Copyright © 1989 by the Genetics Society of America

Use of Pulsed Field Gel Electrophoresis and Transposon Mutagenesis to Estimate the Minimal Number of Genes Required for Motility in *Caulobacter crescentus*

Bert Ely and Tracey W. Ely

Department of Biological Sciences, University of South Carolina, Columbia, South Carolina 29208

Manuscript received July 5, 1989
Accepted for publication September 13, 1989

ABSTRACT

To facilitate the mapping of transposon insertion mutations in *Caulobacter crescentus*, we have used pulsed field gel electrophoresis to construct a detailed physical and genetic map of the *C. crescentus* genome. Restriction fragments were generated by *DraI*, *AseI*, or *SpeI* which cleave the *C. crescentus* 40, 13, and 26 times, respectively, and Tn*5* insertions were used to align the restriction fragments generated by each of the enzymes. The utility of the resulting map was demonstrated by determining the chromosomal locations of a collection of flagellar mutations. As a result of this study, we were able to identify ten new flagellar genes at various locations on the chromosome. Thus, at least 48 genes are required for the assembly of a functional flagellum in *C. crescentus*.

THE aquatic bacterium *Caulobacter crescentus* differentiates during its cell cycle, and cellular motility is a transient phenomenon found in the flagellated daughter cell. These bacteria flourish under nutrient poor conditions and are characterized by a stalk and a holdfast which allows them to attach to a substrate in their environment. When an attached cell divides, the stalked portion remains in place and a motile daughter cell is released. Thus, motility appears to be used primarily as a means for a daughter cell to move to a new location so that it will not have to compete for a limited food supply. The genes involved in motility and chemotaxis are subject to cell cycle control, and all of them appear to be expressed at the same time in the cell cycle. This coordinated expression of temporally regulated genes provides a simple model system for the study of cellular differentiation.

The bacterial flagellum is a complex organelle, consisting of a helical filament which is mounted on a hook and rod structure. The whole assembly is then attached to the layers of the cell envelope by a series of rings called a basal body. This structure functions like a ship's propeller, rotating the hook and filament to propel the bacterium. *C. crescentus* has a typical flagellum (JOHNSON *et al.* 1979) which is present at one pole of the daughter swarmer cells but is not present on the stalked cells. When this flagellum rotates in a clockwise direction, the cell swims forward, and when it rotates in a counterclockwise direction, the cell swims backwards (ELY *et al.* 1986). In the enteric bacteria, multiple flagella are present on each cell. When these flagella rotate in a counterclockwise direction, they form a unified bundle and the bacteria swim smoothly. In contrast, when the flagella rotate in a clockwise direction, coordination of the flagella is lost and the cell reorients itself by tumbling.

In *Escherichia coli*, 36 genes have been identified which are required for the assembly and function of the bacterial flagellum (MACNAB 1987). An additional 20 to 30 genes are involved in the chemotactic response to nutrients in the environment. Since the *E. coli* genome has a coding capacity of about 4000 genes, this means that approximately 15% of the genome is involved in motility or chemotaxis. Thus, it is clear that these bacteria have committed a lot of resources to the maintenance and regulation of their ability to swim. By implication, the ability to swim and locate nutrients must be quite valuable in natural environments to be worthy of this level of commitment. In fact, long-term storage of stocks of enteric bacteria in a nutrient-rich, anaerobic, semi-solid medium results in the accumulation of nonmotile mutants. Presumably, there is no advantage to swimming under these conditions and the bacteria can conserve considerable amounts of energy if they do not make flagella.

Our initial studies of *C. crescentus* demonstrated that more than 30 genes are involved in motility (ELY, CROFT and GERARDOT 1984). However, we suspected that many flagellar genes remained to be discovered. Additional motility mutants were readily obtained by transposon mutagenesis, but the genetic analysis of these mutants proved to be quite tedious. Recently, we began to use pulsed field gel electrophoresis (PFGE) as a genetic tool (ELY and GERARDOT 1988).

The publication costs of this article were partly defrayed by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

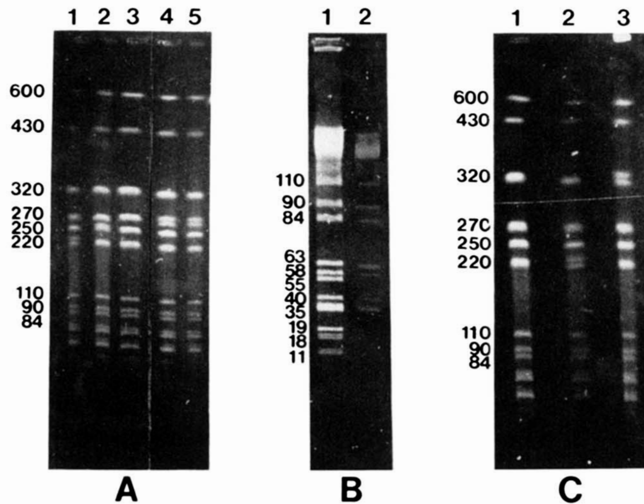

FIGURE 1.—PFGE of *Spe*I restriction digests. (A) The gel was run at 200 V for 14 hr with a 20-sec pulse time. Lane 1, SC1123, the Tn*5* insertion is in one of the 220 kb bands; lane 2, SC2193, the Tn*5*-132 insertion is in one of the 250-kb bands; lane 3, SC3470, the Tn*5*-132 insertion is in one of the 270-kb bands; lane 4, SC2674, the Tn*5* insertion is in one of the 270-kb bands; lane 5, SC2675, the Tn*5* insertion is in one of the 270-kb bands. (B) The gel was run at 240 V for 10 hr with a 3-sec pulse time. Lane 1, the wild-type strain CB15, lane 2, SC2252, the Tn*5* insertion is in the 55-kb band. (C) The gel was run at 200 V for 18 hr with a 20-sec pulse time. Lane 1, SC1994, the Tn*5* insertion is in one of the 320-kb bands; lane 2, SC1046, the Tn*5* insertion is in one of the 220-kb bands; lane 3, SC1582, the Tn*5* insertion is in one of the 320-kb bands.

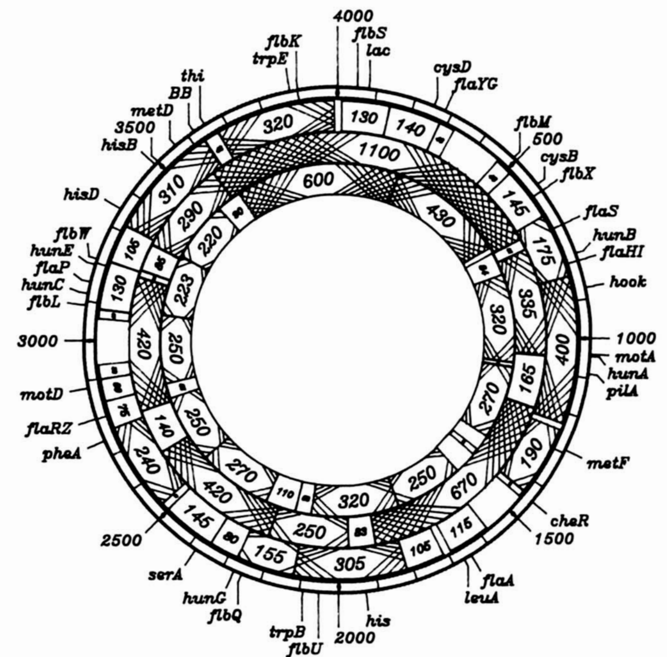

FIGURE 2.—A physical and genetic map of the *C. crescentus* chromosome. Fragment sizes are indicated in kilobases. The circles are restriction maps for *Dra*I, *Ase*I and *Spe*I, respectively, with *Spe*I being the innermost.

PFGE gives one the ability to analyze large fragments of DNA (3 kg to 10,000 kb) in agarose gels (SCHWARTZ and CANTOR 1984). Thus, it is possible to generate a restriction map of a bacterial genome using enzymes which cleave the DNA infrequently (SMITH *et al.* 1987). DNA fragments are separated by PFGE and identified by the presence of an insertion or deletion mutation or by hybridization to radioactive probe DNA. If the genetic locations of the mutations and the probes are known, the location of the restriction fragments can be correlated with the genetic map. We have identified three restriction enzymes, *Dra*I, *Ase*I, and *Spe*I, which cleave the *C. crescentus* genome infrequently (13–40 times) and have shown that they can be used as a means for the rapid determination of the map location of Tn*5* insertions (ELY and GERARDOT 1988; B. ELY, T. W. ELY, C. J. GERARDOT AND A. DINGWALL, submitted for publication). In this paper, we describe the use of PFGE to generate a detailed physical map of the *C. crescentus* genome and to provide a rapid means of determining the map location of a collection of 82 flagellar mutations. In the process, we demonstrated the presence of ten previously uncharacterized flagellar genes.

MATERIALS AND METHODS

Genetic techniques: The growth of *C. crescentus* and the genetic techniques used in this study have been described previously (ELY, CROFT and GERARDOT 1984). Techniques for PFGE analysis were described by ELY and GERARDOT (1988). Bacterial flagella were observed by light microscopy after fixing with phenol and tannic acid and staining with crystal violet (HEIMBROOK, WANG and CAMPBELL 1986).

Transposon mutagenesis: Transposon insertion mutations were isolated as described by ELY and CROFT (1982) using pJB4JI as a suicide vector for transposon Tn*5* mutagenesis (BERINGER *et al.* 1978) and pBEE132 as a suicide vector for a derivative of Tn*5*, Tn*5*-132, which confers tetracycline resistance (ELY 1985). The introduction of pBEE132 into a strain containing a Tn*5* insertion resulted in a recombination event between the chromosomal Tn*5* element and the Tn*5*-132 located on the plasmid. Selection for the tetracycline resistance encoded by Tn*5*-132 and screening for the loss of the kanamycin resistance conferred by Tn*5* resulted in the isolation of strains where the chromosomal Tn*5* element had been replaced by Tn*5*-132.

RESULTS

Generation of a *Spe*I restriction map: Previous experiments had demonstrated that *Spe*I cleaves the *C. crescentus* genome into a small number of fragments (B. ELY, T. W. ELY, C. J. GERARDOT and A. DINGWALL, submitted for publication). Furthermore, the largest fragment (600 kb) contained genes from both ends of the genetic map, providing evidence that the *C. crescentus* chromosome is circular. In order to correlate the remainder of the restriction fragments with the genetic map, we used *Spe*I to digest the DNA from a number of mutants and subjected the resulting fragments to PFGE. Using pulse times of 20 sec and 3 sec, we were able to resolve 18 bands (Figure 1). However, the 19-kb and the 35-kb bands probably

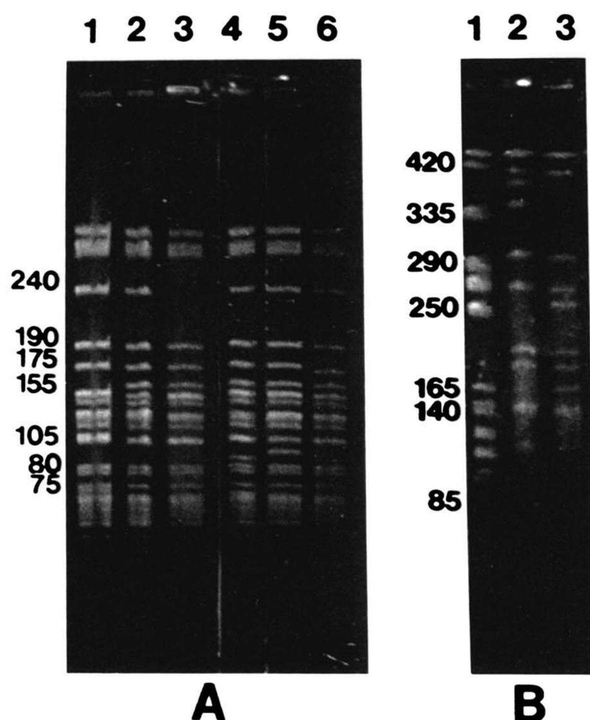

FIGURE 3.—PFGE of restriction enzyme digested *C. crescentus* DNA. (A) The DNA was restricted with *Dra*I and run on a gel at 240 V for 14 h with 10 sec pulse time. Lane 1, SC3566, the Tn*5*-132 insertion is in the 155-kb band; lane 2, SC3567, the Tn*5*-132 insertion is in one of the 145-kb bands; land 3, SC2185, the Tn*5*-132 insertion is in the 240-kb band; lane 4, SC3599, the Tn*5*-132 insertion is in one of the 130-kb bands; lane 5, SC3595, the Tn*5*-132 insertion is in one of the 105-kb bands; lane 6, SC3593, the Tn*5*-132 insertion is in the 115-kb band. (B) The DNA was restricted with *Ase*I and run on a gel at 200 V for 14 hr with a 20-sec pulse time. Lane 1, SC3594, the Tn*5*-132 insertion is in one of the 420-kb bands; lane 2, SC3593, the Tn*5*-132 insertion is in the 335-kb band; lane 3, SC3600, the Tn*5*-132 insertion is in the 335-kb band.

contained two fragments since they were more intensely stained. After calculating the size of each band, we were surprised to find that the sum of the *Spe*I bands was approximately 2700 kb compared to a genomic size of 4000 kb (B. ELY, T. W. ELY, C. J. GERARDOT and A. DINGWALL, submitted for publication). Further experiments indicated that the 220-, 270-, and 320-kb bands each contained two restriction fragments (Figure 1) and that the 250-kb band contained three restriction fragments (data not shown). Since all of the bands in this size class contained multiple fragments, the presence of multiple fragments within a band could not be detected by differences in band intensity. In the case of the 270-kb band, the two fragments apparently have slightly different sizes. If the larger of the two fragments contains a 5.7-kb Tn*5* insertion, the two fragments are resolved into two bands (Figure 1A, lanes 4 and 5). However, if the smaller fragment contains the insertion, a single band is observed at a slightly higher position in the gel (Figure 1A, lane 3). A similar situation occurred with the 320-kb and 220-kb bands. However, we were unable to solve the three 250-kb fragments into discrete bands. Once the extra fragments were identified, the sum of the restriction fragments was approximately 4000 kb, in good agreement with the previous determinations.

The map location of the *Spe*I restriction fragments was determined by screening Tn*5* mutations located at various sites around the genome. Once a particular restriction fragment was identified by the presence of an inserted Tn*5* element, the location of the fragment was determined by comparison to the location of the Tn*5* insertion on the genetic map (Figure 2). The location of the 18-kb fragment was determined by hybridization to a clone containing the gene for the pilin structural protein *pilA* (F. MCNALLY and N. AGABIAN, personal communication). Fortuitously, the *pilA* clone contains a *Spe*I site so it hybridized to two bands, the 18-kb band and a 270-kb band. Thus, the 18-kb band must be adjacent to the 270-kb band. Using these approaches, we were able to determine the map location of all of the large bands and 5 of the 12 smaller bands (Figure 2).

Generation of a composite restriction map of the *C. crescentus* genome: Previous work had resulted in correlations of the *Dra*I and the *Ase*I restriction maps with the genetic map (ELY and GERADOT 1988; B. ELY, T. W. ELY, C. J. GERARDOT and A. DINGWALL, submitted for publication). Therefore, we wanted to generate a composite map showing the relative positions of all three sets of restriction fragments. A crude alignment could be obtained simply by matching all of the fragments to the same set of Tn*5* insertion mutations. However, Tn*5* insertions could not be used to determine the precise position of a restriction fragment since the insertion could be anywhere within the restriction fragment. In contrast, Tn*5*-132 contains both a *Dra*I and an *Ase*I restriction site. Thus, insertion of Tn*5*-132 into the chromosome will result in the cleavage of the corresponding *Dra*I and *Ase*I restriction fragments at the point of the insertion (Figure 3). This information can then be used to determine the relative positions of the two fragments. Much of the data needed for the alignment of the *Dra*I and *Ase*I restriction maps was obtained from the mapping of *fla*::Tn*5*-132 mutations (see below). In addition, Tn*5* insertions at critical locations were converted to Tn*5*-132 elements by homologous recombination (see Materials and Methods), and then subjected to PFGE to determine the precise map location of the insertion. Once the *Dra*I and *Ase*I maps were aligned with each other relative to the genetic map, the *Spe*I map could be aligned relatively easily and provided additional data for a more precise alignment of the other two maps (Figure 2).

Characterization of flagellar mutants: More than

TABLE 1

Distribution of flagellar mutations

Map designation	Map location	Restriction fragments[a]	Mutant strains
flbS	50	130 D 1100 A 600 S	SC2201 (*flbS636*)
flaYG cluster	280	140 D 48 D 1100 A 430 S	SC1031 (*fla-193*), SC1047 (*flaG617*), SC1062 (*flaE178*), SC1065 (*flbA604*), SC1121 (*flaE183*), SC1133 (*fla-184*), SC1165 (*fla-605*), SC1962 (*fla-646*), SC2189 (*fla-626*), SC2196 (*fla-631*), SC2197 (*fla-632*), SC2199 (*fla-634*), SC3597 (*fla-663*)
flbM	490	48 D 1100 A 430 S	SC3351 (*flbM667*)
flbX	600	145 A 1100 A 430 S	SC2204 (*flbX639*)
flaHI	800	175 D 335 A 320 S	SC1048 (*fla-170*), SC1126 (*fla-186*), SC3091 (*fla-152*), SC3600 (*fla-664*)
Hook cluster	870	400 D 395 A 320 S	SC889 (*fla-654*), SC1028 (*fla-188*), SC1032 (*flbD198*), SC1038 (*fla-166*), SC1042 (*fla-601*), SC1043 (*fla-616*), SC1045 (*fla-169*), SC1049 (*flaN616*), SC1052 (*flb0172*), SC1054 (*fla-173*), SC1060 (*fla-176*), SC1061 (*fla-177*), SC1114 (*fla-607*), SC1132 (*flbF608*), SC1134 (*flaN603*), SC1135 (*flbG602*), SC1166 (*fla-609*), SC1953 (*fla-619*), SC1967 (*fla-620*), SC2190 (*fla-627*), SC2208 (*fla-655*), SC2249 (*fla-656*), SC3525 (*fla-659*), SC3592 (*fla-612*), SC3598 (*fla-661*)
motA	1040	400 D 165 A 320 S	SC3346 (*motA123*)
flaA	1650	115 D 670 A 250 S	SC886 (*fla-611*), SC1128 (*flaA613*), SC2193 (*flaA628*)
flbU	2050	305 D 260 A 320 S	SC1055 (*flbU610*)
flbQ	2250	155 D 420 A 110 S	SC3594 (*flbQ668*)
flaRZ	2800	75 D 420 A 55 S	SC1030 (*fla-618*), SC1127 (*fla-614*), SC1979 (*fla-648*), SC2198 (*flbV633*), SC2202 (*fla-637*), SC3090 (*fla-651*)
motD	2800	60 D 410 A 55 S	SC2252 (*motD122*)
flaP	3150	130 D 420 A 223 S	SC1123 (*flbY190*), SC3529 (*flbY665*)
Basal body cluster	3620	310 D 1100 A 90 S or 600 S	SC1033 (*fla-197*), SC1041 (*fla-187*), SC1044 (*fla-168*), SC1051 (*fla-171*), SC1058 (*fla-174*), SC1059 (*fla-175*), SC1066 (*flb0179*), SC1116 (*fla-192*), SC1117 (*flbN194*), SC1120 (*fla-191*), SC1125 (*fla-189*), SC1131 (*flb0196*), SC1963 (*fla-647*), SC2188 (*fla-625*), SC2203 (*fla-638*), SC2250 (*fla-656*), SC3528 (*fla-662*)
flbK	3900	320 D 1100 A 600 S	SC2205 (*flbK640*), SC2207 (*flbK641*)

[a] Abbreviations: D, *Dra*I; A, ASE *Ase*I; S, *Spe*I.

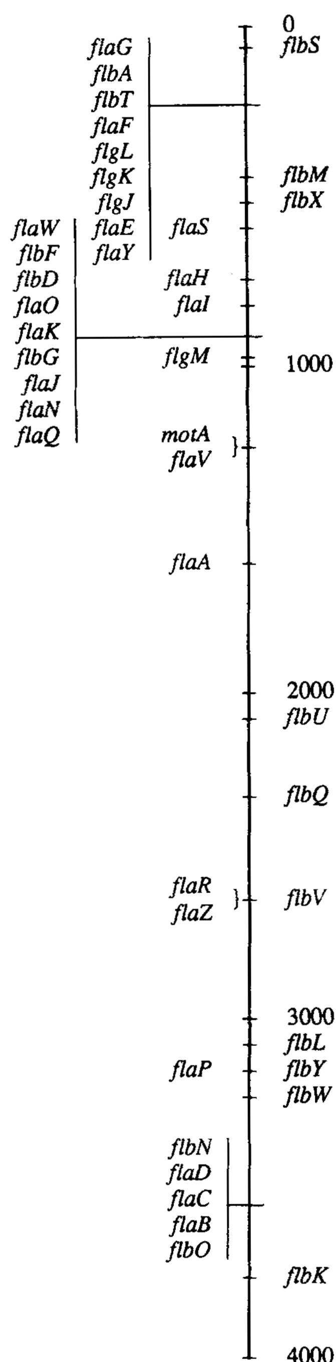

FIGURE 4.—A simplified genetic map showing the position of the newly identified flagellar genes (right) with respect to the previously identified flagellar gene (left).

80 flagellar mutations were isolated by screening for colonies which failed to form swarms after insertion mutatenesis with either Tn*5* or Tn*5*-132. Each mutant was examined in the light microscope and those which were nonmotile and had wild type cell morphology were designated flagellar mutants. Those which were motile and had wild type cell morphology were considered to have defects in chemotaxis (ELY *et al.* 1986), and those which did not have wild-type cell morphology were considered to have defects affecting cell division or polar organelle development (B. ELY, P. V. SCHOENLEIN and P. SHARMA, manuscript in preparation). All of the flagellar mutants were stained for the presence of flagella, and three were found which formed flagellar filaments. Since these mutants were non-motile even though they had apparently normal flagella, they were considered *mot* mutants with a paralyzed flagellum (JOHNSON and ELY 1979).

Use of PFGE to determine the map locations of flagellar mutations: Initially, we resorted to restriction mapping and Southern hybridization, rather than relying on genetic crosses, to provide a rapid means of mapping the flagellar mutants. Using this approach, the map location of several mutants often could be determined by a single gel. The initial experiments involved Southern hybridization of a Tn*5*-containing probe to gels containing *Eco*RI-digested DNA. Since the size of several of the *Eco*RI fragments containing clusters of flagellar genes was known, many of the mutations could be assigned to specific gene clusters based on the size of the *Eco*RI fragment containing the Tn*5* insertion. The assignments were then confirmed by hybridization to the appropriate clone. In this manner, we identified a number of Tn*5* insertions in the vicinity of the hook gene (OHTA *et al.* 1984) and in the cluster of basal body genes (HAHNENBERGER and SHAPIRO 1987). However, once we had generated a *Dra*I restriction map using PFGE, it became relatively simple to map any Tn*5* insertion. Therefore, we used PFGE to map all of the remaining flagellar mutations. DNA from each mutant was cleaved with *Dra*I, *Ase*I, or *Spe*I and subjected to PFGE. The map position of the mutation was determined by observing which band had altered migration. Flagellar mutations generated by Tn*5*-132 insertions were particularly useful since they resulted in the cleavage of both the *Dra*I and the *Ase*I fragments at the point of insertion, and thus, they allowed us to determine the precise location of the gene (Figure 3).

Analysis of 82 flagellar mutations indicated that they were located at 16 positions scattered around the chromosome (Figure 2). Two-thirds of the mutations were located in one of three clusters (Table 1). The hook gene cluster had the most mutations (26) and has been shown to contain at least nine genes (OHTA *et al.* 1984). Seventeen mutations were located in the basal body cluster which contains at least six genes (ELY, CROFT and GERARDOT 1984, HAHNENBERGER and SHAPIRO 1987), and 13 mutations were located in the *flaYG* gene cluster which contains six flagellar genes and three genes which encode the flagellin protein (SCHOENLEIN, GALLMAN and ELY 1989). Of the remaining 26 mutations, 16 were located at previously identified positions (ELY, CROFT and GERARDOT 1984) and the rest were located at eight new positions on the genetic map (Figure 4). Thus, at least

eight new genes were identified. In addition, complementation experiments indicated that the presence of previously unidentified genes in both the *flaRZ* cluster (*flbV*) and the *flaP* cluster (*flbY*). Therefore, this analysis resulted in the identification of 10 new genes in addition to those identified as part of the studies of the major gene clusters.

DISCUSSION

We have used PFGE to construct an integrated physical and genetic map of the *C. crescentus* genome using three restriction enzymes which cleave the genome infrequently. As a consequence, the map location of new mutations can be determined in a fraction of the time that was required previously. In addition, the physical map indicates that the *C. crescentus* chromosome is circular even though the genetic map is linear (B. Ely, T. W. Ely, C. J. Gerardot and A. Dinwall, submitted for publication). These studies suggest that the terminus for chomosome replication is located between the ends of the genetic map and that high frequencies of recombination in this region disrupts genetic linkage.

The flagellar apparatus is very complex and a large number of genes are needed to construct a functional flagellum. In *C. crescentus*, six genes have been identified which encode the flagellin proteins found in the filament (Gill and Agabian 1983). In addition, these studies indicate that at least 38 genes are required for the construction of the flagellum and that mutations in any one of four *mot* genes result in a paralyzed flagellum. Thus, at least 48 genes are involved in the production of a functional flagellum. By analogy to the enteric bacteria (Macnab 1987), we estimate that approximately 20 of these genes encode structural components of the flagellum. The function of the other genes remains to be determined but is likely to include genes involved in the assembly of the flagellum and genes which function in a regulatory capacity. The current evidence suggests that the expression of the flagellar genes is coordinated in a complex fashion and experiments are in progress to identify not only the regulatory interactions between the flagellar genes, but also the mechanism which controls the timing of flagellum biogenesis.

This work was supported by grant GM33580 from the National Institutes of Health.

LITERATURE CITED

Beringer, J. E., J. L. Brynon, A. V. Buchanon-Wallaston and A. W. B. Johnston, 1978 Transfer of the drug resistance transposon Tn*5* to *Rhizobium*. Nature **276:** 633–634.

Ely, B., 1985 Vectors for transposon mutagenesis of non-enteric bacteria. Mol. Gen. Genet. **200:** 302–304.

Ely, B., and R. H. Croft, 1982 Transposon mutagenesis in *C. crescentus*. J. Bacteriol. **149:** 620–625.

Ely, B., R. H. Croft and C. J. Gerardot, 1984 Genetic mapping of genes required for motility in *Caulobacter crescentus*. Genetics **108:** 523–532.

Ely, B., and C. Gerardot, 1988 Use of pulsed field gradient gel electrophoresis to construct a physical map of the *Caulobacter crescentus* genome. Gene **68:** 323–330.

Ely, B., C. J. Gerardot, D. L. Fleming, S. L. Gomes, P. Frederikse and L. Shapiro, 1986 Genetic nonchemotactic mutants of *Caulobacter crescentus*. Genetics **114:** 717–730.

Gill, P. R., and N. Agabian, 1983 Genomic organization of and sequence homology between the developmentally regulated flagellin genes of *Caulobacter crescentus*. Fed. Proc. **42:** 1969.

Hahnenberger, K. M., and L. Shapiro, 1978 Identification of a gene cluster involved in flagellar basal body biogenesis in *Caulobacter crescentus*. J. Mol. Biol. **194:** 91–103.

Heimbrook, M. E., W. L. Wang and J. Campbell, 1986 Easily made flagella stains. ASM Annual Meeting (abstract), p. 240.

Johnson, R. C., and B. Ely, 1979 Analysis of non-motile mutants of the dimorphic bacterium *Caulobacter crescentus*. J. Bacteriol. **137:** 627–634.

Johnson, R. C., M. P. Walsh, B. Ely, and L. Shapiro, 1979 Flagellar hook and basal complex of *Caulobacter crescentus*. J. Bacteriol. **138:** 984–989.

Macnab, R. M., 1987 Flagella, pp. 70–83 in *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, edited by F. C. Neidhardt, J. Ingraham, K. B. Low, B. Magasanik, M. Schaechter and H. E. Umbarger. American Society for Microbiology, Washington, D.C.

Ohta, N., E. Swanson, B. Ely and A. Newton, 1984 Physical mapping and complementation analysis of transposon Tn*5* mutations in *Caulobacter crescentus*: organization of transcriptional units in the hook gene cluster. J. Bacteriol. **158:** 897–904,

Schoenlein, P. V., L. M. Gallman and B. Ely, 1989 Organization of the *flaFG* gene cluster and the identification of two additional genes involved in flagellum biogenesis in *Caulobacter crescentus*. J. Bacteriol. **171:** 1544–1553.

Schwartz, D. C., and C. R. Cantor, 1984 Separation of yeast chromosome, sized DNAs by pulsed field gradient gel electrophoresis. Cell **37:** 67–75.

Smith, C. L., J. G. Economc, A. Schutt, S. Klco and C. R. Cantor, 1987 A physical map of the *Escherichia coli* K12 genome. Science **236:** 1448–1453.

Communicating editor: G. Mosig