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Role of Quorum Sensing and Antimicrobial Component Production by *Serratia plymuthica* in Formation of Biofilms, Including Mixed Biofilms with *Escherichia coli*[▽]

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We have previously characterized the *N*-acyl-L-homoserine lactone-based quorum-sensing system of the biofilm isolate *Serratia plymuthica* RVH1. Here we investigated the role of quorum sensing and of quorum-sensing-dependent production of an antimicrobial compound (AC) on biofilm formation by RVH1 and on the cocultivation of RVH1 and *Escherichia coli* in planktonic cultures or in biofilms. Biofilm formation of *S. plymuthica* was not affected by the knockout of *splI* or *splR*, the *S. plymuthica* homologs of the *luxI* or *luxR* quorum-sensing gene, respectively, or by the knockout of AC production. *E. coli* grew well in mixed broth culture with RVH1 until the latter reached 8.5 to 9.5 log CFU/ml, after which the *E. coli* colony counts steeply declined. In comparison, only a very small decline occurred in cocultures with the *S. plymuthica* AC-deficient and *splI* mutants. Complementation with exogenous *N*-hexanoyl-L-homoserine lactone rescued the wild-type phenotype of the *splI* mutant. The *splR* knockout mutant also induced a steep decline of *E. coli*, consistent with its proposed function as a repressor of quorum-sensing-regulated genes. The numbers of *E. coli* in 3-day-old mixed biofilms followed a similar pattern, being higher with *S. plymuthica* deficient in SplI or AC production than with wild-type *S. plymuthica*, the *splR* mutant, or the *splI* mutant in the presence of *N*-hexanoyl-L-homoserine lactone. Confocal laser scanning microscopic analysis of mixed biofilms established with strains producing different fluorescent proteins showed that *E. coli* microcolonies were less developed in the presence of RVH1 than in the presence of the AC-deficient mutant.

Biofilms are microbial communities that attach to and grow on solid surfaces, mostly in contact with a liquid phase. Bacterial biofilms can develop a complex architecture, consisting of microcolonies embedded in a self-produced matrix, interspersed with water channels that allow the transport and exchange of nutrients and waste products between the depths of the biofilm and the environment (16). Natural biofilms consist of a heterogeneous community of different microbial populations, which engage in complex cell-to-cell interactions. These interactions may be mutually beneficial, as in the case of cooperation for amassing nutrients and cross feeding (27), but they can also be antagonistic if the production of antimicrobial components is involved (1, 28). Studies with dual-species biofilm models have suggested that the mode of interaction between different populations in a biofilm determines their spatial organization: while mutual metabolic dependence tends to bring the partners together (7), antagonism based on the production of antibacterial components drives them apart (36).

In planktonic (liquid culture) cocultivation systems, the production of an antimicrobial compound by one population will ultimately lead to the disappearance of sensitive partners (30). In biofilms, however, the outcome of such interactions is difficult to predict because it also depends on the spatial relationships in the biofilm. Tait and Sutherland (36), using a batch system for biofilm formation, found that a bacteriocin-producing strain more easily gained a foothold in an existing biofilm of bacteriocin-sensitive bacteria than vice versa. Nevertheless, the bacteriocin-sensitive strains were not completely eradicated from the biofilms. Rao et al. (28) studied dual-species biofilms formed in a continuous flow chamber with *Pseudoalteromonas tunicata*, a marine bacterium that produces the antibacterial protein AlpP, and found that the organism could completely outgrow competitor bacteria that were very sensitive to this protein, but not those that were only moderately sensitive or those that themselves produced an antimicrobial compound to which *P. tunicata* was sensitive. These examples illustrate the important role of antimicrobial compound production in shaping mixed-species biofilms.

A factor adding to the complexity of interactions among biofilm bacteria is quorum sensing, a cell density-related communication mode between one or more species by means of small, diffusible signal molecules. The best-studied quorum-sensing system, first described for the marine bacterium *Vibrio fisheri* and widespread in gram-negative bacteria, employs *N*-acyl-L-homoserine lactones (AHLs) as signals. In this system, an AHL synthase, homologous to LuxI of *V. fisheri*, produces one or more AHL signals that are either secreted (13) or passively diffused from the cell. Upon reaching a quorum, the signals bind a response regulator, which in turn activates or

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TABLE 1. Strains and plasmids used in this study

Strains and plasmids	Relevant genotype or description[a]	Reference[b]
Strains		
Chromobacterium violaceum		
CV026	*cviI*::mini-Tn*5* derivative of strain ATCC 31532, Km^r, AHL^-	24
Escherichia coli		
CAG18439	*lacZ*118(Oc) *lacI*3042::Tn10 F^- λ^- *rph-1*	34
ESS	β-Lactam supersensitive	18
MG1655	F^- λ^- *rph-1*	15
MG1655 *lacZ*::*Tc*	*lacZ*::Tc	This study
MP1	MG1655 *lacI*3042::Tn*10* F^- λ^- *rph-1*	This study
MP2	MP1, random insertion of mini-Tn*5*-Km-$P_{A1/04/03}$-RBSII-*dsRed*-T_0-T_1	This study
S17-1 λ*pir*	*recA thi pro hsd*R^-M^+RP4: 2-Tc:Mu:Km Tn*7* λ *pir*, Tp^r Sm^r	33
Serratia plymuthica		
RVH1	Natural isolate	40
RVH1-1	RVH1 *splI*::*aacC1*, Gm^r	*
RVH1-2	RVH1 *splR*::*aacC1*, Gm^r	*
RVH1-Gfp	RVH1 *gal*::mini-Tn*5*-$P_{A1/04/03}$-*gfp*mut3*, Km^r, Cm^r	This study
RVH1-1-Gfp	RVH1-Gfp *splI*::*aacC1*, Km^r, Cm^r, Gm^r	This study
RVH1-2-Gfp	RVH1-Gfp *splR*::*aacC1*, Km^r, Cm^r, Gm^r	This study
RVH1-5-Gfp	RVH1-Gfp, rendered deficient in antimicrobial component production by mini-Tn*10*-Gm insertion, Km^r, Cm^r, Gm^r	This study
Plasmids		
pBSL182	Mini Tn*10* carrying Gm cassette with replicon R6K	2
pJBA28	*oriR6K mobRP4* mini-Tn*5*-Km-$P_{A1/04/03}$-RBSII-*gfp*mut3*-T_0-T_1, Ap^r; Km^r	4
pSF100	Pir-dependent replication	31
pRVH14	*splI*::*aacC1* in pSF100, Ap^r, Km^r, Gm^r	*
pRVH15	*splR*::*aacC1* in pSF100, Ap^r, Km^r, Gm^r	*
pSM1833	*oriR6K mobRP4* mini-Tn*5*-Km-$P_{A1/04/03}$-RBSII-*dsRed*-T_0-T_1, Ap^r; Km^r	37

[a] Resistance markers: Km, kanamycin; Sm, streptomycin; Tc, tetracycline; Sp, spectinomycin; Gm, gentamicin.
[b] *, R. Van Houdt et al., submitted for publication.

represses specific target genes (14). Quorum sensing can play an important role in the formation of fully developed mature biofilms in several bacteria. For example, a loss of AHL production results in the early arrest of biofilm development and the lack of cellular differentiation into filaments or aggregates in *Serratia marcescens* MG1 (formerly *S. liquefaciens* MG1), resulting in flatter and less voluminous biofilms (21). In *Pseudomonas aeruginosa*, the situation is less clear. Davies et al. (9) reported AHL-deficient *P. aeruginosa* to form flat and undifferentiated biofilms lacking the typical mushroom-shaped appearance of the wild type. However, the mushroom morphology was later shown to be strongly medium dependent, and *P. aeruginosa* biofilm formation does not always to depend on quorum sensing (17, 19). Interestingly, quorum sensing can also control the production of antibacterial compounds, like the antibiotics carbapenem and prodigiosin in *Serratia* sp. strain ATCC 39006 (12) or several bacteriocins in *Streptococcus mutans* (38). In addition to biofilm formation and antimicrobial component (AC) production, many other cellular properties are controlled by cell-to-cell signaling in a wide range of bacteria. In complex communities, communication signals of different bacteria may interfere, and moreover, some bacteria have evolved mechanisms to exploit or avoid this cross talk (43).

From a food-processing environment, we previously isolated a biofilm-forming strain that was identified as *S. plymuthica* RVH1 (39, 40). In this strain, the production of an antimicrobial component, an extracellular protease, chitinase, and nuclease, and butanediol fermentation are under the control of an AHL-dependent quorum-sensing system (R. Van Houdt et al., submitted for publication) (42). In this work, we investigated the role of this quorum-sensing system and that of the antimicrobial component in biofilm formation by RVH1 and on competition in planktonic and biofilm-mixed cultures of RVH1 and *Escherichia coli*. This species was chosen because of its sensitivity toward the AC and because it occurs in the same food-processing environment. Moreover, we have shown in other work that *E. coli* can react to AHLs by means of its LuxR homolog (41). To distinguish the influence of AC production and other quorum-sensing-regulated properties, we first isolated a mutant of RVH1 that is deficient in AC production but not in quorum sensing and we used this strain in addition to earlier-constructed knockout strains in the *luxI*/*luxR* homologs in mixed-culture experiments with *E. coli*.

MATERIALS AND METHODS

Bacterial strains, plasmids, standard culture conditions, and chemicals. The strains and plasmids used in this study are listed in Table 1. *E. coli*, *S. plymuthica* RVH1, and *Chromobacterium violaceum* strains were cultured at 30°C in Luria-Bertani (LB) broth or agar (1.5% agar) or in M9 minimal medium (32). The following antibiotics and concentrations (AppliChem, Darmstadt, Germany) were used when appropriate: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; chloramphenicol, 30 μg/ml; gentamicin, 20 μg/ml; and tetracycline, 20 μg/ml. The synthetic AHL used in this study, *N*-hexanoyl-L-homoserine lactone (HHL), was purchased from Sigma (Bornem, Belgium).

Recombinant DNA techniques. Unless otherwise specified, standard techniques were used for the isolation of plasmid DNA, transformation, electroporation, agarose gel electrophoresis, DNA recovery from agarose gels (32), and conjugation (10). The oligonucleotides used in this study were *FW-splI*, 5′-TTG GCTGCAGTGTGTTCGCATGACCG-3′; *REV-splI*, 5′-CCTCTCTAGAACG GACGCAGACA AACCCA-3′; *FW-splR*, 5′-TGTTGAGCTCTCGCTGCCGG TGTAATAAGT-3′; and *REV-splR*, 5′-GGGCTCTAGACGGGCTATAATTC GTAAG-3′. They were synthesized by Eurogentec (Seraing, Belgium).

Construction of green fluorescent *Serratia plymuthica* strains. To construct an *S. plymuthica* RVH1 clone with a fluorescence phenotype, plasmid pJBA28 (4),

containing a mini Tn*5*-Km cassette that encompasses a fusion of the strong LacI-repressible synthetic promoter $P_{A1/O4/O3}$ to an S2R-modified version of the *gfpmut3* gene (8), was transformed into *E. coli* S17-1 λ*pir* and then conjugated into wild-type *S. plymuthica* RVH1, where the plasmid cannot replicate. Colonies were selected on minimal medium containing kanamycin, 0.4% glycerol, 0.1% Casamino Acids, 8 mg/liter biotin, and 0.2% 2-deoxy-D-galactose. The latter compound allows the selection for galactokinase deficiency (caused by *galK* in *E. coli*) because it will prevent the accumulation of toxic phosphorylated metabolites of 2-deoxygalactose (26). A kanamycin-resistant and ampicillin-sensitive exconjugant was selected and confirmed to have a decreased growth rate in minimal medium containing galactose as sole carbon source and to constitutively express *gfp*. This mutant was designated *S. plymuthica* RVH1-Gfp.

For the construction of the fluorescent *S. plymuthica* RVH1 *splI* and *splR* insertion mutants, pRVH14 (carrying *splI*::*aacC1*) and pRVH15 (carrying *splR*::*aacC1*), respectively, were transformed into *E. coli* S17-1 λ*pir* and then conjugated into *S. plymuthica* RVH1-Gfp. Gentamicin-resistant exconjugants were selected and confirmed by PCR to carry genuine chromosomal *splI*::*aacC1* and *splR*::*aacC1* alleles. These strains were designated *S. plymuthica* RVH1-1-Gfp and *S. plymuthica* RVH1-2-Gfp, respectively. For the *splI* mutant loss of AHL, production was confirmed by the *N*-acyl-L-homoserine lactone bioassay.

Construction of a red fluorescent *E. coli* strain. First, we constructed a $LacI^-$ derivative of *E. coli* MG1655 by transducing the *lacI*::Tc^r allele from donor strain CAG18439 (34) to MG1655 by P1 transduction and selecting for tetracycline resistance and β-galactosidase production to ensure no cotransduction of the *lacZ*118(Oc) allele. This strain was designated MP1. Subsequently, plasmid pSM1833 (37), containing a mini Tn*5*-Km cassette with a fusion of the strong LacI-repressible synthetic promoter $P_{A1/O4/O3}$ to the *dsRed* gene (23), was transformed into *E. coli* S17-1 λ*pir* and then conjugated into *E. coli* MP1. A kanamycin-resistant, ampicillin-sensitive exconjugant that constitutively expressed *dsRed* was designated *E. coli* MP2. A verification of growth curves revealed no differences between MP2 and MG1655 in LB broth or AB trace minimal medium supplemented with glucose and thiamine (data not shown), allowing us to assume that the transposon had been inserted in a neutral spot for our experiments.

***E. coli lacZ*::Tc.** This strain was constructed by transformation with Kleckner λTc (20) and screening for *lac*-negative colonies on LB supplemented with tetracycline and with 40 μl of a 20 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside solution (MP Biomedicals, Inc., Solon, Ohio). Selected clones were then subjected to a β-galactosidase Miller assay using *o*-nitrophenyl-β-D-galactopyranoside (Acros Organics; Geel, Belgium) as a substrate (25). One clone that was negative, even upon induction with isopropyl-β-D-thiogalactopyranoside (Acros Organics; Geel, Belgium), was selected and designated as MG1655 *lacZ*::Tc.

Isolation of an *S. plymuthica* RVH1 mutant deficient in AC production but not in quorum sensing. Screening for an AC-deficient strain was carried out with the green fluorescent *S. plymuthica* RVH1-Gfp to allow confocal laser scanning microscopy (CLSM) analysis of this strain in subsequent biofilm experiments. A random transposon insertion library was constructed by the conjugation of pBSL182, a *pir*-based suicide plasmid carrying a minitransposon with a gentamicin resistance cassette, to this strain. Circa 2,000 gentamicin-resistant colonies were screened by stab inoculation on soft agar seeded with *E. coli* ESS. After growth for 24 h at 30°C, clones lacking an inhibition zone were isolated and tested for AHL production by using the *C. violaceum* biological assay. One strain exhibiting wild-type AHL production was further tested by PCR analysis to confirm that the quorum-sensing genes *splI* and *splR* were not disrupted (data not shown). Other quorum-sensing-regulated phenotypes, such as nuclease and protease production, were also unaffected (data not shown). This strain was designated *S. plymuthica* RVH1-5-Gfp.

***N*-Acyl-L-homoserine lactone bioassays.** The biosensor strain *C. violaceum* CV026 (24) produces the purple pigment violacein in response to specific AHLs and was shown earlier to be a suitable reporter for the signals produced by *S. plymuthica* RVH1 (R. Van Houdt et al., submitted). Here, AHL production was detected in a cross-feeding assay by stabbing the strains to be tested onto LB agar plates (0.7% agar) seeded with *C. violaceum* CV026 and inspecting for purple pigment production during incubation at 30°C.

Biofilm formation in flow chamber experiments. Biofilms were grown at 30°C in three-channel flow chambers (BioCentrum DTU, Technical University of Denmark, Kgs. Lyngby, Denmark), with individual channel dimensions of 1 by 4 by 40 mm (5), that were covered with a microscope glass coverslip (st1; Knittel Gläser, Braunschweig, Germany). The setup (6) makes use of a 16-channel peristaltic pump (205S; Watson-Marlow Zellik, Belgium) that feeds each channel with a flow of 3 ml/h (flow rate of 0.2 mm/s) of AB-trace medium [2 g/liter $(NH_4)_2SO_4$; 6 g/liter $Na_2HPO_4 \cdot 2H_2O$; 3 g/liter KH_2PO_4; 3 g/liter NaCl; 1 mM $MgCl_2$; 0.1 mM $CaCl_2$ and 0.1 ml/liter trace metals (200 mg/liter $CaSO_4 \cdot 2H_2O$; 200 mg/liter $FeSO_4 \cdot 7H_2O$; 20 mg/liter $MnSO_4 \cdot H_2O$; 20 mg/liter $CuSO_4 \cdot 5H_2O$; 20 mg/liter $ZnSO_4 \cdot 7H_2O$; 10 mg/liter $CoSO_4 \cdot 7H_2O$; 10 mg/liter $NaMoO_4 \cdot H_2O$; 5 mg/liter H_3BO_3)] supplemented with 0.3 mM glucose and 1 μg/ml thiamine dichloride. Bubble traps were placed in each channel before the flow cell to remove air bubbles.

Before use, the flow system was sterilized by flushing with a solution of 0.5% sodium hypochlorite for 4 h and rinsed with approximately 0.2 liters of sterile water before the medium was pumped in. Bacterial cultures for inoculation were prepared by diluting an overnight LB broth culture to 1/100 in fresh LB medium and regrowing it for 4 h at 30°C. For mixed-species biofilm experiments, cultures of *S. plymuthica* and *E. coli* obtained in this way were mixed in appropriate ratios prior to inoculation. To inoculate the flow cells, the medium flow was stopped, flow chambers were turned with the glass coverslip down, and 250 μl of the diluted cell suspension was carefully injected through the silicon tubes into each flow channel with a small syringe. After 1 h, to allow adsorption of the cells to the coverslip surface, the flow channels were turned upright and the flow was resumed. Biofilms were analyzed either by CLSM or by the enumeration of attached cells by plating. In the latter case, the flow cells were disconnected and the cells in the flow channel were collected by vigorously pipetting up and down with 250 μl potassium phosphate buffer (10 mM, pH 7.0). The obtained cell suspensions were vortexed, diluted, and plated on LB agar plates supplemented with antibiotics if appropriate. Confocal images were collected with a Zeiss LSM 510/ConfoCor II confocal laser scanning microscope using a C-Apochromat ×40/1.2 water immersion lens. Alternating HeNe-1 and argon lasers were used to scan the dual-species biofilms. For each image, the noise was reduced by using the average of two scans.

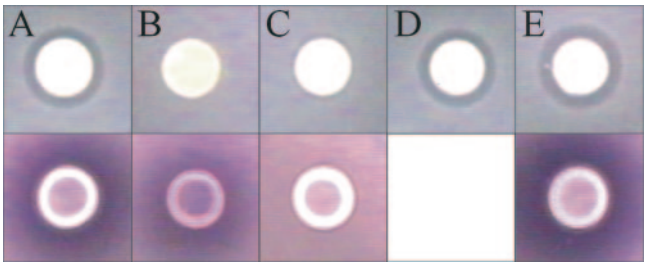

FIG. 1. Bioassays showing AC production as an inhibition zone on a lawn of *E. coli* (top row) and the production of AHL signal molecules as pigment production by a lawn of *Chromobacterium* CV026 (bottom row). The different *S. plymuthica* strains are the wild type (A), a strain deficient in AC production (B), the *splI* knockout (C), the *splI* knockout complemented with 5 μM synthetic HHL added to agar (D), and the *splR* knockout (E). Supplementation with HHL was not performed for the AHL assay because this would result in the pigmentation of the reporter strain all over the plate.

RESULTS

Isolation of an *S. plymuthica* RVH1 mutant deficient in AC production but not in quorum sensing. We previously showed that the production of an AC in *Serratia plymuthica* RVH1 is quorum sensing regulated and that SplR, the LuxR homolog of *S. plymuthica* RVH1, acts as a repressor of AHL-regulated genes (R. Van Houdt et al., submitted). Here, to investigate the role of AC production in single- and dual-species biofilms, we performed random transposon mutagenesis on the green fluorescence strain RVH-1 and selected a knockout strain that no longer produces this AC but that is unaffected in its quorum-sensing system. Figure 1 shows the results of bioassays for the production of AC and AHLs for this mutant in comparison to the wild-type strain and its quorum-sensing mutants. The slightly weaker response of the AHL reporter to the AC-deficient strain than that to the wild-type strain in this assay

(Fig. 1B and A, respectively, bottom row) can be attributed to the observation that the loss of AC production makes the mutant less competitive and results in less vigorous growth on the lawn of the *C. violaceum* CV026 reporter.

Effect of quorum sensing and antimicrobial component production on monospecies biofilms of *S. plymuthica*. Biofilms were grown in flow cells as described in Materials and Methods with *S. plymuthica* strains RVH1 (wild type), RVH1-1 (*splI* knockout), RVH1-2 (*splR* knockout), and RVH1-5-Gfp (AC production knockout). After 3 days, cell densities reached approximately 7.3 log CFU/cm^2, with no significant differences between the strains (data not shown). CLSM analysis of 3-day-old biofilms grown in the same way with the Gfp-labeled strains *S. plymuthica* RVH1-Gfp, RVH1-1-Gfp, RVH1-2-Gfp, and RVH1-5-Gfp also did not reveal any differences in biofilm cell density or in size or shape of biofilm cells and microcolonies (data not shown).

Effect of *S. plymuthica* quorum sensing and antimicrobial component production on planktonic mixed culture with *E. coli* MG1655. Stationary-phase cultures of the different *S. plymuthica* strains and *E. coli* MG1655 were mixed in fresh LB broth at dilutions of 1/10,000 and 1/100, respectively. This resulted in a starting inoculum of about 5.7 log CFU/ml for *S. plymuthica* and 7.4 log CFU/ml for *E. coli*. The growth of both strains in these mixed cultures, incubated at 30°C with shaking, was then followed by plating on LB at regular times (0, 4, 8, 12, and 24 h). *S. plymuthica* and *E. coli* colonies can be easily distinguished by morphology. Figure 2 shows the parallel evolution of cell numbers (as log CFU/ml) for each *S. plymuthica-E. coli* MG1655 combination. During the first 8 h of cocultivation, the colony counts of both partners increased and this evolution was not affected by the genetic background of the *S. plymuthica* strain used. The numbers of *E. coli* and *S. plymuthica* cells reached 9.5 and 8.5 log CFU/ml, respectively, after 8 h. Beyond that point, the numbers of *S. plymuthica* cells continued to increase equally for each strain used, to 8.7 log CFU/ml after 12 h and 9.4 log CFU/ml after 24 h. The numbers of cocultivated *E. coli* cells, however, evolved very differently depending on the *S. plymuthica* partner strain. With the wild-type *S. plymuthica* RVH1 strain as a partner, the *E. coli* numbers still showed a small increase after 12 h (9.5 log CFU/ml), but then steeply declined to 7.5 log CFU/ml after 24 h. With the *splI* knockout or the mutant deficient in AC production as a partner, there was also a small increase after 12 h, but only a small reduction of about 0.2 log CFU/ml after 24 h. On the other hand, with the *S. plymuthica splR* knockout, the *E. coli* counts were decreased already at 12 h and the decrease at 24 h was at least 3 log units. Due to the specific method of counting *S. plymuthica* and *E. coli* cells, reductions higher than 3 log units could not be detected. The addition of 10 μM *N*-hexanoyl-L-homoserine lactone to the medium during cocultivation of the *splI* knockout resulted in an even more rapid decrease of *E. coli* counts by at least 3 log units.

Effect of *S. plymuthica* quorum sensing and antimicrobial component production on the formation of mixed biofilms with *E. coli* MG1655. The next set of experiments was designed to investigate whether *S. plymuthica* quorum sensing or AC production affect the incorporation and establishment of *E. coli* in an *S. plymuthica* biofilm. In a first experiment, the effect of the relative cell densities of both partners was analyzed by inoculating the flow cells with different proportions of *E. coli* MG1655 *lacZ*::Tc and *S. plymuthica* RVH1 or *S. plymuthica* RVH1-5-Gfp and enumerating biofilm cells after 3 days of continuous growth. The *lacZ*::Tc marker was introduced to allow differential counting of *E. coli* to much lower cell densities than that based on colony morphology, and it was assumed that the *lacZ* knockout would not affect the behavior of *E. coli* in our biofilm experiments. In general, the cell density of *S. plymuthica* in the mixed biofilms was not (for wild-type *S. plymuthica*) or was only slightly (for the *S. plymuthica* AC knockout) affected by the different *S. plymuthica*-to-*E. coli* inoculation ratios used (1/100, 1/1, and 100/1) (Fig. 3). On average, *S. plymuthica* reached 7.72 log CFU/cm^2. The AC knockout strain established slightly less well (7.23 ± 0.02 log CFU/cm^2) in the presence of a 100-fold excess of *E. coli* cells, probably due to competition for nutrients. On the other hand, the *E. coli* numbers in the biofilm differed significantly depending on the inoculation ratio and depending on AC production of the *S. plymuthica* partner (Fig. 3). At an *S. plymuthica*-to-*E. coli* initial ratio of 1/100, *E. coli* reached 6.12 (±0.67) log CFU/cm^2 when cocultivated with RVH1 and 9.34 (±0.05) log CFU/cm^2 with RVH1-5-Gfp (AC$^-$). Inoculation ratios of 1/1 and 100/1 led to *E. coli* numbers of 4.78 (±1.27) log CFU/cm^2 and 9.07 (±0.23) log CFU/cm^2 for RVH1 and 0.43 (±0.67) log CFU/cm^2 and 5.93 (±0.52) log CFU/cm^2, respectively, for RVH1-5-Gfp.

In a second experiment, we studied the effect of AC pro-

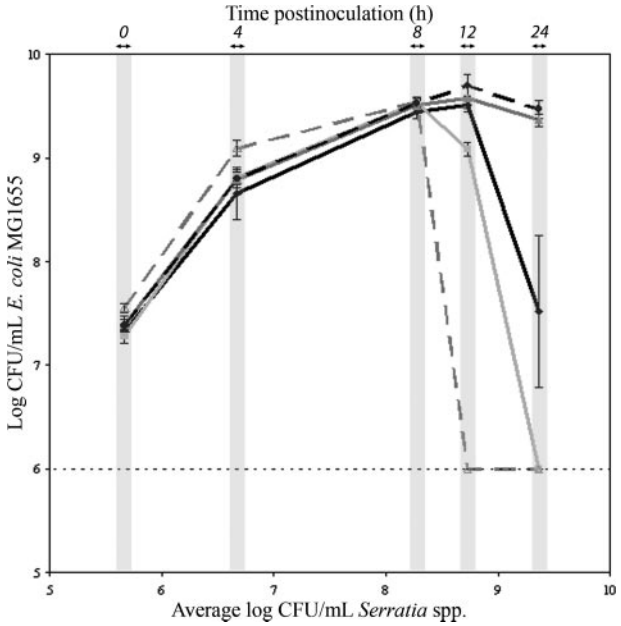

FIG. 2. Relative growth measurements (log CFU/ml) of *E. coli* MG1655 and different *S. plymuthica* strains during cocultivation in planktonic culture in LB broth. Black line, wild-type *S. plymuthica* RVH1; black dashed line, RVH1-5-Gfp (AC production knockout); dark gray line, RVH1-1 (*splI* knockout); dark gray dashed line, RVH1-1 complemented with HHL; light gray line, RVH1-2 (*splR* knockout). The lower detection limit for *E. coli* was 6 log CFU/ml. Each data point is the mean of three independent experiments, and error bars represent standard deviations. Measurements were taken at 0, 4, 8, 12, and 24 h of cocultivation. Data points taken at the same time points are grouped in gray rectangles.

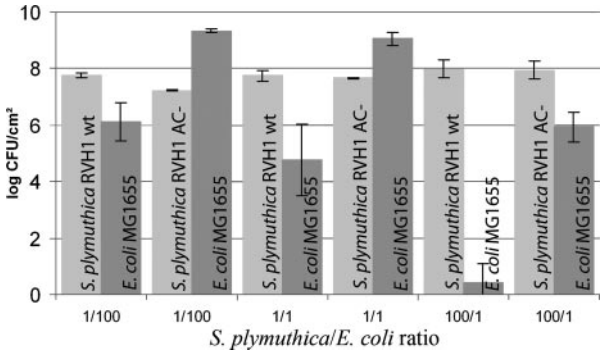

FIG. 3. Counts of biofilm cells of *S. plymuthica* (light gray) and *E. coli* (dark gray) in two-species biofilms established at different inoculation ratios (1/100, 1/1, and 100/1). Two different *S. plymuthica* strains were used: the wild-type strain RVH1 and its isogenic knockout in AC production. Mean values ± standard deviations (error bars) from three independent experiments are shown.

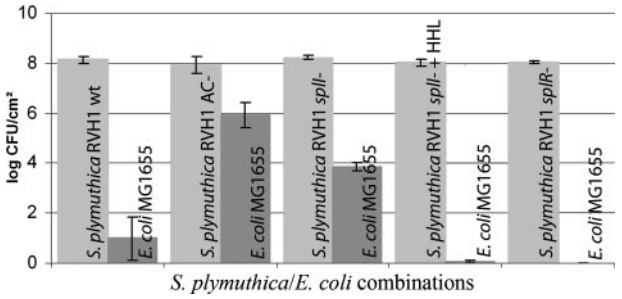

FIG. 5. Counts of biofilm cells of *S. plymuthica* (light gray) and *E. coli* (dark gray) in two-species biofilms established at a 100/1 inoculation ratio. Cell densities (log CFU/cm^2) of *E. coli* MG1655 *lacZ*::Tc with the *S. plymuthica* partners wild type, AC$^-$ strain, *splI* knockout strain, *splI* knockout strain complemented with 5 μM HHL, and *splR* knockout strain (no *E. coli* recovered from biofilm). Mean values ± standard deviations (error bars) were derived from three independent experiments.

duction by *S. plymuthica* in mixed biofilms with *E. coli* by CLSM. *S. plymuthica* RVH1-Gfp and RVH1-5-Gfp (both exhibiting green fluorescence) were mixed with *E. coli* MP2 (exhibiting red fluorescence) in a 1/1 ratio, and these mixtures were inoculated in separate flow cells. A 1/1 ratio was chosen based on the results in Fig. 3 because it results in clearly different biofilm establishments of *E. coli* with *S. plymuthica* RVH1-Gfp and RVH1-5-Gfp, but the incorporation of *E. coli* is still sufficient for microscopic visualization, together with both *S. plymuthica* strains. CLSM analysis of 3-day-old dual-species biofilms showed that *S. plymuthica* RVH1-Gfp and RVH1-5-Gfp developed similar large mushroom-shaped colonies interspersed with water channels (Fig. 4). However, in accordance with the results obtained in plating experiments (Fig. 3), *E. coli* was far more abundant in biofilms formed with RVH1-5-Gfp, which does not produce AC, than in biofilms formed with wild-type *S. plymuthica*. In addition, the *E. coli* colonies were smaller and more separated in the presence of wild-type *S. plymuthica* RVH1. Thus, it seems

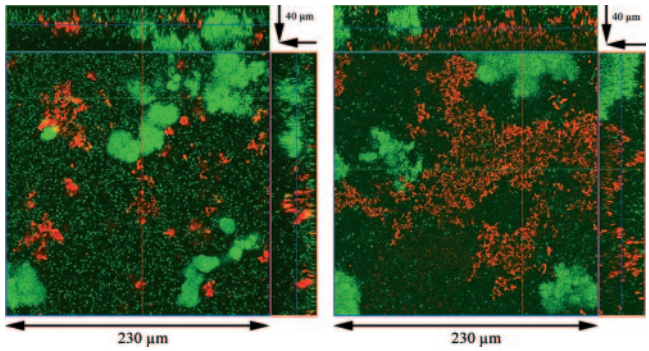

FIG. 4. Confocal laser scanning microscopic images of *S. plymuthica* and *E. coli* dual-species biofilms. *S. plymuthica* and *E. coli* are labeled with green and red Gfp variants, respectively. Left panel, *S. plymuthica* RVH1-Gfp (wild type) and *E. coli* MP2 (red). Right panel, *S. plymuthica* RVH1-5-Gfp (AC$^-$) and *E. coli* MP2 (red). Images are 230-μm squares. Lines in the *xy* plane depict the location of *z* projections, 40 μm deep, shown on the sides of the images. The top and right side of each image depict where biofilms are attached to the coverslip.

that AC production inhibits the outgrowth of the *E. coli* microcolonies and prevents them from merging together. Neither *S. plymuthica* strain formed mixed-species colonies with *E. coli*.

In a third and final experiment, we investigated the effect of quorum sensing on the establishment of *E. coli* in a dual-species biofilm with *S. plymuthica* RVH1. Since the production of AC is quorum sensing dependent in *S. plymuthica*, the elimination of AHL signal production by the knockout of *splI* is expected to support stronger development of *E. coli* in mixed biofilms. However, the situation may be more complex because quorum sensing also affects other properties of *S. plymuthica* that may interfere with the establishment of *E. coli* and because *E. coli* itself can respond to AHL signals, although it does not produce them (41). Biofilms were grown with either *S. plymuthica* RVH1, RVH1-1 (*splI*::*aacC1*), RVH1-2 (*splR*::*aacC1*), or RVH1-5-Gfp (AC$^-$), combined with *E. coli* MG1655 *lacZ*::Tc in a proportion of 100/1. After 3 days of continuous growth, no differences were seen in the biofilm cell densities of the *S. plymuthica* strains. In contrast, the *E. coli* biofilm cell densities varied strongly depending on the *S. plymuthica* partner strain (Fig. 5). The effect of AC production was similar to that shown in Fig. 3, with *E. coli* MG1655 densities of 0.98 (±0.89) and 5.93 (±0.52) log CFU/cm^2 in mixed biofilms with *S. plymuthica* RVH1 (wild-type) and RVH1-5-Gfp (AC$^-$), respectively ($P = 0.0011$). As expected, significantly more *E. coli* cells (3.84 ± 0.18 log CFU/cm^2) were recovered from biofilms with *S. plymuthica* RVH1-1 (SplI$^-$) than with wild-type RVH1 ($P = 0.0054$), but the *E. coli* levels remained below those reached in combination with RVH1-5-Gfp ($P = 0.0028$). The addition of 5 μM synthetic HHL to the feed solution during the growth of a biofilm containing RVH1-1 resulted in an almost total absence of *E. coli* MG1655 (0.03 ± 0.06 log CFU/cm^2). In combination with *S. plymuthica* RVH1-2 (SplR$^-$), *E. coli* biofilm levels were below the detection limit. The levels of *E. coli* in biofilms with wild-type *S. plymuthica* RVH1 were higher than those in biofilms with RVH1-1 plus HHL ($P = 0.1395$) and with RVH1-2 ($P = 0.1288$).

DISCUSSION

S. plymuthica RVH1 is a strong biofilm-forming isolate from a food-processing environment that also produces a variety of quorum-sensing signaling molecules (39, 40). The production of an AC, an extracellular protease, chitinase, and nuclease and butanediol fermentation in this strain are under the control of a LuxI-LuxR-type quorum-sensing system consisting of an AHL synthase (SplI) and an AHL-responsive repressor (SplR) (R. Van Houdt et al., submitted; 42). Based on these properties, RVH1 is an interesting model organism for studying bacterial interactions in biofilms. The objective of the current work was to investigate the role of quorum sensing in biofilms of *S. plymuthica* RVH1 and, more specifically, of AC production on the interaction of RVH1 with *E. coli* MG1655. To be able to separate the effect of AC production from other quorum-sensing-dependent phenotypes, we first isolated a knockout strain of RVH1 that is AC deficient but unaffected in quorum sensing and then compared the behavior of this strain and the already available *splI* and *splR* derivatives with that of the wild-type RVH1 strain in various experimental setups.

CLSM analysis revealed that single-species biofilms of the RVH1 wild-type and *splI* knockout strains formed similar biofilms within our flow cell system that consisted of mushroom-shaped colonies interspersed with water channels comparable to those observed for *Pseudomonas aeruginosa* (35). No differences in biofilm structure or cellular morphology could be observed, indicating that the SplIR-dependent quorum-sensing system does not play an obvious role in biofilm formation. In contrast, in *S. marcescens* MG1 (formerly *S. liquefaciens* MG1), biofilm cells occur in characteristic chains of long filamentous cells which are formed in an AHL-dependent manner (21), although the latter is nutrient dependent and classical biofilms consisting of microcolonies are formed under reduced carbon or nitrogen conditions (29).

In planktonic mixed cultures with *E. coli* MG1655, all *S. plymuthica* strains (RVH1 and *splI*, *splR*, and AC^- mutants) showed similar growth curves throughout the entire cocultivation experiment. During up to 8 h of cocultivation, the *E. coli* strain also grew equally well with each of the *S. plymuthica* strains, reaching a cell density of about 9.6 log CFU/ml, which corresponds to the cell density of a stationary-phase *E. coli* culture. This indicates that both species showed little or no interaction during their exponential growth phase in cocultivation. Beyond 8 h of cocultivation, however, the *E. coli* curves diverged. In cocultivation with the *splI* knockout or the knockout in AC production, which are deficient in AC production (Fig. 1), the *E. coli* numbers still showed a small increase between 8 and 12 h, followed by a small reduction of about 0.3 log units. In combination with the wild-type RVH1, there was also still a small increase between 8 and 12 h, followed by a more substantial 2-log-unit decline. Finally, a decline of at least 3 log units occurred in cocultivation with the *splR* mutant or with the splI mutant in the presence of HHL. Thus, the expected level of AC production by *S. plymuthica* (Fig. 1) is well correlated with the observed antagonistic effect towards cocultivated *E. coli* in this experiment. The small reduction in *E. coli* numbers with the *splI* knockout strain and the AC^- strain between 12 and 24 h suggests that additional but minor mechanisms of antagonism might also be involved. However, if such mechanisms exist, they are not quorum sensing controlled.

In biofilms, antagonistic interactions are more complex because the formation of microcolonies may enhance the persistence of a sensitive species against harmful compounds produced by the antagonist (28). In our biofilm experiments, the interaction of *S. plymuthica* and *E. coli* was very similar to that in planktonic culture (Fig. 5). While the establishment of *S. plymuthica* in the biofilm was again independent of the strain used, the establishment of *E. coli* was again correlated with the level of AC production by *S. plymuthica*. In spite of the anticipated protective effect of the microcolonies, *E. coli* became completely eliminated from mixed biofilms with the *splR* mutant after 3 days. With wild-type *S. plymuthica* RVH1, which produces slightly less AC, complete elimination was observed after 6 days of cocultivation (data not shown). The observation that the SplI knockout strain shows an intermediate phenotype relative to the wild type and the AC mutant suggests either incomplete repression by SplR or additional regulation of AC production.

So far, only a few studies have investigated how the production of antimicrobial compounds can contribute to the shaping of mixed-species biofilms. In some cases, a complete elimination of sensitive species was observed, similar to that in our own study (28), while in other cases, only partial suppression occurred (36). However, in the above systems, the production of the antibacterial compounds was not quorum sensing dependent. In a recent study, An et al. (3) studied mixed biofilms of *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens*. The former species produces rhamnolipids, cyanide, and pyocyanin as antibacterial compounds in a quorum-sensing-dependent manner. However, *A. tumefaciens* could establish and survive during extended periods in mixed cocultures and biofilms with *P. aeruginosa*, and the elimination of the production of these three antimicrobial compounds did not affect the interaction between both species. Therefore, our study provides the case in which AHL-dependent quorum-sensing regulation of an antimicrobial component seriously affects the coexistence of a sensitive species in a dual-species biofilm.

Since quorum sensing contributes to the virulence of many pathogenic bacteria, interference with quorum sensing as a possible strategy to prevent or cure bacterial diseases has received considerable attention during recent years. This idea has been inspired by examples from nature, such as the halogenated furanones from the red alga *Delisea pulchra*, signal analogs which bind to LuxR activator proteins and block the downstream pathway (22), or the AHL lactonases from *Bacillus* sp. and some other bacteria (11), which degrade the AHL signals. While these approaches may be very promising and lead to the development of novel types of antibacterial compounds, their effect on complex microbial communities is difficult to predict. Indeed, the use of chemical quorum-sensing antagonists to combat biofilms, for example, may potentially result only in a replacement of species that use quorum sensing for biofilm establishment with species that do not, such as the *S. plymuthica* strain studied here. In addition, as illustrated in this work, interference with quorum sensing may reduce the ability of biofilm bacteria to exclude competitors and, in this way, cause a shift in the natural biofilm composition. In a

context of antibacterial therapy, such side effects may have undesirable consequences.

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