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# Differentiation of *Serratia liquefaciens* into Swarm Cells Is Controlled by the Expression of the *flhD* Master Operon

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**The velocity with which a swarming colony of *Serratia liquefaciens* colonizes the surface of a suitable solid substratum was controlled by modulating the expression of the *flhD* master operon. In liquid medium, the stimulation of *flhD* expression resulted in filamentous, multinucleate, and hyperflagellated cells that were indistinguishable from swarm cells isolated from the edge of a swarm colony. Thus, expression of the *flhD* master operon appears to play a central role in the process of swarm cell differentiation.**

A liquid culture of *Serratia liquefaciens* induces the synthesis of both extracellular hydrolytic enzymes and flagella in the late logarithmic phase of growth (10, 11). The synthesis of at least one extracellular enzyme, the phospholipase, is genetically coupled to the synthesis of flagella. Control is governed by the *flhD* operon, which contains two genes, *flhC* and *flhD*. This operon constitutes a master regulatory system that controls the entire flagellar regulon in *Escherichia coli* and *Salmonella typhimurium* (17). By placing the *flhD* operon under the control of a foreign inducible promoter, we previously demonstrated that stimulation of this operon results in the induction of phospholipase and flagellar synthesis in *S. liquefaciens* (10). Furthermore, the speed of cellular motility was found to directly correlate with the inducer concentration. However, we recently noticed that *S. liquefaciens* is capable of two different forms of surface translocation, swimming and swarming, depending on whether the growth medium is solid or liquid. In this report we demonstrate that both kinds of motility depend on a functional *flhD* locus and demonstrate that by stimulation of the master operon, swarm cell differentiation can be initiated in liquid culture without the otherwise obligatory requirement of surface contact.

**Swimming and swarming behavior of *S. liquefaciens*.** When grown in a rich liquid medium, cells of *S. liquefaciens* appear as 1.5- to 3.0-μm-long rods carrying 3 to 15 flagella, depending on the growth phase (10). These cells display characteristic swimming behavior which is made up of alternating periods of smooth runs and brief reversals or spins. Swimming motility is also observed in semisolid Difco agar (0.2 to 0.4%), where cells swim through the water-filled channels in the agar, producing typical chemotactic rings (Fig. 1). When *S. liquefaciens* is grown on 0.5 to 1.2% Difco agar, it exhibits a form of multicellular behavior referred to as swarming motility, a form of surface translocation that has also been observed with *Serratia marcescens* (1). *S. liquefaciens* cells inoculated onto a suitable agar medium initially grow as a typical colony until, after a certain period (depending on medium composition), they differentiate at the periphery of the colony into long, filamentous, aseptate swarm cells that rapidly move outward atop the agar surface (Fig. 1). These swarm cells often maintain close cell-to-cell contact along their long axis and migrate coordinately in groups or rafts away from the colony until they stop and undergo reductive divisions, thus reverting into the short vegetative cell form, a process termed consolidation (3, 6). Cells from both the center and the edge of a swarm colony propagated on Luria-Bertani (LB) medium (7) solidified with 0.7% agar for 12 h were washed from the agar surface and prepared for electron microscopy as described previously (10). The long (5 to 50 μm) swarm cells from the edge of the swarm colony were found to be profusely flagellated, while cells from the center were short rods with only a few flagella (data not shown). Stage-specific cells were also stained with 4,6-diamidino-2-phenylindole (DAPI) as described previously (18). Swarm cells were multinucleate with nucleoids evenly distributed along the lengths of the filaments, while cells taken from the internal regions of the colony contained only a single nucleoid (data not shown). These observations clearly add *S. liquefaciens* to a rapidly growing list of bacteria for which swarming motility has been demonstrated, including members of the genera *Proteus*, *Vibrio*, *Chromobacterium*, *Bacillus*, *Clostridium* (3, 13), and more recently *Escherichia* and *Salmonella* (14).

**Factors which influence swarming.** Rich medium such as LB medium, brain heart infusion (Difco), or nutrient broth (Difco) generally supported swarming motility. Swarming was also observed with AB minimal medium (8) supplemented with either 1% tryptone, 0.5% yeast extract, or 1% peptone. AB minimal medium supplemented with either 0.4% glucose, 0.4% fructose, 0.4% sucrose, 1% glycerol, 0.4% succinate, 0.4% gluconate, or 10 mM citrate was unable to promote swarming migration. However, the addition of Casamino Acids at concentrations as low as 0.065% to AB medium in combination with any of the mentioned carbon sources restored the swarming response. Noteworthy is the fact that AB minimal medium supplemented with 0.065% Casamino Acids alone did not support swarming motility. The addition of a mixture of all 20 naturally occurring amino acids (1 mM each) to AB medium containing either 0.4% glucose or 1% glycerol as well as the addition of Casamino Acids induced a swarming response. To investigate whether a single amino acid could serve as a specific inducer for swarm cell differentiation, as has been demonstrated for glutamine in the case of *Proteus mirabilis* (4), we tested all 20 amino acids individually. However, none of the individual amino acids promoted swarming migration. Since the doubling time of *S. liquefaciens* grown in liquid AB minimal medium supplemented with 0.4% glucose was significantly increased by the addition of even low amounts of Casamino Acids (37 min with 0.065% Casamino Acids added versus 66 min without Casamino Acids), we speculate that the require-

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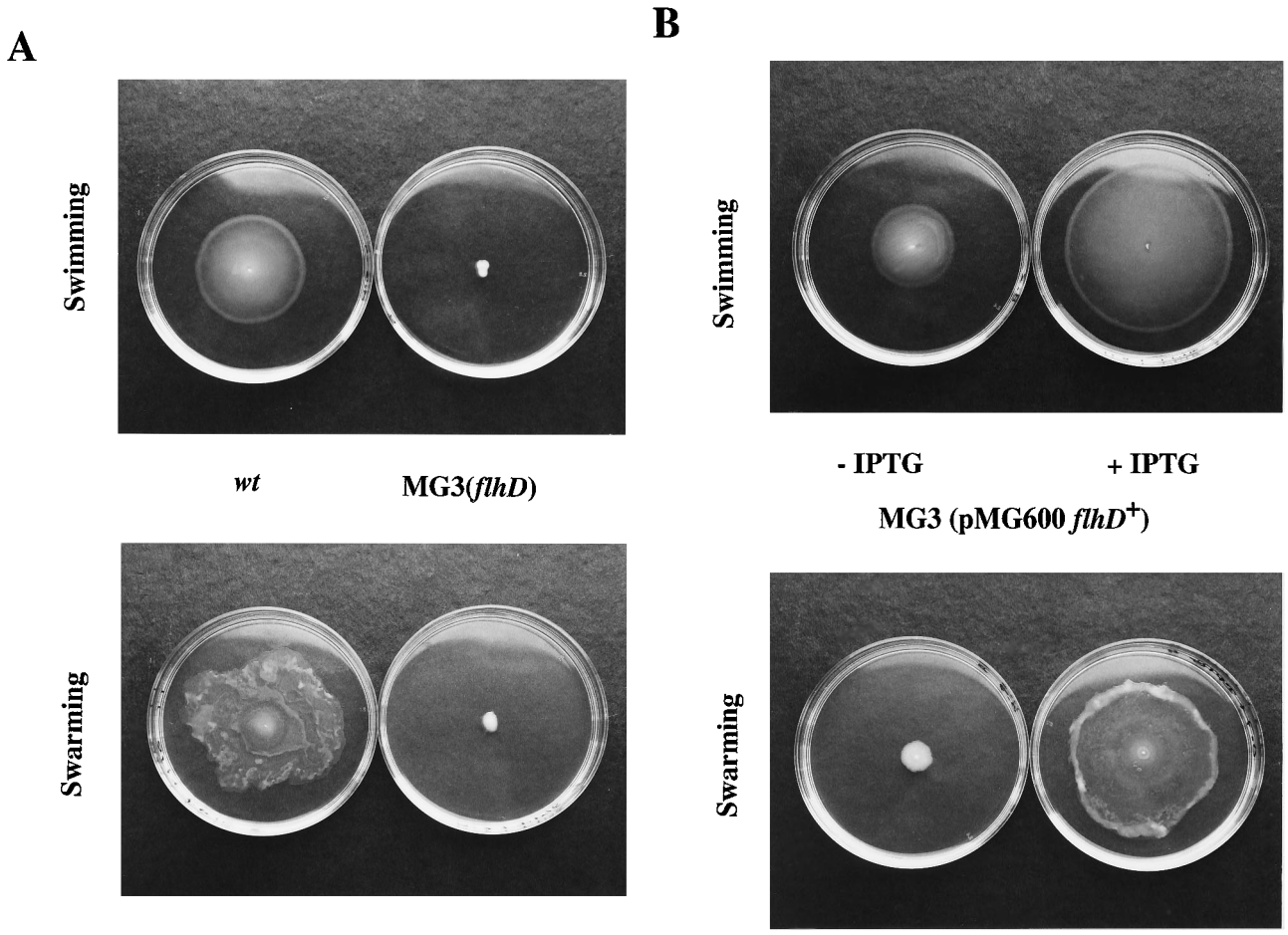



FIG. 1. Swimming and swarming behavior of wild-type (wt) *S. liquefaciens* and strain MG3 (*flhD*) (A) and strain MG3 harboring plasmid pMG600 (*flhD*$^+$) in the absence or presence of 50 μM IPTG (B). Swimming and swarming motilities were assayed in LB medium containing 0.25 and 0.6% agar, respectively. Plates were photographed after 12 h of incubation at 30°C.

ment of adding amino acids to initiate swarming may be attributed to the stimulation of growth. The swarming motility of *P. mirabilis* is strongly promoted by media supporting high growth rates (9, 15).

Temperature also affected swarming and was found to be optimal at 30°C. Interestingly, no swarming could be observed at 37°C whereas the strain was still capable of swimming at this temperature.

**Both swimming and swarming motility are under control of the *flhD* master operon.** A *flhD* null mutant strain of *S. liquefaciens*, MG3, is capable neither of swimming nor of swarming motility and is thus completely nonmotile (Fig. 1A). This is in agreement with our previous observation that this strain is unable to synthesize flagella (10). The introduction of plasmid pMG600, which contains the entire *flhD* operon under control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *Ptac* promoter, restored both forms of motility, although swarming was of rather poor quality. The addition of 25 to 75 μM IPTG greatly stimulated both swimming and swarming motility (Fig. 1B), and the speed of migration was found to correlate with inducer concentrations. However, IPTG concentrations higher than 100 μM were found to be rather inhibitory. Since the presence of high concentrations of IPTG did not affect the motility of the wild type, the reduction of migration speed observed with *S. liquefaciens* MG3 harboring plasmid pMG600 may be the result of extensive cell differentiation. Thus, properly controlled expression of the master regulator appears to be crucial for both the swimming and swarming behaviors of *S. liquefaciens*.

The initiation of swarm cell differentiation was found to be strictly dependent on surface contact, and under no conditions tested so far could swarm cells be observed in liquid medium. However, if the signal transduction system that triggers initiation of swarm cell formation operates via the *flhD* operon, the exogenous control of this operon (which can be achieved by the addition of IPTG to a culture of *S. liquefaciens* MG3 harboring pMG600) might uncouple swarm cell differentiation from its surface dependence. Indeed, when a culture of *S. liquefaciens* MG3(pMG600) growing exponentially in liquid LB medium was induced with IPTG, cells began to elongate and to produce vast numbers of flagella, thus strongly resembling swarm cells. Both cell length and the number of flagella per cell increased dramatically with increasing inducer concentrations (Fig. 2). The increase in flagellation was also found to correlate well with the increase of flagellin levels as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). Staining these long (up to 100 μm) filamentous cells with DAPI demonstrated that they were multinucleate, often with distinctly spaced nucleoids along the entire length of the cell (Fig. 3B). In contrast, cells from an uninduced control

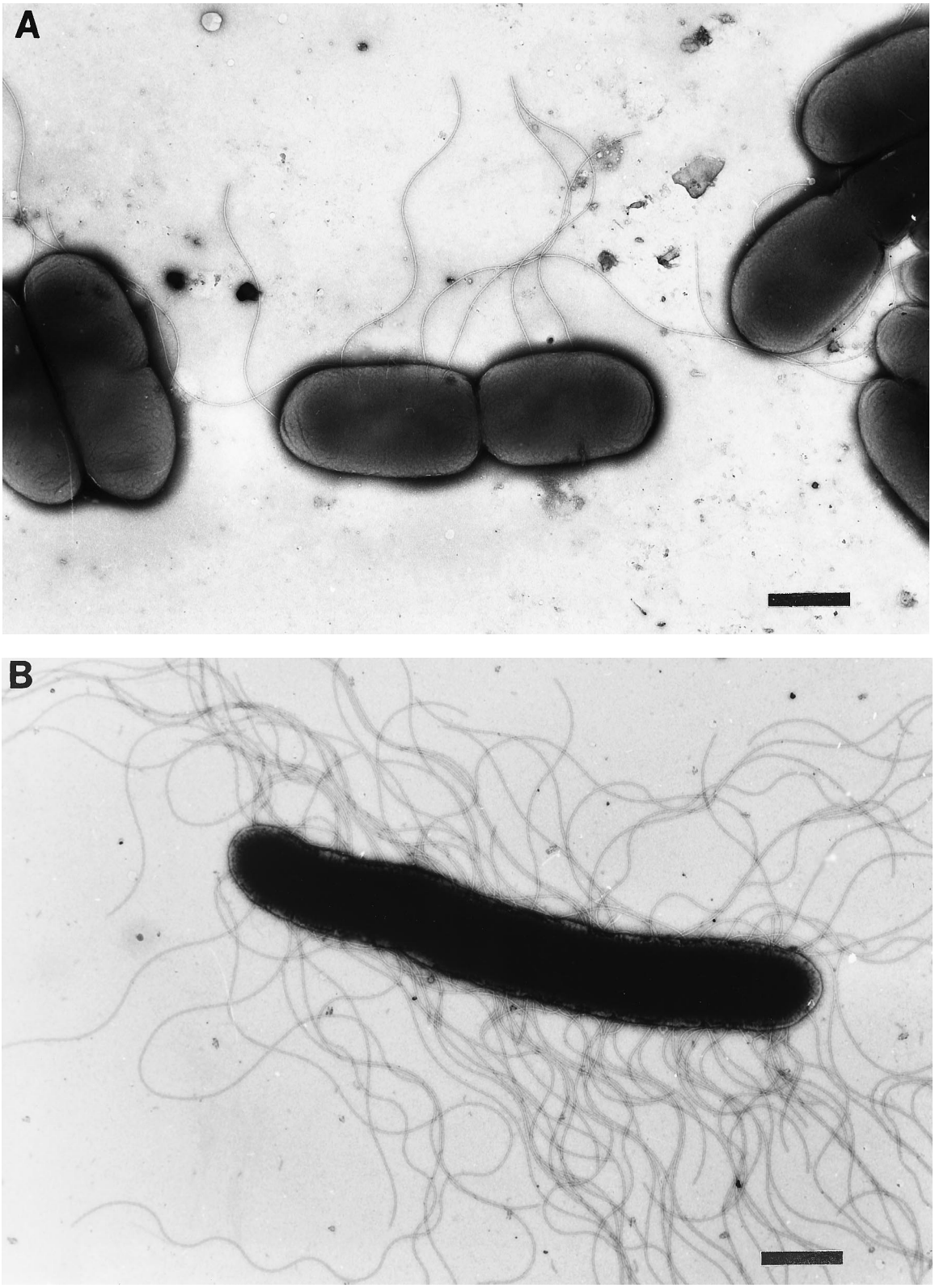
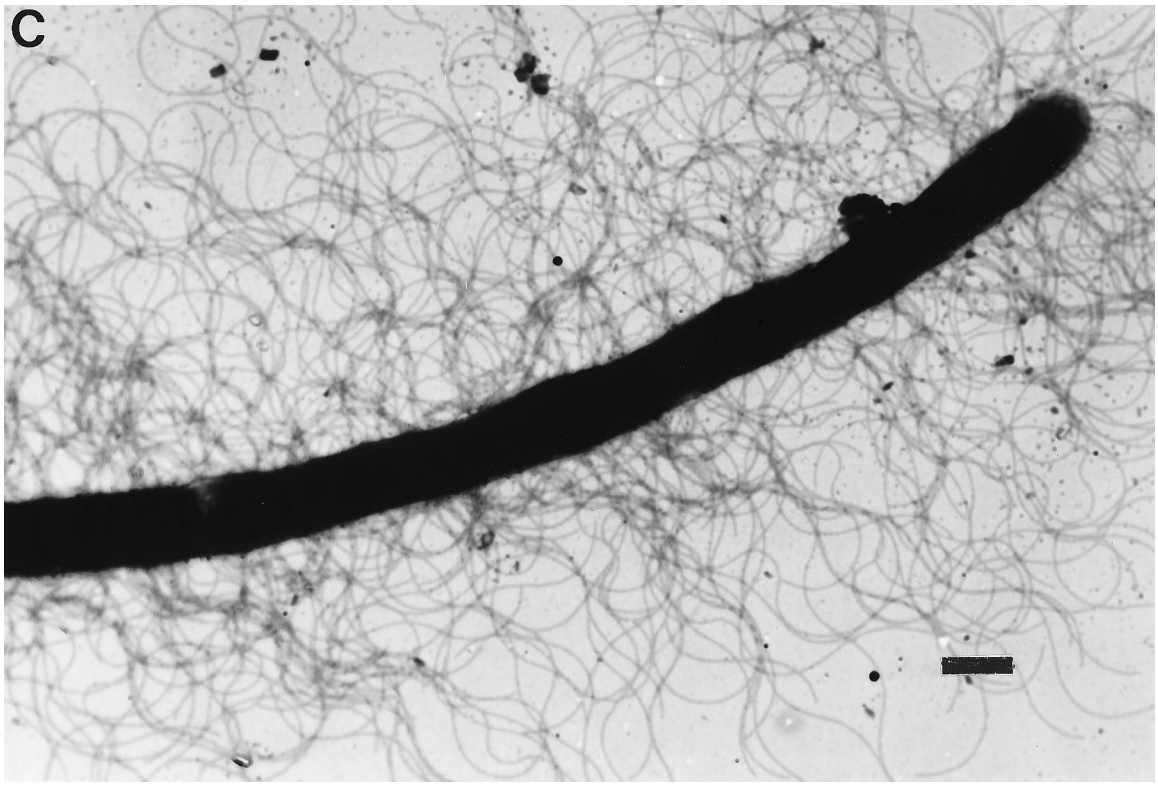



FIG. 2. Electron microscopic inspection of swarm cell differentiation in liquid medium. *S. liquefaciens* MG3 harboring plasmid pMG600 (*flhD*$^+$) was grown exponentially in LB medium for at least five generations before the culture was diluted to an optical density at 450 nm of 0.001 in LB medium containing either no IPTG (A), 100 μM IPTG (B), or 1 mM IPTG (C). Samples for electron microscopy were taken when cultures reached an optical density at 450 nm of 0.5 to 1.0. Bars, 1 μm.




culture resembled typical swimmer cells, i.e., short rods that carry three to five flagella and contain a single nucleoid (Fig. 2A and 3A).

When this induction experiment was performed in glucose-supplemented AB minimal medium, severe growth inhibition and only poor cell elongation were observed. However, the addition of 0.2% Casamino Acids restored the formation of elongated swarm cells, an observation that is consistent with the results from the motility assays performed with swarming plates. The apparently indispensable requirement of adding amino acids may reflect a high demand for both building blocks and energy to synthesize and operate the hundreds of flagella produced during swarming differentiation. The synthesis and operation of the only 5 to 10 flagella of a swimming *E. coli* cell have been estimated to utilize about 2% of the total energy budget of the cell (16).

**Swarm cell differentiation is accompanied by a substantial increase in the level of phospholipase activity.** On the basis of the presented data, we suggest that swarm cell differentiation in *S. liquefaciens* can be triggered by the stimulation of expression of the *flhD* master operon. Since the phospholipase is a member of the flagellar regulon, one prediction of this hypothesis would be that swarm cell differentiation is linked to differential production of this hydrolytic enzyme. Expression of phospholipase was assessed by determining specific β-galactosidase activities expressed from *S. liquefaciens* wild-type cells harboring plasmid pMG102K (11). This plasmid contains a translational fusion of *lacZ* to the phospholipase gene. Swarm cells collected from the colony edge produced significantly higher levels of β-galactosidase than vegetative cells taken from the colony core (Fig. 4). Given that, because of the isolation technique, swarm cell preparations contained a considerable number of short cells (30 to 40% as determined by microscopic inspection), the observed level of induction must underrepresent actual levels of induction. Hence, these results are in accordance with the suggestion that in swarm cells the entire flagellar regulon is up-regulated.

The possibility that the short cells, collected from the center of the swarm colony, demonstrated a low level of β-galactosidase activity because they were in stationary phase was ruled unlikely by the finding that run-out cultures in liquid medium had high levels of marker enzyme activity (Fig. 4). This finding is in agreement with previous studies demonstrating that phospholipase expression (and thus the production of β-galactosidase from pMG102K) occurs in the transition from exponential growth to stationary phase and that the phospholipase–β-galactosidase hybrid protein is stable in *S. liquefaciens* (11).

**Conclusions.** Initiating swarm cell differentiation in *S. liquefaciens* appears to be complex and does not depend on an individual signal or event but rather requires the integration of diverse environmental and intracellular signals involving temperature, metabolic potential (medium composition), and contact with a surface. The task of signal integration and the interpretation of all of the information from the various sensory transducing pathways require a key regulator whose concentration or activity status determines whether or not a colony swarms. The results presented in this report identify the *flhD* master operon as encoding a possible candidate for such a function. Artificial stimulation of this operon overrides the requirement of surface exposure for swarm cell differentiation, indicating that at least this sensory pathway is channelled through FlhC-FlhD. In rich liquid medium the degree of *flhD* stimulation apparently determines whether cells swim or differentiate into swarm cells.

It has been established that the swarming behavior of *P. mirabilis* is closely associated with the pathogenicity of this bacterium. Both the ability to invade human urothelial cells and the expression of virulence factors such as intracellular

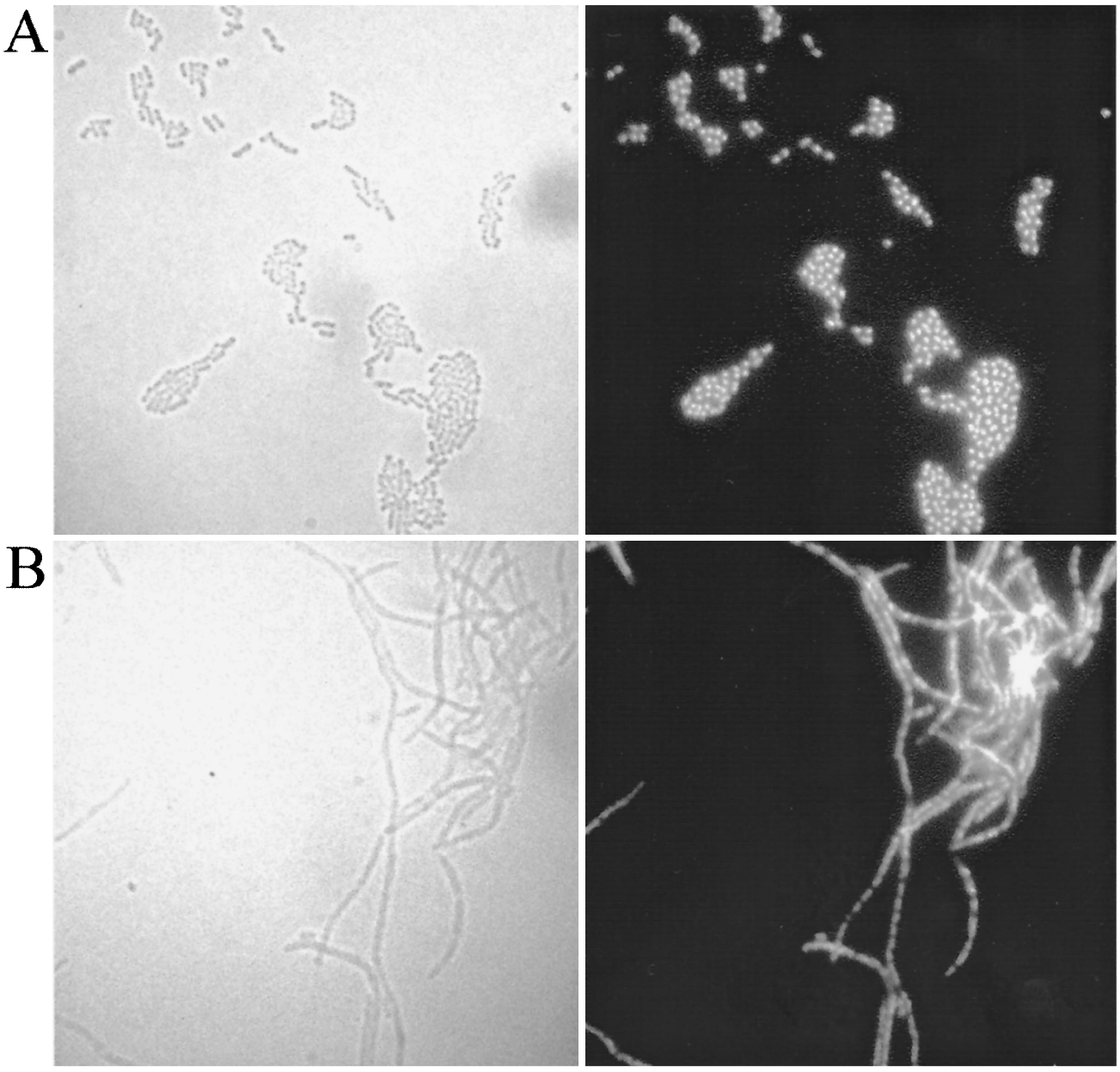



FIG. 3. Arrangement of nucleoids in cells of *S. liquefaciens* MG3 harboring plasmid pMG600 (*flhD*$^+$) growing in LB medium containing no IPTG (A) and 1 mM IPTG (B). Left panels show bright-field images, and right panels show DAPI-stained fluorescence images of the same cells after UV excitation. Bar, 10 μm.

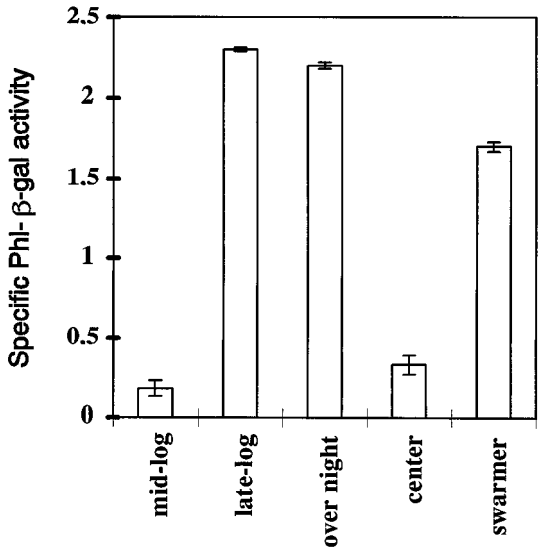



FIG. 4. Determination of specific PhlA–β-galactosidase (Phl-β-gal) activity of *S. liquefaciens* wild-type cells harboring plasmid pMG102K isolated from either the edge or the center of an advancing swarm colony propagated on LB medium containing 0.7% agar. The levels of specific activity of mid-log (optical density at 450 nm = 0.6), late-log (optical density at 450 nm = 3.0), and stationary-phase (overnight incubation) cultures grown in liquid LB medium are also shown. Error bars indicate standard deviations.

urease, extracellular hemolysin, and metalloprotease were demonstrated to be coupled with the swarm cell differentiation of *P. mirabilis* (2, 5). The swarming migration of the opportunistic pathogen *S. liquefaciens* was found to be accompanied by an increased level of expression of the phospholipase gene, a potential virulence determinant (12). Since the phospholipase gene is a member of the flagellar regulon, it is tempting to speculate that the regulatory system encoded by the *flhD* master operon links swarm cell differentiation with the expression of virulence factors.

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