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The *Yersinia enterocolitica* Motility Master Regulatory Operon, *flhDC*, Is Required for Flagellin Production, Swimming Motility, and Swarming Motility

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The ability to move over and colonize surface substrata has been linked to the formation of biofilms and to the virulence of some bacterial pathogens. Results from this study show that the gastrointestinal pathogen *Yersinia enterocolitica* can migrate over and colonize surfaces by swarming motility, a form of cooperative multicellular behavior. Immunoblot analysis and electron microscopy indicated that swarming motility is dependent on the same flagellum organelle that is required for swimming motility, which occurs in fluid environments. Furthermore, motility genes such as *flgEF*, *flgMN*, *flhBA*, and *fliA*, known to be required for the production of flagella, are essential for swarming motility. To begin to investigate how environmental signals are processed and integrated by *Y. enterocolitica* to stimulate the production of flagella and regulate these two forms of cell migration, the motility master regulatory operon, *flhDC*, was cloned. Mutations within *flhDC* completely abolished swimming motility, swarming motility, and flagellin production. DNA sequence analysis revealed that this locus is similar to motility master regulatory operons of other gram-negative bacteria. Genetic complementation and functional analysis of *flhDC* indicated that it is required for the production of flagella. When *flhDC* was expressed from an inducible p*tac* promoter, flagellin production was shown to be dependent on levels of *flhDC* expression. Phenotypically, induction of the p*tac-flhDC* fusion also corresponded to increased levels of both swimming and swarming motility.

The survival of bacteria that exist both free living and in association with a susceptible host depends on the appropriate coordination of physiological responses to environmental changes such as temperature, pH, osmolarity, and nutrient availability. *Yersinia enterocolitica* is a gastrointestinal pathogen of humans and a variety of other mammals (12). It has a bipartite life cycle distinguished by a free-living phase and a host-dependent phase. During the free-living phase, *Y. enterocolitica* survives for extended periods in terrestrial and aquatic environments where ambient temperatures are less than 28°C (12). At temperatures above 35°C in a susceptible host, *Y. enterocolitica* phenotypically expresses a range of proteins that allow it to evade the immune response and parasitize essential nutrients (13, 60). During the free-living phase, *Y. enterocolitica* expresses genes such as *inv*, *yplA*, and the *ure* gene cluster (encoding invasin, phospholipase, and urease respectively) which contribute to pathogenesis at the earliest stages of an infection (14, 45, 50, 59). In vitro these genes are highly expressed at low temperatures but generally are repressed at temperatures that are found in the host (15, 44, 61). Phenotypically, all of these genes are also expressed in a growth phase-specific manner when *Y. enterocolitica* is grown in liquid cultures. Genes which are required for the production of flagella (such as *fliA* and the *fleABC* cluster) exhibit a similar phase-specific expression pattern (28, 29). The role of motility in pathogenesis has remained enigmatic for many bacteria, but genetic loci that encode factors involved with bacterium-host interactions are often coordinately regulated by environmental stimuli which also regulate motility (41, 43). For *Y. enterocolitica*, while there is no evidence defining a direct role for motility in bacterium-host interactions, the similarity between the expression patterns of motility, invasin, phospholipase, and urease suggests that regulation of genes encoding these factors may be coordinated. Moreover, this correlation is strengthened by recent evidence demonstrating that some mutations that affect the expression of invasin also affect motility (9). To determine if common regulatory pathways or direct links exist between the motility regulon and the expression of genes encoding early-stage virulence factors, we need to understand and characterize the motility regulon in greater detail.

For motility, *Y. enterocolitica* produces peritrichously arranged flagella that are probably synthesized in the same way as occurs in other peritrichously flagellated gram-negative bacteria. The paradigm for the flagellum organelle biosynthetic pathway in this group of bacteria comes from studies of *Escherichia coli* and *Salmonella typhimurium* (1, 36). In these bacteria, biosynthesis of the motive organelle involves the sequential expression, localization, and assembly of subunits leading to a mature flagellum. It has also been shown that regulation of the flagellum biosynthetic pathway in *E. coli* and *S. typhimurium* occurs at the transcriptional level where more than 40 genes, including those encoding the chemosensory apparatus, are organized in a cascade. These motility genes are expressed in a hierarchical fashion, allowing the bacteria to stringently control the production and assembly of flagellum subunits in response to environmental signals and by sensing organelle structural intermediates. The entire cascade is ultimately governed by the products of the motility master regulatory operon consisting of *flhD* and *flhC* (10, 35).

Originally, *flhDC* was considered to play an exclusive role in regulating the production of flagella for swimming motility

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(53), but recent studies of this locus suggest that it plays a more global role in regulating diverse physiological processes (46, 47). It is required for the expression of *phlA* of *Serratia liquefaciens*, which encodes an extracellular phospholipase (23). Interestingly, PhlA is related to the virulence-associated phospholipase of *Y. enterocolitica* encoded by *yplA* (50). Expression of *flhDC* in *Proteus mirabilis* and *S. liquefaciens* is required for both the production of flagella and the differentiation of these bacteria from vegetative cells into swarm cells (17, 18, 22). Swarm cell differentiation is required for a form of multicellular behavior where bacterial communities cooperatively migrate over and colonize surface substrata (24). Swarming motility is a flagellum-dependent behavior that allows bacteria to move over solid surfaces and is distinct from swimming motility, which occurs in fluid environments. This multicellular behavior has been implicated in the formation of biofilms and in bacterial pathogenesis (24). Motility and swarm cell differentiation also have been shown in *P. mirabilis* to correlate with increased expression of several virulence-associated phenotypes including invasion of urothelial cells and the production of urease, protease, and hemolysin (3, 5). This study focuses on defining the conditions that affect the expression of motility, including swarm behavior, by *Y. enterocolitica* and characterization of the motility master regulatory operon. With these results, it will be possible to investigate how this locus integrates signals to regulate flagellum production, multicellular behavior, and the expression of virulence factors contributing to the initial stages of pathogenesis.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. Bacteria were generally grown in Luria-Bertani (LB) broth and maintained on LB agar plates (42). Except where indicated, semisolid medium for the examination of swimming motility contained 0.35% Difco agar and that for the examination of swarming motility (surface migration) contained 0.6% Difco agar. T medium consisted of 1% (wt/vol) tryptone (Difco) containing agar as indicated. Minimal medium was M9 (51), M63 (51), or 20 mM phosphate (pH 7). Media were supplemented with Casamino Acids (Difco), arabinose, glucose, glycerol, lactose, rhamnose, sucrose, or yeast extract as indicated. When necessary for selection in *E. coli*, antibiotics were included in the media at the following final concentrations: chloramphenicol, 15 μg/ml; gentamicin, 50 μg/ml; kanamycin, 50 μg/ml; nalidixic acid, 20 μg/ml; and tetracycline, 15 μg/ml. For *Y. enterocolitica*, the antibiotic concentrations were reduced to half the concentrations listed above except for gentamicin and nalidixic acid, which were used at the same concentrations as those listed above. All *Y. enterocolitica* strains were grown at 26°C, and *E. coli* strains were grown at 37°C. *E. coli* DH5α, LE392 and S17-1λ(*pir*) were used for cloning experiments. *E. coli* S17-1λ(*pir*) was used to deliver mobilizable plasmids to *Y. enterocolitica*.

Assays for motility. Phenotypic assays for swimming and swarming were initiated by spotting 2 μl of an overnight culture at the center of agar plates containing 0.35% agar to evaluate swimming motility and 0.6% agar to evaluate swarming motility. The plates were analyzed after at least 16 h of incubation at 26°C. Swimming motility was confirmed by microscopic examination of bacteria under a phase-contrast microscope.

Molecular genetic techniques. Plasmid preparations and in vitro DNA manipulations such as restriction enzyme digestion, ligation, and electroporation were done by standard procedures (7). All restriction endonucleases and DNA ligase were purchased from New England Biolabs or Gibco BRL. The 5′ overhangs were made blunt by a fill-in reaction with Deep Vent Exo$^+$ DNA polymerase (New England Biolabs) as specified by the manufacturer. Southern hybridization was done essentially as described previously (55) with DNA probes labeled with [α-^{32}P]CTP (Amersham Pharmacia Biotech). DNA sequencing was performed by an automated procedure for both strands of the *flhDC* region reported in this study at Bio S&T (Lachine, Quebec, Canada) and by standard procedures by the authors. Ambiguities were clarified by resequencing specific regions of *flhDC*, which was done by the authors by standard procedures. The oligonucleotides used to resolve sequence ambiguities were purchased from Gibco BRL. DNA sequence analysis was performed with the GCG software package, version 9.0 (University of Wisconsin) and the BLAST programs available through the National Center for Biotechnology Information.

Cosmid library construction and cloning of the *flhDC*, *flhBAE*, and *flgEFG* loci. A cosmid library of *Y. enterocolitica* JB580v was constructed in vector pLAFR2 (11) and in λ EMBL3. The λ EMBL3 cosmid library was described previously (28). For the library constructed in pLAFR2, genomic DNA was isolated from strain 8081v and partially digested with *Sau*3AI. Random fragments of DNA were ligated into pLAFR2, packaged into bacteriophage λ by using the Gigapack II in vitro phage-packaging system (Stratagene), and recovered in *E. coli* LE392. To clone the *Y. enterocolitica flhDC* locus, the pLAFR2 cosmid library was transferred by triparental mating to *E. coli* MC4100, which harbors a point mutation mapping to *flhD*. Complementing clones were identified as *E. coli* MC4100 strains that received *Y. enterocolitica* genomic DNA that restored motility. To ensure that the entire *Y. enterocolitica* locus was present, candidate cosmid clones were also transformed into *E. coli* YK4131 (harboring a different point mutation mapping to *flhDC*) and MC1000 *flhD*::Kn (*flhD* and *flhC* due to polarity). This procedure resulted in a cosmid, designated pGY5-21, that was used as the source of DNA for the functional analysis described in this study (Table 1). In addition, an independent clone of the *flhDC* locus was isolated from the λ EMBL3 cosmid library by hybridization analysis with sequences corresponding to *flhDC* of *S. typhimurium* and was shown to contain the same chromosomal region present in pGY5-21.

The *flgDEFG* and *flhBAE* gene clusters were cloned by screening the λ EMBL3 cosmid library with DNA probes corresponding to *S. typhimurium flhA* and *flgE* sequences (54). Both of the clones obtained from the λ EMBL3 library were confirmed by DNA sequence analysis and designated EMBL3-C (*flgDEFG* clone) and EMBL3-M (*flhBAE* clone). A 4-kb *Bam*HI-*Sal*I fragment containing *flgDEF* was subcloned from EMBL3-C into pACYC184. Subsequently, a gentamicin resistance cassette was inserted into this plasmid, resulting in the final construct, pACYC4GM (Table 1), which was used for complementation analysis. A 7-kb *Eco*RV-*Bam*HI fragment was subcloned from EMBL3-M into pACYC177. This plasmid, designated pMS*flh* (Table 1), was used for complementation analysis.

Construction of the *flgE*, *flgF*, *flhA*, and *flhB* insertion mutations. Insertion mutations were constructed in *flgE*, *flgF*, *flhA*, and *flhB* by allelic exchange (Table 1). For the *flhA* mutant, a 7-kb *Eco*RV-*Bgl*II fragment containing *flhBAE* was subcloned from the cosmid EMBL3-M (described above) into pBluescript KS+ (Stratagene, La Jolla, Calif.) digested with *Eco*RV and *Bam*HI. A chloramphenicol acetyltransferase gene (*cat*), derived by *Bam*HI digestion of pPY1034 (58), was cloned into the single *Bam*HI site in *flhA*. Additionally, a 3.8-kb *Xba*I fragment containing *nptI* (Kan^r) and *sacB* (sucrose sensitivity), derived from plasmid pRL250 (34), was inserted into the single *Xba*I site. The recombinant plasmid, designated pMSΩ*flhA*, was recovered by electroporation of *E. coli* DH5α. For allelic exchange, pMSΩ*flhA* was electroporated into *Y. enterocolitica* JB580v with selection for Cm^r and Kan^r. In *Y. enterocolitica*, the plasmid could not be reisolated by standard plasmid purification procedures. This suggested that it had integrated into the chromosome by homologous recombination, resulting in a cointegrate. These recombinants were then plated on LB agar lacking NaCl but containing 5% sucrose and chloramphenicol to select for a second recombination event. Sucrose-tolerant, Cm^r mutants were then screened for Kan^s and loss of motility (due to loss of the plasmid sequences and the wild-type *flhA* locus). One mutant with the appropriate phenotype was retained for further analysis and designated YMS12 (Table 1). A similar strategy was used to construct *flhB*, *flgE*, and *flgF* mutations, except that the *cat* cassette was inserted into unique *Cla*I, *Xba*I, and *Eco*RV sites, respectively (plasmids pMSΩ*flhB*, pCKΩ*flgE*, and pCKΩ*flgF* [Table 1]). The insertion mutations were introduced into the *Y. enterocolitica* chromosome by allelic exchange (as described above), and the mutant strains were designated YMS13, YCK10, and YCK11 (Table 1).

Construction of an *flhDC* insertion mutation and an *flhDC* internal deletion. An insertion mutation was constructed in the *flhDC* locus by using the suicide vector pEP185.2 (30). To construct the insertion mutation, the 4.3-kb *Eco*RI fragment from plasmid pGY10 was gel purified and then digested to completion with *Sau*3AI. These fragments were cloned into the suicide vector pEP185.2 and transferred to *Y. enterocolitica* JB580v. Strains harboring plasmids that integrated into the chromosome by homologous recombination were recovered by selecting for Cm^r. Recovered colonies were then screened for motility on T plates containing 0.35% agar. One colony was obtained that was completely defective for motility and was called GY357 (Table 1). The mutation harbored by GY357 was characterized by determining the target sequence of the suicide vector. The target sequence was determined by recovering the inserted suicide plasmid by a method designated in vivo conjugative cloning (see below for details). The DNA sequence of the target clone was then determined by standard procedures with oligonucleotides corresponding to the flanking ends of the plasmid.

A deletion within *flhDC* was constructed by subcloning the 0.9-kb *Bam*HI fragment encompassing the internal deletion from pGY11 into pEP185.2. This plasmid, designated pGY22, was transferred to *Y. enterocolitica* JB580v with selection for Cm^r. Recovered colonies containing plasmid cointegrates were identified by screening for the loss or reduction of both motility and phospholipase activity, which was recently shown to require functional *flhDC* for expression (61). These candidate cointegrates were then grown overnight in LB media without selection. Each day for 7 days, the bacteria were subcultured into fresh LB media and grown overnight. Subsequently, the cultures were harvested and subjected to cycloserine enrichment as described previously (30). Candidate strains that were cured of the integrated suicide plasmid but retained the deletion in the *flhDC* locus were phenotypically identified as Cm^s, nonmotile, and phospholipase negative. The deletion was then confirmed by Southern hybrid-

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant feature(s)	Source or reference
Y. enterocolitica		
8081v	Wild type	30
JB580v	8081v Δ*yenR* (R^- M^+)	30
GY357	JB580v *flhDC*::pEP185.2	This study
GY460	JB580v *flhDC*	This study
JO1v	JB580v *flgM*::Kan	29
VK1	JB580v *fliA*::Str	29
YCK10	JB580v *flgE*::Cm	This study
YCK11	JB580v *flgF*::Cm	This study
YMS12	JB580v *flhA*::Cm	This study
YMS13	JB580v *flhB*::Cm	This study
E. coli		
LE392	F^- *mcrA hsdR514* (R^- M^+) *supE44 supF58 lacY1 galK2 galT22 metB1 trpRJS*	37
DH5α	F′/*endA1 hsdR17* (R^- M^+) *supE44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*)*U169*, *deoR* [ϕ80d *lac*Δ(*lacZ*)M15]	37
MC4100	F^- *araD139* Δ(*lacZYA-argF*)*U169 rpsL150 relA1 flhD5301 deoC1 ptsF25 rbsR*	51
MC1000	F^- *araD139 galU galK* Δ(*lacZYA-argF*)*U169 rpsL150*	47
MC1000*flhD*::Kan	MC1000 but *flhD*::Kan	47
S17-1 λ(*pir*)	*pro thi hsdR514* (R^+ M^-), Δ*recA*, integrated RP4 2-Tc::Mu-Kn::Tn*7* (Tp^r Str^r)	52
YK4131	F^- *araD139* Δ(*lacZYA-argF*)*U169*	32
Plsamids		
pACYC177	Medium-copy cloning vector, Kn^r	37
pACYC184	Medium-copy cloning vector, Cm^r Tet^r	37
pBluescript KS+	High-copy cloning vector	Stratagene
pEP185.2	*mob*$^+$, *pir*-dependent *ori*R6K, Cm^r	30
pLAFR2	Cosmid, Tet^r	11
Litmus 28	High-copy cloning vector	New England Biolabs
pRK2013	*mob*$^+$ *tra*$^+$, self-transmissible plasmid, Kan^r	21
pACYC4GM	pACYC184 containing a 4.0-kb *flgDEF* clone and a gentamicin resistance cassette	This study
pCKΩ*flgE*	pBluescript KS+ with *flgE*::*cat*, *nptI*, and *sacB*	This study
pCKΩ*flgF*	pBluescript KS+ with *flgF*::*cat*, *nptI*, and *sacB*	This study
pJB222	pTM100 containing *fliA*, Tet^r	8
pMG600	pVLT33 with the *S. liquefaciens flhDC* locus cloned downstream of the p*tac* promoter as an *Eco*RI fragment, Kan^r	23
pMS*flh*	pACYC177 with a 7-kb *flhBAE* clone	This study
pMSΩ*flhA*	pBluescript KS+ with *flhA*::*cat*, *nptI*, and *sacB*	This study
pMSΩ*flhB*	Litmus 28 with *flhB*::*cat*, *nptI*, and *sacB*	This study
pSWIM1	pTM100 containing *flgMN*	29
PTM100	*mob*$^+$, derivative of pACYC184, Cm^r Tet^r	39
pVLT33	*mob*$^+$, low-copy vector containing an inducible p*tac* promoter, Kan^r	16
pWKS130	Low-copy cloning vector, *ori* pSC101, Kan^r	56
pGY5-21	pLAFR2 cosmid containing a ca. 10-kb insert of *Y. enterocolitica* chromosomal DNA containing the *flhDC* locus, Tet^r	This study
pGY10	pTM100 with a 4.3-kb *Eco*RI clone of *flhDC* originating from pGY5-22	This study
pGY11	Δ*Hpa*I of pGY10	This study
pGY12	Δ*Sac*II of pGY10	This study
pGY13	Δ*Bam*HI of pGY15	This study
pGY14	Δ*Nco*I of pGY10	This study
pGY15	Δ*Eco*RV to *Bst*Z17-I of pGY10	This study
pGY16	Δ*Eco*RI to *Pvu*II of pGY15	This study
pGY17	Δ*Eco*RI to *Bst*EII of pGY15	This study
pGY18	Δ*Eco*RI to *Spe*I of pGY15	This study
pGY19	pWKS130 with 1.4-kb *Bam*HI *flhDC* fragment from pGY10	This study
pGY20	pVLT33 with 1.4-kb *Bam*HI *flhDC* fragment from pGY10 placing *flhDC* under control of an inducible p*tac* promoter	This study
pGY21	ca. 100-bp *Sau*3AI fragment from pGY10 cloned into the suicide vector pEP185.2	This study
pGY22	ca. 500-bp *Bam*HI fragment encompassing the *flhDC* deletion from pGY11 cloned into the suicide vector pEP185.2	This study

ization analysis (data not shown). One strain harboring the *flhDC* internal deletion, designated GY460 (Table 1), was retained for further study.

In vivo conjugative cloning for the characterization of the chromosomal integration site of a suicide plasmid. The above-mentioned procedure, in vivo conjugative cloning, allows suicide plasmids to be recovered without in vitro manipulation of DNA. A similar procedure was previously reported and is adapted here for use in *Yersinia enterocolitica* (48). In vivo conjugative cloning was done by triparental mating with *E. coli* DH5α/pRK2013, *E. coli* S17-1λ(*pir*), and *Y. enterocolitica* GY357, which were coincubated overnight at 26°C on LB agar plates. The plasmid was recovered by being mobilized into the replication-permissive recipient strain, *E. coli* S17-1λ(*pir*), by selection for Str^r and Cm^r. The recovered plasmid, designated pGY21 (Table 1), contained a copy of the chromosomal target, which was then sequenced by standard procedures and compared to the complete *flhDC* sequence (see Fig. 5).

Construction of plasmid-located deletions of the *flhDC* locus. Plasmid pGY10 was constructed by subcloning a 4.3-kb fragment of DNA from cosmid pGY5-27 into the *Eco*RI site of pTM100 (Table 1). The series of plasmid-encoded *flhDC* deletions were derived from pGY10 by standard procedures (Table 1). All plasmids derived from pGY10 were recovered by electroporation of *E. coli* S-17λ(*pir*) with selection for Tet^r and transferred to *Y. enterocolitica* by conjugation as described previously (52).

Plasmid pGY20 was derived by subcloning the 1.4-kb *Bam*HI fragment from pGY10 into the *Bam*HI site of pVLT33 (Table 1) (16). Plasmid recombinants were recovered after electroporation of *E. coli* S17-1λ(*pir*) with selection for Kan^r. Plasmid pGY20 was transferred to *Y. enterocolitica* by conjugation as described previously (52). Plasmid pGY19 was derived by subcloning the 1.4-kb *Bam*HI fragment from pGY10 into the *Bam*HI site of the low-copy-number cloning vector pWKS130 (Table 1) and recovered after electroporation of *E. coli* DH5α with selection for Kan^r. Plasmids derived from pWKS130 were transferred to *Y. enterocolitica* by electroporation (30).

Protein gel and immunoblot analysis. Supernatant fractions containing flagellin were prepared from cultures grown in T broth at 26°C on a roller drum. To harvest flagellin and extracellular proteins, the cultures were vortexed for 3 min and then centrifuged at 8,000 × *g* for 3 min. The supernatant fraction was collected and filtered through a 0.2-μm-pore-size nonpyrogenic filter (Gelman Sciences, Ann Arbor, Mich.), and proteins were concentrated by precipitation with ice-cold trichloroacetic acid at a final concentration of 10% (wt/vol) for 30 min. After centrifugation at 8,000 × *g* for 15 min, the protein pellet was washed with ice-cold acetone and incubated on ice for 10 min. The final protein pellet was collected by centrifugation at 8,000 × *g* for 15 min. Protein samples were resuspended in sample buffer containing β-mercaptoethanol (37) and normalized with respect to the optical density at 600 nm (OD_{600}) of the culture. For samples harvested directly from agar plates, bacteria were collected by resuspension in phosphate-buffered saline, the OD_{600} was determined, and concentrated sample buffer containing β-mercaptoethanol was added. After being heated to 95°C for 10 min, all the samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5% polyacrylamide) (33). The gels were then stained with Coomassie brilliant blue, or the proteins were transferred to a nitrocellulose membrane for immunoblot analysis (7). Immunoblot analysis was performed with monoclonal antibody 15D8 (Igen Inc.) as the primary antibody to detect flagellin proteins (28). Reactive antigen was detected with goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase (Sigma) as the secondary antibody and visualized with a chemiluminescent substrate (Amersham).

Microscopy. Bacteria were examined for general cell morphology by phase-contrast microscopy (Olympus BH-2 microscope). Samples for phase-contrast microscopy were prepared as wet mounts in T medium or phosphate-buffered saline. For photographic purposes, bacteria were examined by phase-contrast microscopy with an Olympus BS60 microscope. Bacteria examined by electron microscopy to confirm cell morphology and the presence of flagella were resuspended from solid media in phosphate-buffered saline. Samples were then negatively stained with uranyl acetate and examined by electron microscopy (Zeiss 902A microscope).

Nucleotide sequence accession number. The compiled sequence of *flhDC* was submitted to GenBank under accession no. AF081587.

RESULTS

Medium ionic strength and availability of a carbon source affect *Y. enterocolitica* motility. Previous studies of swimming motility with LB or tryptic soy media established that flagellar biogenesis by *Y. enterocolitica* occurs only at temperatures below 28°C (19, 27–29). Regulation of this process occurs at the transcriptional level due to repression of *fliA* transcription at temperatures above 28°C (28, 29). Ultimately, we would like to know the role of motility in *Y. enterocolitica* pathogenesis because initial bacterium-host interactions might be affected by flagella on the bacterial surface or as a result of coordinate regulation of motility and virulence factor expression. Either hypothesis requires an understanding of growth conditions that best stimulate the expression of motility. A survey of motility on semisolid agar (0.35%) revealed that LB (1% tryptone, 0.5% yeast extract, 1% NaCl) was a poor medium for expression of this phenotype compared to T medium (1% tryptone) (Fig. 1). It is possible that motility was greater in T medium as a result of nutrient depletion and subsequent chemotaxis, which might be delayed in the nutrient-rich LB. To address this, yeast extract (0.5%) was added to T medium, effectively making it as nutrient-rich as LB medium. However, this did not result in any significant phenotypic decrease in motility. In contrast, increasing the ionic strength of the medium did affect motility. When NaCl (1%) was included in T medium, motility was inhibited (Fig. 1). Replacement of NaCl with KCl had a similar inhibitory effect (data not shown), but there was no inhibition of motility by replacement with the sugars lactose or rhamnose, which are not metabolized by *Y. enterocolitica* (Fig. 1); this indicated that expression of motility was sensitive to increased ionic strength of the medium rather than to a general increase in osmolarity. Other media examined that promoted motility included minimal M9 supplemented with 100 mM glycerol or 100 mM glucose, 20 mM phosphate (pH 7.0) and T medium supplemented with 100 mM arabinose or 100 mM glucose. Interestingly, the presence of arabinose or glucose in T medium stimulated *Y. enterocolitica* to translocate over the surface of the agar plate (Fig. 1) (see below). This surface translocation appeared to be phenotypically similar to the cooperative multicellular surface migration, designated swarming, that has been described for other bacteria such as *E. coli*, *P. mirabilis*, *S. typhimurium*, and *Serratia* species (2, 18, 25, 57).

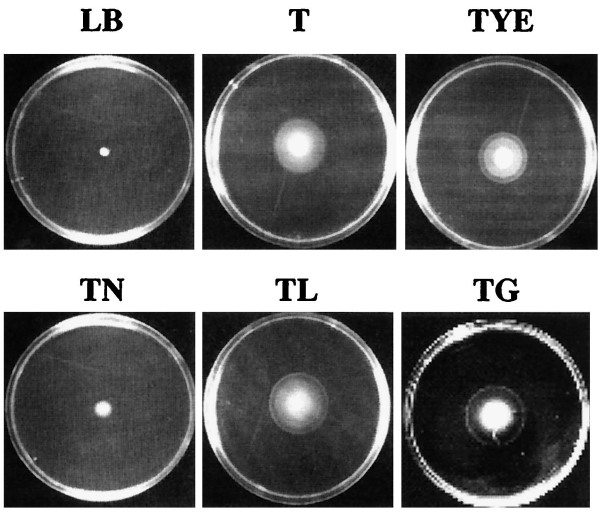

FIG. 1. Growth conditions affect *Y. enterocolitica* motility. Each panel shows an image of *Y. enterocolitica* JB580v grown at 26°C for 20 h on medium solidified with 0.35% Difco agar. The plates were inoculated by spotting 2 μl of culture into the center. LB medium (LB), T medium (T), T medium plus yeast extract (TYE), T medium plus NaCl (TN), T medium plus lactose (TL), and T medium plus glucose (TG) plates are shown.

***Y. enterocolitica* colonizes the surface substratum by swarming motility.** Surface translocation on media with more than 0.45% agar has been designated swarming motility. This distinguishes the phenomenon from swimming motility, which occurs in media containing less than 0.45% agar (24). *Y. enterocolitica* exclusively exhibited swarming motility on T medium containing 100 mM glucose (TG medium) when the agar concentration was increased above 0.45%. Under these conditions, the morphology of an advancing colony is similar to that of colonies of other bacterial genera known to translocate over agar surfaces (Fig. 2). Swarming motility by *Y. enterocolitica* was gradually inhibited by increasing agar concentrations, and inhibition was complete at 1.2% agar (data not shown). Further examination of swarming motility was done with media containing 0.6% agar. On TG medium, *Y. enterocolitica* showed progressive swarming. On T medium alone, *Y. enterocolitica* appeared to initiate swarming motility at the periphery of the colony but swarming was never progressive, indicating that a utilizable carbon and energy source was a necessary stimulus for colony migration. Progressive swarming was also

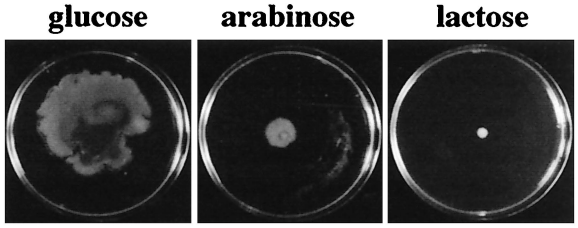

FIG. 2. Swarming motility is influenced by growth conditions. Each panel shows an a image of *Y. enterocolitica* grown at 26°C for 24 h on T medium solidified with 0.6% Difco agar and supplemented with carbon sources (indicated above each panel) at a final concentration of 100 mM. The plates were inoculated by spotting 2 μl of an overnight culture of *Y. enterocolitica* JB580v into the center.

stimulated by carbon sources other than glucose such as sucrose, arabinose, glycerol, and maltose (Fig. 2 and data not shown). Carbon and energy sources not metabolized by *Y. enterocolitica*, such as lactose and rhamnose, failed to stimulate swarming motility (Fig. 2 and data not shown). The addition of yeast extract to TG medium enhanced swarming by *Y. enterocolitica* but was not sufficient to stimulate translocation when added alone to T medium (data not shown). No surface translocation was observed on chemically defined glucose-containing media such as M9, M63, or a 20 mM phosphate base, even when they were supplemented with an exogenous source of amino acids in the form of Casamino Acids or tryptone. Swarming motility, like swimming motility, was inhibited by increasing the ionic strength of the media by adding NaCl or KCl (data not shown).

Mutations in flagellar genes affect both swimming and swarming motility. Swarming motility in bacteria closely related to *Y. enterocolitica* is flagellum dependent (2, 25, 57), suggesting that genes known to be required for the expression of swimming motility in *Y. enterocolitica* were likely to be essential for swarming motility. To examine this possibility, mutant strains of *Y. enterocolitica* that were unable to produce flagella were tested for swarming motility on TG medium containing 0.6% agar (Table 2). The results of this analysis showed that mutations in the flagellar gene clusters *flgEFG* (encoding subunits of the flagellum hook and rod), *flgMN* (encoding a flagellum-specific negative regulator and an accessory protein for filament assembly), and *flhBAE* (encoding subunits of the flagellum type III export machinery) or *fliA* (encoding the flagellum-specific alternate sigma factor) prevented both swimming and swarming motility (Table 2). Complementation of the *fliA*, *flgEF*, and *flhBA* mutations by a plasmid-located copy of the corresponding locus restored both forms of motility to wild-type levels. The mutation affecting *flgMN* expression was partially restored for swarming motility when complemented by a plasmid-located copy of *flgMN*. These results indicated that swarming motility by *Y. enterocolitica* is a flagellum dependent activity.

Cells induced for swarming are elongated compared to cells fully induced for swimming. For other bacteria such as *P. mirabilis*, a swarming colony is thought to go through cycles of expansion followed by consolidation (4, 57). During an expansion phase, a subset of vegetative cells at the periphery of the colony differentiate into swarmer cells, distinguished by cell elongation and a substantial increase in the number of flagella per cell. Movement over the bacterial surface appears to require swarmer cells which cooperatively migrate over surfaces en masse at the colony edge or as rafts of migrating swarmer cells temporarily leaving the colony behind. Visual examination of *Y. enterocolitica* under swarm-inducing conditions revealed similar behavioral patterns (Fig. 3A). Cells in a swarming colony were often organized into distinct terraces (Fig. 3A, top left). Cells in the first terrace were at the front of an advancing colony and appeared to move in parallel to each other. Cells in the second terrace overlaid those in the first and moved randomly. Surface migration at the colony periphery was generally en masse with occasional rafts of cells that temporarily migrated away from the colony forefront (Fig. 3A, top middle). Always preceding an expanding colony was a clear layer of material or slime layer (Fig. 3A, top left). Some individual *Y. enterocolitica* cells isolated from the periphery of a swarming colony appeared elongated compared to cells isolated from media that did not induce swarming motility (Fig. 3A). Often, the elongated cells appeared to be septated, possibly reflecting the frequency at which swarmer cells convert (dedifferentiate) to the vegetative form. Quantitation by immunoblot analysis of flagellin produced by bacteria resuspended directly from plates revealed that conditions that stimulated swarming motility (TG medium) resulted in high levels of flagellin production (Fig. 4). Similar levels of flagellin were detected for bacteria isolated from T medium plates, conditions that stimulate swimming but not swarming motility. In contrast, less flagellin was produced by bacteria isolated under conditions that do not stimulate motility. Also notable is the fact that the same flagellins were detected for bacteria isolated under swarm-induced and swarm-repressed conditions. Direct examination of bacteria isolated from swarming colonies by electron microscopy confirmed the results of immunoblot analysis (Fig. 3B). Bacteria isolated from T plates and TG plates generally had 8 to 10 flagella per cell. Occasionally, bacteria from TG medium had as many as 15 flagella per cell. By comparison, cells isolated under other conditions had zero to five flagella per cell.

Cloning of the *Y. enterocolitica flhDC* locus and characterization by DNA sequence analysis. In other bacteria, flagellum-dependent swarming involves bacterial differentiation, which requires increased expression of the flagellum biosynthetic pathway; this is usually modulated by the motility master regulatory operon, *flhDC* (18, 22, 26). Previously cloned *Y. enterocolitica* motility genes have a high similarity to motility genes of other gram-negative bacteria, indicating that the motility regulon is highly conserved (19, 27–29). Therefore, it was likely that *Y. enterocolitica* maintains a functional analogue of the motility

TABLE 2. Flagellar genes are required for swarming motility

Strain	Mutation	Plasmid	Swimming phenotype[a]	Swarming phenotype[b]
YCK10	*flgE*::Cm	None	−	−
		pAYCY4GM	+	+
YCK11	*flgF*::CM	None	−	−
		pACYC4GM	+	+
JO1v	*flgM*::Kan	pTM100	−	−
		pSWIM1	±	±
YSM12	*flhA*::Cm	pACYC177	−	−
		pMS*flh*	+	+
YSM13	*flhB*::Cm	pACYC177	−	−
		pMS*flh*	+	+
VK1	*fliA*::Str	pTM100	−	−
		pJB22	+	+

[a] Swimming was determined as migration from the source of inoculation on TG medium containing 0.35% agar at 26°C. Swimming was scored as positive (+), negative (−), or variable (±).

[b] Swarming was determined as surface migration on TG medium containing 0.6% agar at 26°C. Swarming was scored as positive (+), negative (−), or variable (±).

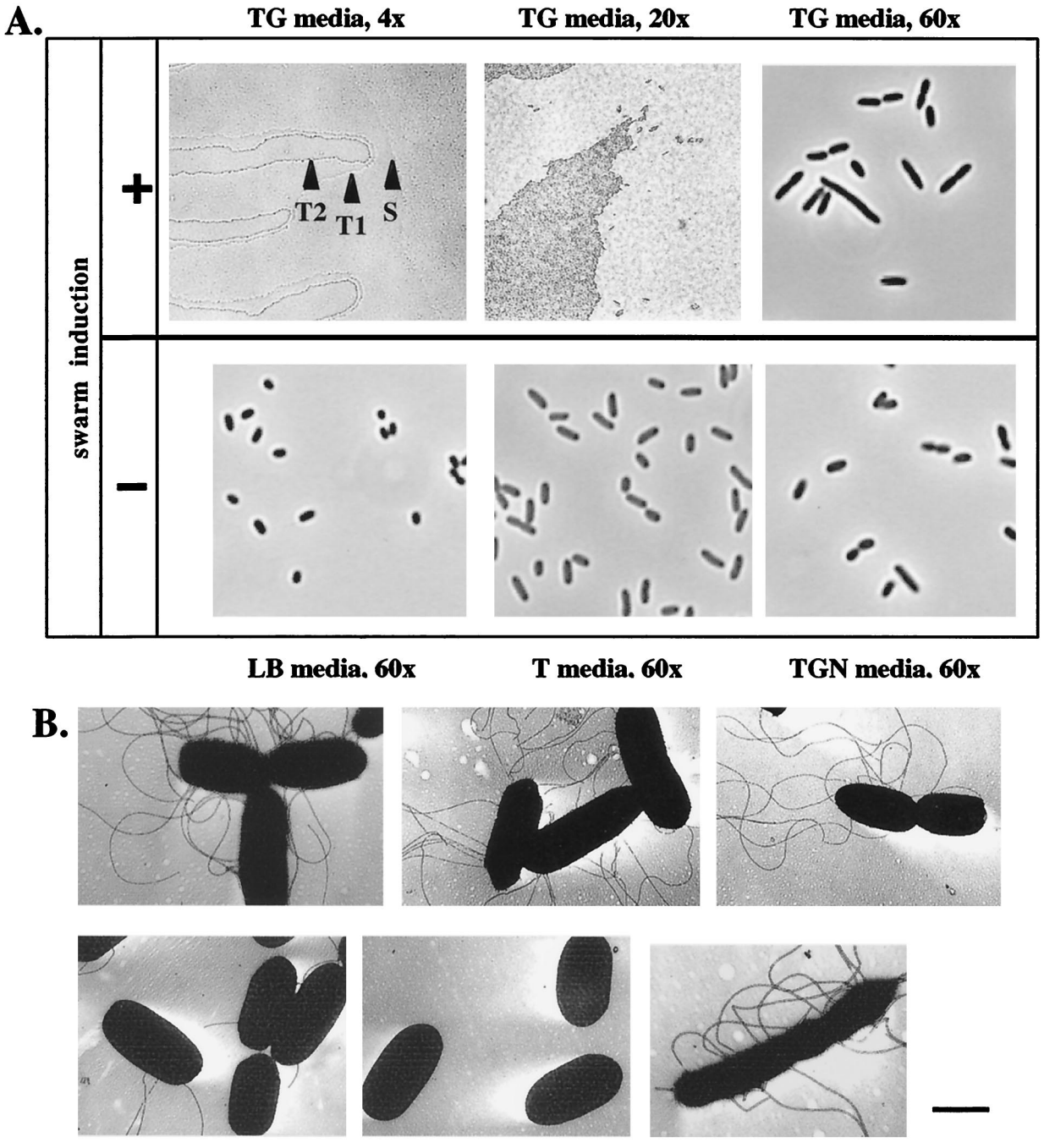

FIG. 3. Images of colony and cellular morphology of swarming bacteria suggest that *Y. enterocolitica* differentiates into cells that cooperatively migrate over agar surfaces. (A) The top row shows images of the morphology of an advancing colony of bacteria grown on a TG plate at 26°C, conditions that induce swarming motility. (Top left) Image of a colony at ×4 magnification; S indicates the location of the slime layer preceding the front of migrating colony; T1 indicates the first terrace of migrating cells in the swarming colony; T2 indicates the location of the second terrace of cells that appear to overlay cells forming the first terrace. (Top middle) Image of the same colony at ×20 magnification, showing rafts of cells that often advance ahead of a colony. (Top right) Image of elongated cells resuspended from a swarming colony taken at ×60 magnification. The bottom row shows images (at ×60 magnification) of bacteria grown on LB plates, T plates, or T plates containing 100 mM glucose and 1% NaCl (TGN) at 26°C, conditions that influence swimming motility but do not induce swarming motility. (B) Electron micrographs of bacteria isolated under conditions that influence motility. Bacteria were isolated from agar plates grown for 16 h at 26°C. (Top left) Cells isolated under conditions that induce swimming motility on T medium. (Top middle) Cells isolated under swarm-induced conditions on TG medium. (Top right) A single swarm cell that appears to be dedifferentiating. (Bottom left) Cells isolated from LB medium, which does not induce motility. (Bottom middle) cells isolated from TGN medium, which does not induce motility. (Bottom right) A single elongated swarm cell isolated under inducing conditions on TG medium. Bar, 1 μm.

master regulatory operon. To clone the *Y. enterocolitica flhDC* locus, a cosmid-located genomic library of *Y. enterocolitica* was transferred to *E. coli* MC4100, which harbors a point mutation mapping to *flhD* (see Materials and Methods for details). This procedure identified a single cosmid (pGY5-21) which complemented all the *E. coli flhDC* mutations tested. A ca. 4.3-kb *Eco*RI fragment retaining the ability to complement the different *E. coli* mutations was subcloned (pGY10) and charac-

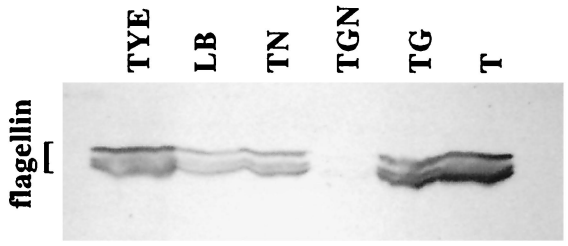

FIG. 4. Detection of flagellin proteins produced by cells isolated from media that influence the expression of swimming and swarming motility. Proteins were detected by immunoblot analysis with an anti-flagellin-specific monoclonal antibody. Each lane contains cells at 0.25 OD_{600} units per ml, isolated from 0.6% agar plates incubated for 18 h at 26°C. Lanes: TYE, T medium supplemented with yeast extract; LB, LB medium; TN, T medium supplemented with 1% NaCl; TGN, TG medium supplemented with 1% NaCl; TG, TG medium; T, T medium. The bracket at the left of the figure indicates the location of the detected flagellin.

terized by DNA sequence analysis. Examination of the DNA sequence revealed four open reading frames (ORFs) with similarity to the previously characterized *E. coli* genes *uspA*, *flhD*, *flhC*, and *motA* (Fig. 5A). The highest degree of identity for the predicted FlhD and FlhC proteins was to FlhD and FlhC of *P. mirabilis* (84 and 81%, respectively) and *Serratia liquefaciens* (85 and 86%, respectively). Given the high degree of identity and the conserved organization between the *Y. enterocolitica* and *E. coli* loci, these four ORFs were designated *uspA*, *flhD*, *flhC*, and *motA*. Transcription of *uspA* is divergent compared to *flhD*, with an intergenic region of 1,017 bp (Fig. 5A). The *flhD* and *flhC* ORFs are situated in the same orientation and are separated by only three nucleotides, suggesting that they form an operon. Upstream of *flhD* there is no obvious −10 promoter region, but five palindromic sequences that could function as *cis*-acting regulatory sites were identified centered 19, 44, 334, 398, and 684 bp upstream of *flhD* (Fig. 5B). The palindromic sequence centered 334 bp upstream of *flhD* is identical to the proposed consensus *E. coli* cyclic AMP receptor protein binding site ($TGTGAN_6TCACA$), suggesting that expression of *flhDC* may be influenced by growth conditions affecting the levels of cyclic AMP. The *motA* gene is located 137 bp downstream of and transcribed in the same direction as *flhDC*.

Construction of *flhDC* mutations. A mutation within the *flhDC* locus was constructed by site-directed insertion mutagenesis with the suicide plasmid pEP185.2 as described in Materials and Methods; this mutant was designated GY357. Southern blot analysis showed that the suicide plasmid had inserted into a region corresponding to the 5′ end of *flhD* (data not shown). To verify the mutation, the integrated plasmid was recovered by in vivo conjugative cloning from *Y. enterocolitica* and the DNA sequence of the fragment that served as the insertion target was determined (Fig. 5B; see Materials and Methods for details). These results showed that the target sequence was 130 bp and mapped immediately upstream of *flhD*. This mutant was defective for swimming motility, swarming motility, and flagellin production (data not shown). Consistent with the DNA sequence analysis, these results suggest that the promoter region for *flhDC* includes sequences more than 208 bp upstream of the *flhD* ORF. However, to be certain that the phenotype of the insertion mutation was due to the loss of *flhDC* expression, a deletion mutant, GY460, was constructed (see Materials and Methods for details). GY460 has a deletion removing the final 165 bp of the *flhD* ORF and the initial 352 bp of the *flhC* ORF. This strain was phenotypically indistinguishable from the strain carrying the upstream insertion mutation (data not shown).

Complementation analysis shows that *flhDC* is required for expression of swimming and swarming motility. The functional boundaries of the *flhDC* locus were examined by constructing a series of plasmid-encoded deletion mutants (Fig. 5A). Each deletion was assayed for the ability to phenotypically complement the chromosomal *flhDC* mutations in *trans*. From the 3′ end (downstream region) of *flhDC*, complementation was maintained as long as the deletion did not extend into the *flhC* ORF. Any deletion removing sequences that included the *flhD* or *flhC* ORF resulted in a complete loss of complementation for motility. Deletions of *uspA* had no effect on complementation. However, all complementation was eliminated when the 5′ deletions eliminated sequences extending within the 308 bp of *flhD*. These results show that the chromosomal mutations of *flhDC* used in this study are recessive to a functional copy of *flhDC* when provided in *trans* on a plasmid. Consistent with the results of the DNA sequence analysis and the phenotype of the insertion mutant, these results also indicate that the region required for complementation extends more than 308 bp upstream of the *flhD* ORF, suggesting that this region is required for regulation of *flhDC* expression. There is no obvious ORF present in this sequence, and additional experiments are required to determine the function of this *cis*-acting regulatory site. Additional evidence implicating a regulatory role for the upstream region came from the observation that complementation was restored when the *flhDC* ORFs were cloned downstream of an exogenous p*tac* promoter (Fig. 5A) (see below).

Flagellin protein synthesis and motility rate are regulated by expression of *flhDC*. To establish whether flagellin production is dependent on *flhDC* expression, DNA containing the *flhDC* ORFs was cloned immediately downstream of the p*tac* promoter on a low-copy-number plasmid (pGY20). This transcriptional fusion can be induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to the media. When examined in strain GY357, increased expression of p*tac-flhDC* resulted in increased flagellin synthesis (Fig. 6A). No flagellin was detected for GY357 containing only the cloning vector. As a positive control, GY357 was transformed with pMG600, which contains the *S. liquefaciens* 5′ region and the *flhDC* locus cloned downstream of the p*tac* promoter. *Y. enterocolitica* GM357 transformed with pMG600 resulted in constitutive levels of flagellin (Fig. 6A). Induction of the *S. liquefaciens*-derived p*tac-flhDC* by the addition of IPTG resulted in a superinduction of flagellin synthesis (data not shown). In addition to the flagellin proteins, three other *flhDC*-dependent proteins were produced in culture supernatants (Fig. 6A). These three proteins are immunologically distinct from the flagellins since they do not react with a flagellin-specific monoclonal antibody (data not shown). The function of these proteins is not known, but preliminary studies have indicated that the 35 kDa protein is the virulence-associated phospholipase, YplA (61). Examination of these strains for swimming and swarming motility revealed that increased levels of flagellin corresponded to an increase in swimming and swarming motility (Fig. 6B and C). Taken together, these results show that levels of *flhDC* expression directly influence both swimming and swarming motility. They also show that FlhD and FlhC proteins from *S. liquefaciens* and *Y. enterocolitica* are functionally interchangeable. Interestingly, microscopic examination of *Y. enterocolitica* GY357 harboring pGY20 or pMG600 (induced with as much as 1 mM IPTG) did not result in unusual cell elongation (data not shown) as has been reported for *S. liquefaciens* (18). This may reflect differences in the roles of *flhDC* in elaborating swarm cell development in these bacteria.

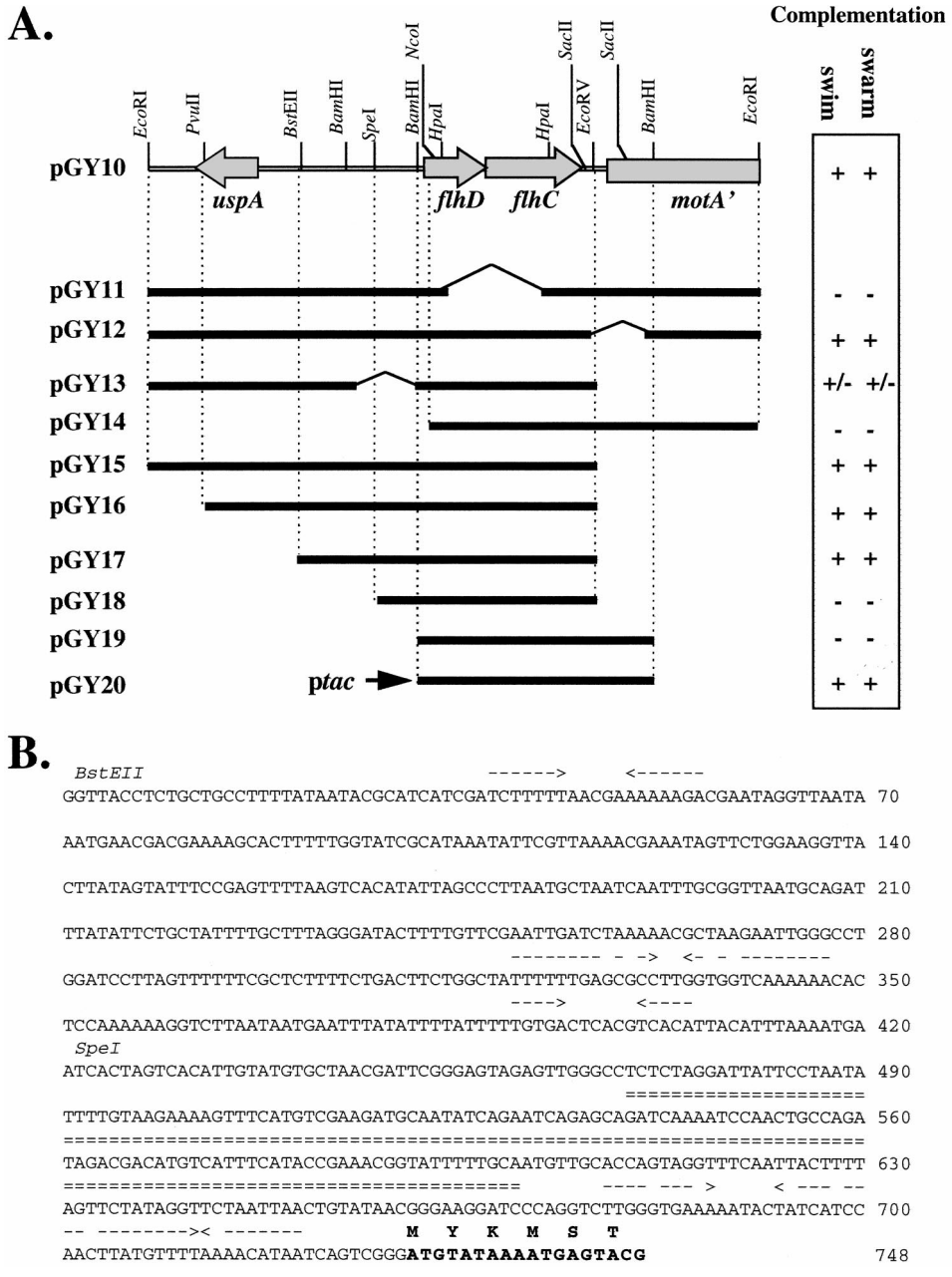

FIG. 5. Genetic and functional analysis of the *Y. enterocolitica* chromosomal region encompassing the motility master regulatory operon. (A) Schematic representation of the *flhDC* locus. Shaded boxes indicate the locations and directions of the ORFs identified by DNA sequence analysis, and relevant restriction sites are shown above the diagram. The series of plasmid deletions are shown below. Heavy black lines indicate the DNA present in each clone, and thin black lines indicate internal regions that were deleted. Plasmids pGY11 to pGY18 are derived from pGY10. Plasmids pGY19 and pGY20 contain a fragment of DNA subcloned from pGY10 into pWKS130 and pVLT33, respectively. The box at the right shows the results of functional complementation by each plasmid for the chromosomal *flhDC* mutations described in this study. A positive result (+) for swimming motility or swarming was determined as migration extending away from the point of inoculation on TG medium containing 0.35 or 0.6% agar, respectively. A negative result (−) was determined as bacterial growth at the point of inoculation but no apparent migration. An intermediate result (+/−) was determined as significantly reduced levels of migration. All of the functional assays were compared to the swimming and swarming motility of the wild-type strain, JB580v. (B) The DNA sequence of the chromosomal region upstream of *flhD*. The upstream nucleotide sequence is shown in standard type, and the bold type shows the first 18 nucleotides of *flhD*. Above the DNA sequence, single bold letters show the first 6 amino acid residues predicted for FlhD based on the DNA sequence; convergent dashed lines indicate palindromic sequences that may serve as *cis*-acting regulatory sites; the double dashed line indicates the location of the insertion for the chromosomally integrated suicide vector in *Y. enterocolitica* GY357; italic type indicates the locations of relevant restriction sites.

DISCUSSION

Y. enterocolitica is a gastrointestinal pathogen that infects a variety of mammals including humans (12). This bacterial pathogen has a life cycle consisting of repeated passage through the host environment followed by periods of free living in terrestrial and aquatic environments. During its life cycle, *Y. enterocolitica* appears to adjust to its surroundings by adjusting its physiology to enhance its ability to survive these environmental changes. In natural environments, these pathogenic

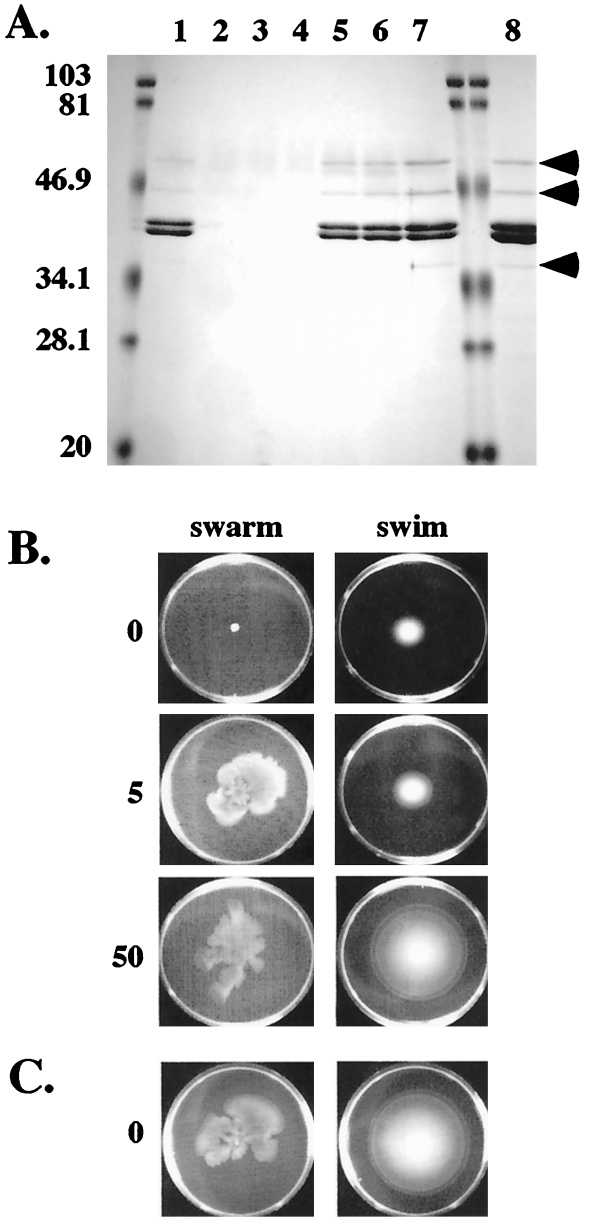

FIG. 6. Extracellular protein production, swimming motility, and swarming motility are affected by the levels of *flhDC* when expressed under the control of a p*tac* promoter. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5% polyacrylamide) of extracellular protein samples stained with Coomassie brilliant blue showing the production of flagellins and several other extracellular proteins (arrowheads) induced by increased levels of IPTG in the growth medium. Lanes: 1, wild-type strain JB580v grown in T broth; 2 to 4, GY357/pVLT33 grown in T broth with 0, 5, and 50 μM IPTG, respectively; 5 to 7, GY357/pGY20 grown in T broth with 0, 5, and 50 μM IPTG, respectively; 8, GY357/pMG600 grown in T broth. Unlabeled lanes contain protein molecular mass standards with their corresponding size in kilodaltons indicated on the left of the gel. (B) Swimming motility (right) and swarming motility (left) for GY357/pGY20. Cells were grown on TG medium with 0, 5, and 50 μM IPTG (indicated on the left of the figure). All the plates were inoculated by spotting 2 μl of an overnight culture in the center and were incubated at 26°C for 24 h. (C) Swimming (right) and swarming (left) motility of GY357/pMG600 on TG medium with no IPTG. The culture conditions were the same as those described for panel B.

bacteria interact with other bacteria, the surfaces that they contact during free-living growth, and the surfaces that they contact during growth in a susceptible host. In each case, flagella may influence these interactions. The results of this study show that swarming motility and, more specifically, flagella are required for migration over and colonization of surfaces by *Y. enterocolitica*. The existence of multicellular behavior in this organism raises many questions about the ecological niche that *Y. enterocolitica* occupies. It is not known to be motile in the laboratory at temperatures found in the host (37°C), suggesting that swarming behavior is important for survival only during the free-living phase. However, it is possible that expression of swarm behavior is induced by other stimuli in the host. Indeed, repression of some genes such as *inv* and *yst* by temperature can be relieved by other environmental factors (40, 44). Even if motility is repressed in the host environment, the presence of flagella or swarm cell differentiation may influence initial pathogen-host interactions, as has been documented for *P. mirabilis* (3, 5).

The swarming response of *Y. enterocolitica* is similar to that which occurs for other gram-negative enteric bacteria on media solidified with relatively low agar concentrations ranging from 0.4 to 1.2% (2, 25). The growth conditions that promote swimming and swarming motility in *Y. enterocolitica* are similar to the conditions that stimulate these activities in other bacteria (24). Our results indicate that swimming motility is sensitive to environmental conditions such as temperature and ionic strength. Swarming motility is also dependent on the availability of appropriate nutrients such as a utilizable carbon and energy source such as glucose. Swarming colonies of *Y. enterocolitica* appear mucoid and are surrounded by a clear material or slime layer that always precedes multicellular migration. It is not clear whether these exogenous carbon sources supply the bacteria with a pool of intermediate compounds necessary for the production of a slime layer or provide growth conditions resulting in metabolic changes that trigger a swarming response.

The same motive organelle appears to be required for swimming and swarming movement because bacteria fully induced for swimming and swarming are similarly flagellated. In addition, the genes required for the production of flagella are also required for surface translocation. This is similar to other members of the *Enterobacteriaceae* (24) but differs from *Vibrio parahaemolyticus*, which has two distinct motive organelles: a polar flagellum dedicated to swimming motility and lateral flagella dedicated to swarming motility (38). Swarming colonies of *Y. enterocolitica* are often well organized, since cells near the periphery were aligned and moved parallel to one another and toward the center of the colony the bacteria appeared to be layered in terraces where bacteria were highly motile and moved in random directions. Swarm colonies were always mucoid and occasionally had the macroscopic appearance of concentric rings of growth, but there were no obvious consolidation cycles that have been associated with swarm cell dedifferentiation in *P. mirabilis*. Cellular morphology was different for cells isolated from swarming colonies of *Y. enterocolitica*. These cells were generally more elongated than cells isolated from colonies under other conditions, although the number of flagella per swarm cell was not greater than that observed for a fully induced swim cell. This indicates that swarm cell differentiation involves cell elongation but not necessarily hyperflagellation as occurs for other bacteria (24) or that this cell type was unusually rare under the conditions tested. Alternatively, increased flagellation is necessary but not sufficient for surface migration, which probably also requires conditions that induce the production of a slime layer.

In other enteric bacteria, expression of the motility master regulatory operon serves as a key control point for flagellation and swarm cell differentiation (17, 18, 22). The results presented in this study show that *Y. enterocolitica* has a similar

locus that is required for both flagellation and the elaboration of multicellular behavior. Cloning and DNA sequence analysis revealed that the *Y. enterocolitica* motility master regulatory operon consists of two genes designated *flhDC* due to their high degree of identity to genes previously characterized for other gram-negative bacteria. The addition of the DNA sequence of *flhDC* to the expanding list of characterized *Y. enterocolitica* motility genes which are analogous to genes from *E. coli* and *S. typhimurium* indicates that both structural and regulatory components of the flagellum biosynthetic pathway are highly conserved among the members of the *Enterobacteriaceae*. Sequence conservation reflects functional conservation of FlhD and FlhC, since different mutations in the *Y. enterocolitica flhDC* locus can be complemented by *S. liquefaciens flhDC*. Also, *Y. enterocolitica flhDC* (this study), *S. liquefaciens flhDC* (23), and *P. mirabilis flhDC* (22) functionally complement *flhDC* mutations in *E. coli*.

This study has focused on the essential role played by the motility master regulatory operon in governing the production of a functional motive organelle and the movement of *Y. enterocolitica* in response to chemical and physical features of the environment. Temperature was known to be an important environmental signal in *Y. enterocolitica* for regulating the production of flagella (28, 29), and this study shows that flagellation is also affected by nutrient availability and the ionic strength of the environment. Additional studies are required to determine if regulation of flagellum production and multicellular behavior in response to environmental cues is integrated through pathways that affect *flhDC* expression, as occurs in other related bacteria (17, 36). In other bacteria, *flhD* and *flhC* (referred to as class I genes) are at the top of the motility regulatory cascade (36). Expression of these two genes results in the activation of all class II genes. Subsequently, class III genes are expressed and the bacteria become motile. However, this regulatory organization for motility gene expression remains to be clarified in *Y. enterocolitica* because not all class II genes respond to the same environmental stimulus. Other studies have established that temperature affects the production of flagella by affecting transcription of the class II gene *fliA*, which encodes an alternate sigma factor. FliA subsequently directs transcription of the class III genes such as *fleA*, *fleB*, and *fleC* (28, 29). In contrast, temperature does not appear to affect the transcription of other *Y. enterocolitica* class II genes such as the *flhBAE* operon (19).

A role for motility in host-pathogen interactions has been shown for some pathogenic bacteria such as *Campylobacter jejuni* and *Helicobacter pylori* but has remained obscure for many bacterial pathogens which do not have well-defined experimental animal models of infection (43). Recent studies of *Vibrio cholerae* and *Pseudomonas aeruginosa* have implicated motility as a factor that affects interactions with the host and the production of virulence factors (6, 20, 31, 49). Despite the correlation between swarm cell differentiation and virulence factor production in *P. mirabilis*, there is still no clear consensus about a role for motility in pathogenesis (43). A previous report has suggested that motility itself is not a virulence factor for *Y. enterocolitica* O:9 (27). However, preliminary data from experiments based on this work have shown that normal expression of motility in *Y. enterocolitica* O:8 is required for proper expression of the virulence factors invasin and phospholipase (61). In conclusion, this study has defined in vitro conditions that stimulate motility and multicellular behavior in *Y. enterocolitica*, which will allow issues regarding roles for motility in pathogenesis to be more precisely addressed for this organism. By using well-defined in vitro tissue culture systems and an in vivo murine model of infection for *Y. enterocolitica*, future studies will focus on elucidating how the expression of this complex set of phenotypes affects initial bacterium-host interactions.

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