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# Iron Regulation of Swarmer Cell Differentiation of *Vibrio parahaemolyticus*

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***Vibrio parahaemolyticus* has two distinct cell types, the swimmer cell and the swarmer cell, adapted for locomotion in different circumstances. The swimmer cell, produced when the bacterium is grown in liquid media, is a short rod with a single sheathed polar flagellum. The swarmer cell, produced when *V. parahaemolyticus* is grown on solidified media, is greatly elongated and synthesizes, in addition to the polar flagellum, numerous unsheathed lateral flagella which are responsible for translocation over surfaces. We are interested in understanding how this bacterium differentiates in response to contact with surfaces and have determined in earlier work that the polar flagellum acts as a tactile sensor which controls transcription of genes (*laf*) encoding the swarmer cell phenotype. Surface recognition involves sensing of forces that obstruct movement of the polar flagellum. In this report we show that a second signal, iron limitation, is also required for swarmer cell differentiation. Production of lateral flagella occurred only when polar flagellar function was perturbed and iron-limiting growth conditions were imposed. The same conditions were required to induce light production in strains of *V. parahaemolyticus* in which a *laf* gene was transcriptionally fused to the *lux* operon encoding the enzymes for bioluminescence. The *lafA* gene encoding the lateral flagellin subunit was cloned and used in Northern (RNA) blot measurements. Examination of mRNA levels revealed that transcription of *lafA* is dependent on growth in iron-depleted media. The control of differentiation by multiple environmental stimuli is discussed.**

When *Vibrio parahaemolyticus*, a gram-negative marine organism, is grown in a liquid environment, its behavior resembles that of *Escherichia coli*: a straight rod, it swims in stretches of smooth runs interspersed with brief tumbles. Swimming is propelled by a single sheathed polar flagellum (Fla). When grown on solid media, *V. parahaemolyticus* differentiates into a swarmer cell, changing both its morphology and its mode of locomotion (1, 19). The swarmer cell is highly elongated, often up to 30 μm in length, and the cell body is covered with numerous lateral flagella (Laf). Lateral flagella are not sheathed and have a different subunit composition from polar flagella (13, 18). Movement of the swarmer cell across a solid surface, or swarming, results in progressive spreading of the bacterial colony and colonization of a surface (9, 22).

What are the signals required for induction of the swarmer cell phenotype? Expression of lateral flagella is induced by physical conditions that restrict the movement of the polar flagellum, for example, cultivation on solid media or in liquid media of high microviscosity (e.g., media supplemented with Ficoll or polyvinylpyrrolidone) and agglutination with antibody (4, 13). In measuring forces that influence its movement, the polar flagellum appears to act as a dynamometer, or tactile organelle. Introduction of mutations into the genes for the polar organelle confirms the regulatory interaction between the polar and lateral flagellar gene systems. Mutants with *flaC* defects, which do not possess functional polar flagella, no longer exhibit surface-dependent lateral flagella gene expression; i.e., strains with mutations in *flaC* produce lateral flagella in liquid media (13). However, genetic or physical interference with polar flagellar rotation is not sufficient to cause expression of lateral flagellar genes. In this report, we show that swarmer cell differentiation is also controlled by a second signal, limitation for iron in the growth medium.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The strains, plasmids, and cosmids used for this work are presented in Table 1. For propagation of *V. parahaemolyticus* strains, the following media were used: 2216 (28 g of marine broth 2216 [Difco Laboratories] per liter; broth was filtered after autoclaving to remove precipitate) and HI (25 g of heart infusion broth [Difco] and 20 g of NaCl per liter). Solidified marine medium was prepared by using 2% Bacto-Agar (Difco). For propagation of lambda, NZYM medium was used (12), and 0.7% agarose was used in NZYM top agar for plaque blots. Antibiotics and iron chelators (Sigma Chemical Co.) were added to the media at the following concentrations: 80 μg of ampicillin, 50 μg of kanamycin sulfate, 10 μg of tetracycline, 1 mg of conalbumin, 6 μg of ethylenediamine-di(*o*-hydroxyphenylacetic acid) (deferrated by the method of Rogers [17]), and 1 mg of human transferrin per ml. Preparation of media containing polyvinylpyrrolidone (PVP-360; Sigma) and Ficoll (type 400; Sigma) has been described before (4, 13). Viscosities were measured with a Cannon-Fenske calibrated viscometer (Thomas Scientific) at 27°C.

**Immunological techniques.** Antibody production was elicited in New Zealand White rabbits. The antigens for antibodies 127 and 129 were the protein subunits of lateral and polar flagella, respectively. These were isolated from flagellar preparations (13) after separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 2), by excising appropriate slices from the gel and electroeluting the flagellin subunits. Rabbits were inoculated, intramuscularly and subcutaneously, with 25 μg and boosted with 16 μg of antigen in Freund incomplete adjuvant (Difco) at 0, 4, 7, and 11 weeks. Rabbits were bled at 8 weeks and later.

For Western analysis, proteins in sample buffer (11) were

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TABLE 1. Bacterial strains and plasmids

| Strain or plasmid | Description[a] | Origin (reference) |
|---|---|---|
| *V. parahaemolyticus* | | |
| BB22 | Wild type | 4; R. Belas |
| LM899 | *flaC1*::*kan*[b] | 13 |
| LM1017 | *laf-313*::*lux* (Tet$^s$) | 13 |
| LM1024 | *laf-313*::*lux* (Tet$^s$) *flaC1*::*kan* | 13 |
| Plasmid or cosmid | | |
| pLM1453 | Ap$^r$; pUC-8 with 0.7-kb *Pst*I-*Sal*I insert from pLM1457 encoding truncated lateral flagellin (17 kDa) | This work |
| pLM1457 | Tet$^r$; pLAFRII with 7.3-kb *Hin*dIII-*Bam*HI insert encoding *V. parahaemolyticus* lateral flagellin (27 kDa) | This work |
| pLM139.40 | Tet$^r$; pLAFRII cosmid from a *V. parahaemolyticus* bank encoding a 41-kDa outer membrane protein | L. McCarter |
| pLM140.20 | Tet$^r$; pLAFRII cosmid from a *V. parahaemolyticus* bank encoding a 36-kDa outer membrane protein | L. McCarter |
| pLAFRII | Tet$^r$; pLAFRI with polylinker | 7; F. Ausubel |
| pUC-8 | Ap$^r$ | 23; R. Ogden |

[a] Tet$^s$ or Tet$^r$, Tetracycline sensitive or resistant; Ap$^r$, ampicillin resistant; kDa, kilodaltons.
[b] The mutation is caused by insertion of a restriction fragment from Tn*5* encoding kanamycin resistance.

separated by SDS-PAGE. The resolving gels were 12% acrylamide. Electrophoretic transfer of proteins (21) to Nytran after SDS-PAGE was for 2 h at 100 Volts in 0.096 M glycine–0.125 M Tris base–20% methanol. Western blots (immunoblots) were blocked with saturant (5% nonfat dry milk–0.2% $NaN_3$ in phosphate-buffered saline [20 mM sodium phosphate, pH 7.3, 100 mM NaCl]), reacted with antiserum diluted in saturant, washed with phosphate-buffered saline, incubated with $^{125}$I-labeled protein A ($2 \times 10^5$ cpm/ml of saturant; Dupont, NEN Research Products), washed with phosphate-buffered saline, dried, and exposed to X-ray film (Kodak XAR-5).

To screen for immunopositive lambda clones, the library was plated on *E. coli* P2392 (from Stratagene) to yield 300 to 500 plaques per plate (5). After an overnight incubation at 30°C, the plates were overlaid with nitrocellulose circles (BA85, 82 mm; Schleicher & Schuell) and incubated at 37°C for 4 h. The filters were then removed and blocked in saturant for 3 h. Subsequent treatment of filters was identical to that for Western blots. Diluted antisera in saturant was preadsorbed with a cell extract of strain P2392 (1 mg of extract per ml of saturant). For identification of subclones producing flagellar antigen, the colony filter blot method of Meyer et al. (15) was adopted, and hybridization conditions for colony blots were similar to those for plaque blots.

**Measurement of luminescence in *laf*::*lux* fusion strains.** Mini-Mu*lux* is a transposon that can generate fusions which couple transcription of a target gene to that of the transposon's reporter genes such that expression of a *lux* fusion results in the production of light (6). The target gene for the particular fusion used in this work (*laf-313*::*lux*) is an unmapped gene in the lateral flagella system (4, 13). Overnight cultures, grown in HI medium, were diluted 1:1,000 into HI or HI supplemented with 1 mg of conalbumin per ml and grown at 30°C with aeration. When the cultures reached an optical density at 600 nm of 1, bioluminescence of diluted cultures was measured in an LKB1211 minibeta scintillation counter, using the single photon event, or chemiluminescence, mode, and it is reported as specific light units, which are scintillation counts per minute normalized to 1 ml of culture at an optical density at 600 nm of 1.

**Recombinant DNA techniques.** Transformations, ligations, and other general cloning procedures were performed by the methods of Maniatis et al. (12). Restriction endonucleases and T4 DNA ligase were from Boehringer Mannheim Biochemicals, and were used according to the instructions of the supplier. A lambda library of *V. parahaemolyticus* DNA was constructed with the EMBL3 cloning vector (8), using the protocol provided by the supplier (Stratagene). Chromosomal DNA was isolated by the spooling technique (20; with omission of the freezing step). Chromosomal DNA, partially digested with *Sau*3A, was size fractionated by agarose gel (0.4%) electrophoresis and electroelution, using the electroeluter device of International Biotechnologies, Inc. Sized DNA (0.5 μg in the 17- to 25-kilobase [kb] range) was ligated with 1 μg of predigested EMBL3 arms (*Bam*HI/*Eco*RI) and packaged with the Gigapack Plus lambda packaging extracts of Stratagene, yielding $2 \times 10^7$ recombinants. Lambda phage were manipulated by the method of Silhavy et al. (20).

**RNA analysis.** Exponentially growing cultures were harvested at an optical density at 600 nm of 1.0 by rapid chilling on dry ice-ethanol. Total RNA was isolated by the method of von Gabain et al. (24). RNA was denatured with formaldehyde and formamide and fractionated in 1% agarose gels containing 0.66 M formaldehyde (16). Transfer of RNA to Nytran (0.45 μm; Schleicher & Schuell) and hybridization of Northern blots with nick-translated probes were as described for Southern blots (12, 14).

## RESULTS

**Regulation of lateral flagella synthesis.** Lateral flagella are synthesized when *V. parahaemolyticus* is cultivated on the surface of solidified medium. Expression of the many genes in the lateral flagellar system is controlled by the polar flagellar organelle. We have previously shown that recognition of surfaces results from physical antagonism of rotation of the polar organelle (4, 13). Moreover, interference with Fla function can be achieved by genetic means. A mutation in *flaC*, a polar flagellar gene, has pleiotropic effects: it causes loss of polar flagellum function and releases expression of *laf* genes from surface dependence (13). However, we have found that *laf* expression in *flaC* mutants is still conditional. Mutants with defects in *flaC* produced lateral flagella when grown in 2216 marine broth, but these mutants did not synthesize lateral flagella when grown in HI broth.

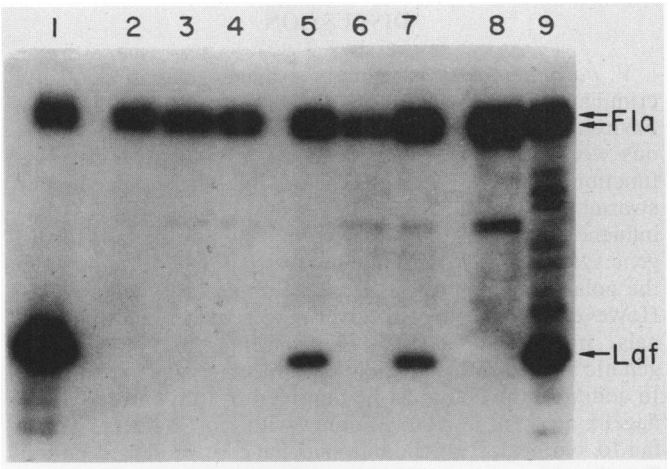


FIG. 1. Western blot analysis of flagella synthesis in *V. parahaemolyticus*. Overnight cultures or cells suspended from plates were diluted into sample buffer for SDS-PAGE. BB22 is the wild-type strain and LM899 is a strain with a *flaC* defect. (Although a strain with a *flaC* defect still makes polar flagellar filament, the filament is not functional.) Lanes: 1, BB22 grown on 2216 agar plate; 2, BB22 in 2216 broth; 3, BB22 in HI broth; 4, BB22 in HI with 1 mg of conalbumin per ml; 5, LM899 in 2216 broth; 6, LM899 in HI broth; 7, LM899 in HI with 1 mg of conalbumin per ml; 8, BB22 in HI–20% Ficoll (19 cP); 9, BB22 in 2216–18% Ficoll (15 cP). The immunoblot was reacted with pooled antisera 127 and 129, which are directed against subunits of lateral (Laf) and polar (Fla) flagella, respectively, and subsequently with iodinated protein A.

This difference in lateral flagella production was quantitated by Western blot analysis with antibody specific for the flagellin subunit of the lateral flagella filament (Fig. 1, lanes 5 and 6). One prominent difference in the composition of the two media is the availability of iron, a conclusion supported by the observation that iron-starvation-inducible outer membrane proteins of *V. parahaemolyticus* were expressed when growth was in 2216 broth but not when it was in HI broth (data not shown). It should be noted that cells grown on HI plates also behaved as though they were iron deprived; i.e., the iron-regulated outer membrane proteins were produced. Addition of iron chelators (10), including ethylenediamine-di(*o*-hydroxyphenylacetic acid) and the more selective biological chelators conalbumin (ovotransferrin) and human transferrin, to HI broth resulted in synthesis of lateral flagella by mutants with *flaC* defects (Fig. 1, lane 7 for conalbumin). Furthermore, growth of the wild type in viscous, iron-rich broth (HI-Ficoll) did not result in the production of lateral flagella, but growth of the wild type in viscous, iron-poor broth (2216-Ficoll) did elicit lateral flagella synthesis (Fig. 1, lanes 8 and 9). Therefore, synthesis of lateral flagella requires iron limitation in addition to interference with rotation of the polar flagellum. Iron deficiency alone was not sufficient to induce swarmer cell differentiation since lateral flagella synthesis was not induced when the wild-type strain was grown in iron-limited broth (Fig. 1, lane 2).

**Effect of iron limitation on luminescence in *laf*::*lux* fusion strains.** Gene fusions, which couple transcription of reporter genes to promoters of interest, have simplified analysis of gene regulation, especially for the study of swarmer cell development which involves morphological changes that are complex and difficult to measure. Strains with fusions in the lateral flagellar gene system generated by the transposon mini-Mu*lux*, e.g., *laf-313*::*lux* in strain LM1017, do not swarm, fail to synthesize lateral flagella, and exhibit surface-dependent expression of luminescence (4). The specific nature of the *laf-313* defect has not been defined, although we know that *laf-313*::*lux* does not map in or near the lateral flagellin structural gene (data not shown). Fusions such as *laf-313*::*lux* were used to show that physical factors, such as high viscosity and antibody agglutination, affect expression of *laf* genes (4, 13). Expression of fusion *laf-313*::*lux* is also affected by *flaC* mutations: when grown in 2216 broth, liquid cultures of *laf*::*lux* strains with *flaC* defects are bright, while a strain with only the *laf*::*lux* defect is dark (13). The availability of iron in the growth medium plays a key role in determining expression of the *laf-313*::*lux* fusion. Strain LM1017 failed to produce significant light when grown in HI broth ($3 \times 10^8$ specific light units) and light production was not greatly affected when the iron chelator conalbumin was included in the growth medium ($6 \times 10^8$ specific light units). However, when polar flagellar function was disrupted by introduction of the *flaC*::*kan* mutation into LM1017, iron limitation did affect *laf* gene expression: strain LM1024 produced 100-fold more light when grown in HI plus conalbumin compared with growth in HI ($5 \times 10^{10}$ versus $2 \times 10^8$ specific light units). So, as is the case for synthesis of lateral flagella, transcription of a gene in the *laf* system is dependent on two signals: iron limitation and interference of polar flagellar function.

**Cloning of the lateral flagellin structural gene (*lafA*).** Antibody directed against lateral flagellin was used to screen an EMBL3 lambda library of *V. parahaemolyticus* DNA. There were approximately 5 immunopositive plaques per every 1,000 plaques plated. DNA clone analysis of three purified recombinant lambda phage showed independent, but overlapping clones with inserts of approximately 20 kb. In Western blot analysis, each of the lambda clones directed synthesis of an immunoreactive protein that comigrated with *V. parahaemolyticus* lateral flagellin (molecular weight, 27,000). Subcloning from one of the original lambda phage into the *Hin*dIII-*Bam*HI restriction sites of pLAFRII yielded cosmid pLM1457. This cosmid contained a 7.3-kb insert of bacterial DNA, and it produced lateral flagellin in *E. coli* (Fig. 2, lane 3). Subclone pLM1453, containing an insert of 0.7 kb in the *Pst*I-*Sal*I sites of pUC-8, revealed the location of the structural gene (*lafA*). A strain carrying plasmid pLM1453 produced a truncated, immunoreactive product of molecular weight 19,000 (Fig. 2, lane 4). A restriction map of the *lafA* locus is shown in Fig. 3.

**Iron limitation regulates transcription of lateral flagellin mRNA.** We examined the effect of iron limitation on transcription of lateral flagellin mRNA, using the cloned *lafA* gene as a probe of Northern blots. Blot A in Fig. 4 shows that the *lafA* message is expressed in liquid culture only when *flaC* mutant strain LM899 is grown under iron-limiting conditions, i.e., 2216 broth (Fig. 4, lane 3) or HI with conalbumin (Figure 4, lane 5), and not when it is grown under iron-sufficient conditions (HI; Fig. 4, lane 4). The wild-type strain does not synthesize lateral flagellin message when grown in iron-rich or iron-poor media (Fig. 4, lanes 1 and 2), and it only synthesizes hybridizing message in viscous medium that is deficient in available iron (2216-polyvinylpyrrolidone; Fig. 4, lane 7) and not in viscous, iron-rich media (HI-polyvinylpyrrolidone; Fig. 4, lane 6). Probes for two different mRNA transcripts were used as controls in the Northern blot experiment shown in Fig. 4. One control probe, cosmid pLM139.40, which was hybridized againt blot B, detects mRNA for an outer membrane protein that is expressed under the opposite growth conditions required for lateral flagella expression, i.e., HI but not

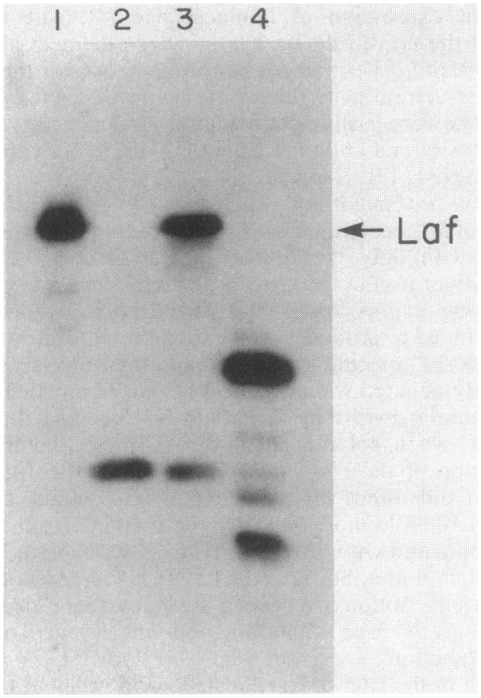


FIG. 2. Western blot of lateral flagellin producing clones in *E. coli*. Overnight cultures were diluted 1:2 into sample buffer for SDS-PAGE. The reference sample was wild-type *V. parahaemolyticus* BB22 harvested from a plate. Lanes: 1, BB22; 2, *E. coli* YMC10*recA* (14); 3, YMC10*recA* with pLM1457; 4, YMC10*recA* with pLM1453. The immunoblot was reacted with antibody 127, which is directed against lateral (Laf) flagellin subunit, and subsequently with iodinated protein A.

2216 medium. The second probe, cosmid pLM140.20, which was hybridized againt blot C, shows levels of message for a constitutively expressed outer membrane protein. It is evident that nonspecific degradation of mRNA was not occurring in some of the samples. The levels of *lafA* mRNA observed in Fig. 4 correlated with the amount of lateral flagellin protein detected in Fig. 1. Thus, iron regulation of lateral flagellin synthesis acts at the level of transcription. Synthesis of lateral flagellin mRNA requires both iron limitation and interference with polar flagellar function.

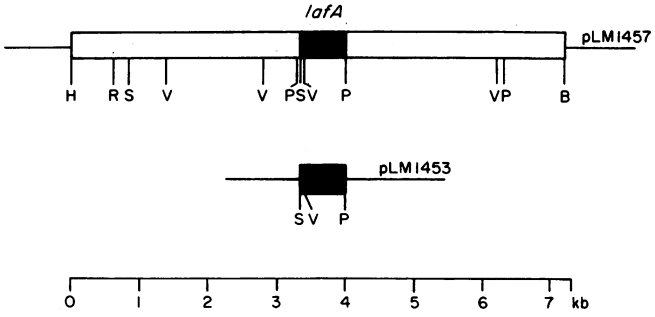


FIG. 3. Restriction map of the *lafA* locus. Open bar indicates bacterial insert DNA. Closed bars indicate coding region for lateral flagellin. Solid lines indicate vector DNA, which is pLAFRII for pLM1457 and pUC-8 for pLM1453. Abbreviations for restriction endonuclease sites are as follows: B, *Bam*HI; R, *Eco*RI; S, *Sal*I; V, *Eco*RV; P, *Pst*I; H, *Hin*dIII. Cosmid pLM1457 produces lateral flagellin and plasmid pLM1453 produces a truncated, immunoreactive product.

## DISCUSSION

*V. parahaemolyticus* exhibits surface recognition: it discriminates between life on a surface and life in liquid environments and modifies its cell type accordingly. Previous work has shown that interference with polar flagellar function leads to expression of the surface-dependent swarmer cell phenotype. By sensing external forces that influence its motion and conveying this information to the gene system (*laf*) responsible for swarmer cell development, the polar flagellum acts as a tactile organ or dynamometer. However, commitment to swarming requires multiple signals. Impairment of the polar flagellum by physical or genetic means is not sufficient to induce *laf* gene expression. In addition, the cell must be deprived of iron before lateral flagella are synthesized. Mutants with *flaC* defects, which fail to synthesize functional polar flagella, produced lateral flagella when grown in 2216 broth but not when grown in HI broth. The particular ingredient responsible for the difference in *laf* expression is iron, which is available for utilization in HI but not in 2216 broth. Addition of iron chelators such as conalbumin to HI broth confirmed the iron effect: *flaC* mutants synthesized lateral flagella in this medium. Similarly, the wild-type strain produced lateral flagella when grown in viscous iron-poor media but not when grown in viscous iron-rich media.

Regulation by iron occurs at the level of transcription. The iron effect could be measured by using *laf*::*lux* fusions. Transcriptional fusions coupling the promoter or regulatory region of a gene in the *laf* system to the genes encoding bioluminesence (*lux*) produced 100-fold more light when grown in low-iron compared with high-iron medium, provided that polar flagellar function was also disrupted. In strains with functional polar flagella, deprivation of iron in the growth medium did not significantly increase levels of light production. Cloning of the structural gene (*lafA*) for lateral flagellin provided a specific probe for examination of mRNA levels. The amount of *lafA* message produced under various growth conditions for the wild type and a strain with a *flaC* defect correlated directly with the amount of lateral flagellin protein synthesized. Neither iron limitation nor interference with polar flagellar function alone was sufficient to allow production of lateral flagellin mRNA. Both conditions were required to detect *lafA* gene transcription in Northern blots.

*V. parahaemolyticus* fails to produce lateral flagella in liquid HI medium even when polar flagellar function is perturbed. However, cells do synthesize lateral flagella and swarm on HI plates. So, HI plate-grown cells react as though they were iron starved, and indeed they produce outer membrane proteins similar to those iron uptake proteins induced in many bacteria in response to "iron stress" (10). Although iron is abundant in liquid HI medium, provision of iron to cells growing on the surface of an HI agar plate apparently is not adequate to provide for the needs of the cell. Possibly, diffusion of iron to cells on the surface is limiting. Adaptation of bacteria to life on a surface probably requires recognition of distinctive features of that habitat, and iron limitation could be one indicator of growth in a biofilm or similar configuration. Perhaps deficiencies in other nutrients or the accumulation of metabolic by-products could also serve as indicators of life on a surface.

The swarmer cell is a highly elongated cell that produces great numbers of lateral flagella per cell. Examination of total cellular protein on SDS-PAGE clearly revealed lateral flagellin to be the most abundant protein produced by the cell

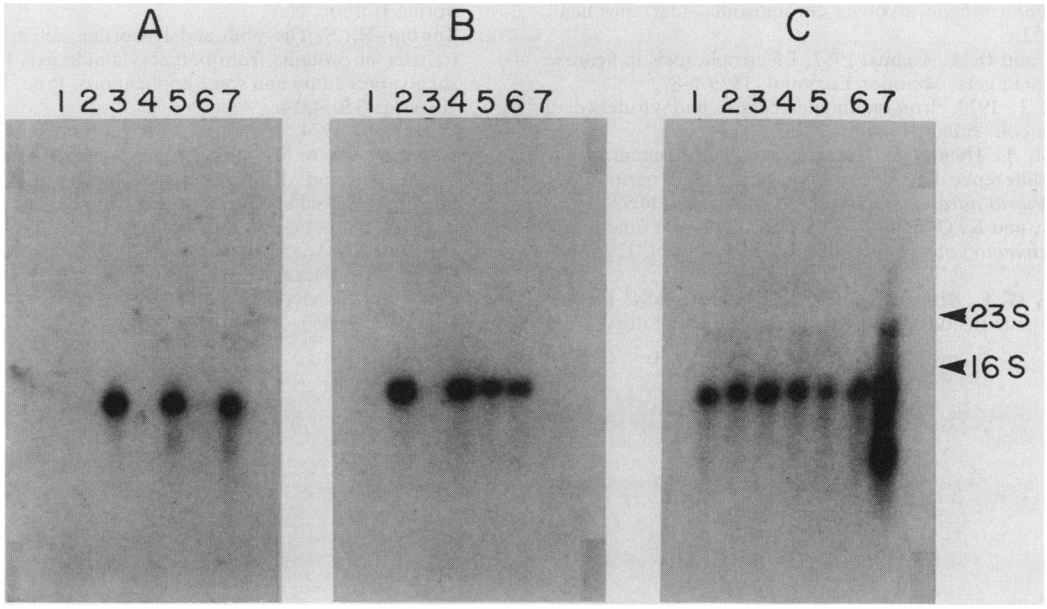


FIG. 4. Regulation of lateral flagellin mRNA. Northern blot A was probed with radiolabeled DNA encoding lateral flagellin (pLM1453). RNA was prepared from wild-type strain BB22 and *flaC* mutant strain LM899 grown as indicated below. Approximately 5 μg of total RNA was loaded in each lane. Lanes: 1, BB22 grown in 2216 broth; 2, BB22 in HI broth; 3, LM899 in 2216 broth; 4, LM899 in HI broth; 5, LM899 in HI broth with 1 mg of conalbumin per ml; 6, BB22 in HI broth with 10% polyvinylpyrrolidone; and 7, BB22 in 2216 broth with 10% polyvinylpyrrolidone. Control Northern blots B and C were identical to blot A and were probed with cosmids pLM139.40 and pLM140.20, respectively. Cosmid pLM139.40 encodes an outer membrane protein that is expressed when cells are grown in HI but not 2216 medium. Cosmid pLM140.20 encodes an outer membrane protein that is expressed under both growth conditions. The positions to which the 23S and 16S rRNA species migrated are designated with arrows, and from these markers the approximate length of the *lafA* transcript was calculated to be 1 kb.

(L. McCarter, unpublished data). Induction of a large number of genes, some of which must be very highly expressed, is required for differentiation to the swarmer cell. Specifically, 1% of all transposon-generated mutants fail to swarm (3, 4). So, the *laf* mutational target is relatively large. Therefore, in terms of cellular economy the commitment to swarm represents a large investment in genes, proteins, and energy to generate flagellar rotation, and the appropriate response to the specific environmental conditions signalling swarming could best be accomplished by basing the "decision" to differentiate on multiple inputs. One input alone, such as the detection of iron deprivation, probably could not be used to discriminate the specific circumstances for which swarming is beneficial, i.e., an overpopulated community of cells attached by a substratum, while with multiple inputs recognition of such particular situations could be achieved. And so we find that at least two channels of information, surface recognition and nutritional limitation for iron, are required to signal swarmer cell differentiation.

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