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Assessment of *flhDC* mRNA Levels in *Serratia liquefaciens* Swarm Cells

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We reported previously that artificial overexpression of the *flhDC* operon in liquid-grown *Serratia liquefaciens* resulted in the formation of filamentous, multinucleated, and hyperflagellated cells that were indistinguishable from surface-induced swarm cells (L. Eberl, G. Christiansen, S. Molin, and M. Givskov, J. Bacteriol. 178:554–559, 1996). In the present report we show by means of reporter gene measurements, Northern analysis, and in situ reverse transcription-PCR that the amount of *flhDC* mRNA in surface-grown swarm cells does not exceed the maximum level found in nondifferentiated, vegetative cells. This suggests that surface-induced *S. liquefaciens* swarm cell differentiation, although dependent on *flhDC* gene expression, does not occur through elevated *flhDC* mRNA levels.

Swarming motility is an intrinsically linked surface and cell density phenomenon. Surface exposure leads to a differentiation process that transforms cells into multinucleate, aseptate, and profusely flagellated swarm cells that are highly elongated (see reference 16 for a review). The cells of a swarming colony have the ability to migrate coordinately in rafts away from the center of the colony. The combined action of cells involved in motility and cells involved in cell division and growth results in colony expansion and rapid colonization of all of the available surface. In the uropathogen *Proteus mirabilis* it has been demonstrated that swarming behavior is closely associated with modulation of virulence characteristics and the ability to invade human urothelial cells (1–3). In *Vibrio parahaemolyticus* differentiation into swarm cells plays an important role in adsorption to and colonization of chitinaceous shells of crustaceans (4). For the opportunistic pathogen *Serratia liquefaciens*, evidence that expression of the phospholipase gene (*phlA*), which encodes a potential virulence determinant, is differentially expressed in swarm cells has been presented (6). In *S. liquefaciens* MG1, the swarming phenomenon is linked to high cell density by means of a quorum-sensing mechanism (7, 14). Quorum sensing controls production of the extracellular biosurfactant serrawettin W2, which is required for swarm cells to travel atop the agar surface and which enables swarm colony expansion (21).

The *flhDC* operon appears to play a crucial role in the swarm cell differentiation process. In both *S. liquefaciens* and *P. mirabilis* artificial and prolonged overexpression of the *flhDC* operon dramatically enhances cell elongation and causes increased flagellation (6, 10). Moreover, for *P. mirabilis* it has been demonstrated (by Northern analysis) that the amount of *flhDC* mRNA is increased more than 30-fold in swarm cells compared to the amount found in vegetative cells (10). Previously it was shown that *S. liquefaciens* swarm cells carry more flagella and express higher levels of phospholipase (PhlA) than their vegetative counterparts (6). Since artificial overexpression of *flhDC* in *S. liquefaciens* also leads to cell elongation and increased phospholipase and flagellar expression (6, 13), the apparent synchronization of swarm cell differentiation with *phlA* and flagellum expression would be most readily explained by assuming that, as in *P. mirabilis*, the level of *flhDC* mRNA is specifically increased in swarm cells. However, here we present evidence that the level of *flhDC* mRNA in *S. liquefaciens* swarm cells is within the range of levels found in vegetative cells, which suggests that surface-induced *S. liquefaciens* swarm cell differentiation, although dependent on *flhDC* gene expression, does not occur through elevated *flhDC* mRNA levels.

MATERIALS AND METHODS

Strains and plasmids. The following isogenic *S. liquefaciens* strains were used: MG1, the wild type (12); MG3 (*flhD*), in which the *flhDC* operon is inactivated by insertion of a 1.5-kbp DNA fragment carrying the streptomycin resistance marker (13); and TAK69 (*flhD*::*luxAB*), which carries a *luxAB* transposon (18) insertion in the *flhD* gene localized 120 bases from the translation start site. Plasmid pMG600 carries a P_{tac}-*flhDC* transcriptional fusion and *lacI* so that expression of the *flhDC* operon can be controlled by IPTG (isopropyl-β-D-thiogalactopyranoside) addition to the medium (13). pMG600 confers kanamycin resistance to the host. Plasmid pAC33 is a derivative of pMG600 that carries the *aacC1* gene which confers gentamicin resistance to the host.

Medium and growth conditions. Luria-Bertani (LB) medium (5) was used throughout. LB medium solidified with 0.6% agar was used as the medium for swarming bacteria. In the experiments involving cells harboring plasmids, kanamycin at a concentration of 20 μg/ml and gentamicin at 15 μg/ml was used to select for plasmid maintenance. IPTG was supplied to the growth medium as indicated below. The temperature of all incubations was 30°C.

Measurements of bioluminescence. Bioluminescence was quantified in a Bio-Orbit 1253 luminometer by measuring 1-ml samples of cultures appropriately diluted to give a linear response. One microliter of *n*-decanal was mixed with each sample prior to measuring. Luminescence is reported in specific light units, which are calculated as relative light units per second per unit of optical density at 450 nm (OD_{450}).

Electrophoresis and immunoblotting. Cells were harvested and resuspended to an OD_{450} of 1.0. The proteins were heat denatured in sodium dodecyl sulfate (SDS)-containing sample buffer and separated by a standard SDS-polyacrylamide gel electrophoresis procedure (19). The proteins were transferred to an Immobilon-P membrane (Millipore) by means of a semidry blotting apparatus and subjected to immunoblotting analysis (Western blotting) using rabbit antibodies directed against *S. liquefaciens* flagellar protein followed by visualization by the binding of secondary alkaline phosphatase-labeled anti-rabbit immunoglobulin G using *p*-nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate.

Oligonucleotides. Two sets of primers for seminested PCR amplification of the *S. liquefaciens phlA* and *flhDC* genes were designed based on the published sequences (11, 13): PhlA902F (forward primer targeting nucleotide positions 902 to 922), 5′-CCGGTGGCCTCTCCCTCTCAG-3′; PhlA1056i (internal forward

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primer targeting nucleotide positions 1056 to 1079), 5′-TGGCCAAGGACGTT TACTCACTCA-3′; PhlA1629R (reverse primer targeting nucleotide positions 1629 to 1606), 5′-ATGCCGGTCAGGGTATCGTTGTTG-3′; FlhDC295F (forward primer targeting nucleotide positions 295 to 319), 5′-CGATGTTCCGC CTTGGTATTGATGA-3′; FlhDC529i (internal forward primer targeting nucleotide positions 529 to 552), 5′-GCCCGACGAAGAAAAGAGCCTGAT-3′; FlhDC1052R (reverse primer targeting nucleotide positions 1052 to 1036), 5′-CGCGGGATGGGGGTTGG-3′. The two primers PhlA1056i and FlhDC529i were labeled with biotin in the 5′ end during automated synthesis and were subsequently purified by reverse-phase high-performance liquid chromatography. All primers were tested by PCR with *S. liquefaciens* MG1 DNA as the template and were found to give PCR products of the expected sizes.

Northern analysis. The PCR product generated by use of the FlhDC295F and FlhDC1052R primers, with *S. liquefaciens* MG1 DNA as the template, was used as a probe in Northern analysis to target the *flhDC* mRNA molecules. Preparation of total RNA from 1-ml cell suspensions (OD_{450} = 1), preparation of [α-^{32}P]dCTP-labeled probe, Northern blotting, and quantification of radioactive signals from specific bands were done as previously described (17).

Detection of specific mRNA in individual cells. Cell fixation, cell wall permeabilization, in situ reverse transcription-PCR (RT-PCR), and detection of intracellular PCR products were done essentially as described earlier (26). Cell wall permeabilization was done by the use of 1 mg (instead of 0.5 mg) of lysozyme/ml. Since biotin-labeled internal primers were used (instead of fluorescein-labeled internal primers), biotinylated intracellular PCR products were detected using a streptavidin-horseradish peroxidase conjugate (DuPont, NEN Research Products).

Microscopy and image processing. Microscopic examinations were done with an Axioplan microscope (Carl Zeiss, Oberkochen, Germany), using a Ph3 Plan-NEOFLUAR 63×/1.25 oil objective (Carl Zeiss). Digital images were captured with a slow-scan charge-coupled device CH250 camera (Photometrics, Tucson, Ariz.) mounted on the microscope. The exposure time was 25 ms for phase-contrast micrographs and 1,000 ms for epifluorescence micrographs. Captured digital images were processed with PMIS software, and cell size distributions were obtained by the use of an image analysis program written in MATLAB, version 5.1 (MathWorks Inc., Natick, Mass.).

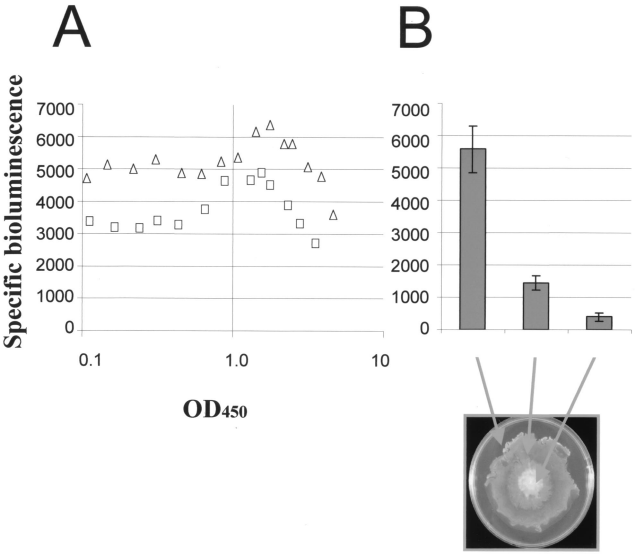

FIG. 1. Expression of bioluminescence from a chromosomal *flhD*::*luxAB* transcriptional fusion in strain TAK69. (A) Samples of TAK69 (squares) and TAK69 carrying plasmid pAC33 (p_{tac}-*flhDC*) (triangles) were taken from liquid cultures throughout the growth cycle; (B) samples taken from a swarming colony of TAK69 carrying plasmid pAC33 (mean values of eight individual samples with error bars indicating deviations). All media were supplemented with 20 μM IPTG.

RESULTS AND DISCUSSION

In order to test the hypothesis that *flhDC* expression is specifically increased in *S. liquefaciens* swarm cells, we employed the reporter strain TAK69, which carries a chromosomal insertion of the promoterless *luxAB* genes in *flhD* (see Materials and Methods for details). When monitored through the growth cycle, the transcriptional activity of the *flhD-luxAB* fusion was found to be constitutive during the log phase of growth. As the growth rate decreased, the transcriptional activity gradually increased until it reached its maximum value at a cell density of approximately 1.5 OD_{450} units. Following this, activity declined during the entry into stationary phase (Fig. 1A). Due to the *luxAB* insertion in *flhD* the TAK69 strain is unable to synthesize flagella and unable to swim and swarm. However, IPTG-induced expression of a P_{tac}-*flhDC* fusion carried on the plasmid pAC33 complemented the inactivated chromosomal *flhDC* operon. As judged from the expansion rate of swarming colonies, the presence of 20 μM IPTG restored the swarming motility of TAK69/pAC33 to the level of the MG1 wild-type strain (not shown). Microscopic inspection of the cells present in the outer swirling layer of the swarming colony revealed the presence of elongated cells similar to those from a wild-type colony (data not shown). In liquid culture, induction of the *flhDC* operon with 20 μM IPTG did not affect growth, nor did the bacteria differentiate into swarm cells (data not shown). Transcriptional activity of the chromosomal *flhD-luxAB* fusion was slightly upregulated (1.3-fold) in the presence of the IPTG-induced *flhDC* operon (Fig. 1A). However, the transcriptional activity of the *flhD-luxAB* fusion followed the same pattern in the presence or absence of the plasmid-borne IPTG-induced P_{tac}-*flhDC* fusion, demonstrating that the *flhDC* operon of *S. liquefaciens* is not subject to autogenous control. The finding that swarm cell differentiation takes place in surface-grown cells with constitutive P_{tac}-driven *flhDC* expression but apparently does not take place in liquid-grown cells with similar induction of the P_{tac}-*flhDC* fusion is not in agreement with the hypothesis that swarm cells have increased levels of *flhDC* transcription. Furthermore, expression from the *flhDC* promoter was not specifically increased in the surface-grown cells relative to that in liquid-grown cells (Fig. 1B).

Complementation of the inactivated chromosomal *flhDC* operon in the reporter strain with a plasmid-borne IPTG-inducible *flhDC* operon is an artificial system, which might give rise to artifacts. We therefore analyzed the content of *flhDC* mRNA in *S. liquefaciens* MG1 (wild type). RNA extracted from cells isolated from the outer layer of a swarming colony, or from a liquid culture at the mid-log (OD_{450} = 0.4) and late log (OD_{450} = 1.2) phases was subjected to *flhDC* mRNA Northern analysis. In concurrence with the results obtained with the reporter strain, Fig. 2 shows that the late-log-phase cells and the cells isolated from the edge of a swarming colony had similar *flhDC* mRNA levels. The RNA in each lane was isolated from samples normalized to identical OD_{450} values (as described in Materials and Methods). As shown in Fig. 2B this normalization resulted in samples containing similar amounts of rRNA. The finding that *P. mirabilis* swarm cells have an *flhDC* mRNA content more than 30-fold higher than that of vegetative cells was also based on a Northern analysis of samples with the same amounts of rRNA (10). Our results therefore strongly suggest that surface-induced swarm cells of *S. liquefaciens* differ considerably from those of *P. mirabilis* with respect to *flhDC* transcription.

The original hypothesis that *S. liquefaciens* swarm cells have an increased level of *flhDC* mRNA was put forward because artificial overexpression of *flhDC* leads to elongation of liquid-grown cells so that they resemble swarm cells (6). Using strain MG3 and plasmid pMG600, which are similar to strain TAK69 and plasmid pAC33 (see Materials and Methods), it was shown that, for liquid-grown cells, induction with 100 μM IPTG caused a slight cell elongation, while induction with 1 mM IPTG was required in order for the liquid-grown cells to differentiate into fully elongated cells resembling swarm cells (6).

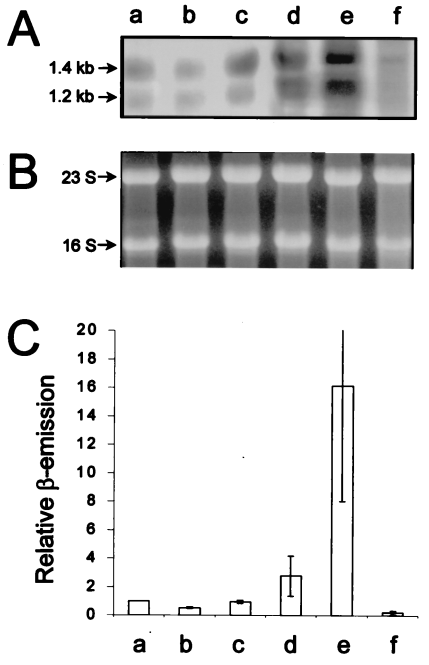

FIG. 2. Northern analysis of *flhDC* mRNA in *S. liquefaciens* (performed as described in Materials and Methods). (A) The Northern blot contains RNA from MG1 cells isolated from the edge of a swarming colony (lane a), liquid-grown MG1 mid-log-phase (OD_{450} = 0.4) cells (lane b), liquid-grown MG1 late-log-phase (OD_{450} = 1.2) cells (lane c), liquid-grown MG3/pMG600 mid-log-phase cells sampled 20 min after addition of 50 μM IPTG (lane d), liquid-grown MG3/pMG600 mid-log-phase cells sampled 20 min after addition of 200 μM IPTG (lane e), and liquid-grown MG3/pMG600 mid-log-phase cells grown in the absence of IPTG (lane f). The sizes of the two transcripts are indicated at the left. (B) Photograph of the ethidium-stained gel used for Northern blotting. 16S and 23S rRNA bands are indicated. (C) Radioactive signals emitted from specific bands and quantified by the use of an electronic instant-imager device. The relative values are averages of two independent Northern blots. Bars, standard deviations.

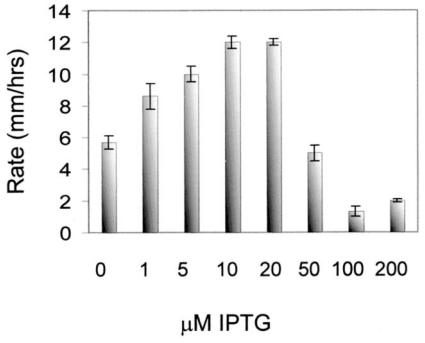

FIG. 3. Expansion rates of MG3/pMG600 colonies on 0.6% agar medium supplemented with different IPTG concentrations.

In order to assess the amount of *flhDC* mRNA that causes liquid-grown cells to differentiate, samples for Northern analysis were taken from an exponential-phase MG3/pMG600 culture at OD_{450} = 0.3, 20 min after the culture had received IPTG to a final concentration of 50 or 200 μM. As shown in Fig. 2, the level of *flhDC* mRNA in cells grown in liquid culture in the presence of 50 μM IPTG was approximately 3-fold higher than the level found in the wild-type swarm cells, while the amount of *flhDC* mRNA in the cells induced with 200 μM IPTG was approximately 16-fold higher than the level in the wild-type swarm cells. These results suggest that differentiation of liquid-grown cells occurs only in response to artificially high *flhDC* mRNA levels, which are not found in surface-grown swarm cells. Figure 3 shows that the swarming motility of MG3/pMG600 is inhibited at IPTG concentrations above 50 μM, indicating that high expression of *flhDC* is not compatible with swarming motility on agar surfaces.

As shown in Fig. 2, *flhDC* transcripts of two different sizes (approximately 1.4 and 1.2 kb) were detected in the Northern analysis. For lanes a to c the *flhDC* mRNA is transcribed in the wild-type strain, while for lanes d to f the level of *flhDC* mRNA is controlled from the IPTG-inducible P_{tac} promoter in the MG3/pMG600 strain. Since the intensities of both bands are increased by IPTG induction in strain MG3/pMG600, we believe that the smaller transcript (1.2 kb) originates from post-transcriptional processing of the larger transcript (1.4 kb).

The presence of differentiated and nondifferentiated cells in the outer region of a swarming *S. liquefaciens* colony (see below) raises the question of whether the determined levels of *flhDC* transcription (Fig. 1) and *flhDC* mRNA content (Fig. 2) represent an average of a high level in the swarm cells and a low level in the nondifferentiated cells. However, since swarm cells can be distinguished from nondifferentiated cells by their morphology, the problems associated with population-level analysis can be overcome by monitoring gene expression in individual cells. Using *Salmonella enterica* serovar Typhimurium as a model organism, we have previously demonstrated the ability of in situ RT-PCR to monitor the presence or absence of specific mRNA in individual cells (26, 27) and have shown that this method can be used to monitor different levels of specific mRNA in individual bacterial cells semiquantitatively (17). In order to monitor *flhDC* mRNA levels in individual *S. liquefaciens* bacteria, MG1 cells sampled from the outermost layer of a swarming colony and from a mid-log-phase (OD_{450} = 0.4) culture were subjected to *flhDC* mRNA-targeted in situ RT-PCR. The in situ RT-PCR was carried out with reporter molecule-labeled primers, and subsequently intracellular reporter molecules were detected by the use of a fluorogenic assay. As shown in Fig. 4A and B, the swarm cells and vegetative cells did not exhibit significant differences in fluorescence intensity after *flhDC* mRNA-targeted in situ RT-PCR, suggesting that the swarm cells contained about the same amount of *flhDC* mRNA as the vegetative cells. To exclude the possibility that in situ RT-PCR is inhibited in the swarm cells, we performed in situ RT-PCR targeting the *phlA* mRNA. Expression of the phospholipase, determined as transcription of a *phlA-lacZ* fusion, was previously shown to be specifically increased in swarm cells (13), and, as shown in Fig. 4C, this was also visualized using in situ RT-PCR.

The cell populations taken from the growing culture or from the edge of a swarming colony were heterogeneous with respect to the intensity of the fluorescence emitted from the cells after *flhDC* mRNA-targeted in situ RT-PCR (Fig. 4A and B). This could mean that the *flhDC* mRNA levels in the cells were at the limits of detection. However, in general the dividing cells and the small daughter cells emitted little fluorescence, while the nondividing (growing) cells emitted more fluorescence. This pattern of fluorescence heterogeneity indicates cell cycle-regulated expression of the *flhDC* operon, with lowest levels in dividing and newborn cells, which is in concurrence with the results of Prüss and Matsumura (25) demonstrating that expression of the *flhDC* operon in liquid-grown *Escherichia coli* cells peaks in the middle of the cell cycle and is lowest at the time of cell division. Nevertheless, we found it worthwhile to ensure that different levels of *flhDC* mRNA could be visualized

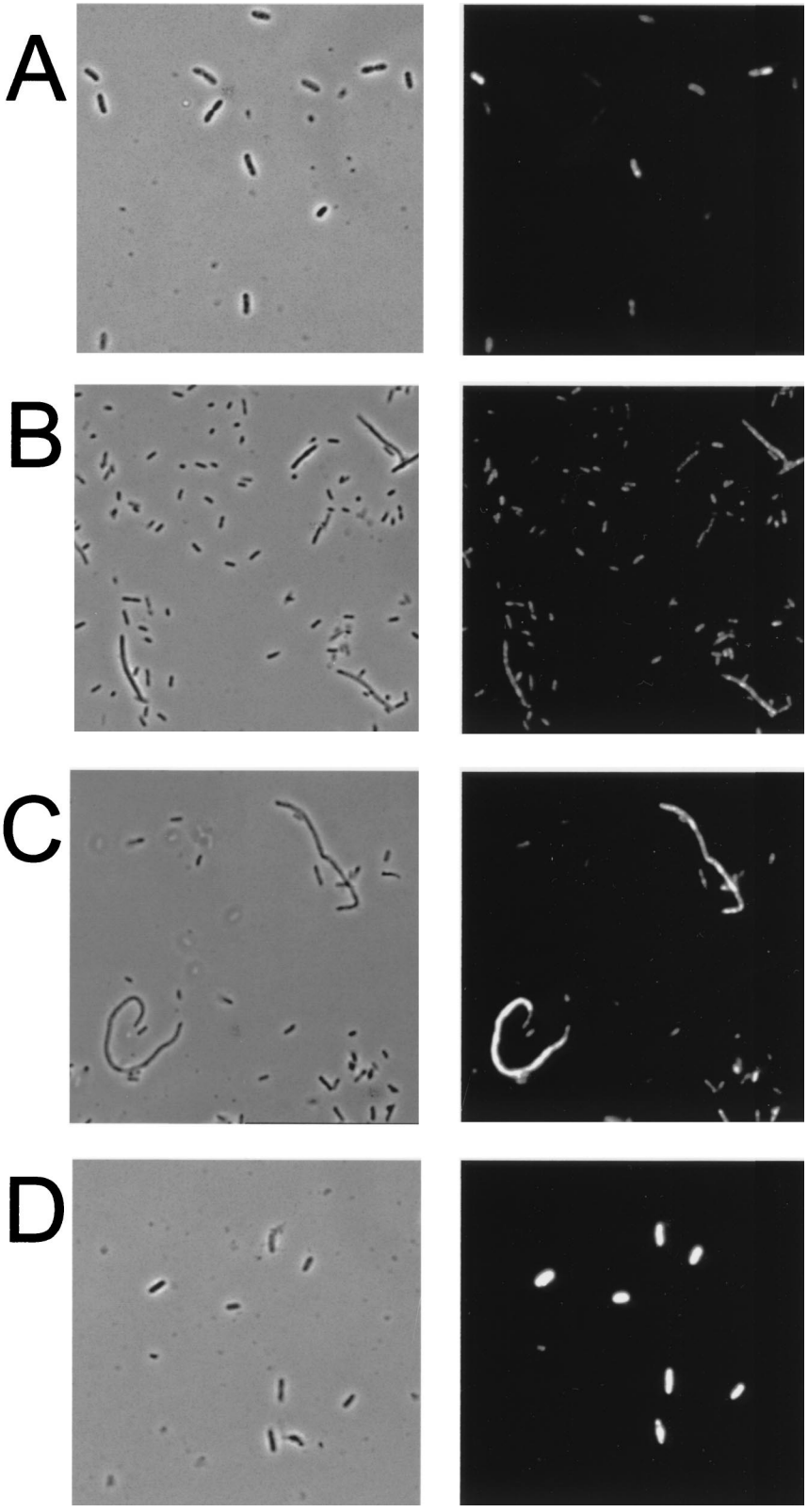

FIG. 4. In situ RT-PCR targeting *flhDC* mRNA (A, B, and D) or *plhA* mRNA (C) in *S. liquefaciens*. (A) Liquid-grown MG1 mid-log-phase (OD_{450} = 0.4) cells. (B and C) MG1 cells isolated from the edge of a swarming colony. (D) Liquid-grown MG3/pMG600 mid-log-phase cells sampled 20 min after addition of 200 μM IPTG. Phase-contrast photomicrographs (left) and epifluorescence photomicrographs (right) of the same viewing fields are shown.

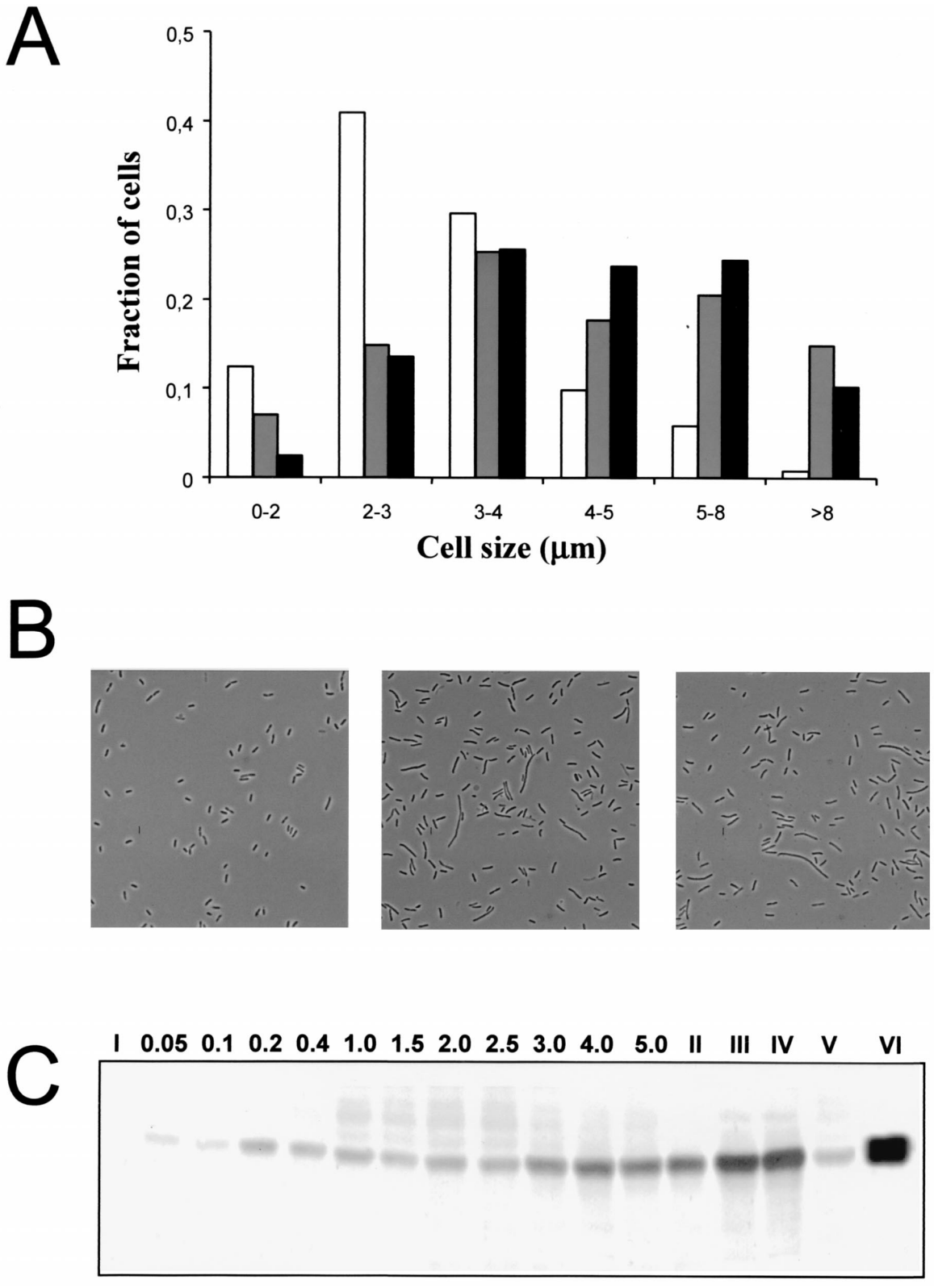

FIG. 5. Cell size and flagellar content in liquid cultures and a population isolated from the edge of a swarming colony. (A) Cell size distributions of an *S. liquefaciens* MG1 population from a mid-log-phase (OD_{450} = 0.4) culture (white bars), an *S. liquefaciens* MG1 population from the edge of a swarming colony (gray bars), and an *S. liquefaciens* MG3/pMG600 population from an IPTG-induced (1 mM) mid-log-phase culture (black bars). (B) Micrographs of MG1 cells from a mid-log-phase culture (left), MG1 cells from the edge of a swarming colony (middle), and MG3/pMG600 cells from an IPTG-induced (1 mM) mid-log-phase culture (right). (C) Western blot with flagellum-specific antibodies. Lane I, MG3 (*flhD*); lanes II, III, and IV, samples from the edge of a swarming MG1 colony loaded 3-fold diluted, 1.5-fold diluted, and undiluted, respectively; lane V, sample from the center of a swarming MG1 colony; lane VI, sample from an IPTG-induced (1 mM) MG3/pMG600 mid-log-phase culture; lanes 0.05 to 5.0, samples from an exponentially growing MG1 culture at OD_{450} values equal to the lane numbers.

using the in situ RT-PCR method. Furness et al. (10) reported that *P. mirabilis* swarm cells contained at least 30-fold more *flhDC* mRNA than vegetative cells. Since the Northern analysis indicated that liquid-grown *S. liquefaciens* MG3/pMG600 cells induced with 200 μM IPTG contain approximately 16 times more *flhDC* mRNA than wild-type vegetative cells (Fig. 2, lanes a and e), we could determine whether such differences in *flhDC* mRNA levels could be detected by use of the in situ RT-PCR method. After being subjected to *flhDC* mRNA-targeted in situ RT-PCR, liquid-grown *S. liquefaciens* MG3/pMG600 cells induced with 200 μM IPTG did indeed fluoresce much more than wild-type vegetative and swarm cells (Fig. 4A, B, and D).

It was of interest to investigate the extent of flagellation of *S. liquefaciens* swarm and swim cells. Cell samples taken from the outer region of a swarming *S. liquefaciens* colony contain a mixture of differentiated swarm cells and nondifferentiated cells as shown in Fig. 5. This renders collection of pure populations of *S. liquefaciens* swarm cells difficult. However, approximately 65% of the cells isolated from the edge of a swarming colony consisted of elongated cells, and Western analysis demonstrated that the average content of flagella per cell mass (OD_{450}) in this population was approximately threefold higher than the content of liquid-grown cells in the late log phase (Fig. 5). We also investigated liquid-grown *S. liquefaciens* MG3/pMG600 cells induced with different IPTG concentrations (0, 10, 25, 50, 100, 250, 500, and 1,000 μM). The cell size distribution and the content of flagella per cell mass were determined in samples taken from these cultures, which were all grown from an OD_{450} of 0.02 to 0.3 (data not shown). The cell size analysis supported our previous report (6) that liquid-grown *S. liquefaciens* MG3/pMG600 cells induced with 1 mM IPTG closely resemble wild-type *S. liquefaciens* swarm cells isolated from the edge of a swarming colony (Fig. 5A and B). However, the Western analysis suggested that the population of liquid-grown *S. liquefaciens* MG3/pMG600 cells induced with 1 mM IPTG had approximately twice the amount of flagella found on the wild-type swarm cell population (Fig. 5C).

Taken together the results presented here demonstrate that surface-induced swarm cell differentiation in *S. liquefaciens* is not accompanied by a substantial increase in *flhDC* transcription or *flhDC* mRNA content. Cells do, however, elongate, and therefore the average swarm cell carries many more flagella than a swim or vegetative cell, but the cells are, strictly speaking, not hyperflagellated. In contrast to this, *P. mirabilis* swarm cell differentiation is evidently accompanied by a 30-fold increase in *flhDC* mRNA content and a 50-fold increase in flagellation (10, 20). Our results obtained with IPTG-controlled *flhDC* expression demonstrate that a similar increase in the level of *S. liquefaciens flhDC* mRNA would switch off the swarming motility of surface-grown cultures. Since differentiation of liquid-grown *S. liquefaciens* into swarm cells requires a substantially higher *flhDC* mRNA content than that found in wild-type (surface-grown) swarm cells, it might be speculated that swarm cell differentiation on the surface occurs through posttranscriptional regulation of the *flhDC* operon. If posttranscriptional modification occurs specifically in response to stimuli present in a swarming colony (for example, surface exposure), then liquid-grown cells may differentiate only in response to artificially elevated *flhDC* mRNA contents, resulting in an increased FlhDC amount and activity that under physiological conditions is found only in swarm cells.

The ability of the cells comprising the swim and the swarm subpopulations to go through cycles of differentiation and dedifferentiation is considered a major factor determining expansion of the moving culture (8, 9). However, it must be emphasized that *Serratia* and *Proteus* display different kinds of swarming behavior. In *P. mirabilis* the swarm-swim interconversion cycles occur in a synchronized fashion, and as a result the colony either grows or expands. Unlike *Proteus*, *Serratia* secretes serrawettins, making continuous spreading of the growing culture possible (21–24). Gfp tagging and single-cell analysis were recently used to dissect a swarming *Serratia* colony (8). This analysis suggested that the behavior of the differentiated rafted *Serratia* swarm cells causes the formation and spreading of a surface-conditioning film and circulates cells between the subcultures of swarm and vegetative cells present at the border and the more central parts of the colony, respectively (8). This continuously creates new zones of growth and abolishes the formation of distinct consolidation-motility phases, as seen with *P. mirabilis*. On the other hand, *P. mirabilis* produces a capsular polypeptide that reduces surface friction and enables the differentiated cells to move on growth media with high agar content (15). *Proteus* swarms on agar concentrations from 0.5 to 2.5%, whereas *Serratia* swarming takes place in the narrow range of 0.5 to 1% agar. The difference in control of *flhDC* gene expression and cell differentiation probably relates to the obvious mechanistic and behavioral differences in swarming motility of the two related enteric bacteria *Serratia* and *Proteus*.

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