Autoinducer 2-Dependent *Escherichia coli* Biofilm Formation Is Enhanced in a Dual-Species Coculture

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**ABSTRACT**  Biofilms in nature typically consist of multiple species, and microbial interactions are likely to have crucial effects on biofilm development, structure, and functions. The best-understood form of communication within bacterial communities involves the production, release, and detection of signal molecules (autoinducers), known as quorum sensing. Although autoinducers mainly promote intraspecies communication, autoinducer 2 (AI-2) is produced and detected by a variety of bacteria, thus principally allowing interspecies communication. Here we show the importance of AI-2-mediated signaling in the formation of mixed biofilms by *Enterococcus faecalis* and *Escherichia coli*. Our results demonstrate that AI-2 produced by *E. faecalis* promotes collective behaviors of *E. coli* at lower cell densities, enhancing autoaggregation of *E. coli* but also leading to chemotaxis-dependent coaggregation between the two species. Finally, we show that formation of such mixed dual-species biofilms increases the stress resistance of both *E. coli* and *E. faecalis*.

**IMPORTANCE**  The role of interspecies communication in the development of mixed microbial communities is becoming increasingly apparent, but specific examples of such communication remain limited. The universal signal molecule AI-2 is well known to regulate cell-density-dependent phenotypes of many bacterial species but, despite its potential for interspecies communication, the role of AI-2 in the establishment of multispecies communities is not well understood. In this study, we explore AI-2 signaling in a dual-species community containing two bacterial species that naturally cooccur in their mammalian hosts, i.e., *Escherichia coli* and *Enterococcus faecalis*. We show that active production of AI-2 by *E. faecalis* allows *E. coli* to perform collective behaviors at low cell densities. Additionally, AI-2- and chemotaxis-dependent coaggregation with *E. faecalis* creates nucleation zones for rapid growth of *E. coli* microcolonies in mixed biofilms and enhances the stress resistance of both species.

**KEYWORDS**  autoinducer 2, biofilms, mixed communities, quorum sensing

Living in dense, structured, multicellular communities, such as surface-attached biofilms, generally provides bacteria with a number of fitness advantages, compared to a solitary planktonic lifestyle (1). Bacterial biofilms are present in most ecological niches, including the human body, where they can consist of hundreds of species (2–4). The complexity of multispecies biofilms is accompanied and regulated by a number of interactions within and between species, ranging from cooperation to predation (5–8). The best-understood coordination mechanism of bacterial behavior within a community is cell-density-dependent chemical communication called quorum sensing (QS) (9). QS is based on production of, secretion of, and subsequent concentration-dependent responses to signal molecules (autoinducers). This process plays a role in various types of collective bacterial behaviors, including biofilm formation and also colonization of plant and animal hosts by symbiotic or pathogenic
bacteria. An array of different QS molecules and response systems exist, allowing bacteria to establish relationships on both intraspecies and interspecies levels (10).

The best-described broad-range interspecies signaling molecule is autoinducer 2 (AI-2) (11). AI-2 is produced by a range of Gram-positive and Gram-negative bacteria (11, 12) and regulates bioluminescence, biofilm formation, motility, and virulence (13). Although most of these functions have been investigated in communities of individual species, several studies suggested the importance of interspecies communication mediated by AI-2 for establishment of mixed biofilms and development of dental plaque (14–16). Furthermore, AI-2 is also produced by many gut-associated bacteria (17–19), and it was shown to affect the composition of the gut microbiota, favoring Firmicutes while hindering Bacteroides in an antibiotic-treated mouse model (20).

AI-2 is the only known QS molecule produced by the enteric bacterium Escherichia coli, and its production and uptake were shown to affect several E. coli phenotypes, including biofilm formation, motility, and virulence (21, 22). Previous work showed that, during autoaggregation or biofilm formation by E. coli, AI-2 serves as a chemoattractant that recruits planktonic cells to growing cell aggregates (23–25). However, it remained unclear whether and how E. coli could use this AI-2-mediated autoaggregation in mixed microbial communities in which it represents only a minor fraction of the population.

E. coli and the Gram-positive bacterium Enterococcus faecalis both inhabit the human gastrointestinal tract, and they cooccur in catheter-associated urinary tract infections (26, 27). It was shown recently that E. faecalis augments E. coli growth under iron-limited conditions, as found within the host, by secreting L-ornithine, which induces siderophore synthesis in E. coli (28). This suggests that these two species might generally interact in the host during polymicrobial infection. In this study, we describe another level of interaction between E. coli and E. faecalis during the formation of mixed biofilms. We show that AI-2 produced locally by E. faecalis aggregates attracts E. coli cells, leading to enhanced aggregation and microcolony formation by E. coli and to increased stress resistance of both species. Moreover, AI-2 production by E. faecalis allows the E. coli population to maintain the induced state of its QS system, despite low cell densities. Together, these results demonstrate that E. coli can use AI-2 produced by other species to promote its QS-regulated collective behavior at low cell densities. We propose that such interspecies signaling may provide fitness advantages to E. coli or other bacterial species in ecological niches where their relative abundance is low, such as the human gastrointestinal tract.

RESULTS

E. faecalis enhances biofilm formation by E. coli. To investigate possible effects of interspecies communication on biofilm formation, we cocultivated E. coli with E. faecalis in microtiter plates. Such static cultures of E. coli are known to form robust biofilms, in which intercellular interactions are mediated primarily by a major adhesin, antigen 43 (Ag43), at 37°C (as used here) (24, 29, 30) or by curli filaments at 30°C (24, 31, 32).

We observed that, under these conditions, E. coli cultures reached optical density at 600 nm (OD600) values of ~0.8 at 10 to 12 h postinoculation, whereas the growth of E. faecalis was limited to maximal OD600 values of ~0.15 (Fig. 1A). Consistently, cocultivation of E. coli with E. faecalis had little effect on the overall growth (Fig. 1A), with E. faecalis being quickly overgrown by E. coli and constituting about 10 to 14% of the biofilm biomass after 24 h of cocultivation (Fig. 1B). Nevertheless, we observed that E. coli biofilms formed under these conditions were apparently more structured when grown in cocultures with E. faecalis (Fig. 2A and B; also see Fig. S1A in the supplemental material). Notably, microcolonies formed by E. coli and E. faecalis apparently colocalized within these mixed biofilms (Fig. 2B and C). Image quantification confirmed that E. coli biofilms in mixed communities consisted of significantly larger microcolonies (Fig. 2D).

AI-2 secretion by E. faecalis aggregates attracts chemotactic E. coli cells. To further understand the underlying mechanisms, we monitored the early stages of
biofilm formation for single- and dual-species cultures using fluorescence microscopy. In accordance with previous work (24), *E. coli* cells rapidly formed small and relatively unstable cell aggregates at the surface of the well, with the number and size reaching $8 \pm 2$ aggregates/1,000 $\mu$m$^2$ and $80 \pm 20$ $\mu$m$^2$, respectively, during the first hour of incubation (Fig. 3A and D). These aggregates grew slowly during the first 3 h and eventually merged to form larger structures, with $\sim 5 \pm 1$ aggregates/1,000 $\mu$m$^2$ and an average size of $200 \pm 20$ $\mu$m$^2$.

In the mixed cultures, *E. faecalis* cells could be observed as chains of single cells or aggregates after 1 h (Fig. 3B and C). These aggregates also seemed to incorporate *E. coli* cells (Fig. 3B and C), which dramatically increased the growth of *E. coli* aggregates. Already after 2 h, the aggregates of *E. coli* cells coinoculated with *E. faecalis* were on average 3 times larger than aggregates in *E. coli*-only cultures (Fig. 3A, B, and D).
Such apparent recruitment indicated that *E. faecalis* aggregates might chemotactically attract *E. coli* cells. Since AI-2 was identified previously as the autoaggregation-mediating chemotactic signal in *E. coli* (23, 24, 33), we hypothesized that it might also promote chemotaxis-mediated coaggregation of different species. Indeed, *E. faecalis*...
cells are known to secrete AI-2 in the exponential phase of growth (34), and we confirmed that the strain used in this study was an active producer of AI-2 (Fig. S2). Consistent with the role of AI-2 chemotaxis in the observed coaggregation, a non-AI-2-producing mutant of *E. coli* (Δ*luxS*) did not form larger microcolonies in *E. coli*-*E. faecalis* mixed biofilms (Fig. S3). This suggests that secretion of AI-2 by *E. coli* is required for aggregation, although interpretation of this phenotype is complicated by the known pleiotropic nature of the luxS deletion, which also affects motility (13, 24).

**FIG 3** Aggregation of *E. coli* during early stages of biofilm formation in single- or double-species cultures. (A to C) Aggregates formed at the well surface by *E. coli* cells (expressing EGFP) grown in monoculture (A) or cocultured with unlabeled *E. faecalis* (B and C). Cells of *E. faecalis* can be seen in the phase-contrast channel as distinct chains of round cells or as parts of *E. coli*-*E. faecalis* aggregates. Scale bars, 30 μm (A and B) or 20 μm (C). White arrows in panel C indicate chains and aggregates of *E. faecalis*. (D) Sizes of *E. coli* aggregates in monoculture or in coculture with *E. faecalis*. Means of at least four independent replicates are shown; error bars indicate standard deviations. *P* values for the differences between single- and double-species biofilms were calculated using Mann-Whitney tests. **, *P* < 0.005; ns, not significant.
More conclusively, deletion of either the key chemotaxis protein CheY, which generally abolishes chemotaxis, or a periplasmic protein (LsrB) that mediates Al-2 signaling to the chemotaxis system (23) also abolished coaggregation and enhanced formation of mixed biofilms (Fig. 4; also see Fig. S5A and B in the supplemental material). These phenotypes strongly support our hypothesis that Al-2 chemotaxis is essential for the observed enhancement of aggregation. Furthermore, coaggregation required the self-interacting E. coli adhesin Ag43 (Fig. S4 and S5C), indicating that E. faecalis aggregates do not interact directly with E. coli cells but rather serve as local sources of Al-2 that attract E. coli and initiate its autoaggregation.

Cocultivation with E. faecalis promotes Al-2 signaling in E. coli. In E. coli, the lsr operon, which includes lsrB, is positively regulated by Al-2. As a consequence, the operon is repressed at low cell density but becomes activated in the mid-exponential to late exponential phase, when the concentration of extracellular Al-2 becomes sufficiently high to relieve the repression. This leads in turn to enhanced AI-2 internalization and depletion from the medium, as the lsr operon encodes a high-affinity Al-2 importer (13).

The LsrB-dependent growth of E. coli aggregates in mixtures with E. faecalis already during the early exponential phase thus seemed surprising, as the population density in the initial stages of biofilm growth should normally be too low to allow induction of the lsr operon. Indeed, upon dilution of the overnight culture in fresh medium to an OD_{600} of 0.03, lsr operon activity was rapidly inhibited in most E. coli cells during the first hour of monoculture incubation (Fig. 5A and F). The subsequent growth resulted in gradual induction of expression, with 96% of the population expressing the lsr operon after 5 h.

In contrast, only about one-third of E. coli cells coinoculated with E. faecalis switched off the Al-2 system upon reinoculation (Fig. 5B and F). Thus, LsrB expression is indeed maintained in a large fraction of E. coli cells in early mixed biofilms. This effect of E. coli-E. faecalis cocultivation on the activity of the Al-2 QS system of E. coli was not contact dependent, since it was also observed in cells lacking Ag43 (Fig. 5G). Such increased (compared to E. coli monocultures) expression of the lsr operon during early growth was apparently correlated with the elevated levels of Al-2 in the mixed cultures (Fig. S2). Indeed, lsr expression at the early time points was also above the control values for E. coli grown in conditioned medium from E. faecalis (Fig. 5C and F), as well as E. coli grown in conditioned medium from E. coli or in cultures with 50 μM synthetic 4,5-dihydroxy-2,3-pentanedione (DPD)/Al-2 (Fig. 5D, E, and F). All of these findings suggest that greater lsr expression in E. coli-E. faecalis cocultures, compared to E. coli monocultures, is indeed due to the elevated levels of Al-2. Cocultivation of E. coli with E. faecalis or the addition of synthetic DPD/Al-2 did not, however, affect the level of E. coli luxS promoter activity (Fig. S7), suggesting that production of Al-2 by E. coli was not altered in the presence of E. faecalis.

Lower levels of Al-2 seem not to be the only reason for decreased expression of the lsr operon in the early stages of growth, as substantial decreases were observed even in the presence of E. faecalis or externally added Al-2. This is in agreement with previous reports suggesting that other factors, such as the metabolic state of the cells, also contribute to lsr activation (35–38).

Further supporting the connection between lsr induction and increased aggregation of E. coli, stimulation by conditioned media from E. faecalis or E. coli or by synthetic DPD/Al-2 was sufficient to enhance microcolony formation in mature E. coli biofilms (Fig. 6A and B). As neither addition of synthetic DPD/Al-2 nor cocultivation with E. faecalis affected Ag43 expression or the percentage of Ag43-producing E. coli cells (Fig. S8), interspecies Al-2 signaling promotes aggregation largely by stimulating Al-2 chemotaxis of E. coli. Nevertheless, because E. coli-E. faecalis coaggregation resulted in faster aggregate growth in the earlier stages of biofilm formation than did stimulation with Al-2 or with conditioned medium (Fig. 6C), we conclude that E. faecalis enhances E. coli biofilm formation at low cell densities both globally, by
FIG 4 Dependence of coaggregation and mixed biofilm formation on AI-2 chemotaxis. (A) Confocal laser scanning microscopy of static biofilms of *E. coli ΔcheY* and *ΔlsrB* (expressing mCherry) grown in monoculture or mixed with *E. faecalis* (expressing EGFP), initially inoculated at a 1:1 ratio. Scale bars, 40 μm. (B) Distribution of microcolony volumes in the biofilms. The *P* values for the differences between single- and double-species biofilms were calculated using unpaired *t* tests (the data distribution was confirmed to be normal). ns, not significant. (C) Time-lapse fluorescence microscopy of *E. coli ΔlsrB* (expressing EGFP) grown with *E. faecalis* (unlabelled). The white arrows indicate an aggregate of *E. faecalis*.
relieving *lsr* operon inhibition, and locally, by nucleating formation of *E. coli* aggregates.

**Formation of mixed biofilms enhances stress resistance.** Aggregation and biofilm formation are generally known to enhance the stress resistance of bacteria, and it was shown previously that Ag43-mediated autoaggregation of *E. coli* provides protection against oxidative stress (1, 24, 39, 40). Since *E. coli* forms more structured biofilms with larger microcolonies when it is cocultured with *E. faecalis*, we hypothesized that such enhancement might promote stress resistance of *E. coli*, and possibly also of *E.
FIG 6  *E. coli* biofilm formation in conditioned media and in the presence of exogenous DPD/AI-2. (A) Confocal laser scanning microscopy of static *E. coli* (expressing EGFP) biofilms grown in TB, in conditioned medium (CM) from *E. coli* or *E. faecalis*, or in TB supplemented with 50 μM synthetic DPD/AI-2, as indicated. Scale bars, 40 μm. (B) Distribution of microcolony volumes in the indicated biofilms. *P* values for the differences from *E. coli* biofilms grown in TB

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Indeed, the survival rate of *E. coli* upon H$_2$O$_2$ treatment increased from ~33% in a single-species biofilm to >50% in a mixed biofilm (Fig. 7). Moreover, the coaggregation in a mixed biofilm also greatly enhanced the survival rate of *E. faecalis*, confirming that *E. faecalis* cells in these biofilms are covered with *E. coli* microcolonies and thus are less exposed to the oxidative stress.

**DISCUSSION**

In recent years, bacterial biofilms have been increasingly viewed as primarily multispecies communities, with elaborate spatial structures and complex interspecies interactions (4, 8). One of the most extensively studied forms of these interactions is a small-molecule-based mechanism of cell-cell communication known as quorum sensing (QS) (9). Because bacteria in biofilms are packed into dense aggregates, it seems obvious that QS must be relevant in natural communities. However, although there is clear evidence that QS plays distinct roles in biofilm formation by individual species, including the initial attachment phase and biofilm maturation and dispersal (41), the importance of QS in multispecies biofilms remains largely unexplored (4, 14, 16, 42, 43).

In this respect, the QS signaling mediated by AI-2 is an attractive candidate for interspecies communication, since AI-2 production and sensing is widespread among various taxonomic groups of bacteria (13, 44, 45). Indeed, the influence of AI-2 on multispecies oral biofilms (14–16, 46) and community composition in the mouse gut (20) was demonstrated, although the details of the underlying regulation remain unknown. Here we provide direct evidence that AI-2 signaling between different species can enhance biofilm formation, and we further characterize the mechanism of this enhancement.

Recent work showed that AI-2 plays a major role in *E. coli* biofilm formation, by

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**FIG 7** Survival of *E. coli* and *E. faecalis* in single- or double-species biofilms under oxidative stress. Single-species or mixed (Ec:Ef) biofilm cultures of *E. coli* and *E. faecalis* were exposed to 0.5% H$_2$O$_2$ as described in Materials and Methods. *E. coli* cultures incubated under nonaggregating conditions (shaking at 270 rpm) were used as controls. Means of at least five independent replicates are shown; error bars indicate standard deviations. *P* values for the differences between single- and double-species biofilms were calculated using Mann-Whitney tests. ***, *P* < 0.0002.

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**FIG 6** Legend (Continued)

were calculated using unpaired t tests (the data distribution was confirmed to be normal). (C) Aggregate sizes (assayed as in Fig. 2) of *E. coli* cells grown in TB (in monoculture or in coculture with *E. faecalis*), in conditioned medium from *E. coli* or *E. faecalis*, or in TB supplemented with 50 μM synthetic DPD/AI-2, as indicated. Means of at least three independent replicates are shown; error bars indicate standard deviations. *P* values for the differences from *E. coli* biofilms grown in TB or between indicated cultures were calculated using Mann-Whitney tests. ****, *P* < 0.0001; ***, *P* < 0.0002; **, *P* < 0.005; ns, not significant.
mediating chemotaxis toward growing cell aggregates (24, 25). During Ag43-dependent autoaggregation, initial *E. coli* aggregates formed by random cell collisions secrete AI-2, which attracts other planktonic cells. In this study, we demonstrate that *E. coli* can also use AI-2 chemotaxis for coaggregation with *E. faecalis*, resulting in enhanced *E. coli* microcolony formation and subsequent biofilm formation in a mixed community. One apparent benefit of such coaggregation is to enable an individual species (in this case, *E. coli*) to aggregate at lower cell density than in monoculture, and we indeed observed that formation of *E. coli* aggregates occurred already during the first hours of growth in cocultures. Moreover, the formation of mixed aggregates also promoted stress resistance of both species, which could be explained by the formation of larger *E. coli* aggregates and the protection of *E. faecalis* cells incorporated in those aggregates.

Another key factor in this AI-2 dependent enhancement of collective behavior in mixed cultures is sustained induction of the *E. coli lsr* operon by *E. faecalis*. This induction is important, because AI-2 chemotaxis requires LsrB protein, which is also a part of the cell-density-dependent Lsr system for AI-2 internalization and degradation (13, 23). As a consequence, in *E. coli* monocultures, AI-2-mediated autoaggregation emerges only as a population enters the mid-exponential to late exponential growth phase and the AI-2 concentration in the medium is high enough to cause derepression of the *lsr* operon (24). In contrast, greater Lsr expression in the mixed cocultures, apparently due to the additional AI-2 that is secreted by *E. faecalis*, enables autoaggregation of *E. coli* already in early stages of growth. Consistently, both sustained induction of the *lsr* operon and enhanced autoaggregation and biofilm formation could also be achieved with the addition of exogenous DPD/AI-2 to *E. coli* monocultures. Since induction of the *lsr* operon is the only known effect of AI-2 on gene expression in *E. coli*, we conclude that this induction is indeed the major cause of enhanced biofilm formation in the cocultures. However, the most prominent enhancement was observed when both factors, i.e., LsrB induction and nucleation zones provided by *E. faecalis*, were present (Fig. 8).

Besides providing clear evidence for the importance of cell-cell communication for the formation of mixed biofilms, our work also resolves two apparent paradoxes,
namely, (i) why *E. coli* uses AI-2, an interspecies QS molecule, for autoaggregation and (ii) how such autoaggregation can occur in the human intestine, where *E. coli* constitutes a minority population and is unlikely to reach cell densities high enough either to activate the AI-2 QS system or to aggregate on its own. The use of AI-2 produced by *E. coli* might provide important means to reach local densities that are sufficient for collective behaviors such as QS induction or biofilm formation. Particularly for minor species within the community, coaggregation strategy of chemotaxis-driven coaggregation might be common in mixed microbial communities. Moreover, we hypothesize that the strategy of chemotaxis-driven coaggregation might be common in mixed microbial communities. Particularly for minor species within the community, coaggregation might provide important means to reach local densities that are sufficient for collective behaviors such as QS induction or biofilm formation.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** The strains and plasmids used in this study are listed in Table 1. *E. coli* W3110 (RpoS+) and *E. faecalis* ATCC 29212 were grown in liquid tryptone broth (TB) (10 g tryptone and 5 g NaCl per liter), supplemented with antibiotics when necessary.

A genomic reporter construct for assessing Ag43 (flu) expression (48) was introduced into the *E. coli* W3110 genome via P1 transduction (49). This construct enables amplification of the signal from the flu promoter by replacing its coding sequence with the T7 RNA polymerase gene. The resulting strain, LeoL194, was then transformed with the pHL32 plasmid carrying the promoterless flu::gfpmut3.1; Kms 24.

Fluorescent labeling of *E. faecalis*. The reporter plasmid pLeoL7 was constructed in order to visualize *E. faecalis* cells in mixed biofilms. The –350 to +17 nucleotide region of the *E. faecalis* constitutively expressed rplL gene was cloned into the p85U100 shuttle vector carrying a promoterless copy of egfp (51). Transformation of *E. faecalis* cells was performed as described elsewhere (52). The fluorescence signal from this plasmid was low and only allowed imaging using confocal laser scanning microscopy.

**TABLE 1 Strains and plasmids used in this study**

| Strain or plasmid | Relevant genotype or phenotype | Reference or source |
|-------------------|--------------------------------|---------------------|
| **Strains**       |                                |                     |
| *E. coli* W3110   | W3110 derivative with functional RpoS, rpoS396(Am) | 55                   |
| *E. faecalis* ATCC 29212 | Wild-type strain isolated from urine | Leibniz Institute DSMZ German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) |
| VS823             | W3110 ΔluxS; Km+ | 24 |
| VS824             | W3110 Δflu; Km+ | 24 |
| VS825             | W3110 ΔluxB; Km+ | 24 |
| VS695             | W3110 ΔcheY; Km+ | 24 |
| CHNL13B           | MC4100 flu:flu0–48 bp-T7RNApol; Cam+ | 48 |
| LeoL194           | W3110 flu:flu0–48 bp-T7RNApol; Cam+ | This study |
| **Plasmids**      | Expression vector; pBR ori, pTrc promoter, IPTG inducible; Amp′ | 56 |
| pUA66             | Expression vector; SC101 ori, GFPmut2 under control of promoter of interest; Km+ | 50 |
| pBSU100           | Expression vector; pUC ori, pAmS1 ori, promoterless egfp; Spc′ | 51 |
| pVS1515           | egfp in pTrc99A, IPTG inducible; Amp′ | 24 |
| pOB2             | mCherry in pTrc99A, IPTG inducible; Amp′ | 31 |
| pLeol7           | Prpl-egfp in p85U100; Spc′ | This study |
| pLeol8           | PluxS-egfp in pUA66; Km+ | This study |
| pVS1723          | Pslr-egfp in pUA66; Km+ | 24 |
| pHLS2            | P(T7)-gfpmut3.1; Km+ | 48 |

*Kms*, kanamycin sensitive; *Km*, kanamycin resistant; *Cam*, chloramphenicol resistant; *Amp′*, ampicillin resistant; *Spc′*, spectinomycin resistant.

For two-color labeling, overnight cultures of *E. coli* carrying plasmid pOB2 carrying mCherry under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible trc promoter and *E. faecalis* carrying egfp under the control of the constitutively expressed rplL promoter were diluted in TB containing 5 μM IPTG, to a final OD 600 of 0.03. For single-color labeling of *E. coli*, plasmid pVS1515 carrying egfp under the control of the trc promoter was used. For dual-species biofilm cultivation, the same amounts of *E. coli* and *E. faecalis* cells were plated on LB agar plates at 37°C for 24 h.
coincubated, resulting in a final OD_{600} of 0.06; 400 μl of each sample was cultivated for 24 h at 37°C in 8-well glass-bottom slides (μ-Slide, 8-well glass bottom; ibidi). The biofilms were visualized using a Zeiss LSM-800 microscope (apochromat 40× objective), and z-stack images were acquired and analyzed using ZEN Black software (Zeiss).

Three-dimensional structures of mature *E. coli* biofilms (green fluorescent protein [GFP] positive) were quantified using the 3D objects counter plugin for ImageJ (53). The plugin allows quantification of the volumes of patches formed by connected fluorescent cells.

**Flow cytometry.** Levels of *lsr* operon induction were assayed using a plasmid-based reporter containing the 217-nucleotide region upstream of the *lsrA* gene fused to *egfp* (24). Samples were prepared as described above and diluted 1:20 in tethereal buffer (10 mM KH_{2}PO_{4}, 100 μM EDTA, 1 μM L-methionine, and 10 μM lactic acid [pH 7.0]), and fluorescence was measured every hour with a BD LSRFortessa SORP cell analyzer (BD Biosciences, Germany).

For quantification of Al-2 levels in supernatants, a non-Al-2-producing biosensor strain was used, and the quantification was performed as described previously (24). Where indicated, synthetic DPD (obtained from Rita Ventura, ITQB, Oeiras, Portugal) (54) solution was added to the samples; it is referred to as DPD/Al-2 because of its spontaneous conversion into Al-2. Cell-free conditioned medium was prepared by filtration, through 0.2-μm filters, of supernatants collected from statically grown cultures (1 h at 37°C).

Biofilm growth rates were determined by counting the GFP-positive (*E. coli*) and GFP-negative (*E. faecalis*) cells in the samples (1:400 dilution in tethering buffer). The biofilms were disrupted by pipetting and subsequent vortex-mixing of the samples. During cell counting, the flow rate was set to 6 μl/min, with an acquisition time of 10 s.

**Fluorescence microscopy.** The initial stages of biofilm formation in 8-well glass-bottom slides were assessed with a Nikon Eclipse Ti-E microscope equipped with an Andor Zyla 4.2 sCMOS camera (Andor Technology Ltd., UK), a plan apochromat 40× objective, and a filter set for GFP. Images were acquired every hour using NIS-Elements AR software (Nikon) and were analyzed using the particle analysis tool (ImageJ) to determine the size of aggregates (the detection threshold was set to 5 μm²).

**Hydrogen peroxide treatment.** Cell survival upon treatment with H_{2}O_{2} was determined as described previously (24, 40). Briefly, *E. coli*, *E. faecalis*, or *E. coli*-*E. faecalis* static biofilms were grown in 400 μl TB for 24 h at 37°C. Subsequently, 5.7 μl of 35% H_{2}O_{2} was added to each sample, resulting in a final H_{2}O_{2} concentration of 0.5%, and samples were incubated for 15 min at room temperature. CFU for each sample before and after treatment were counted by plating serial dilutions of cultures. For mixed biofilms, CFU of *E. coli* and *E. faecalis* could be easily counted separately based on differences in their colony morphology on LB plates. The survival rate was determined as (posttreatment CFU/initial CFU) × 100%.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02638-17.

**SUPPLEMENTAL FILE 1**, PDF file, 1.4 MB.

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