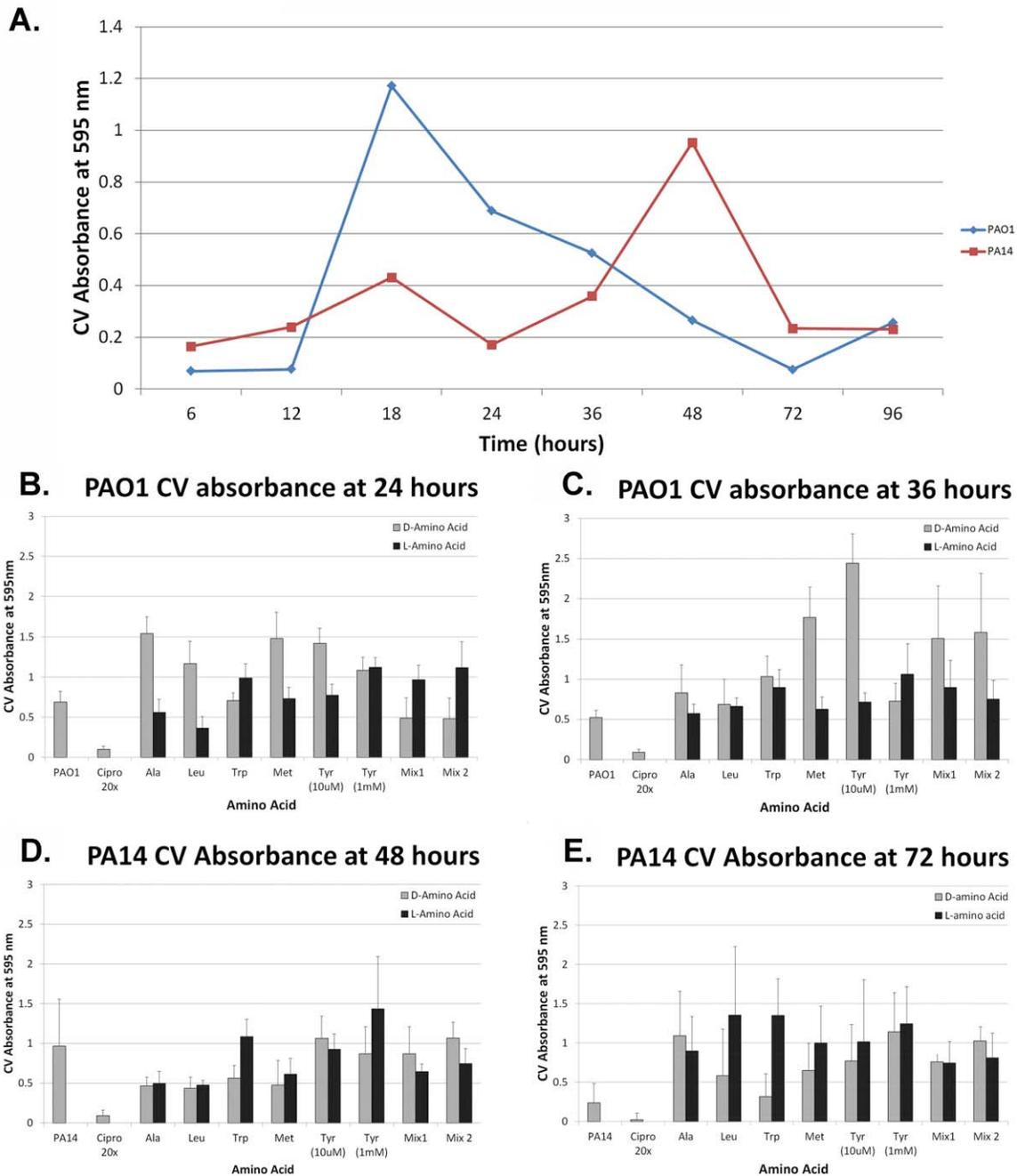
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Fig. 3. Effect of D-amino acids on biofilm formation by PA01 and PA14 in 96-well plates. (A) Graphic depiction of PAO1 and PA14 biofilm development over the course of 96 hours. PAO1 biofilm growth was assayed in the presence of ciprofloxacin versus various amino acids at 24 hours (B) and 36 hours (C). PA14 biofilm growth was assayed in the presence of ciprofloxacin versus various amino acids at 48 hours (D) and 72 hours (E).

PA14

We then examined biofilm formation by a second *P. aeruginosa* strain: PA14. In the absence of amino acid supplementation, PA14 formed biofilms similarly to PA01 (Fig. 2A). In contrast to PA01, individual D-amino acids delayed biofilm formation up to 72 hours (Fig. 2B) but did not ultimately prevent biofilm formation (Fig. 2C). Thus, for both PA01 and PA14, D-amino acids do not prevent biofilm formation.

Although it was clear that D-amino acids do not prevent biofilm formation, there was some inconsistency

in the crystal violet staining in the 24-well plates (Fig. 1 and Fig. 2). This may have been due to the loose adherence of the biofilms to the surface of the plate, resulting in loss of material during the wash prior to staining with crystal violet (compare dark field photographs and crystal violet stains in Fig. 1 and Fig. 2). Because of this inconsistency, we quantitatively re-examined biofilm formation in a 96-well assay in which the biofilms were more tightly adherent to the well surface.

Careful examination of biofilm formation by the two strains revealed that the two strains formed biofilms at

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different rates (Fig. 3A). Notably, PAO1 formed biofilms more quickly than PA14 (Fig. 3A). Because of this observation, we analyzed biofilm formation at various time points to ensure that we detected peak biofilm formation. Specifically, we measured biofilm formation by PA01 at 24 and 36 hours (Fig. 3B and 3C) and PA14 at 48 and 72 hours (Fig. 3D and 3E), respectively. In this experiment, we included the antibiotic ciprofloxacin as a known control that prevents biofilm formation. In contrast to ciprofloxacin, D-amino acids did not prevent biofilm formation by PA01 or PA14. In fact, the presence of certain D-amino acids appeared to enhance biofilm formation by PA01.

DISCUSSION

Because the use of D-amino acids might be a well-tolerated antibiofilm factor in the treatment of biofilm infections, we chose to study a variety of these molecules in their ability to inhibit two common laboratory strains of *P. aeruginosa*. In this study, we looked at the formation of biofilms by PA01 and PA14 in the presence and absence of D- and L-amino acids using both a 24-well and 96-well assay. Although we observed a modest delay in biofilm formation by both strains in the presence of D-amino acids, this effect dissipated in all conditions by 72 to 168 hours. Thus, we were unable to reproduce a previously published result, suggesting that D-amino acids inhibit biofilm formation by *P. aeruginosa*.

The effect of D-amino acids on biofilm formation has recently come under scrutiny because the original result showing biofilm dissolution in *B. subtilis* was shown to be due to a mutation in the strain used in that study.[21] Although Kolodkin-Gal et al. also reported that 10 µM D-tyrosine or 5 nM mixtures of several D-amino acids were able to inhibit biofilm formation in *P. aeruginosa* in their original study,[20] they did not repeat their experiments on *P. aeruginosa* biofilm in their subsequent publication.[21] Several other studies have examined the effect of D-amino acids on biofilm formation by *P. aeruginosa*. However, these studies were unable to convincingly show an effect. For example, Brandenburg et al. showed that both D- and L-tryptophan inhibited biofilm formation in *P. aeruginosa*.[22] Sanchez et al.[23–25] investigated the effect of D-methionine, D-phenylalanine, and D-tryptophan on biofilm dispersal in six strains of *P. aeruginosa* and found that biofilm dispersal was strain-dependent. Moreover, in contrast to the Kolodkin-Gal et al. study,[20] Sanchez et al.[23] clearly demonstrated that D-tyrosine was ineffective in biofilm dispersal of *P. aeruginosa*. Finally, Sanchez et al.[25] also reported toxic effects of D-amino acids on *P. aeruginosa* viability, thus confounding the interpretation of their results.

Considering that the inhibitory effects of D-amino acids on biofilm formation were first observed using a *B. subtilis* mutant strain that is sensitive to the presence of D-amino acids, this has raised questions about the validity of this finding. In a careful review of the literature, D-amino acids occasionally have an effect on biofilm formation that is not replicable between studies. In our study, we systematically examined the effect of D-amino

acids on biofilm formation by *P. aeruginosa* and were unable to reproduce earlier reports.

CONCLUSION

Our results show that D-amino acids did not prevent *P. aeruginosa* biofilm formation. This was true for two *P. aeruginosa* strains, PAO1 and PA14, in the presence of D-alanine, D-methionine, D-leucine, D-tryptophan, D-tyrosine, and D-amino acid mixtures. Thus, we conclude that D-amino acids do not represent a viable therapeutic option for treatment of *P. aeruginosa* biofilm-related infections.

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