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The Last Gene of the *fla*/*che* Operon in *Bacillus subtilis*, *ylxL*, Is Required for Maximal σ^D Function

H. Werhane,[1] P. Lopez,[1] M. Mendel,[1] M. Zimmer,[2] G. W. Ordal,[2] and L. M. Márquez-Magaña[1]*

Department of Biology, San Francisco State University, San Francisco, California 94132,[1] and Department of Biochemistry, University of Illinois, Urbana, Illinois 61801[2]

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***ylxL* was found to be the last gene of the *fla*/*che* operon in *Bacillus subtilis* and is cotranscribed with the gene for the flagellum-specific alternate sigma factor, σ^D. The *ylxL* gene was disrupted by insertional mutagenesis, and the resultant mutant strain was found to be compromised for σ^D-dependent functions.**

In *Bacillus subtilis*, the structural genes that encode the hook-basal body complex (HBB), several genes controlling chemotaxis, and the gene for the alternate sigma factor, σ^D, are found adjacent to one another in a 26-kb region of the bacterial chromosome called the *fla*/*che* region (15). Several studies have demonstrated that these genes comprise a single operon (2, 10, 16, 18), while genes encoding these functions in the enteric bacterium are found in at least seven operons located throughout the bacterial chromosome (7). Many of the genes within the *fla*/*che* operon have been cloned, sequenced, and characterized (1, 15); however, the 3′ end of the operon has yet to be mapped, and the function of the *ylxL* gene product has not been analyzed.

Genetic organization of *ylxL*. An open reading frame, originally referred to as *orfC* and later renamed *ylxL* as a result of the *B. subtilis* genome project (3), was identified immediately downstream of the structural gene for σ^D, *sigD* (Fig. 1). While *sigD*, located 26 kb from the major promoter for this operon, has been shown to be part of *fla*/*che*, (2, 16), it was not known if *ylxL* is part of the *fla*/*che* transcription unit. Previous studies demonstrated that insertions between *sigD* and *ylxL* resulted in defects in chemotaxis (9, 18), swimming (9), and motility (10). Additionally, sequence analysis of this intergenic region failed to identify a transcriptional terminator, leading to the speculation that *ylxL* is part of the *fla*/*che* operon. Furthermore, the *rpsB* gene immediately downstream of *ylxL* is known to encode a ribosomal protein (5) and appears to be monocistronic with its own promoter (http://genolist.pasteur.fr/SubtiList), suggesting that *ylxL* may be the final gene of the *fla*/*che* operon.

RPA of *ylxL*. To determine whether *ylxL* is the last gene of the *fla*/*che* operon, RNase protection assays (RPA) were performed using the RPA II kit from Ambion. Riboprobes were synthesized for this analysis by first cloning the intergenic regions highlighted in Fig. 1 into plasmid pGEM-Zf7(+) from Stratagene. These intergenic regions were amplified by PCR and cloned into the plasmid in the orientation that allows for production of antisense RNA in an in vitro transcription reaction. T7 RNA polymerase and [α-^{32}P]UTP were used in such a reaction to yield body-labeled riboprobes. Full-length protection of the riboprobes generated from plasmids pHW1 and pHW2 was obtained (data not shown), confirming that *sigD* is part of the *fla*/*che* operon and demonstrating that *ylxL* is cotranscribed with *sigD*. The data obtained using the riboprobe generated from pHW3 were less straightforward.

The 416-base riboprobes synthesized from pHW3 include nonhomologous sequence from the vector and 368 bases of sequence complementary to the region spanning the *ylxL* and *rpsB* genes (Fig. 1). These riboprobes were used to identify the 3′ end of the *fla*/*che* operon. The *rpsB* gene encodes the 30S ribosomal protein S2 (5) and is not considered to be part of the *fla*/*che* operon. Total RNA was extracted from wild-type *B. subtilis* cells (LMB7) grown in 2XSG sporulation medium and harvested at T_0 and $T_{0.5}$, when *sigD* expression is known to be maximal (13). Riboprobes were incubated with isolated RNA to allow annealing of complementary strands. Single-stranded portions of the riboprobes were digested with RNase provided with the RPA II kit, while regions of riboprobes complexed in the RNA duplex with *ylxL-rpsB* mRNA were protected from digestion and resolved by electrophoresis on a 5% denaturing gel. The resulting autoradiograph of the dried gel showed three primary products of 368, 190, and 119 nucleotides (Fig. 2A).

The 368-base fragment is consistent with the lack of transcription termination by RNA polymerase at the end of *ylxL* and read-through into the *rpsB* gene: this is the predicted size for protection of homologous sequences contained within the riboprobes synthesized from pHW3. The significantly more abundant 119-base fragment is of the expected length for *rpsB* transcripts initiating at its own promoter, according to the SubtiList website (http://genolist.pasteur.fr/SubtiList). The protected 190-base fragment, however, suggests the termination of transcription at the end of the *ylxL* coding sequence prior to this promoter. In order to confirm the last two assignments, an end-labeled probe of the pHW3 region used for riboprobe synthesis was generated. The appropriate region of pHW3 was amplified by PCR and 5′ end labeled in a kinase reaction using [γ-^{32}P]dATP. The resulting DNA probe was incubated with *B. subtilis* RNA and subjected to S1 nuclease digestion. As with the riboprobe assay, protection of the DNA

* Corresponding author. Mailing address: Department of Biology, San Francisco State University, 1600 Holloway Ave., San Francisco, CA 94132. Phone: (415) 338-1548. Fax: (415) 338-0927. E-mail: marquez@sfsu.edu.

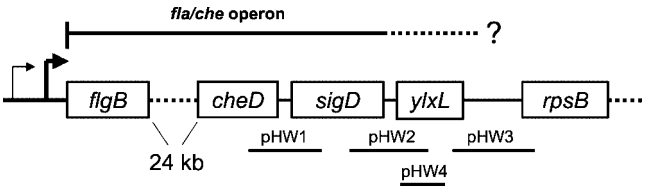


FIG. 1. Schematic diagram of the *fla*/*che* operon that starts with dual promoter elements dependent on σ^D and σ^A holoenzymes. The σ^D-dependent promoter (P_{D-3}) lies 133 bp upstream of the σ^A-dependent promoter (*fla*/*che* P_A), which lies 70 bp upstream of the translational start codon for the first gene in the *fla*/*che* operon, *flgB*. The *sigD* gene is found 26 kb downstream of these promoters, between the *cheD* and *ylxL* genes. DNA sequences amplified by PCR and cloned into pGEM-Zf7(+) for riboprobe synthesis or pJM102 for insertional mutagenesis are indicated by a bold line below the region that was amplified. Plasmids pHW1, pHW2, and pHW3 contain inserts that span the intergenic regions indicated, while the insertional plasmid pHW4 contains a fragment internal to the *ylxL* gene.

probe from digestion requires that it anneal to complementary mRNA emanating from the *ylxL-rpsB* intergenic region, but it would be detected by autoradiography only if the 5′ end of the DNA probe were found within the duplex. While the 368- and 119-base fragments were still detected following S1 digestion, the 190-base fragment was no longer evident (Fig. 2B). These results demonstrate that the 5′ end of the probe is not found within the 190-base protected fragment, and this is likely due to protection of the 3′ end of the probe by *ylxL* mRNA. Taken together, the data obtained from the RPA show that *ylxL* is the last gene of the *fla*/*che* operon, although some read-through into the downstream *rpsB* gene is evident.

Studies of *ylxL* function. *ylxL*, a 501-bp gene found at the 3′ end of the *fla*/*che* operon in this study, encodes a protein of unknown function. BLAST analysis showed that the predicted protein product of the *ylxL* gene has low similarity to other proteins, also of unknown function. However, due to previous genetic studies of the *ylxL* region (9, 18) and its determined location within the *fla*/*che* operon, we postulated that it might play a role in flagellar and chemotaxis functions. To study its predicted function, an insertional disruption of *ylxL* was generated by transformation with the disruptional plasmid pHW4 into the wild-type strain LMB7, yielding LMB231 (Table 1). pHW4 contains a 169-bp insert complementary to the 5′ end of the *ylxL* open reading frame immediately downstream of its translational start codon. Insertion of this plasmid disrupts *ylxL* without affecting expression of *sigD* as determined by anti-σ^D immunoblot analysis, described previously (16). σ^D protein levels in the *ylxL* mutant LMB231 were found to be comparable to those found in the wild-type strain (Fig. 3A) and were undetectable in the σ^D null mutant, LMB10. Furthermore, the phenotype of the *ylxL* mutant was found to be the result of the insertional disruption in the *ylxL* gene and not to an unlikely polar effect on *rpsB* expression. Insertion of the identical plasmid sequences that are found in the *ylxL* disruption between *ylxL* and *rpsB* in strain LMB281 did not produce the mutant *ylxL* phenotype (data not shown).

Flagellin expression. To determine the biological importance of *ylxL* in flagellin gene expression, strain LMB237 bearing both the *ylxL* disruption and a *hag-lacZ* translational fusion (Table 1) was grown in 2XSG sporulation medium, and β-galactosidase activity was measured as described previously (13). Under these conditions, flagellin gene expression increases throughout exponential growth and peaks shortly thereafter (13), as found for *hag-lacZ* expression in the wild-type strain, LMB25 (Fig. 3B). However, the reporter strain bearing the

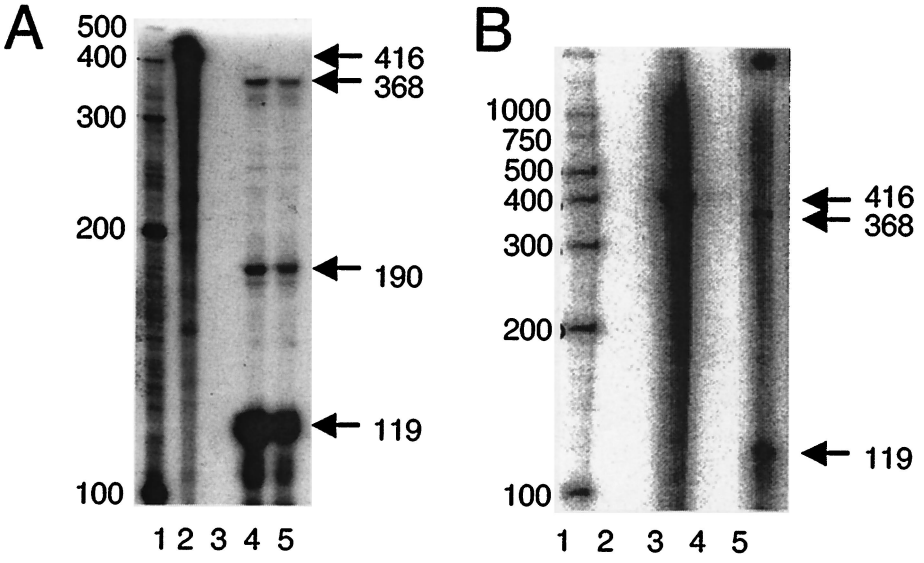


FIG. 2. Results of RPA for the intergenic region between *ylxL* and *rpsB* using a body-labeled riboprobe (A) or an end-labeled DNA probe (B). The intact probe is 416 bases, and when fully hybridized to *B. subtilis* mRNA it gives rise to a protected fragment of 368 nucleotides upon digestion. (A) Results of RPA using body-labeled riboprobe and RNase digestion. Lane 1, RNA molecular weight marker; lane 2, intact riboprobe, no RNase added; lane 3, riboprobe incubated with RNase; lane 4, RPA using mRNA from the wild-type strain at T_0 when flagellin gene expression is induced (13); lane 5, RPA using mRNA from wild-type strain at $T_{0.5}$ when flagellin expression is maximal (13). (B) Results of RPA using end-labeled probe and S1 nuclease. Lane 1, DNA molecular weight marker; lane 2, empty; lane 3, intact probe, no S1 added; lane 4, RPA with yeast RNA; lane 5, RPA using mRNA from wild-type strain at $T_{0.5}$.

TABLE 1. *B. subtilis* strains used in this study

Strain	Genotype	Derivation (source or reference)[a]
LMB4	*his leu fliM1*	OI2553[b] (18)
LMB7	*trpC2 pheA1*	JH642[b] (J. Hoch)
LMB10	*trpC2 pheA1 sigD*	CB100[b] (9)
LMB25	*trpC2 pheA1 hag-lacZ* (Ery^r)	Transform [LMB7:pDM632Ery, Ery^r] (13)
LMB231	*trpC2 pheA1 ylxL*::*cat*	Transform [LMB7:pHW4, Cm^r] (this study)
LMB237	*trpC2 pheA1 ylxL*::*cat hag-lacZ* (Ery^r)	Transform [LMB231:LMB25, Ery^r] (this study)
LMB281	*trpC2 pheA1 ylxL-rpsB*::*cat*[c]	Transform [LMB7:pLO3[d], Cm^r] (this study)

[a] Transformations are shown as follows: [recipient strain:plasmid DNA (pHW4) or chromosomal DNA from listed strain used for transformation, and selection for resistance].
[b] Previous name of strain.
[c] The insertion is between *ylxL* and *rpsB* and does not disrupt either gene.
[d] The same insert found in pHW3 (Fig. 1) was cloned into the integrational plasmid, pJM102, to yield pLO3.

ylxL mutation, LMB237, displayed a drastic decrease in β-galactosidase activity (Fig. 3B). In this strain, *hag-lacZ* expression ranged from 50 to 100 Miller units throughout growth, indicating that *ylxL* is required for high-level flagellin gene expression that is developmentally regulated.

These results were confirmed and extended by antiflagellin immunoblot analysis performed as described previously (16). The level of flagellin protein was significantly reduced in the *ylxL* mutant LMB231 when compared to levels found in the wild-type strain (Fig. 3C). Nonetheless, flagellin protein was easily detectable in this strain and absent in the *sigD* null mutant. Therefore, while the alternate sigma factor encoded by *sigD*, σ^D, is absolutely required for expression of the flagellin gene as previously determined (11), *ylxL* appears to play a critical role in up-regulating its expression.

Motility and chemotaxis function. Having demonstrated that the *ylxL* mutant is capable of synthesizing flagellin protein, we sought to test whether it is motile and able to demonstrate chemotactic behavior. Semisolid agar (0.3% agar) plates were used as described previously (9) to monitor the ability of the mutant to swim away from the site of inoculation towards areas of greater nutrient concentration. Disruption of the *ylxL* gene

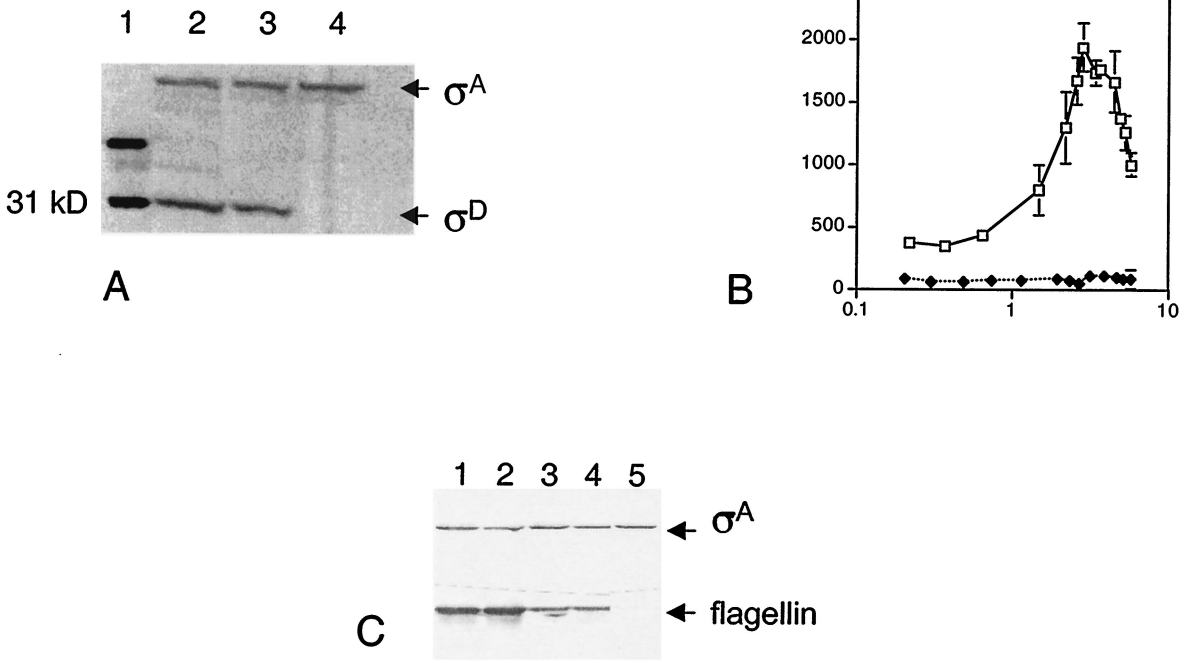
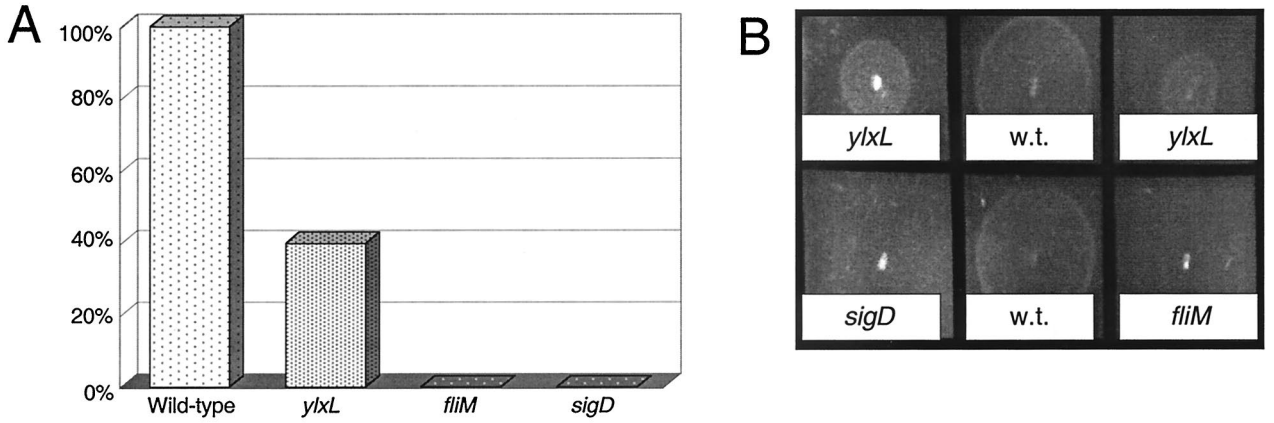


FIG. 3. σ^D and flagellin gene expression in the *ylxL* mutant. (A) σ^D protein levels were measured in 50 μg of total protein extract by immunoblot analysis, using σ^A expression as an internal control. Lane 1, ladder; lane 2, wild-type strain (LMB7); lane 3, *ylxL* strain (LMB231); lane 4, *sigD* null mutant (LMB10). (B) β-Galactosidase activity was monitored in a strain bearing a *hag-lacZ* reporter construct throughout growth in complex, sporulation medium. The *y* axis is β-galactosidase activity expressed in Miller units, and the *x* axis is absorbance at 600 nm. Symbols: open squares, wild type (LMB25); filled diamonds, *ylxL* (LMB237). The data presented are the results of several experiments. (C) Flagellin protein levels were measured in total protein extract by immunoblot analysis, using σ^A expression as an internal control. Lane 1, wild-type strain (LMB7) at T_0; lane 2, wild-type strain (LMB7) at $T_{0.5}$; lane 3, *ylxL* strain (LMB231) at T_0; lane 4, *ylxL* strain (LMB231) at $T_{0.5}$; lane 5, *sigD* null mutant (LMB10) at $T_{0.5}$.



C

	Blank	Asparagine	Proline	Mannitol
ylxL (LMB231)	33.5	531	444	198.5
Wild-type (LMB1)	71	1183	1211.5	385
% of wild-type	47%	45%	37%	52%

FIG. 4. Results of chemotaxis assays for the *ylxL* mutant. (A) Bar graph of the average data obtained for several experiments in the plate assay shown in panel B. Results are given as a percentage of the diameter of growth obtained for the wild-type strain (LMB7). (B) Plate assay for chemotaxis and motility in the *ylxL* strain (LMB231), the wild-type strain (LMB7), and two nonmotile strains, the *sigD* strain (LMB10) and the *fliM* strain (LMB4). (C) Results of capillary assay for *ylxL* mutant (LMB231) compared to wild-type strain (LMB7) for several attractants (asparagine, proline, and mannitol) used at a concentration of 10^{-5} M. The data given are the average of two experiments for the number of bacteria per capillary (minus the blank).

resulted in impaired swimming motility and/or chemotaxis, as demonstrated by a smaller diameter of growth found for this strain when compared to the wild-type strain (Fig. 4A and B). The *ylxL* mutant, LMB231, exhibited 40% of the diameter of growth found for the wild-type strain, LMB7, but was clearly capable of swimming motility when compared to two nonmotile strains bearing null mutations in the flagellum-specific alternate sigma factor, *sigD*, and the flagellar switch protein, *fliM*.

The chemotactic efficiency of the *ylxL* mutant towards three attractants (asparagine, proline, and mannitol) was monitored by capillary assays that were performed as previously described (14, 17). Several concentrations of each attractant were assayed (data not shown), and the results obtained by use of 10 μM attractant are presented, since they are consistent with the data obtained for all concentrations tested (Fig. 4C). Accumulation of *ylxL* mutant cells in capillaries containing the three attractants tested ranged from 37 to 52% of the number of wild-type cells that accumulated under identical conditions. While this could be interpreted as compromised chemotactic behavior, a similar difference in the level of accumulation (47%) was found for the blank that contained no attractant (Fig. 4C). Therefore, our results suggest that the reduced diameter of growth found on the semisolid agar plates (Fig. 4A and B) was due to compromised swimming motility and not a defect in chemotaxis. This result is consistent with a role of the *ylxL* gene product in enhancing σ^D function. The genes for flagellin and the motility proteins (11, 12) are σ^D dependent, while most of the chemotaxis genes are found in the *fla*/*che* operon (1) and are not dependent on the alternate sigma factor for expression (16).

Autolysin activity. The genes for several autolysins contain σ^D-dependent promoters (4, 8), and cells lacking σ^D function grow as long filaments due to decreased autolytic activity necessary for cell separation (9). Microscopic observation of the *ylxL* mutant compared to the wild-type strain demonstrated filamentous growth, but the filaments were significantly shorter than those found for the *sigD* null mutant and included a mixture of short and long filaments (Fig. 5). These data further support the postulate that the *ylxL* gene product is required for maximal σ^D activity in the cell.

Summary. In this work we have demonstrated that the *ylxL* gene is the last gene of the *fla*/*che* operon that apparently encodes a protein required for maximal σ^D function. Sequence analysis of the intergenic region between *ylxL* and *rpsB* has failed to detect a factor-independent transcriptional terminator, suggesting that the termination of transcription identified in this study is factor dependent. The location of *ylxL* at the end of the *fla*/*che* transcription unit, coupled with its demonstrated role in enhancing σ^D function and read-through into *rpsB*, provides for the intriguing possibility that it acts as a sensor for complete *fla*/*che* transcription and/or couples flagellin transcription and translation.

While BLAST analysis using the predicted protein product of *ylxL* as the query sequence showed only weak matches to proteins of unknown function, a search of the *B. subtilis* ge-

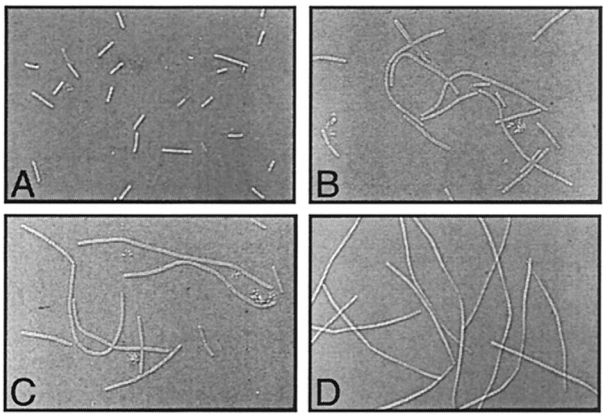


FIG. 5. Microscopic observation of the *ylxL* mutant using Nomarski optics (×1,250). (A) Wild-type strain LMB7; (B and C) *ylxL* mutant LMB231; (D) *sigD* null LMB10.

nome using products of several enteric *flhC* genes as the query identified a single match, the *ylxL* gene product (2). The *flhC* gene is part of the master operon (7) that is required for expression of the flagellar regulon in enteric bacteria. This gene encodes part of a transcriptional activator complex absolutely required for expression of the genes that form the HBB and the flagellum-specific sigma factor in that system (6). However, *ylxL* does not appear to be required for *sigD* expression, since a strain disrupted for *ylxL* in this study was able to display limited σ^D functions. Interestingly, the similarity between the *ylxL* gene product and the enteric FlhC proteins is limited to a third of the protein and suggests a different domain structure. Nonetheless, the *ylxL* gene product may function as a transcriptional activator of σ^D-dependent genes. Further, its unique location at the end of the *fla*/*che* transcription unit may allow this activity to be coupled to complete transcription of the operon. Finally, read-through from *ylxL* to *rpsB* may suggest a link between σ^D-dependent transcription of the *hag* gene and its subsequent translation.

Recent work from the Hughes laboratory (P. Aldridge, H. R. Bonifield, J. Gnerer, J. E. Karlinsey, and K. T. Hughes, Abstr. BLAST VII Bact. Locomotion Signal Transduction, p. 42, 2003) suggests that *hag* mRNA in *Salmonella enterica* serovar Typhimurium may be targeted to the HBB complex for translation. The product of the *rpsB* gene encodes a component of the ribosome found to be localized to the poles of cells (5). The genetic organization of this gene immediately downstream of *ylxL* and the low level of read-through found in this study suggest such a link, but much greater investigation is required to support this prediction.

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