JOURNAL OF BACTERIOLOGY, June 1991, p. 3580–3583
0021-9193/91/113580-04$02.00/0
Copyright © 1991, American Society for Microbiology
Vol. 173, No. 11

NOTES

Genetic Analysis of the *flaA* Locus of *Bacillus subtilis*

P. M. HAUSER,[1,2] W. D. CRABB,[3] M. G. FIORA,[1] F. SCOFFONE,[4] AND A. GALIZZI[1]*

Dipartimento di Genetica e Microbiologia "A. Buzzati-Traverso," Università degli Studi di Pavia,[1] and Istituto di Genetica Biochimica ed Evoluzionistica, Consiglio Nazionale delle Ricerche,[4] 27100 Pavia, Italy; Institut de Génétique et de Biologie Microbiennes, Université de Lausanne, 1005 Lausanne, Switzerland[2]; and Genencor, South San Francisco, California 94080[3]

Received 14 January 1991/Accepted 13 March 1991

We isolated two clones of recombinant lambda bacteriophage with overlapping inserts of *Bacillus subtilis* chromosomal DNA corresponding to part of the *flaA* locus. The *flaA4* and *flaA15* mutations were localized on the physical map by marker rescue experiments. The *flaA* locus and the *flaB* (*sigD*) gene were mapped in transduction crosses, and the order *glnA polC flaB flaA* was determined. *FlaB* was linked to *polC* in transformation crosses.

A large number of genes involved in flagellar synthesis and assembly have been described in both *Escherichia coli* and *Salmonella typhimurium*. Extensive genetic and molecular analyses have shown that about 3% of the genetic information in these bacteria is devoted to synthesis and assembly of flagella, to their functioning (proteins of the motor and of the motor switch), and to the chemotactic response (10, 11). Although *Bacillus subtilis* has been used as a model system to study the energetics of the flagellar motor (9, 17), little is known about the genetics of the system. Few mutants of *B. subtilis* affected in motility have been isolated and characterized (7). On the basis of transformation and transduction mapping, they have been assigned to four loci: *flaA*, *flaB*, *flaC*, and *flaD* (1, 15); *flaA* and *flaB* map between *thyA* and *pyr*, *flaC* is linked to *hisA*, and *flaD* is linked to *aroD*. The *flaD* locus has been cloned (16), and *flaB* has recently been shown to be an allele of *sigD* (12). More advanced is the genetics of the chemotactic response of *B. subtilis*, for which a large number of mutants have been isolated and attributed to at least 21 complementation groups (13, 14, 18). A number of chemotaxis genes are linked to *pyr* and thus map in the same region as *flaA* and *flaB*. We isolated two overlapping clones, λUF7 and λA21 (Fig. 1A), from a λCharon 4A *B. subtilis* chromosomal library (2, 5). The *B. subtilis* strains used are described in Table 1. On the basis of restriction maps, the two clones appeared to be identical to the two clones described by Ordal et al. (13) and reported to contain most of the *che* genes. The two clones span the *flaA* locus.

Clone λUF7 contains the wild-type alleles of *flaA4* and *flaA15*. On the basis of previous mapping experiments (6), it appeared that the *B. subtilis* DNA insert of λUF7 was derived from a region of the chromosome linked to the *pyrD* marker, i.e., not far from the *flaA* locus. We thus tested the lambda clone for its ability to restore the wild-type phenotype to *flaA4* and *flaA15* mutant strains. Marker congression experiments (Fig. 1B) showed that both *flaA* markers could be rescued by DNA obtained from clone λUF7. Further subcloning of the lambda insert definitely demonstrated that *flaA4* and *flaA15* are located in different portions of the cloned DNA (Fig. 1B). The *flaA4* mutation was rescued by a DNA fragment of 2,570 bp (pUF102). We were unable to assign the position of the mutation more precisely. The shortest fragment that could restore the motility phenotype to strains with the *flaA15* mutation had a length of 655 bp (carried by plasmid pUFH-B). As deduced from the nucleotide sequence of the 8.3-kb *Eco*RI fragment (2), the *flaA15* mutation corresponds to open reading frame 2 (ORF2), whereas the *flaA4* mutation corresponds to a different ORF (ORF8, ORF9, or ORF10). Mutations *flaA4* and *flaA15* were found to be closely linked in transformation crosses and, for this reason, were both named *flaA* (15). Our data show that the two mutations affect two different cistrons.

The *flaA15* mutation appears to be in a gene involved in the synthesis of a switch protein, whereas *flaA4* may effect the rod, the hook length, or unknown functions, depending on its exact location. We reevaluated the phenotype of strains PB5060 (*flaA15*), PB5070 (*flaA4*), and PB5071 (*flaA4* in a different genetic background). Examination by electron microscope of samples from cultures at the end of exponential growth and in the stationary phase showed that both PB5060 and PB5070 were completely devoid of flagellar filaments. Under the same conditions, cells of control strains were surrounded by many flagellar filaments. Most strain PB5071 cells were without flagella, and less than 10% had few, bent filaments. The leakiness of the *flaA4* mutation was observed on a swarm plate as well. We conclude that both *flaA4* and *flaA15* mutations affect the assembly of flagellar filaments.

Integration plasmids containing various DNA fragments derived from λUF7 were used in transformation experiments with selection for chloramphenicol resistance. Good transformation efficiencies were obtained with all of the integrational plasmids analyzed, and the only phenotype associated with chloramphenicol resistance was invariably the inability of the colonies to swarm on gelatin-agar plates. These results suggest the possibility that the insert of λUF7 is part of a single transcription unit involved in flagellar biosynthesis and confirm the finding of Zuberi et al. (19) that scoring for the chemotaxis deficiency phenotype showed that the transcription unit starts upstream of the 8.3-kb *Eco*RI fragment and ends in the 10.9-kb *Eco*RI fragment or downstream of it.

* Corresponding author.

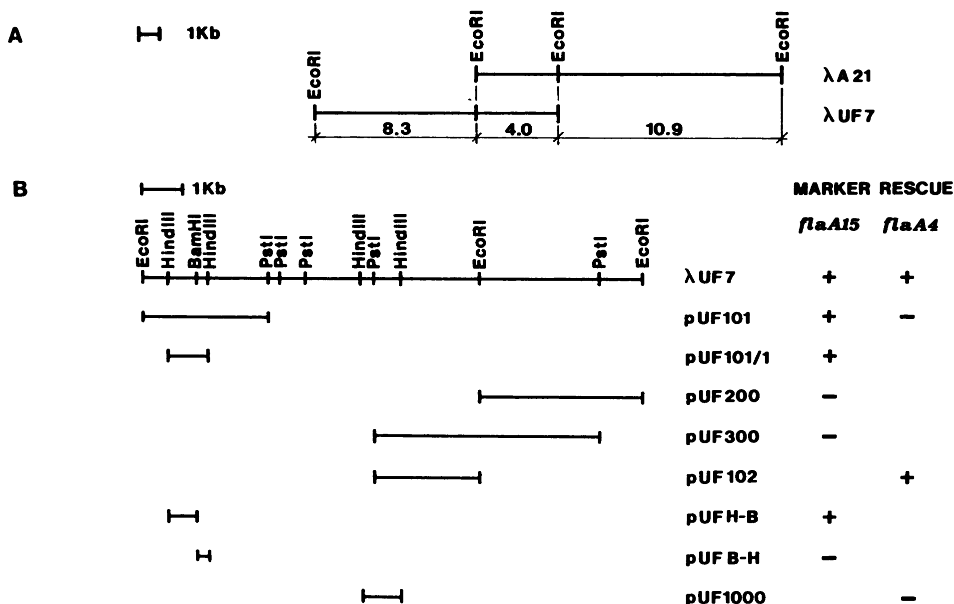

FIG. 1. (A) Physical organization of *B. subtilis* DNA cloned in phage lambda. (B) Localization of *flaA4* and *flaA15* mutations. The locations of the two mutations were determined by marker congression in transformation. The plasmids were all derivatives of pGEM-4Z (Promega Biotec, Madison, Wis.). Symbols: +, the fragment gave motile transformants; −, no motile transformants were observed.

Genetic mapping of the *flaA* and *flaB* loci. The *flaA* locus was mapped by transduction crosses between *pyrD* and *thyA* (15). We performed transduction experiments aimed at finer mapping of *flaA* in regard to the markers *glnA* and *polC*. To facilitate the genetic mapping, we used integrational vector pJH101 (4), whose chloramphenicol resistance determinant could be employed as a useful selective marker. All integrational derivatives of clone λUF7 were nonmotile and thus not suitable for transduction mapping with flagellum-tropic bacteriophage PBS1. Zuberi et al. (19) showed that an integrational vector carrying a 2-kb *Pst*I-*Eco*RI fragment at the end of the insert of phage λ14.9 (corresponding to our phage clone λA21) could generate motile *B. subtilis* transformants. We transformed our parental strain, PB1424, with a similarly constructed plasmid (pJH2000) and confirmed that the Cm^r transformants were motile. One such transformant was used as the donor in transduction experiments. The chloramphenicol resistance determinant (*cat*) was taken as a genetic marker of *flaA*. The recipient was PB1814, and selection was for GlnA$^+$, chloramphenicol resistance, and Ts$^+$ (the *polC* allele *dnaF69* of strain PB1814 confers a temperature sensitivity phenotype). The results of the three-factor crosses (Table 2 and Fig. 2) are consistent with the order *glnA polC cat* (*flaA*). This order is in agreement with the previous mapping of *flaA* of Pooley and Karamata (15) and with the more recent data of Zuberi et al. (19), who located *cheF* and *cheM*, two genes of the *flaA* locus, between *spcB* and *pyrD*. According to the data in Table 2, the *flaA* locus is 87% linked to *polC* in transduction. The *flaA15* marker was not linked to *polC* in transformation. It

TABLE 1. *B. subtilis* strains used

Strain	Genotype	Source
PB1669	*trpC2 dnaF69*	G. Mazza
PB1814	*dnaF69 glnA100*	A. Galizzi
PB5058	*flaB2 trpC2 thyA1 thyB1 ilvA1*	L5310; H. M. Pooley
PB5059	*flaA15 thyA1 thyB1 xin-15 trpC2*	Ni15; H. M. Pooley
PB5060	*flaA15 purA16 ilvA1*	L5256; D. Karamata
PB5061	*flaB2 purA16 ilvA1 metB5*	L5298; H. M. Pooley
PB5070	*flaA4 ilvA1 trpC2 thyA1 thyB1*	L5395; H. M. Pooley
PB5071	*flaA4 hisA1 argC4 pyrA1*	L5404; D. Karamata
PB5077	*hisB2 trpC2 metD4 flaA*::pJH2000	This work
PB5079	*hisB2 trpC2 metD4 flaB*::pLM3	This work

TABLE 2. Mapping of *flaA* (*cat*): three-factor[a] transduction crosses involving *glnA100* and *polC* (*dnaF69*)

Selected marker[b]	Phenotype[c]			No. of recombinants[d]
	GlnA	DnaF	Cm^r	
DnaF$^+$ (Ts$^+$)	R	D	D	134
	R	D	R	19
	D	D	D	35
	D	D	R	7
Cm^r (*cat*)	R	D	D	247
	R	R	D	40
	D	D	D	39
	D	R	D	1
GlnA$^+$	D	R	R	106
	D	D	D	20
	D	D	R	15
	D	R	D	1

[a] Deduced order: *glnA polC cat* (*flaA*).
[b] The donor was PB5077 (*flaA*::*cat*), and the recipient was PB1814 (*glnA100 dnaF69*). *dnaF69* is a temperature-sensitive allele of *polC*.
[c] D, donor; R, recipient.
[d] PBS-1 phage-mediated transductions were performed by the method of Hoch et al. (8). Recombinants for auxotrophic markers were selected on minimal medium (3) supplemented with 0.5% glucose and the appropriate required compounds at a concentration of 25 μg/ml. Cm^r recombinants were selected on nutrient agar supplemented with 5 μg of chloramphenicol per ml; Ts$^+$ recombinants were selected on nutrient agar plates incubated at 47°C. The recombinants were picked and reisolated on the same selective medium, and their phenotypes were determined.

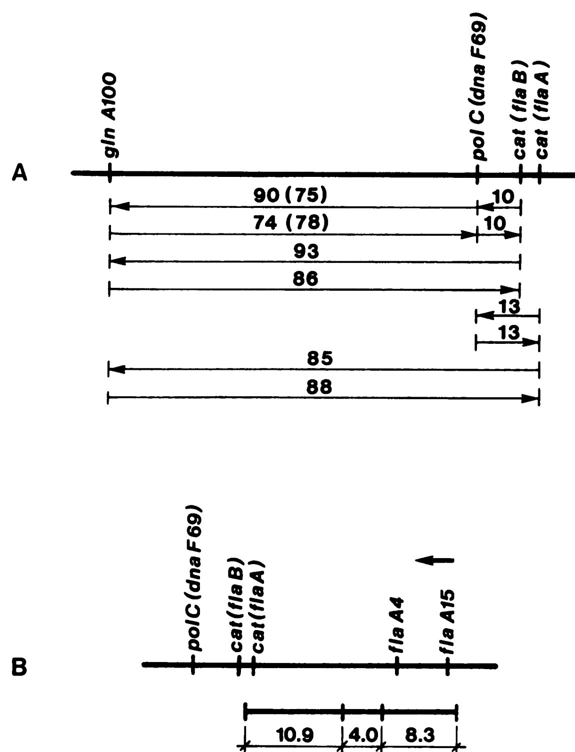

FIG. 2. Genetic map of the *flaA* and *flaB* loci. (A) The map is based on three-factor crosses in transduction experiments, and the distances are expressed as percentages (100% − percent cotransduction). The arrows point to the selected markers. The numbers in parentheses are map distances obtained with *flaA*::*cat*. (B) Relationship between the physical and genetic maps of the *flaA* locus. The genetic map is not drawn to scale. The physical map is oriented with respect to the genetic map on the basis of the present data and the results of Zuberi et al. (19). The arrow indicates the direction of transcription.

should be noted that the strong linkage observed in transduction was between *polC* and the *cat* gene of insertional plasmid pJH2000. The plasmid carried a DNA fragment derived from downstream of the *flaA* locus and, according to the genetic data of Zuberi et al. (19), nearest to the *polC* gene. We thus tested for linkage in transformation between *polC* and the chloramphenicol resistance determinant of pJH2000. Chromosomal DNA isolated from strain PB5077 (*flaA*::pJH2000) was used to transform strain PB1669 to chloramphenicol resistance. Of 72 chloramphenicol-resistant transformants tested, 21 (29%) were found to be Ts^+. This demonstrates linkage in transformation between *polC* and one of the extremes of the *flaA* locus.

The *flaB2* mutation has also been mapped between *thyA* and *pyrD* (15), but the mutation could not be rescued by DNA from λUF7 and λA21 (data not shown). In transformation experiments, we failed to observe linkage between *flaB2* and *flaA15*. This is in agreement with the observations of Pooley and Karamata (15), who could not find a linkage in transformation between *flaA4* and *flaB2*.

It was recently shown that *flaB* is an allele of gene *sigD*, which codes for transcriptional factor σ^D (12). By using integrational plasmid pLM3, containing the 3′ end of gene *sigD* (12), we mapped the *sigD* (*flaB*) gene with regard to *polC*. The downstream integrants obtained with plasmid

TABLE 3. Mapping of *flaB* (*cat*): three-factor[a] transduction cross involving *glnA100* and *polC* (*dnaF69*)

Selected marker[b]	Phenotype[c]			No. of recombinants
	GlnA	DnaF	Cm^r	
$DnaF^+$ (Ts^+)	D	D	D	118
	R	D	D	364
	D	D	R	22
	R	D	R	29
Cm^r (*cat*)	D	D	D	95
	R	D	D	506
	D	R	D	1
	R	R	D	63
$GlnA^+$	D	D	D	27
	D	R	D	1
	D	D	R	11
	D	R	R	340

[a] Deduced order: *glnA polC cat* (*flaB*).
[b] The donor was PB5079 (*flaB*::*cat*), and the recipient was PB1814 (*glnA100 dnaF69*). *dnaF69* is a temperature-sensitive allele of *polC*.
[c] D, donor; R, recipient.

pLM3 have only slightly reduced levels of flagellin expression and are motile (12). We used one such integrant as the donor in PBS1 transduction experiments; the results of the three-factor crosses (Table 3 and Fig. 2) suggested the order *glnA polC sigD* (*flaB*). Thus, the loci *flaB* and *flaA* map on the same side with respect to *polC*. The 90% linkage between *sigD* and *polC* prompted us to check for linkage of the two markers in transformation. Chromosomal DNA isolated from the pLM3 integrant was used to transform strain PB1669 to chloramphenicol resistance. Of 150 chloramphenicol-resistant transformants tested, 146 (97%) were Ts^+, a finding that demonstrates very high linkage between the *polC* locus and *sigD* (*flaB*). We can conclude that despite the absence of linkage in transformation between the *flaB2* and *flaA4* (15) markers and the *flaB2* and *flaA15* markers (see above), the *flaA* and *flaB* loci are both linked to *polC*. This suggests the possibility that they are part of the same operon.

We thank H. M. Pooley and D. Karamata for kindly providing strains and M. J. Chamberlin for providing plasmid pLM3.

P.M.H. was the recipient of a scholarship from the Ministero degli Affari Esteri (Italy). This work was partially supported by CNR and MURST (Rome).

REFERENCES

1. **Akamatsu, T., and J. Sekiguchi.** 1987. Genetic mapping by means of protoplast fusion in *Bacillus subtilis*. Mol. Gen. Genet. **208:**254–262.
2. **Albertini, A. M., T. Caramori, W. D. Crabb, F. Scoffone, and A. Galizzi.** 1991. The *flaA* locus of *Bacillus subtilis* is part of a large operon coding for flagellar structures, motility functions, and an ATPase-like polypeptide. J. Bacteriol. **173:**3573–3579.
3. **Davis, B. D., and E. S. Mingioli.** 1950. Mutants of *Escherichia coli* requiring methionine or vitamin B12. J. Bacteriol. **60:**17–28.
4. **Ferrari, F. A., A. Nguyen, D. Lang, and J. A. Hoch.** 1983. Construction and properties of an integrable plasmid for *Bacillus subtilis*. J. Bacteriol. **154:**1513–1515.
5. **Galizzi, A.** Unpublished data.
6. **Gianni, M., and A. Galizzi.** 1986. Isolation of genes preferentially expressed during *Bacillus subtilis* spore outgrowth. J. Bacteriol. **165:**123–132.
7. **Grant, G. F., and M. I. Simon.** 1969. Synthesis of bacterial flagella. II. PBS1 transduction of flagella-specific markers in

Bacillus subtilis. J. Bacteriol. **99**:116–124.
8. **Hoch, J. A., M. Barat, and C. Anagnostopoulos.** 1967. Transformation and transduction in recombination-defective mutants of *Bacillus subtilis*. J. Bacteriol. **93**:1925–1937.
9. **Khan, S., and R. M. Macnab.** 1980. Proton chemical potential, proton electrical potential and bacterial motility. J. Mol. Biol. **138**:599–614.
10. **Macnab, R. M.** 1987. Flagella, p. 70–83. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
11. **Macnab, R. M.** 1987. Motility and chemotaxis, p. 732–759. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
12. **Marquez, L. M., J. D. Helmann, E. Ferrari, H. M. Parker, G. W. Ordal, and M. J. Chamberlin.** 1990. Studies of σ^D-dependent functions in *Bacillus subtilis*. J. Bacteriol. **172**:3435–3443.
13. **Ordal, G. W., D. O. Nettleton, and J. A. Hoch.** 1983. Genetics of *Bacillus subtilis* chemotaxis: isolation and mapping of mutations and cloning of chemotaxis genes. J. Bacteriol. **154**:1088–1097.
14. **Ordal, G. W., H. M. Parker, and J. R. Kirby.** 1985. Complementation and characterization of chemotaxis mutants of *Bacillus subtilis*. J. Bacteriol. **164**:802–810.
15. **Pooley, H. M., and D. Karamata.** 1984. Genetic analysis of autolysin-deficient and flagellaless mutants of *Bacillus subtilis*. J. Bacteriol. **160**:1123–1129.
16. **Sekiguchi, J., B. Ezaki, K. Kodama, and T. Akamatsu.** 1988. Molecular cloning of a gene affecting the autolysin level and flagellation in *Bacillus subtilis*. J. Gen. Microbiol. **134**:1611–1621.
17. **Shioi, J.-I., S. Matsuura, and Y. Imae.** 1980. Quantitative measurements of proton motive force and motility in *Bacillus subtilis*. J. Bacteriol. **144**:891–897.
18. **Zuberi, A. R., C. Ying, H. M. Parker, and G. W. Ordal.** 1990. Transposon Tn*917lacZ* mutagenesis of *Bacillus subtilis*: identification of two new loci required for motility and chemotaxis. J. Bacteriol. **172**:6841–6848.
19. **Zuberi, A. R., C. Ying, M. R. Weinreich, and G. W. Ordal.** 1990. Transcriptional organization of a cloned chemotaxis locus of *Bacillus subtilis*. J. Bacteriol. **172**:1870–1876.