Inhibition of Biofilm Formation by D-Tyrosine: Effect of Bacterial Type and D-Tyrosine Concentration

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Abstract

D-Tyrosine inhibits formation and triggers disassembly of bacterial biofilm and has been proposed for biofouling control applications. This study probes the impact of D-tyrosine in different biofilm formation stages in both G⁺ and G⁻ bacteria, and reveals a non-monotonic correlation between D-tyrosine concentration and biofilm inhibition effect. In the attachment stage, cell adhesion was studied in a flow chamber, where D-tyrosine caused significant reduction in cell attachment. Biofilms formed by *Pseudomonas aeruginosa* and *Bacillus subtilis* were characterized by confocal laser scanning microscopy as well as quantitative analysis of cellular biomass and extracellular polymeric substances. D-Tyrosine exhibited strong inhibitive effects on both biofilms with an effective concentration as low as 5 nM; the biofilms responded to D-tyrosine concentration change in a non-monotonic, bi-modal pattern. In addition, D-tyrosine showed notable and different impact on EPS production by G⁺ and G⁻ bacteria. Extracellular protein was decreased in *P. aeruginosa* biofilms, but increased in those of *B. subtilis*. Exopolysaccharides production by *P. aeruginosa* was increased at low concentrations and reduced at high concentrations while no impact was found in *B. subtilis*. These results suggest that distinct mechanisms are at play at different D-tyrosine concentrations and they may be species specific. Dosage of D-tyrosine must be carefully controlled for biofouling control applications.

Keywords

D-tyrosine, Biofouling, Biofilm, D-amino acids, Extracellular polymeric substances

1. Introduction

Biofilm is the preferred form of growth for bacteria in most environmental conditions. (Karatan and Watnick, 2009) The biofilm formation process starts with bacterial cells approaching and depositing on the surfaces. (Karatan and Watnick, 2009, O'Toole and Kolter, 1998) The cells attach to the surface then proliferate to form microcolonies and eventually develop into a continuous biofilm. (Mansouri et al., 2010) The undesirable growth of biofilms in water and wastewater systems, referred to as biofouling, can lead to severe operational problems. (Hall-Stoodley et al., 2004, Parsek and Singh, 2003)

Once established, biofilms are very difficult to eradicate because the extracellular polymeric substances including proteins and polysaccharides protect bacteria from hydraulic shear and disinfectants or antibiotics. (Flemming et al., 1997, Herzberg et al., 2009, Mah and O'Toole, 2001) Current practice for biofouling control in water and wastewater systems heavily relies on the use of disinfectants and antibiotics, which has several limitations. Firstly, oxidative disinfectants including chlorine and ozone can form toxic disinfection byproducts. (Shah and Mitch, 2012) In addition, polyamide NF and RO membranes are highly susceptible to oxidative degradation, (Mansouri et al., 2010) thus it is not feasible to maintain a disinfectant residual in NF or RO systems. Therefore, any remaining bacteria in the feed water may eventually lead to biofouling. Finally, use of antibiotics is not desirable due to the wide-spread and increasing antibiotic resistance in bacteria. (Andersson and Hughes, 2014, Michael et al., 2013) On the other hand, it may be undesirable to inactivate bacteria in systems such as membrane bioreactors,

where biological activity must be maintained. (Drews, 2010) Therefore, a non-biocidal approach to biofouling control without inducing resistance is highly desirable.

D-amino acids were recently found to inhibit biofilm formation and trigger self-dispersal of biofilms without affecting bacterial growth. Kolodkin-Gal et al. (Kolodkin-Gal et al., 2010) found four D-amino acids in mature biofilms of *Bacillus subtilis* and showed that they could effectively inhibit biofilm formation at the water-air interface and trigger biofilm disassembly. D-Tyrosine had the highest efficacy with an effective concentration of 3 μ M, while the equimolar mixture of the four D-amino acids showed strong synergy with an effective concentration of 10 nM. (Kolodkin-Gal et al., 2010) D-amino acids were also reported to reduce submerged biofilms formed on solid substrates by *Staphylococci aureus*, *P. aeruginosa* and a mixed culture from activated sludge. (Hochbaum et al., 2011, Kolodkin-Gal et al., 2010, Sanchez et al., 2013, Xu and Liu, 2011b) Considering that D-amino acids are non-toxic and can be produced by bacteria, they present a potentially low cost, non-toxic and non-biocidal approach to biofouling control. Its efficacy for membrane biofouling control was recently demonstrated in bench-scale ultrafiltration and nanofiltration systems.(Xu and Liu, 2011b, Yu et al., 2012)

Further development of biofouling control strategies using D-amino acids requires understanding the specific role of D-amino acids in the inhibition and disassembly of biofilms formed by different bacterial species as well as the effective concentrations needed. However, there is limited knowledge on how extraneous D-amino acids interact with bacteria. Although present at very low quantities, D-amino acids are common constituents of peptidoglycan in bacterial cell wall. (Typas et al., 2011) Bacteria also produce a variety of non-canonical D-amino acids into periplasm that have unique functions. The accumulated concentration of D-amino acids in a bacterial culture in the stationary phase can reach milimolar level (Lam et al., 2009),

which has been suggested to be a biofilm self-regulation mechanism. (Kolodkin-Gal et al., 2010)

Several studies have shown various effects of D-amino acids that contribute to their impact on biofilms, including changing peptidoglycan layer structure (Cava et al., 2011a, Cava et al., 2011b, Kolodkin-Gal et al., 2010, Lam et al., 2009, Romero et al., 2011), interfering with protein synthesis (Leiman et al., 2013) or quorum sensing (Xu and Liu, 2011a, Xu and Liu, 2011b), and inhibiting cell growth (Leiman et al., 2013, Sanchez et al., 2013). Although they all presented strong evidence of D-amino acids' inhibitive effect on biofilms, the physiological changes observed in different studies were very different. One possible cause of such discrepancy is the large difference in D-amino acids concentration used, which ranged from 2.5 nM (Kolodkin-Gal et al., 2010) to 100 mM (Ramon-Perez et al., 2014)). However, the effect of D-amino acid concentration has not been investigated. In addition, several proposed mechanisms are related to bacterial cell wall structure, but D-amino acids were found to inhibit biofilms of both Gram positive (G+) and Gram negative (G-) bacteria, which have distinct cell wall structures. It is therefore important to compare the susceptibility of G+ and G- bacteria to D-amino acids.

The present study systematically examined the effect of D-tyrosine, over a wide concentration range, on the initial cell attachment and subsequent biofilm formation of pure cultures of model G+ and G- bacteria, and characterized the specific effects on each biofilm composition to elucidate the role of D-tyrosine in biofilm inhibition. We show for the first time that D-tyrosine inhibits biofilm formation by both G+ and G- bacteria at concentrations as low as 5 nM. The inhibition is a result of both reduced initial cell attachment and changes in EPS production, although the impact on EPS production varies widely depending on the type of bacteria and D-tyrosine concentration. We also show for the first time that bacterial biofilms respond to D-

tyrosine concentration non-monotonically, suggesting that different mechanisms are involved at different concentrations. The specific mechanisms involved will be discussed in another paper.

2. Materials and methods

2.1. Chemicals

D-Tyrosine (99%) and D-leucine (99%) were purchased from Sigma-Aldrich (St. Louis, MO). D-Tryptophan (99%) and D-methionine (99%) were purchased from Alfa Aesar (Ward Hill, MA) and MP Biomedicals (Santa Ana, CA), respectively. Phosphate buffered saline (PBS) was diluted from PBS 10X concentrate (Fisher Scientific, Pittsburg, PA) with ultra pure water (18.0 Ω , generated by E-pure system, Barnstead, IL). Reagent grade inorganic salts used include K_2HPO_4 (Sigma-Aldrich, St. Louis, MO), NaCl, KH_2PO_4 , $(NH_4)_2SO_4$, $MgSO_4$ and $NaHCO_3$ (Fisher Scientific, Pittsburg, PA). Ampicillin (Sigma-Aldrich, St. Louis, MO) was applied in media and buffers for GFP labeled *E. coli*. Crystal violet was purchased from Fisher Scientific (Pittsburg, PA), and 4', 6-diamidino-2-phenylindole (DAPI), Syto45, Fluorescein (FITC) and conA-tetramethylrhodamine were from Invitrogen (Carlsbad, CA). The organic solvents used include DMSO (Sigma-Aldrich, St. Louis, MO) and ethanol (190 proof, Decon Labs, King of Prussia, PA).

2.2. Bacteria strain and media

One G+ (*Bacillus subtilis* ATCC 6051) and two G- (*Pseudomonas aeruginosa* ATCC 700829 and *Escherichia coli* JM109G) bacteria were used in the study. *Escherichia coli* JM109G (kindly provided by Dr. Sharon Walker) was labeled with GFP for in situ visualization during the attachment experiment. *P. aeruginosa* and *B. subtilis* were grown in TSB media statically at 37 $^{\circ}C$ to late exponential phase until $OD_{600} = 0.8$ ($\sim 5 \times 10^8$ CFU/mL) and washed twice with PBS

prior to use. *E. coli* was grown statically at 37 °C until late exponential phase ($OD_{600} = 1.0$, $\sim 1 \times 10^9$ CFU/mL) in Trypticase soy broth (BD BBL™, Franklin Lakes, NJ) supplemented with 5 µg/mL ampicillin, and washed twice with PBS containing 5 µg/mL ampicillin. The biofilm culturing media used was M63 (28g KH_2PO_4 , 14g K_2HPO_4 , 8g $(NH_4)_2SO_4$ in 1L ultra pure water) supplemented with 5g Casamino acids (Fisher Scientific, Pittsburg, PA), 0.2% or 2% glucose and 1 mM $MgSO_4$. (Kolodkin-Gal et al., 2010)

2.3. Biofilm cultivation

Biofilm cultivation conditions were optimized to produce robust biofilms of *P. aeruginosa* and *B. subtilis* in order to minimize variation caused by sample handling. For most experiments, biofilms were grown in M63 media with 2% glucose in polystyrene 12-well plates. Each well was seeded with 2 mL bacterial suspension with an initial concentration of 5×10^5 CFU/mL. Biofilms were then incubated at 37 °C with agitation on an orbital shaker at 90 rpm (*P. aeruginosa*) or statically (*B. subtilis*) for 24 h, and analyzed for biomass and EPS using methods described below. To evaluate the impact of D-amino acids on preformed biofilms, 2 mL wash solutions containing PBS and D-amino acids were added to biofilms grown in the absence of D-amino acids, and the plates were agitated at 90 rpm on an orbital shaker for 30 min. The remaining biofilms were quantified using the crystal violet assay described below. To characterize the biofilms using CLSM, biofilms were grown on glass slides immersed in 20 mL of bacterial suspensions in modified M63 media with 2% (*P. aeruginosa* and *B. subtilis*) or 0.2% (*P. aeruginosa*) glucose containing different concentrations of D-tyrosine in glass petri dishes. The same incubation conditions were used as samples grown in 12-well plates.

2.4. Bacterial attachment under flow conditions

To investigate the impact of D-tyrosine on initial bacterial attachment, attachment of the three model bacteria on glass surface in the presence and absence of D-tyrosine was characterized in a rectangular parallel plate flow chamber (GlycoTech, Gaithersburg, MD) of 3 in $\times$ 1.5 in $\times$ 0.34 in. D-tyrosine concentration tested for *E. coli* and *B. subtilis* was 100 μ M. D-tyrosine concentrations from 5 nM to 200 μ M, the same as those used in the biofilm experiments, were tested for *P. aeruginosa* to investigate the effect of concentration.

Immediately before the experiment, the flow chamber and the tubings were cleaned with 1% SDS and rinsed thoroughly with DI, followed by sterilization with ethanol and rinse with sterilized DI. The system was then preconditioned with the background solution (PBS with 1% LB). The bacterial stock suspensions were diluted to 4.5×10^5 CFU/mL using PBS supplemented with 1% LB media (5 μ g/mL ampicillin was added for *E. coli*) and various concentrations of D-tyrosine, and pumped through the flow chamber at a rate of 40 μ L/min (shear stress of 2.06×10^{-3} N/m²) for 3 h for *P. aeruginosa* and *B. subtilis* and 3h or 13 h for *E. coli*. Then, the flow chamber was washed with PBS at the same flow rate for 60 min to remove unattached cells. The number of cells attached on the glass surface after the attachment was quantified using an inverted fluorescence microscope (Olympus IX71, Japan). For GFP labeled *E. coli*, the cells were enumerated *in situ*. For *P. aeruginosa* and *B. subtilis*, the flow chamber was disassembled after each experiment and the cells on the glass slide were stained with 10 μ g/mL DAPI for 10 min before imaging. The attached cell number was averaged from 20 images taken along the center streamline. The impact of D-tyrosine on cell detachment was studied in an additional step after attachment of GFP labeled *E. coli* in the flow chamber, in which the flow chamber was flushed with PBS for 30 min at an increased flow rate of 500 μ L/min.

2.5. Cell zeta potential measurement.

The impact of D-tyrosine on cell surface charge was studied by measuring the cell zeta potential. Bacteria cells were incubated in PBS with 1% LB and different concentrations of D-tyrosine for 3 h with initial cell concentration of 1×10^8 CFU/mL and harvested by centrifugation. The cell pellet was washed twice and resuspended in 10 mM KCl. Cell zeta potential was measured by Zetasizer Nano (Malvern Instruments, Worcestershire, UK).

2.6. Biomass quantification

Biofilms grown on 12-well plates were quantified using the crystal violet assay.(Pang et al., 2005) Details of the method including measures taken to improve reproducibility are described in Supplementary data. Six control wells and six D-tyrosine added wells were included in each plate. Twelve replicate wells on two separate plates were run for each condition in every experiment and each experiment was repeated 3 times.

2.7. EPS characterization

The composition and structure of the biofilms were characterized by CLSM using a triple staining method.(Chen et al., 2006, Yu et al., 2011) Biofilms grown on glass slides were gently washed twice with 10 mL PBS and stained with 20 μ M syto 45 in dark for 45 min to label the cells. Excess dye was rinsed off with 1 mL PBS. To protect amine groups, the glass slide was rinsed with 1mL and immersed in 10 mL 0.1 M NaHCO_3 . Proteins were stained by adding 200 μ L of 1 mg/mL FITC dissolved in DMSO. The samples were agitated on an orbital shaker at 50 rpm for 1 h followed by sequential wash with 1 mL 0.1 M NaHCO_3 and PBS. In the last step, alpha-D-glucopyranose in polysaccharides was stained with 200 μ g/mL conA conjugated tetramethylrhodamine for 30 min. After rinse with PBS, the glass slides were covered with a cover slip and sealed with nail polish. Z-stack 3-D images of the biofilms were taken over a 12 μ m depth in 0.5 μ m steps at 3 random locations on the slide using a Nikon A1-Rsi confocal laser

scanning microscope. The excitation laser wavelengths used for syto 45, FITC and conA-tetramethylrhodamine were 401.2, 488.0 and 561.8 nm, respectively. The fluorescence volume of each channel was quantified with the volume analysis function of the NIS Element 4.0 software. The average was calculated from a total of 6 images taken from two independent experiments at random locations on the glass slide.

To quantitatively assess the impact of D-tyrosine on extracellular proteins and polysaccharides, bounded EPS was extracted from *P. aeruginosa* biofilms and analyzed for protein and polysaccharide contents using the BCA assay and the sulfuric acid-phenol method, respectively (see Supplementary data). (Liu and Fang, 2002) Two independent experiments were conducted, and each experiment included two replicates for each condition.

3. Results and Discussion

3.1. Effect on bacterial cell attachment.

The effect of D-tyrosine on bacterial attachment was studied under flow conditions using two G- bacteria, *E. coli* and *P. aeruginosa* and one G+ bacterium, *B. subtilis*. Figure 1 presents the attached cell numbers normalized by the influent cell concentration and image area. GFP labeled *E. coli* showed notably less attachment at 3 h compared to the other two bacteria, around 30 and 25% of that for *B. subtilis* and *P. aeruginosa*, respectively. At 100 μ M, D-tyrosine significantly reduced the surface attachment of both *E. coli* and *B. subtilis*. The effect seemed to be greater on *B. subtilis* (70% reduction) than on *E. coli* (16%) with 3 hours of attachment time, presumably due to the very low attachment of *E. coli*. When the attachment time was increased to 13 hour, notably more *E. coli* cells attached in the control, and the impact of D-tyrosine became much more obvious (74% reduction). Using the GFP labeled *E. coli*, cell detachment was also studied.

A large fraction of cells attached in the presence of D-tyrosine detached while there was no notable detachment of cells attached in the absence of D-tyrosine (Figure S1). The effect was greater at longer attachment time suggesting that the contact time of bacterial cells with D-tyrosine may be important.

The effect of D-tyrosine concentration on cell attachment was investigated using *P. aeruginosa*. The attachment of *P. aeruginosa* was significantly reduced ($p < 0.1$) at a wide range of D-tyrosine concentrations (Figure 1C). However, zeta potential measurements showed that neither L- nor D-tyrosine affected *P. aeruginosa* surface zeta potential (Figure S2), suggesting that the decreased cell attachment cannot be simply attributed to changes in cell surface charge due to adsorption of D-tyrosine. Given the importance of EPS in forming irreversible adhesion (Karatan and Watnick, 2009), it was hypothesized that D-tyrosine reduced EPS production or changed the physicochemical properties of EPS. Interestingly, the effect of D-tyrosine concentration exhibited a non-monotonic pattern. Cell attachment was not affected at 1 and 5 μM D-tyrosine, while both lower and higher concentrations notably reduced *P. aeruginosa* attachment. The same effect was also observed in subsequent biofilm development, and will be discussed later.

3.2. Inhibition on biofilm growth.

The effect of D-tyrosine on biofilm growth of *P. aeruginosa* and *B. subtilis* during the 24 hour incubation is shown in Figure 2. Data are shown as percent reduction in cellular biomass compared to the control as a function of D-tyrosine concentration. Each data point shown represents results from a total of 108 measurements made with 36 extract samples obtained from 12 biofilm samples grown in 3 independent experiments, each experiment including duplicate plates. A total of 324 control biofilms were extracted and analyzed. The biofilm growth of both

P. aeruginosa and *B. subtilis* exhibited interesting, non-monotonic response to D-tyrosine concentration, which was not reported before. At both high (20, 50 and 200 μ M) and low (5 nM and 0.5 μ M) concentrations, the presence of D-tyrosine led to significant ($P < 0.05$) reduction (18 to 28%) in *P. aeruginosa* biofilm. However, the amount of biofilm grown at these different D-tyrosine concentrations was not significantly different from each other. Interestingly, at 1 to 10 μ M, D-tyrosine showed no significant reduction on *P. aeruginosa* biofilm while 1 μ M D-tyrosine even slightly increased biomass. This non-monotonic response to D-tyrosine concentration was similar to that found in the attachment experiments, suggesting that the reduced cellular biomass can be partly attributed to the reduced cell attachment and subsequently delayed biofilm development.

All D-tyrosine concentrations tested significantly reduced *B. subtilis* biofilm formation. Compared to *P. aeruginosa*, D-tyrosine appeared to have stronger inhibitive effects on *B. subtilis*, whose biofilm was reduced by 42% with 5 nM D-tyrosine. The response of *B. subtilis* biofilm to D-tyrosine concentration also exhibited a non-monotonic trend, with the smallest impact of D-tyrosine observed at 1 μ M.

For both bacteria, D-tyrosine effectively reduced biofilm formation at 5 nM. This is the first time that a single D-amino acid was shown to inhibit biofilms at sub-micromolar concentration, suggesting the potential of D-tyrosine as a cost-effective biofilm control chemical. The non-monotonic effect of D-tyrosine concentration on biofilm reduction suggests that different mechanisms may be involved at different concentration levels. It is noted that both *B. subtilis* and *P. aeruginosa* release several D-amino acids including D-tyrosine at micromolar concentration level in the stationary phase. (Lam et al., 2009) At concentrations beyond the physiological production level, D-tyrosine has been shown to replace D-alanine in the

peptidoglycan layer, affecting its thickness and anchorage of surface proteins (in G⁺ bacteria), (Lam et al., 2009, Lupoli et al., 2011) and hence cell-cell or cell-surface interactions. Leiman et al. showed retardation in *B. subtilis* cell growth in the presence of D-tyrosine at concentrations as low as 6 μ M possibly through misincorporation into cellular protein during protein synthesis, which also contributed to the reduced biomass in biofilm.(Leiman et al., 2013) Kolodkin-Gal et al. found that the mixture of D-tyrosine, D-leucine, D-tryptophan and D-methionine inhibited *B. subtilis* biofilm formation at 10 nM.(Kolodkin-Gal et al., 2010) D-amino acids at such low concentrations may serve as signals of factors such as environmental stress for biofilm self-regulation.(Cava et al., 2011b) Depending on the concentration, the presence of D-tyrosine may also lead to defense responses that repair the impaired cell wall and even trigger biofilm formation. D-amino acids can also be utilized as a nutrient, and potentially stimulate growth. (Conrad et al., 1974)

B. subtilis biofilms detached more easily in the presence of D-tyrosine as well as the mixture of D-tyrosine, D-methionine, D-tryptophan and D-leucine with the mixture being more effective in removing *B. subtilis* biofilms (Figure S4). The effective concentration was as low as 10 nM for D-tyrosine and 5 nM for the mixture. However neither D-tyrosine nor the mixture showed significant impact on preformed *P. aeruginosa* biofilms at the concentrations tested (Figure S5).

3.3. Effect on Biofilm Composition.

Figures 3 and S6 present 3-D images of biofilms formed on glass slides by *P. aeruginosa* and *B. subtilis*, respectively, in M63 media supplemented with 2% glucose. *P. aeruginosa* biofilms were also grown with 0.2% glucose (Figure S7). The split views are shown in Figure S8-S10. In both *P. aeruginosa* and *B. subtilis* control biofilms, the cells were aggregated and covered by large amounts of EPS, although *B. subtilis* developed notably less biofilm compared to *P. aeruginosa*

in the same media. Reducing glucose percentage in the media resulted in less biofilm formation by *P. aeruginosa*. The images show the 3-D distribution of cells (blue), proteins (green) and polysaccharides (red) in the biofilm, and the volume of each component is shown in Figure 4.

Both *P. aeruginosa* and *B. subtilis* biofilms responded to D-tyrosine concentration in a non-monotonic fashion: D-tyrosine greatly reduced the quantity of cells at both low and high concentrations, but had no impact at 5 and 1 μ M on *P. aeruginosa* and *B. subtilis*, respectively (Figure 4). This is consistent with the results from the crystal violet assay. In the inhibited biofilms, bacterial cells attached sparsely and there was notably less EPS. At the lowest concentration tested, 5 nM, D-tyrosine reduced the number of cells in the biofilm by 80% and 64% for *P. aeruginosa* and *B. subtilis*, respectively. The lower cell number might be partly attributed to the lower initial attachment (Figure 1), which delayed the biofilm formation. There was notably less *P. aeruginosa* biofilm in the low glucose media (Figure S7). The non-monotonic response was also observed with the ineffective concentration at 0.5 and 50 μ M, presumably a result of the reduced cell number in the biofilm.

At the first look, the data on total proteins and polysaccharides (Figure 4) seemed to suggest effect similar to that on the cell number. A close examination of the amount of proteins and polysaccharides normalized by cell numbers (Figure 5), however, revealed some interesting phenomenon. At low concentrations (5 nM and 0.5 μ M), D-tyrosine greatly reduced extracellular protein production in *P. aeruginosa*, but had no impact on or slightly increased exopolysaccharides production. As a result, the EPS composition was notably different from that of the control, with the protein to polysaccharides ratio lowered from 1.18 to 0.60 - 0.65. At high concentrations (200 μ M or 50 μ M), D-tyrosine decreased production of both EPS components similarly by up to 52%, maintaining a proteins/polysaccharides ration similar to that

of the control. At 5 μ M, however, D-tyrosine had no notable impact on *P. aeruginosa* EPS production or biofilm composition. This is again consistent with the attachment and biofilm growth data.

The effect of D-tyrosine on cell normalized EPS in *B. subtilis* biofilms was completely different. It had no discernable impact on cell normalized exopolysaccharides over the whole concentration range tested, but increased cell normalized extracellular proteins at 5 nM and 50 μ M. This suggests that the decreased biofilm observed is most likely due to the reduced cell attachment in the presence of D-tyrosine (Figure 1B), not reduced EPS production.

To corroborate the CLSM results, *P. aeruginosa* biofilms were also grown on 12-well plates and harvested for extraction and analysis of cellular biomass and EPS. As shown in Figure 6, the results agreed qualitatively with those from CLSM characterization. On the per unit cellular biomass basis, D-tyrosine at 200 μ M reduced extracellular protein production. The degrees of reduction were different from those observed by CLSM, likely because the biofilms were grown on different surfaces (polystyrene vs glass). The cellular biomass excluding the EPS content was reduced at 5 nM D-tyrosine but not at 200 μ M, which was in accordance with the quantitative CLSM results (Figure 4).

4. Conclusions

This study shows that D-tyrosine reduces bacterial attachment and subsequent biofilm formation in both G⁺ and G⁻ bacteria at concentrations as low as 5 nM. This suggests that D-tyrosine can potentially be used as a green biofilm control strategy at low cost. The effect of D-tyrosine follows a bi-modal trend as a function of D-tyrosine concentration. High concentrations from 20 to 200 μ M and low concentrations from 5 to 500 nM significantly reduce cell attachment and

biofilm growth while intermediate concentrations from 1 to 10 μ M have no notable impact. In addition to reducing cell attachment, D-tyrosine induces changes in EPS production, which leads to changes in biofilm structure and composition. The overall effect is reduced biomass accumulation in both G⁺ and G⁻ biofilms at both high and low concentration ranges. However, the specific effects on extracellular protein and polysaccharides vary depending on D-tyrosine concentration and bacterial type, suggesting that multiple biochemical pathways may be involved. The complex behaviors of G⁺ and G⁻ bacterial biofilms in response to D-tyrosine concentration call for further investigation on the specific mechanisms involved in order to ensure proper dosage of D-tyrosine for biofilm control applications, which will be discussed in another paper. In particular, it is important to understand how bacteria mitigate the inhibitive effect of D-tyrosine at intermediate concentrations in order to avoid ineffective dosage. It is also important to determine the distinct mechanisms in G⁺ and G⁻ bacteria so that adequate application strategies can be designed to target a wide range of bacteria.

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Appendix A. Supplementary data.

The following is the supplementary data related to this article:

355 Detachment of *E. coli*; Zeta potential of *P. aeruginosa*; fluorescence images captured in
 356 attachment /detachment experiments under flow conditions; impact of D-amino acids on
 357 detachment of preformed biofilms; split channels in CLSM images for *P. aeruginosa* grown in
 358 M63 (2% glucose); 3-D and split channel images of *B. subtilis* grown in M63 (2% glucose) and
 359 *P. aeruginosa* biofilms grown in M63 (0.2% glucose) are included in the supporting information.

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Figure 1

FIGURES

Figure 1. Effect of D-tyrosine on bacteria attachment under flow conditions on glass substrate. (A) GFP labeled *E. coli*. (B) *B. subtilis*. (C) *P. aeruginosa*. The attached cell number was normalized by the image area and the initial cell concentration. The same volume of ultra pure water was added in the place of D-tyrosine solution into the control. In the attachment experiments, the media was 1% LB in PBS, the influent bacterial concentration was 4×10^5 CFU/mL, and the flow rate was 40 μ L/min. Each data point is average of counts from 20 images, and error bars represent standard deviation. The text labels on data bars represent the results of statistical analysis. Data points bearing the same text label are not statistically significantly different from each other at 90% confidence level. The corresponding fluorescence images are shown in Figure S3.

Figure 2

Figure 2. Effect of D-tyrosine on biofilm formation of (A) *P. aeruginosa* grown in M63 media with 2% glucose, shaken at 90 rpm and (B) *B. subtilis* grown statically in M63 media with 2% glucose. Biofilms were cultivated in 12 well plates, and the biomass was quantified using the crystal violet assay. The x axis is D-tyrosine concentration in logarithmic scale. The text labels represent the results of statistical analysis. Data points bearing the same text label are not significantly different from each other at 95% confidence level.

Figure 3

Figure 3. CLSM z-stack 3D images of *P. aeruginosa* biofilms grown in the presence of 0 (A), 200 μ M (B), 50 μ M (C), 5 μ M (D), 0.5 μ M (E) and 5 nM (F) of D-tyrosine in M63 media with 2% glucose. Bacteria cells were stained blue with syto 45; proteins were stained green with FITC; polysaccharides were stained red with conA-tetramethylrhodamine. The combination of protein (green) and polysaccharides (red) results in yellow; the combination of polysaccharides (red) and cells (blue) results in purple/pink. Magnification is 60x; cross section area is 164 μ m $\times$ 164 μ m.

Figure 4

Figure 4. Volume of cells and EPS in (A) *P. aeruginosa* and (B) *B. subtilis* biofilms calculated from CLSM images. The values significantly different from control were marked with * ($p < 0.05$).

Figure 5

Figure 5. Volume composition of cells and EPS in (A) *P. aeruginosa* and (B) *B. subtilis* biofilms grown on glass surface calculated from CLSM images. The values significantly different from control were marked with * ($p < 0.05$).

Figure 6

Figure 6. (A) Extracellular protein and polysaccharides production per unit biomass; (B) cellular biomass production by *P. aeruginosa* with or without D-tyrosine in M63 media with 2% glucose on 12 well polystyrene plates.