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# Quorum Sensing-Controlled Biofilm Development in *Serratia liquefaciens* MG1

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***Serratia liquefaciens*** **MG1 contains an *N*-acylhomoserine lactone-mediated quorum-sensing system that is known to regulate swarming motility colonization. In this study, we describe for *S. liquefaciens* MG1 the development of a novel biofilm consisting of cell aggregates and differentiated cell types, such as cell chains and long filamentous cells. Furthermore, quorum sensing is shown to be crucial for normal biofilm development and for elaborate differentiation. A mutant of *S. liquefaciens* MG1 that was incapable of synthesizing extracellular signal formed a thin and nonmature biofilm lacking cell aggregates and differentiated cell chains. Signal-based complementation of this mutant resulted in a biofilm with the wild-type architecture. Two quorum-sensing-regulated genes (*bsmA* and *bsmB*) involved in biofilm development were identified, and we propose that these genes are engaged in fine-tuning the formation of cell aggregates at a specific point in biofilm development.**

*N*-Acylhomoserine lactone (AHL)-mediated quorum sensing (QS) controls diverse phenotypic traits in various gram-negative proteobacteria (for a recent review, see reference 29). However, despite the proposed role of QS systems in bacterial colonization of surfaces, to date only a limited number of organisms are known to require a functional AHL-mediated QS system for formation of biofilms. In *Pseudomonas aeruginosa*, *Burkholderia cepacia*, and *Aeromonas hydrophila*, functional AHL regulatory systems are necessary for formation of microcolony structures (5, 13, 18). In *Pseudomonas putida* IsoF, AHL-mediated QS affects the architecture of the biofilm, and a QS mutant forms a heterogeneous microcolony-based biofilm rather than the homogeneous biofilm typical of the wild type (26). In these organisms, a specific QS-regulated determinant for biofilm formation has been identified only in *P. putida* IsoF. An AHL-regulated long-chain fatty acid coenzyme ligase was proposed to alter the fatty acid composition of the cell membrane, changing the surface properties of the cell and ultimately the biofilm structure (26).

*Serratia liquefaciens* is an opportunistic pathogen which is capable of colonizing a wide variety of surfaces in water, soil, the digestive tracts of rodents, plants, insects, fish, and humans (10). *S. liquefaciens* secretes a broad range of hydrolytic enzymes, and strain MG1 can differentiate into specialized swarmer cells capable of rapid surface motility (6). Swarming motility in *S. liquefaciens* MG1 is AHL regulated and is directed through specific control of the *swrA* gene. This gene encodes a peptide synthetase that catalyzes synthesis of the biosurfactant serrawettin W2, which reduces surface tension and allows swarming motility to occur (17). QS is therefore involved in at least one surface colonization process in *S. liquefaciens* MG1. SwrI and SwrR, homologues of the I and R proteins characteristic of AHL-mediated systems, have also been reported in this strain (7). SwrI synthesizes the signal molecules *N*-butanoyl-L-homoserine lactone ($C_4$-HSL) and *N*-hexanoyl-L-homoserine lactone at a 10:1 ratio (7). The signal molecule ($C_4$-HSL or *N*-hexanoyl-L-homoserine lactone) is hypothesized to bind the SwrR transcriptional regulator and affect the expression of at least 28 proteins, as shown by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (9). This suggests that a number of other phenotypes are also regulated by AHL-mediated QS in *S. liquefaciens* MG1.

In this study, we demonstrated that the surface-colonizing bacterium *S. liquefaciens* MG1 forms a unique type of biofilm structure consisting of cell aggregates, differentiated filaments, and cell chains. We showed that normal biofilm formation in *S. liquefaciens* MG1 requires a functional QS system, and we identified two AHL-regulated genes that are proposed to fine-tune the formation of cell aggregates at a specific point in biofilm development.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth.** The bacterial strains used in this study are listed in Table 1. All strains were routinely grown at 30°C in Luria-Bertani broth or Difco minimal broth (DMB) supplemented with 0.2% (wt/vol) glucose and 0.5% (wt/vol) Casamino Acids. For complementation of strains MG44 and MG3633 to MG3651 in the static biofilm assay and flow chamber experiments, 250 nM $C_4$-HSL was added to the medium. In the complementation flow chamber experiments with strains MG3646(pBsmA), MG3646 (pBsmAOp), and MG3651(pBsmB), 250 nM $C_4$-HSL and 30 μg of gentamicin $ml^{-1}$ were added to the medium.

**DNA manipulation and nucleotide sequencing.** Plasmid DNA was prepared by using the Wizard Plus Minipreps DNA purification system (Promega). Cloning, chemical transformation of *Escherichia coli*, and electroporation of *S. liquefaciens* MG1 were performed by using standard procedures (3). Genomic DNA was extracted from cultures of *S. liquefaciens* strains MG3646 and MG3651 by using the XS buffer protocol (28). Identification of transposon mutated genes was carried out by using a combination of two protocols. The first protocol was an

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TABLE 1. Strains and plasmids

| Strain(s) or plasmid | Relevant characteristics[a] | Source or reference |
|---|---|---|
| *Escherichia coli* strains | | |
| DH5α | ϕ80d*lacZ*ΔM15 *recA1 endA1 gyrA96 thi-1 hsdR17* ($r_K^-$ $m_K^+$) *supE44 relA1 deoR* Δ(*lacZYA-argF*)*U169* | Promega |
| MV1190 (λ-*pir*) | Δ(*lac-proAB*) Δ(*srl-recA*)*306*::Tn*10*[F′ *traD36 proAB lacI*$^q$ Δ(*lacZ*)M15] *thi supE*; lysogenized with λ-*pir* phage | 11 |
| HB101 | *recA thi pro leu hsdRM*$^+$ Sm$^r$ | 15 |
| *Serratia liquefaciens* strains | | |
| MG1 | Wild type, Ap$^r$ Tc$^r$ | 8 |
| MG44 | MG1, *swrI* gene disrupted with a streptomycin cassette, Sm$^r$ | 7 |
| MG3633 to MG3651 | MG44, disruption in a $C_4$-HSL responsive gene by a mini-Tn*5* transposon carrying promoterless *luxAB* and a kanamycin marker, Km$^r$ | 17 |
| MG3659 | *gfpmut3**-$T_0$-$T_1$-tagged MG1, Km$^r$ | This study |
| MG3663 | *gfpmut3**-$T_0$-$T_1$-tagged MG44, Km$^r$ | This study |
| Plasmids | | |
| pBluescript II SK | Cloning vector, Ap$^r$ | Stratagene |
| pBR322 | Cloning vector, Ap$^r$ Tc$^r$ | New England Biolabs |
| pUCGM | Gm$^r$ | 24 |
| pJBA28 | Ap$^r$ Km$^r$ | 2 |
| pBRGM | pBR322 bearing the 855-bp gentamicin cassette from pUCGM | This study |
| pML001 | pBluescript II SK bearing a 1.4-kb *bsmA* fragment | This study |
| pML002 | pBluescript II SK bearing a 1.2-kb *bsmB* fragment | This study |
| pML003 | pBluescript II SK bearing a 2.8-kb *bsmA-nifS* fragment | This study |
| pBsmA | pBRGM bearing a 1.4-kb *bsmA* fragment | This study |
| pBsmB | pBRGM bearing a 1.2-kb *bsmB* fragment | This study |
| pBsmAOp | pBRGM bearing a 2.8-kb *bsmA-nifS* fragment | This study |
| RK600 | *ori* ColE1 RK2-Mob$^+$ RK2-Tra$^+$ Cm$^r$; helper plasmid in triparental matings | 15 |

[a] Tc$^r$, tetracycline resistant; Ap$^r$, ampicillin resistant; Sm$^r$, streptomycin resistant; Km$^r$, kanamycin resistant; Gm$^r$, gentamicin resistant.

adaptor ligation PCR protocol described by Siebert et al. (25) and modified by Tillett (27), in which the specific mini-Tn*5* transposon primers P6 (5′-GCCCG GTCGCATTACACCTT-3′) and P7.2 (5′-CGCTTCATCACTTCGGTCTGAG A-3′) were used. The second protocol involved shotgun ligating *Bam*HI-digested chromosomal DNA into pBluescript II SK. Clones bearing inserts carrying the Tn*5* kanamycin resistance marker and chromosomal DNA were selected and sequenced. Both strands of DNA were sequenced by using a primer walking strategy in a thermocycling reaction with BigDye terminators. Sequences were analyzed at the Automated Sequencing Facility at the University of New South Wales. DNA sequences were compared to other sequences in the GenBank database by using the on-line open reading frame (ORF) finder and BLASTP programs at the National Center for Biotechnology Information web site (http://www.ncbi.nlm.nih.gov/).

**Construction of plasmids pBsmA, pBsmAOp, and pBsmB.** Plasmids pBsmA, pBsmAOp, and pBsmB were constructed by amplifying *bsmA*, the *bsmA-nifS* operon, and *bsmB* from *S. liquefaciens* MG1 with *Pwo* polymerase (Roche) by using primers F2ML (5′-TTACCCACCAGAAGCTTGAAGG-3′) (*Hin*dIII) and R2ML (5′-CCAGGTTGGCTCTAGATTTCCC-3′) (*Xba*I), primers F2ML and R7ML (5′-ATCGTCATGGTCTAGAAGGTCG-3′) (*Xba*I), and primers F5ML (5′-CTGGCAACATATCTAGATTACCC-3′) (*Xba*I) and R4ML (5′-GG TTCGGTGAGAATTCAGGAG-3′) (*Eco*RI), respectively; these primers were engineered to code for restriction endonuclease sites (underlined), and the enzyme that cleaved each sequence is indicated in parentheses. Plasmid pBluescript II SK and the PCR-amplified fragments (containing either *bsmA*, *bsmB*, or the *bsmA-nifS* operon) were digested with the corresponding restriction endonuclease sites engineered into the primers and purified, and the fragments were ligated into pBluescript II SK to obtain pML001 (bearing *bsmA*), pML002 (bearing *bsmB*), and pML003 (bearing the *bsmA-nifS* operon). Plasmid pBRGm was then constructed by ligating the 855-bp *Sma*I fragment from pUCGm containing the gentamicin resistance marker into the *Ssp*I site of pBR322. The inserts from pML001, pML002, and pML003 were then transferred to plasmid pBRGm to obtain plasmids pBsmA, pBsmB, and pBsmAOp, respectively.

**Construction of *gfp*-tagged *S. liquefaciens* strains MG3659 and MG3663.** *S. liquefaciens* strains MG1 and MG44 were tagged with *gfpmut3**-$T_0$-$T_1$ by transposition with pJBA28 to create *S. liquefaciens* strains 3659 and 3663, respectively. The transposition procedure was carried out as described previously (2).

**Microtiter plate biofilm screening.** Nineteen previously isolated mutants bearing Tn*5*::*luxAB*::*npt* insertions in QS-regulated genes (17) were screened for biofilm formation in 24-well polystyrene microtiter dishes (Sarstedt Inc., Newton, N.C.) as previously described (21, 22). DMB supplemented with 0.2% glucose and 0.5% Casamino Acids was used, and biofilms were allowed to form for 24 h at 30°C with shaking and then assessed by microscopy for microcolony formation at the base of the wells.

**Luminescence assays.** Induction of the mutated genes in MG3646 and MG3651 was determined by measuring *luxAB* expression after addition of $C_4$-HSL. Samples were taken hourly during growth and measured for luminescence with a Wallac Victor$^2$ 1420 multilabel counter.

**Flow chamber biofilm experiments.** Biofilms of *S. liquefaciens* strains were grown by using DMB supplemented with 0.05% (wt/vol) glucose and Casamino Acids. The flow chamber consisted of a glass base (10 by 5 cm) with two glass slides (7.5 by 2.5 cm) glued on top with a UV-curing glue (3042B; Three Bond Co., Ltd., Tokyo, Japan). A 1-mm gap was left between the slides, and a glass coverslip (6 by 2.4 cm) was glued on top of the slides, resulting in an all-glass flow tunnel. Silicone tubing was connected to each side of the flow cell tunnel and sealed with silica gel. All experiments were carried out at room temperature (approximately 21°C), and the flow chamber was sterilized by autoclaving prior to inoculation. The flow chamber was inoculated by injecting the appropriate strain into the flow channel with a small syringe. Flow into the chamber was halted for 1 h, after which sterile medium was pumped through the flow chamber with a peristaltic pump at a flow rate of 0.18 ml/min. Development of biofilms was monitored by light microscopy and fluorescence microscopy. To monitor biofilm development by fluorescence microscopy, *gfp*-tagged strains MG3659 (wild type) and MG3663 (*swrI* mutant) were used. Images of *S. liquefaciens* biofilms that were fixed with 2% glutaraldehyde and stained with 0.01% acridine orange were obtained with a confocal microscope. To ensure that glutaraldehyde had no effect on biofilm structure, we compared biofilms fixed and stained as described above and biofilms formed with the *gfp*-tagged strains (strains MG3659 and MG3663). Glutaraldehyde fixation had no effect on biofilm structure (data not shown). Thus, fixation and staining were the preferred methods of image capture since biofilms formed by the *gfp*-tagged strains lost green fluorescent protein fluorescence over time.

**Quantification of bacterial aggregates formed in biofilms.** To quantify bacterial aggregation, triplicate 48-h-old biofilms of the *S. liquefaciens* wild type and the *S. liquefaciens swrI* mutant were fixed and stained as described above. Confocal images (magnification, ×400) were captured from 10 random points in each flow chamber, and total aggregates were counted.

**Biofilm COMSTAT analysis.** For statistical evaluation of biofilm structures, triplicate flow cells were prepared for each strain, and 10 image stacks (2-μm intervals) per flow chamber were obtained for the mature biofilm at 72 h postinoculation. The images were analyzed with the biofilm computer program COMSTAT (12).

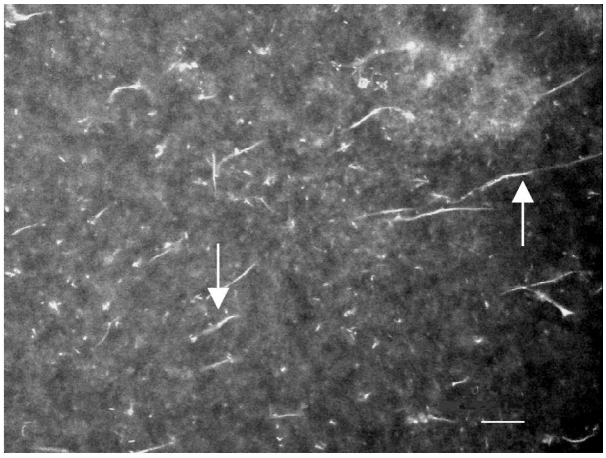
**Microscopy.** Biofilms from the microtiter plate assay were examined with an Olympus IMT2 inverted scanning confocal laser microscope (Olympus Optical Co. Ltd., Tokyo, Japan) equipped with a 40× phase-contrast lens whose numerical aperture was 0.6 under normal light. Biofilm development was observed with an Olympus CH-2 light microscope and with a Leica DMLB epifluorescence microscope (Leica Microsystems, Wetzlar, Germany), both equipped with a 40× lens whose numerical aperture was 0.65. Scanning confocal laser microscopy images of the flow chambers were obtained with an Olympus GB200 microscope fitted with a piezoelectric *z* stage. The microscope was equipped with a 40× lens whose numerical aperture was 0.95 and a 60× oil immersion lens whose numerical aperture was 1.4. Image scanning was carried out with the 488-nm laser line of an argon laser. Captured images were further processed for display by using Photoshop software (Adobe, Mountain View, Calif.).

**Nucleotide sequence accession numbers.** The nucleotide sequences of the *bsmA-nifS* operon and *bsmB* have been deposited in the GenBank database under accession numbers AF537272 and AF537273, respectively.

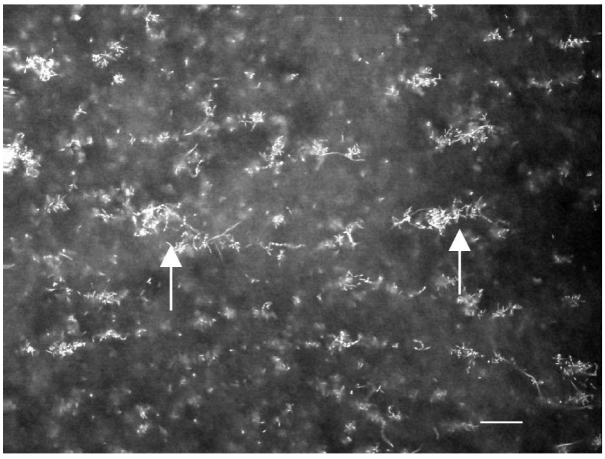
## RESULTS

***S. liquefaciens* MG1 forms a biofilm with a novel structure in flow chambers.** The development of the *S. liquefaciens* MG1 biofilm is distinct from the development of biofilms formed by model biofilm-forming bacteria, such as *P. aeruginosa* and *E. coli*. As a control, we confirmed that *P. aeruginosa* forms a biofilm consisting of microcolonies in our flow chamber setup, which allowed us to make direct comparisons between the two biofilm structures (data not shown). During development of the *S. liquefaciens* MG1 biofilm, elaborate cellular differentiation and structural differentiation were clearly observed. Specifically, at 24 h, long filamentous cells were observed (Fig. 1a); at 48 h, aggregation of vegetative bacteria with the filamentous cells was observed (Fig. 1b); and at 72 h, intertwining cell chains where there was biofilm maturation (Fig. 1c) were observed.

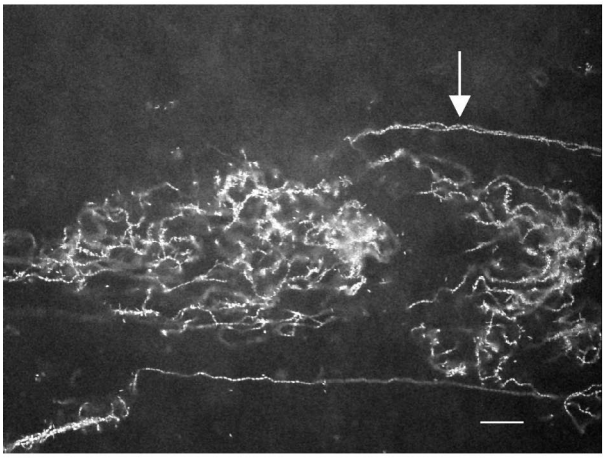
***S. liquefaciens swrI* mutant forms a biofilm that is morphologically distinct from and thinner than the biofilm formed by the wild type.** Compared to the wild-type biofilm, striking differences in biofilm architecture were observed in the *swrI* mutant biofilm. At 24, 48, and 72 h, the *swrI* mutant biofilm was identical to the 24-h wild-type biofilm consisting of long filamentous cells (Fig. 2a). This suggests that development in the mutant biofilm stalled after 24 h. In contrast, the wild-type biofilm exhibited six- to sevenfold more aggregation at 48 h (number of aggregates per field of view for the wild type, 18.6 ± 4.34 [average ± standard deviation]; number of aggregates per field of view for the *swrI* mutant, 2.3 ± 1.9) and contained intertwining cell chains at 72 h (Fig. 2b). The contrast between the mature wild-type and *swrI* mutant biofilms was further supported by COMSTAT analysis (Table 2). The *swrI* mutant biofilm was on average five times thinner than the wild-type biofilm, and the maximum biofilm thickness of the *swrI* mutant biofilm was approximately 19 μm, compared to approximately 52 μm for the wild-type biofilm. The total biovolume of the wild-type biofilm was approximately four times greater than



(a)



(b)



(c)

FIG. 1. Confocal *xy* images of *S. liquefaciens* MG1 biofilm at different times during development. (a) Formation of filamentous cells at 24 h; (b) aggregation with the filamentous cells at 48 h; and (c) mature biofilm of intertwining cell chains at 72 h. The arrows indicate examples of filamentous cells (a), aggregates (b), and intertwining cell chains (c). Bars = 20 μm.

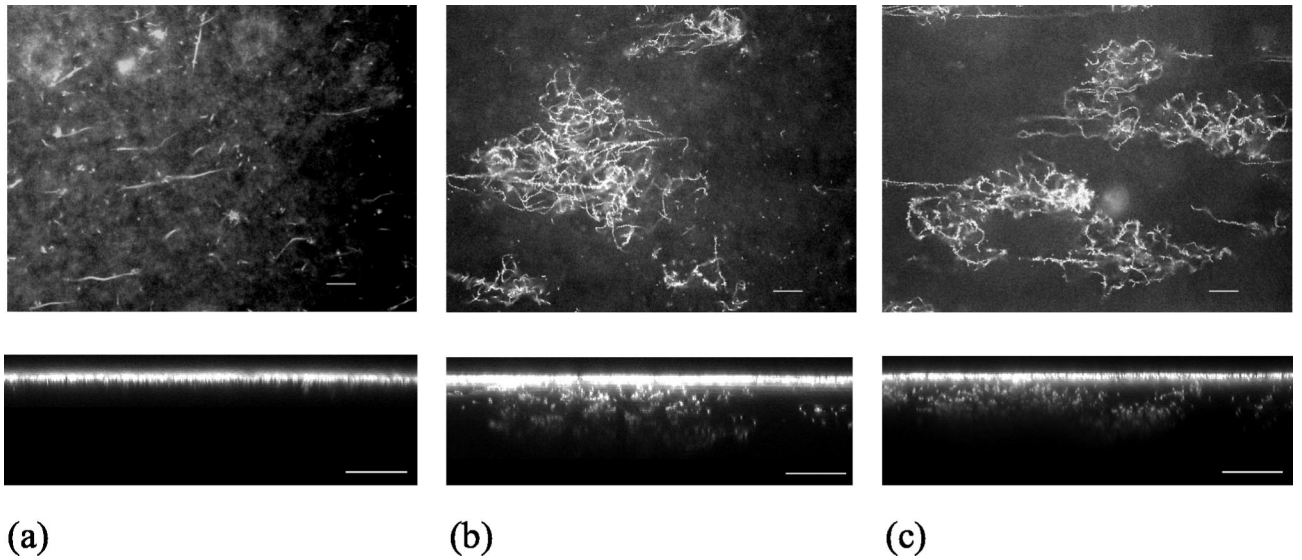



FIG. 2. Confocal *xy* (top panels) and *xz* (bottom panels) images of 72-h flow chamber biofilms formed by the *S. liquefaciens* MG44 *swrI* mutant strain (a), wild-type strain MG1 (b), and *swrI* mutant strain MG44 in the presence of $C_4$-HSL (c). Bars = 20 μm.

that of the *swrI* mutant biofilm, confirming the greater biofilm-forming ability of the wild type. The wild-type biofilm also had slightly greater substratum coverage than the *swrI* mutant biofilm. Addition of 250 nM $C_4$-HSL to the flow medium of the *swrI* mutant biofilm restored the developmental process to that of the wild-type biofilm, as shown in Fig. 2c and Table 2 (COMSTAT analysis). These data were replicated on more than 10 separate occasions, and we therefore concluded that proper biofilm formation by *S. liquefaciens* MG1 requires a functional QS system.

**Identification of *S. liquefaciens* $C_4$-HSL-regulated biofilm genes.** In an effort to identify the specific $C_4$-HSL-regulated genes required for biofilm formation by *S. liquefaciens* MG1, a set of 19 $C_4$-HSL-responsive reporter-transposon *S. liquefaciens* MG44 mutants (17) were screened for reduced biofilm formation in a microtiter plate. Two of the 19 transposon mutants screened, MG3646 and MG3651, were defective in biofilm formation. Included in the 19 transposon mutants was the *swrA* surfactant mutant. Although this mutant is deficient in swarming colonization (17), it adhered and formed a normal biofilm in the static biofilm assay and was not studied further. The growth rates of both MG3646 and MG3651 were similar to that of the parent strain (data not shown), indicating that reduced biofilm formation was not a result of poor growth.

Sequence information from the site of the transposon insertion in MG3646 indicated that the transposon had disrupted a 645-bp ORF, and the deduced sequence of the protein encoded by this ORF exhibited 61% identity and 76% similarity (over 152 amino acid residues) to the sequence of a hypothetical protein (GenBank accession number NP_230828) from *Vibrio cholerae*. This ORF was designated *bsmA* (for "biofilm structure mutant A"). Downstream within the same putative operon and overlapping by 4 nucleotides is a gene coding for a protein with 61% identity and 76.5% similarity to a putative NifS homologue (GenBank accession number NP_230829) from *V. cholerae* (Fig. 3a). Measurement of promoter activity through detection of luminescence after addition of $C_4$-HSL revealed that *bsmA* is induced 2.8-fold at the transition from the logarithmic phase to the stationary phase. While addition of $C_4$-HSL enhanced transcription of *bsmA*, some expression still occurred in the absence of $C_4$-HSL, indicating that other regulatory elements also contribute to regulation of this gene.

Analysis of the sequence data indicated that the sequence of transposon mutant MG3651 was disrupted in a 531-bp ORF designated *bsmB* (Fig. 3b). BLASTP analysis of the translated protein sequence of *bsmB* revealed no similarity to sequences in the GenBank database. This suggests that a novel gene involved in biofilm formation was present. Fifty-three base pairs downstream from *bsmB* is a 318-bp ORF in the opposite orientation, suggesting that *bsmB* is not in an operon. Promoter activity assays for *bsmB* in the presence and absence of $C_4$-HSL revealed that this gene is highly dependent on a functional QS system for expression and is maximally induced 9.8-

TABLE 2. COMSTAT analysis of mature biofilms formed by *S. liquefaciens*[a]

| Strain | Mean thickness (μm) | Maximum thickness (μm) | Biovolume ($\mu m^3/\mu m^2$) | Substratum coverage (%) |
|---|---|---|---|---|
| *S. liquefaciens* MG1 | 34.99 ± 8.10 | 51.60 ± 12.11 | 34.76 ± 7.91 | 99.07 ± 1.41 |
| *S. liquefaciens* MG44 | 7.21 ± 0.85 | 18.93 ± 1.55 | 8.46 ± 0.82 | 94.42 ± 1.96 |
| *S. liquefaciens* MG44 with $C_4$-HSL | 28.21 ± 4.76 | 37.27 ± 4.94 | 29.69 ± 4.83 | 99.36 ± 0.78 |

[a] The values are means ± standard deviations.

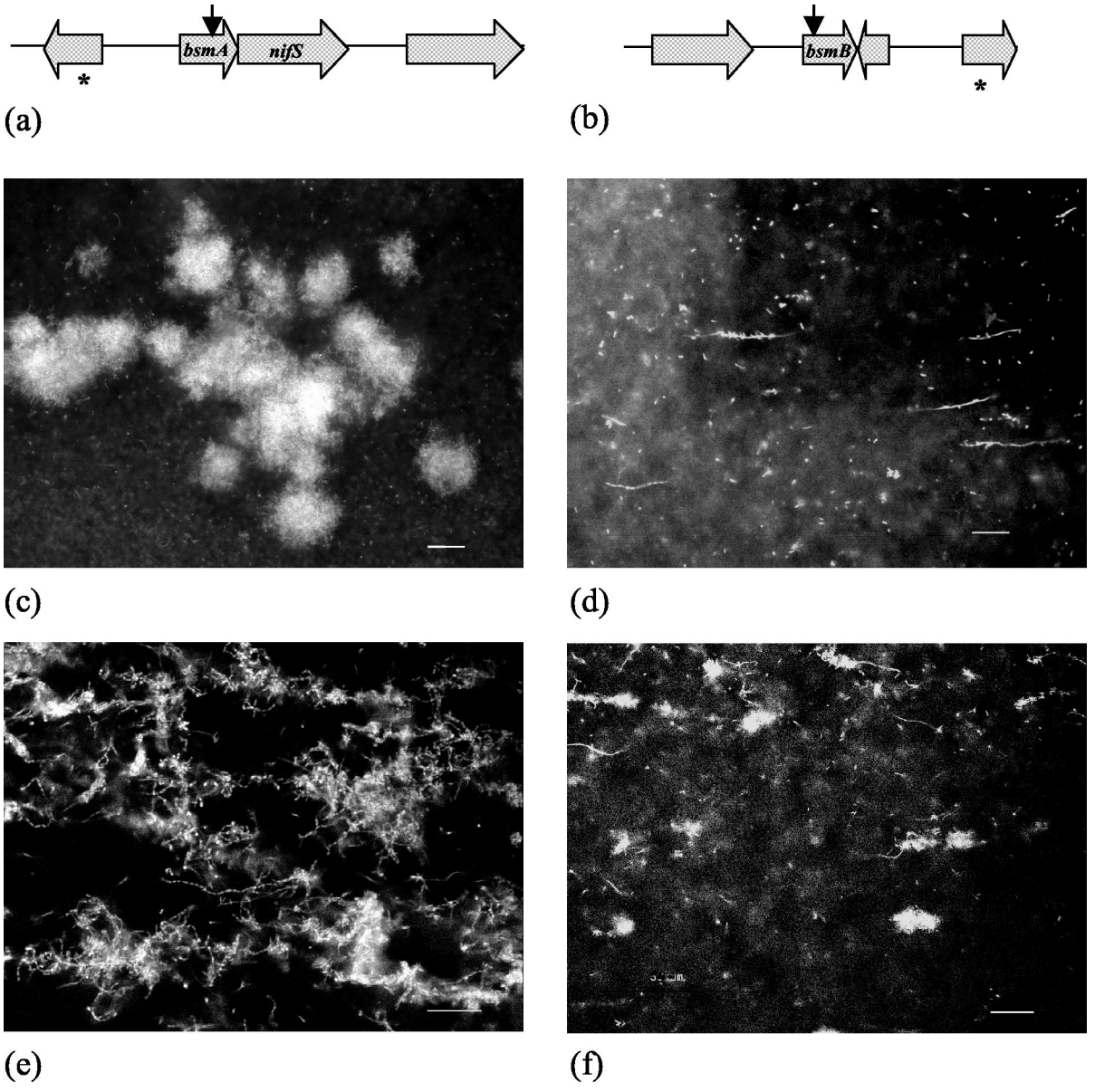



FIG. 3. (a and b) Genetic maps of the site of Tn*5* insertion and surrounding ORFs in biofilm mutant strains MG3646 (a) and MG3651 (b). The vertical arrow indicates the site of Tn*5* insertion, and the asterisk indicates an incomplete ORF. (c to f) Confocal *xy* images of 72-h flow chamber biofilms formed by *S. liquefaciens* MG3646 (c), *S. liquefaciens* MG3651 (d), *S. liquefaciens* MG3646(pBsmA) (e), and *S. liquefaciens*(pBsmB) (f). Bars = 20 μm.

fold at the transition from the logarithmic phase to the stationary phase in the presence of $C_4$-HSL.

**Biofilm formation by MG3646 and MG3651.** Biofilms of mutants MG3646 and MG3651 were established in flow chambers to determine how each mutation affected biofilm structure. Biofilms formed by MG3646 and MG3651 in the absence of $C_4$-HSL were identical to the *swrI* mutant biofilm, with no cell chains and few aggregates (data not shown). However, in the presence of $C_4$-HSL, the MG3646 mutant displayed a biofilm phenotype different from that of the *swrI* mutant and that of the wild type (Fig. 3c). This indicated that advancement of a biofilm developmental process had occurred through addition of $C_4$-HSL, which was attributed to expression of other AHL-controlled biofilm genes, but was incomplete due to the transposon mutations. In the presence of $C_4$-HSL the *bsmA* mutant formed a biofilm with large aggregates (Fig. 3c). Closer inspection revealed that aggregation with the filamentous cells was uncontrolled, which resulted in the formation of large aggregates. In contrast, the *bsmB* mutant biofilm resembled the biofilm of the *swrI* mutant and displayed little or no aggregation with the filamentous cells (Fig. 3d).

**Complementation of MG3646 and MG3651.** Biofilms formed by MG3646(pBsmA) in the presence of 250 nM $C_4$-HSL had the wild-type biofilm structure and contained filamentous cells, cell chains, and aggregates, although there were some differences (Fig. 3e). Large aggregates and gross adhesion to fila-

mentous cells and cell chains by vegetative bacteria were observed. Biofilms formed by MG3646 bearing the *bsmA-nifS* operon in *trans* formed biofilms identical to that of MG3646 bearing pBsmA, suggesting that *nifS* plays no role in biofilm morphology (data not shown). Biofilms formed by MG3651 (pBsmB) in the presence of 250 nM $C_4$-HSL exhibited aggregation with the filamentous cells, although formation of intertwining cell chains was not observed (Fig. 3f).

## DISCUSSION

Biofilm development has been the focus of intense interest recently, and the ultimate goal is to develop methods for biofilm prevention, control, or eradication. In cases where AHL signaling has been found to be important for biofilm formation, the biofilms consist of mushroom-like microcolonies separated by water channels (5, 13, 18, 26). Until now, there has been no evidence for AHL regulation of cellular differentiation in biofilm development, and only one study has linked an AHL-regulated determinant to biofilm architecture (26). In *S. liquefaciens* MG1, surface colonization through swarming motility has previously been shown to require a $C_4$-HSL-regulated peptide synthetase that directs the formation of a surfactant (7, 17). Similarly, in this study we demonstrated that colonization through biofilm formation is also dependent on the $C_4$-HSL regulatory system, and we identified specific genes in the QS regulon necessary for biofilm formation.

In this study we found that biofilms of *S. liquefaciens* MG1 consist of different morphotypes, including differentiated filamentous cells and cell chains. This is in contrast to other previously well-described biofilms of microorganisms such as *P. aeruginosa* and *E. coli*, whose biofilms consist of undifferentiated cells packed together into microcolonies (19, 20, 23). Previous observations of filamentous cells or cell chains have been reported; however, the regulation or form of cellular differentiation has not been addressed previously (4, 16). Given the change in *S. liquefaciens* MG1 biofilm architecture that leads to a mature biofilm over 72 h, it is proposed that biofilm development follows a genetically encoded program. This is clearly demonstrated by the differentiation of specific cells into filamentous cells and cell chains. The filament formation process does not occur when *S. liquefaciens* MG1 is grown in liquid culture, leading to the conclusion that this process is a surface-based phenomenon (data not shown).

Furthermore, in this study we demonstrated that the production of $C_4$-HSL by *S. liquefaciens* MG1 is crucial for the formation of a normal biofilm. Statistical analysis by COMSTAT supported the observation that C4-HSL is necessary for formation of a thick biofilm. Importantly, the *swrI* mutant biofilm exhibited wild-type levels for all COMSTAT parameters tested after addition of the signal. Clearly, $C_4$-HSL is necessary to drive the biofilm through its developmental program. Because COMSTAT was specifically designed for analysis of microcolony-like biofilms, not all of the parameters in the COMSTAT program are applicable for analysis of the *S. liquefaciens* MG1 biofilm. As a result, to distinguish specific differences between the wild-type and *swrI* mutant biofilms, we also relied on careful microscopic observation. Microscopic inspection of the *swrI* mutant biofilm showed that although this biofilm contained filamentous cells at 24 h, very little aggregation of the filamentous cells with undifferentiated rod-shaped cells had occurred at 48 h, and no cell chains were apparent at 72 h. It can be inferred that $C_4$-HSL is required for aggregation with filamentous cells and population differentiation into cell chains. We propose that aggregation is an important developmental step for cell chain differentiation, possibly by providing a framework for cell chain support. To support the significance of the QS system in biofilm formation, attempts were made to mutate *swrR.* However, all efforts to generate an *swrR* mutant have failed, suggesting that such a mutation is lethal.

Two transposon mutants bearing insertions in a $C_4$-HSL-regulated gene (the genes which were mutated were *bsmA* and *bsmB*) were identified as poor biofilm formers. Biofilms formed by both the *bsmA* and *bsmB* mutants in the flow chamber contained no cell chains and had abnormal aggregate formation. The excess aggregation by the *bsmA* mutant and no aggregation by the *bsmB* mutant suggest that aggregation is a highly regulated process, with a gene product necessary for activating aggregation (i.e., BsmB) and a gene product necessary to control the size of aggregation (i.e., BsmA). Similarly, fruiting body formation in *Myxococcus xanthus* requires activators and repressors for proper structure formation (14). The involvement of both $C_4$-HSL-regulated genes in aggregation indicates that QS is involved at a specific stage of biofilm development and is crucial for continued biofilm maturation.

The BsmA protein had the highest level of similarity (as assessed by BLASTP) with a hypothetical protein from *V. cholerae.* Interestingly, in *V. cholerae* the *bsmA* homologue is part of an operon encoding a NifS homologue. The primary activity of NifS-like proteins is the desulfuration of L-cysteine, yielding L-alanine and elemental sulfur (30). The elemental sulfur is then necessary for the building of iron-sulfur (Fe-S) clusters on proteins. It has previously been suggested that protein posttranslational modification in *S. liquefaciens* MG1 may be $C_4$-HSL regulated (9). On a 2D-PAGE gel, the isoelectric points of four proteins from the *swrI* mtuant were reported to shift in response to addition of $C_4$-HSL (9). It is possible that the formation of NifS-mediated Fe-S clusters on these four proteins may affect their isoelectric points, resulting in a shift in their positions on a 2D-PAGE gel.

To ensure that the effects on biofilm formation of the transposon insertions of *bsmA* and *bsmB* were not polar effects, we complemented the corresponding strains with *bsmA* and *bsmB.* Biofilms formed by MG3646(pBsmA) had the wild-type biofilm structure, but there were some differences. While filamentous cells, cell chains, and aggregates were present in the MG3646(pBsmA) biofilm, the aggregates were larger than those produced in the wild-type biofilm and the cell chains and filamentous cells were heavily coated with vegetative bacteria, suggesting that the product of *bsmA* is probably an adhesin. The excess adhesion and aggregation seen in the MG3646 (pBsmA) biofilm were probably a result of overexpression through the multicopy plasmid used for complementation. As mentioned above, the *bsmA* gene product appears to control bacterial aggregation with the filamentous cells. If *bsmA* codes for an adhesion, it is currently unclear how the proposed adhesin mediates the control of bacterial aggregation. However, since MG3646 bearing plasmid pBsmAOp formed a biofilm identical to that formed by MG3646(pBsmA), we concluded that the altered biofilm seen as a result of the transposon

mutation in *bsmA* was due to the disruption of *bsmA* and not to a polar effect on the downstream *nifS*-like gene.

Later stages in biofilms formed by MG3651(pBsmB) did not have the full wild-type biofilm structure. As stated above, the *bsmB* gene is likely to encode a positive effector of bacterial aggregation. The MG3651 transposon mutant was unable to aggregate unless *bsmB* was supplied in *trans*, which restored aggregation. However, biofilm development was not able to progress to formation of the characteristic intertwining cell chains, presumably because the plasmid was a multicopy plasmid. Because *bsmB* is highly dependent on $C_4$-HSL for induction and is induced 10-fold, other $C_4$-HSL-regulated gene expression involved in biofilm formation is diluted, and this prevents further biofilm development. Based on the ORFs surrounding *bsmB* and the ability of *bsmB* in *trans* to restore the mutant biofilm partially, it is highly unlikely that a polar effect on a gene downstream of *bsmB* is responsible for the biofilm deficiency of MG3651. This is particularly evident since a putative gene 53 bp downstream from *bsmB* is present in the orientation opposite that of *bsmB*, indicating that *bsmB* is not in an operon and is expressed monocistronically.

In this study, we found that *S. liquefaciens* MG1 forms has a novel differentiated biofilm structure and that biofilm development is dependent on $C_4$-HSL. We also identified two AHL-regulated genes important for normal biofilm formation. Current work in our laboratory is aimed at determining whether there are other $C_4$-HSL-controlled genetic determinants for normal biofilm development. Furthermore, studies are being directed at understanding the functions inherent to the highly differentiated biofilm formed by *S. liquefaciens* MG1. This includes the expression of virulence traits, the role of the AHL-controlled colonization genes in attachment, and the resistance to predation offered by the elongated cells at the biofilm surface. Previous work has demonstrated that *S. liquefaciens* MG1 cells longer than 15 μm are resistant to grazing by protozoans, suggesting that filamentous cells and cell chains are likely to be a defense mechanism against protozoan predation in the environment (1).

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