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Coaggregating *Delftia acidovorans* facilitates the metabolic activity of partner bacteria in drinking water biofilms

AFONSO A. C.[1,2,3*], GOMES I. B.[1], SAAVEDRA M. J.[2], SIMÕES L.C.[3], SIMÕES M.[1]

[1]ALiCE-LEPABE, Faculty of Engineering, University of Porto, Porto, Portugal
[2]CITAB, University of Trás-os-Montes e Alto Douro, Vila Real, Portugal
[3]CEB-LABBELS, School of Engineering, University of Minho, Braga, Portugal

*corresponding author: A. C. Afonso
e-mail: up202010573@edu.fe.up.pt

Abstract Although bacterial coaggregation has already been identified as a key mechanism in the multispecies biofilms development in diverse environments, little is known about this highly specific type of cell-cell interaction in aquatic systems. In this study, a strain of *Delftia acidovorans* isolated from drinking water (DW) and previously described with coaggregation ability, was studied for its role in biofilm development. Single and dual-species biofilms, of *D. acidovorans* and the DW representative bacteria *Citrobacter freundii* and *Pseudomonas putida*, were grown in 96-well microtiter plates and characterized in terms of metabolic activity. In addition, to prove that *D. acidovorans* can facilitate the metabolic activity of other bacteria, single biofilms were formed in the presence of *D. acidovorans* cell-free supernatant (CFS). The metabolic activity was higher when comparing single with dual-species biofilms. Furthermore, in the presence of CFS, it was evident an increase in the metabolic activity of *C. freundii* and *P. putida* biofilms. These results suggest the production of extracellular metabolites by *D. acidovorans*, that possibly act as public goods. That said, the presence of coaggregating species, namely *D. acidovorans*, in biofilms appears to create a functional cooperative microbial community, providing a favorable metabolic opportunity for partner bacteria.

Keywords: Bacterial fitness; Cell-cell interaction; Coaggregation; Public goods.

1. Introduction

Nowadays, we still see that a significant part of the world's population faces limitations in access to water for basic needs, both in quantity and/or quality (WHO, 2014). Drinking water distribution systems (DWDS) allows the control and management of water, but several problems can occur. From a microbiological point of view, , one of the main problems is biofilm formation (Simões & Simões, 2013). The presence of biofilms in DWDS allows the resistance of bacteria to disinfection, leading to bacterial growth that translates into problems of color, turbidity, odor and corrosion, as well as higher concentrations of pathogens and waterborne outbreaks (Zhu et al., 2020). Although they can exist in the form of monospecies, biofilms formed by a single bacterial species are rare in natural and man-made environments (Kostaki, Chorianopoulos, Braxou, Nychas, & Giaouris, 2012). When associated with surfaces, microorganisms usually appear in the form of complex multispecies communities. The structural and functional dynamics of these multispecies communities are largely due to the interactions between the species (James, Beaudette, & Costerton, 1995; Moons, Michiels, & Aertsen, 2009) and this complexity is often not taken into account. Indeed, interspecies spatial and metabolic interactions play a role in the organization of multispecies biofilms, being able to alter the physiology of individual biofilm species, as well as the functions of the entire community (Wimpenny, Manz, & Szewzyk, 2000). A potential key mechanism in multispecies biofilm formation is coaggregation. Coaggregation, a form of cell–cell interaction, is defined as a highly specific mechanism of recognition and adhesion of different bacterial species to each other (Rickard, Gilbert, High, Kolenbrander, & Handley, 2003), which facilitates the interaction and the integration of bacterial species into biofilms (Rickard, McBain, Ledder, Handley, & Gilbert, 2003). Coaggregation was first recognized among oral bacteria and has been extensively studied in this field, but little is known about this mechanism and its role in the development of biofilms in aquatic systems (Afonso et al., 2021). This study allows us to elucidate the role of coaggregation and how this mechanism contributes to the formation of biofilms of DW bacteria.

2. Materials and Methods

2.1. Bacteria and culture conditions

A *Delftia acidovorans* strain isolated from drinking water (DW) and with known coaggregation ability plus two strains representative of autochthonous species from DW (*Citrobacter freundii* and *Pseudomonas putida*) were selected. Bacterial cells were grown at room temperature (25 ± 2) °C, under agitation (150 rpm; shaker

IKA KS 130 shaker, Sigma-Aldrich, Portugal), overnight, using R2A broth, a validated medium for the study of freshwater bacteria (Reasoner & Geldreich, 1985; L. C. Simões, Azevedo, Pacheco, Keevil, & Vieira, 2006): [peptone 05 g/L (Oxoid, UK), glucose 0.5 g/L (Chem-Lab, Belgium), magnesium sulfate heptahydrate 0.1 g/L (Merck, Germany), sodium pyruvate 0.3 g/L (Merck, Germany), yeast extract 0.5 g/L (Merck, Germany), casein hydrolysate 0.5 g/L (Oxoid, UK), starch soluble 0.5 g/L (Sigma-Aldrich, Portugal) and di-potassium phosphate trihydrate 0.4 g/L (Aplichem Panreac, USA)]. Cells were harvested by centrifugation (10 min at 13,000g), washed in 0.85% (v/v) sterile saline solution and resuspended in R2A broth until achieve a cellular density of 10^8 cells/mL.

2.2. Delftia acidovorans cell-free supernatants

To obtain the cell-free supernatants (CFS), *D. acidovorans* was inoculated in R2A broth and allowed to grow for 72 h, to stabilize the growth phase (Morin, Morrison, Harms, & Dutton, 2022), at (25 ± 2) °C and under agitation. After the incubation period, cell suspensions were centrifuged (20 min at 13,000g) and the supernatants were filter-sterilized using 0.2 µm filters (Whatman, Germany).

2.3. Single and dual-species biofilm formation

Biofilm formation assays were performed according to the Stepanovic et al. (2000) modified microtiter plate test using a sterile 96-well microtiter plate (Tissue Culture Plate, VWR, Portugal). Single-species biofilms were formed for the three strains adding 200 µL of the cell suspensions. For single-species biofilms formed with *D. acidovorans* CFS, the wells were filled with 180 µl of cell suspensions and 20 µl of cell-free supernatant (Lúcia Chaves Simões, Simões, & Vieira, 2011). For the dual-species biofilms, the strains were combined in pairs and 100 µl of each cell suspension was added to the wells (*D. acidovorans – C, freundii; D. acidovorans – P.* putida). Negative control wells contained R2A broth without bacterial cells. After that, the plates were incubated aerobically for 24 and 48 h at (25 ± 2) °C and under agitation. For the 48 h plates, the growth medium was discarded and replaced by a fresh one daily (as for CFS). After each incubation period, the content of the wells was removed. Then, to remove the reversibly adherent bacteria, each well was washed with 0.85% (v/v) sterile saline solution. Plates were air-dried, and the remaining attached biofilms were analysed in terms of metabolic activity.

2.4. Metabolic activity quantification

This assay was performed according to Sarker et al. (2007). For the staining procedure, 190 µL of sterile fresh R2A broth and 10 µL of alamar blue indicator solution (0.4 mM) (Sigma-Aldrich, Merck KGaA, Germany) were added to each well. Then, microtiter plates were incubated for 20 min in darkness, at (25 ± 2) °C and 150 rpm. Finally, fluorescence was measured at λexcitation = 570 nm and λemission = 590 nm (FLUOstar® Omega, BMG LABTECH).

3. Results and discussion

The metabolic activity of 24 h and 48 h-old *C. freundii* and *P. putida* single-species biofilms, dual-species biofilms (*C. freundii - D. acidovorans* and *P. putida – D. acidovorans*) and *C. freundii* and *P. putida* single-species biofilms plus *D. acidovorans* CFS was evaluated.

For biofilms formed with the *C. freundii* strain (Fig. 1), it was observed that from 24 h to 48 h there was an increase in metabolic activity for all cases ($P < 0.01$). Comparing the different types of biofilms at the same time (24 h), higher values of metabolic activity were found for both the dual-species biofilms ($P < 0.01$) and the biofilms exposed to CFS ($P < 0.01$), when compared to *C. freundii* single-species biofilms. At 48 h, there was a slight increase in the metabolic activity values of the dual-species biofilms ($P > 0.05$) and biofilms exposed to CFS ($P < 0.01$), compared to the values of *C. freundii* single-species biofilms. The lowest metabolic activity was always observed for *C. freundii* single-species biofilms both at 24 h and 48 h. The highest value of metabolic activity was observed for dual-species biofilms at 48 h.

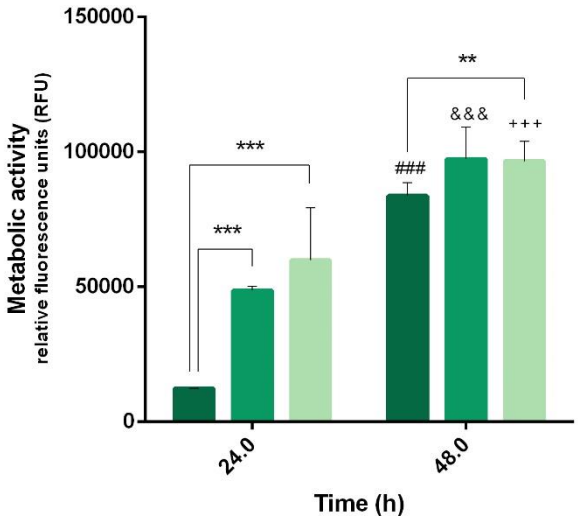

Figure 1. Metabolic activity values at 24 h and 48 h for *C. freundii* single-species biofilms (■), *C. freundii – D. acidovorans* dual-species biofilms (■) and *C. freundii* – CFS biofilms (■). **P < 0.01; *** P < 0.001; ###P < 0.001 (comparison between *C. freundii* single-species biofilms at 24 h and 48 h); &&&P < 0.001 (comparison between *C. freundii – D. acidovorans* dual-species biofilms at 24 h and 48 h); +++P < 0.001 (comparison between *C. freundii* – CFS biofilms at 24 h and 48 h). The standard deviation (SD) of three independent experiments is represented by the error bars..

Similar to *C. freundii*, for biofilms formed with *P. putida*, an increase in metabolic activity was observed from 24 h

to 48 h for all cases (*P. putida* single-species biofilms: P < 0.01; Dual-species biofilms and *P. putida* - CFS biofilms: P < 0.05). For 24 h, there was a higher value of metabolic activity for dual-species biofilms (P < 0.05) when compared to *P. putida* single-species biofilms. For 48 h, there was an increase in the metabolic activity values of the dual-species biofilms (P < 0.05) and biofilms with CFS (P < 0.05) when compared to *P. putida* single-species biofilms. Once again, the lowest metabolic activity was observed for single-species biofilms both at 24 h and 48 h; and the highest value was observed at 48 h for dual-species biofilms.

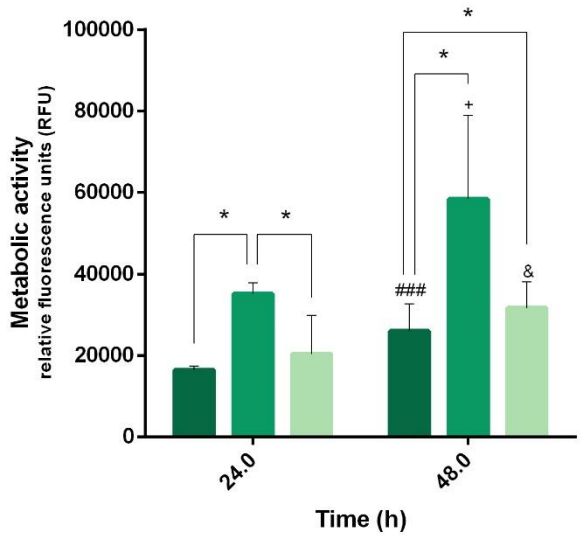

Figure 2. Metabolic activity values at 24 h and 48 h for *P. putida* single-species biofilms (■), *P. putida* – *D. acidovorans* dual-species biofilms (■) and *p. putida* – CFS biofilms (■). *P < 0.05; ###P < 0.001 (comparison between *P. putida* single-species biofilms at 24 h and 48 h); +P < 0.05 (comparison between *P. putida* – *D. acidovorans* dual-species biofilms at 24 h and 48 h); &P < 0.001 (comparison between *P. putida* – CFS biofilms at 24 h and 48 h). The SD of three independent experiments is represented by the error bars.

The fact that the metabolic activity was higher for dual-species biofilms suggests a coexistence behaviour between the coaggregating strain *D. acidovorans* and *C. freundii* and *P. putida* (Oliveira et al., 2020). When comparing these values with those of biofilms plus CFS, a decrease in metabolic activity was observed. This event was already expected since, in dual-species biofilms, molecules continue to be produced by the *D. acidovorans* strain throughout the incubation period, contrary to what happens with the biofilm plus CFS, where these are added at the beginning of the experiment and after the daily-basis culture medium replacement.
On the other hand, single-species biofilms have always been those with the lowest metabolic activity, especially when compared with biofilms containing CFS. The same had already been observed when CFS from the coaggregating strain *Acinetobacter calcoaceticus* was added to biofilms of *Burkholderia cepacia*, *Methylobacterium* sp. and *Mycobacterium mucogenicum*, isolated from a DWDS (Lúcia Chaves Simões et al., 2011). These results show that the production of extracellular molecules by *D. acidovorans* metabolically favors the development of biofilms from other species. Cooperation between bacteria usually occurs through the production of so-called public goods, which consist of metabolically costly molecules produced by certain individuals, and which become available to neighbouring individuals (Özkaya, Xavier, Dionisio, & Balbontín, 2017). This type of cooperation can be exploited by non-producers who benefit from these public goods by saving the cost of their production (cheaters), taking a fitness advantage over producers (cooperators) (Özkaya et al., 2017). Thus, in mixed populations, such as biofilms, cheaters may increase in frequency in the population, relative to cooperators. This becomes especially worrying when cheaters are pathogens or even microorganisms resistant to disinfection or antimicrobials (Rundell, McKeithen-Mead, & Kazmierczak, 2016).

4. Conclusions

To understand the role of bacterial coaggregation in multispecies biofilm formation, biofilms formed by representative species of DW, including a coaggregating strain (*D. acidovorans*), were evaluated for metabolic activity. Furthermore, to assess whether possible extracellular metabolites produced by the coaggregating strain could favour the metabolism of the biofilm, *D. acidovorans* CFS was added to *C. freundii* and *P. putida* single-species biofilms. The overall results showed a higher metabolic activity for dual-species biofilms and single-species biofilms exposed to CFS, compared to single ones. Furthermore, these results reveal the production of extracellular molecules by *D. acidovorans* that favour the development of biofilms from other species. Possibly these molecules could be public goods that benefit the fitness of species other than the producers. This represents a special concern when these cheaters are microorganisms that pose a risk to public health. In conclusion, this study allowed a better understanding of bacterial coaggregation and metabolite production by DW *D. acidovorans* in biofilm formation and development. The identification of coaggregating strains like *D. acidovorans*, which represent a significant contribution to the development and maintenance of the DW consortium, provides new insights to successfully understand the mechanism of coaggregation and its impact on aquatic systems.

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