Identification, Distribution, and Sequence Analysis of New Insertion Elements in *Caulobacter crescentus*

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We describe two insertion elements isolated from *Caulobacter crescentus* that are designated IS298 and IS511. These insertion elements were cloned from spontaneous flagellar (*fla*) gene mutants SC298 and SC511 derived from the wild-type strain CB15 (ATCC 19089), in which they were originally identified as insertions in the *flbG* operon of the hook gene cluster (N. Ohta, E. Swanson, B. Ely, and A. Newton, J. Bacteriol. 158:897-904, 1984). IS298 and IS511 were each present in *C. crescentus* CB2 and CB15 in at least four different positions, but neither was present in strain CB13 or in several *Caulobacter* species examined, including *C. vibrioides*, *C. leidyia*, and *C. henricii*. Nucleotide sequence analysis across the chromosome-insertion element junctions showed that IS298 is located 152 base pairs (bp) upstream from the ATG translation start of the hook protein gene *flaK*, where it is bounded by a 4-bp direct repeat derived from the site of insertion, and that IS511 is inserted at codon 186 of the *flaK* coding sequence, where it is also bounded by a 4-bp direct repeat duplicated from the site of insertion. The *ilvB102* mutation in strain SC125 was also shown to result from insertion sequence IS511, but no duplication of the genomic sequence was present at the insertion element junctions. IS298 contains an imperfect terminal inverted repeat 16 bp long, and IS511 contains a 32-bp inverted repeat at the termini. IS298 and IS511 are the first insertion elements described in *C. crescentus*.

Insertion elements are a class of short, 0.8- to 1.8-kilobase (kb) DNA segments that are found in multiple copies in the chromosomes and episomes of a variety of procaryotes (for reviews, see references 16 and 25). These elements transpose at low frequencies to other locations in the chromosome or to other resident replicons. They can also catalyze a variety of genetic rearrangements such as replicon fusions, deletions, and inversions and change the normal expression of nearby genes by introducing transcription start or stop signals (for reviews, see references 3, 8, and 12). Insertion elements also share a common structure; they carry at least one open reading frame and a short (9- to 40-base-pair [bp]) terminal inverted repeat serving as a *cis*-acting sequence. Both of these sequence features seem to be essential for transposition of the elements. Moreover, a direct repeat of the target sequence, ranging from 3 to 20 bp long, depending on the element, is usually generated at the termini as a consequence of transposition (12, 25).

Physical mapping of the flagellar (*fla*) genes in the hook gene cluster of the aquatic bacterium *Caulobacter crescentus* revealed that the *fla* mutations in strains SC298 and SC511 resulted from DNA insertions designated here as IS298 and IS511, respectively (20). Southern hybridization analysis of genomic DNA from IS298 with nick-translated hook operon DNA showed that the mutant contains an approximately 800-bp insertion in the 0.64-kb *Bam*HI(b)-*Bam*HI(c) fragment of the *flbG* (hook) operon (Fig. 1A) (20). IS511 was identified as an insertion in the spontaneous *fla* mutant strain SC511. This insertion of approximately 1,300 bp is in the 2.3-kb *Bam*HI(c)-*Bam*HI(d) fragment containing *flaK*, which codes for the hook protein (Fig. 1A) (20). IS298

and IS511 are the only insertion elements reported in *C. crescentus*, and because of the potential use of these elements in genetic studies, we have cloned the two sequences, examined their distribution in a number of *Caulobacter* strains, and determined nucleotide sequences at the sites of insertion. The experiments reported here show that IS298 and IS511 contain imperfect terminal inverted repeats of 16 and 32 bp, respectively, and that both elements contain at their ends short direct repeats at the site of insertion in the hook operon. We also found that the *ilvB102* mutation in SC125 was caused by an IS511 insertion with the same inverted terminal repeats as the one cloned from the *flaK* mutation, although no direct repeat of genomic sequences was present at the junction of the insertion. These results suggest that IS298 and IS511 are transposable insertion elements.

MATERIALS AND METHODS

Strains and growth conditions. *Caulobacter* strains were grown in PYE medium (21) at 30°C, *Escherichia coli* strains were grown in L broth (17) at 37°C, and antibiotics (ampicillin at 50 µg/ml and tetracycline at 15 µg/ml) were added when necessary. Lambda bacteriophage EMBL3 (11), M13 bacteriophages mp18 and mp19 (27), and plasmids pBR322 (4) and pUC18 (27) were used as cloning vectors.

Cloning of insertion elements. IS298 was cloned from strain SC298 (14). Genomic DNA was partially digested with *Mbo*I, ligated to lambda phage EMBL3 DNA restricted with *Bam*HI, packaged in vitro (13), and used to infect *E. coli* Q358 (15). Plaques were patched on the lawn of Q358 and transferred to nitrocellulose filters (2), and recombinants were screened with nick-translated (23) plasmid pBRS161, a pBR322 recombinant plasmid that contains the 2.94-kb *Bam*HI(b)-*Bam*HI(d) fragment (Fig. 1A), as a probe.

Among the 1,100 phage isolates that were screened in this assay, three gave positive hybridization signals. Restriction analysis of the DNA from each of these phages showed that

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FIG. 1. (A) Physical and genetic maps of the hook operon. G, J, H, and K correspond to genes *flbG*, *flaJ*, *flbH*, and *flaK*. *flbGp* refers to the start site of transcription for the hook operon (*flbG* operon), and the arrow just below G, J, H, and K indicates the direction of transcription. (B) Location, restriction map, and sequencing strategy of the IS298 element. M13 phages used for sequencing were 18298, starting at the *Sall*(a) site (a); 18298/125 (Bal31 deletion in 18298), starting 189 bp from the right end of IS298 (b); 19298/202 (Bal31 deletion in 19298), starting at bp 239 (c); and 19298, starting at the *Bam*HI(c) site (d). (C) Location, restriction map, and sequencing strategy of the IS511 element. M13 phages used were 18511-2/310 (Bal31 deletion in 18511), starting -21 bp from the left end of IS511 (a); 18511-2/188 (also a Bal31 deletion), starting 289 bp from the right end (b); and 18511-3, starting at the *Sall*(c) site (d). A 17-mer synthetic oligonucleotide corresponding to nucleotides 261 to 277 was used to sequence the stretch designated by c. (D) Location of IS511 in the *ilvB-ilvN* region and restriction map. DNA sequencing was carried out from the restriction sites indicated in a to d. Distances shown are in kilobases. Restriction sites were as follows: A, *Apal*I; B, *Bam*HI; Bg, *Bgl*II; R, *Eco*RI; K, *Kpn*I; P, *Pst*I; S, *Sall*; Sm, *Sma*I; and St, *Sst*I.

FIG. 2. Genomic Southern hybridization analysis of IS298 and IS511 sequences in various *Caulobacter* strains. *Pst*I fragments of genomic DNA from the indicated *Caulobacter* strains were separated on 0.8% agarose gels. Southern blots were prepared (26) and probed with nick-translated plasmid pIS298 DNA (A) and plasmid pUCIS511 DNA (B), respectively (see Materials and Methods). Lanes: 1, *C. leidyia*; 2, *C. vibrioides*; 3, *C. crescentus* CB2; 4, *C. crescentus* CB13; 5, *C. crescentus* CB15; 6, *C. crescentus* SC298; 7, *C. crescentus* SC511; 8, *C. crescentus* SC511 *mot*⁺ revertant. (C) The *Sst*I-*Bam*HI(b) fragment from IS125 was used as a probe. Lanes: 1 and 3, CB15; 2 and 4, SC125. DNAs in lanes 3 and 4 were digested with *Bam*HI. Arrowheads indicate new bands. DNA fragment sizes are in kilobases.

the 0.64-kb *Bam*HI(b)-*Bam*HI(c) fragment from the hook operon, which is normally present in the wild-type genome, was replaced by a fragment approximately 1.4 kb long, as expected for an insertion of 800 bp (data not shown). One of the clones, Cc12,003, was used for further studies. The 1,400-bp *Bam*HI(b)-*Bam*HI(c) fragment (Fig. 1A) containing IS298 was subcloned from bacteriophage lambda clone Cc12,003 in the *Bam*HI site of pBR322 to yield pIS298, from which the 928-bp *Sal*I(a)-*Bam*HI(c) fragment (Fig. 1B) was further subcloned in pUC18 as well as in M13mp18 and M13mp19 to yield pUCIS298, 18298, and 19298, respectively. M13 clones were treated with Bal31 nuclease to generate overlapping deletions for sequencing of the insertion-genomic DNA junctions and the ends of the element (see below).

The DNA fragment bearing the IS511 insertion was cloned from strain SC511 (14) as described above for the cloning of IS298. Of approximately 1,100 hybrid phages, 2, Cc12,004 and Cc12,007, hybridized with the plasmid pBRS161 and contained a 6.1-kb *Eco*RI(a)-*Eco*RI(b) fragment instead of the 4.7-kb *Eco*RI(a)-*Eco*RI(b) fragment present in the wild type (Fig. 1A). The 6.1-kb *Eco*RI(a)-*Eco*RI(b) fragment was subcloned from hybrid lambda clone Cc12,007 into the *Eco*RI site of pBR322 and designated pIS511. Restriction analysis showed that IS511 contains *Bam*HI, *Sal*I, *Sma*I, and *Sst*I sites (Fig. 1C). M13 subclones 18511-2 and 18511-3, which contain the entire IS511 sequence and short flanking wild-type genomic sequences on both sides, were obtained by cloning *Sal*I(b)-*Sal*I(c) from pIS511 in M13mp18 in opposite directions. The same *Sal*I(b)-*Sal*I(c) fragment was also cloned in pUC18, designated pUCIS511, and used as a probe in Southern hybridization experiments (see below). DNA fragments containing IS511 in the *ilvBN* region of strain SC125 were cloned by ligating *Bam*HI-restricted genomic DNA into pUC19 and screening for the insert with plasmid pIS511 DNA (see Results).

Restriction analysis, PFGE, and Southern hybridization analysis. Restriction endonuclease digestion conditions were

those recommended by the vendors. Genomic mapping of the IS298 and IS511 elements in the genomes of various *Caulobacter* strains was carried out by the method of Southern (26). Pulsed-field gel electrophoresis (PFGE) was carried out as described previously (10).

Nucleotide sequence analysis. Sequencing was carried out by the dideoxy-chain termination method developed by Sanger et al. (24) with hybrid phage M13 single-stranded DNA (22). For the characterization of IS511 in SC125, double-stranded plasmid DNA was used as a template to direct DNA sequencing (5) and Sequenase (U.S. Biochemical Co.) was used in place of the Klenow fragment of DNA polymerase I.

RESULTS

Isolation and physical mapping of IS298 and IS511. Insertion elements IS298 and IS511 (Fig. 1A to C) from *flaK* mutants SC298 and SC511 (20) were cloned as described above. Insertion element IS125, which is the mutation in the *ilvB* gene in strain SC125 (J. Tarleton and B. Ely, submitted for publication), was cloned in two parts: a 3.7-kb *Bam*HI(a)-*Bam*HI(b) DNA fragment in plasmid pIS125 and a 0.4-kb *Bam*HI(b)-*Bam*HI(c) DNA fragment in plasmid pIS1 (Fig. 1D) in pUC19 (see Materials and Methods). Restriction analyses of the cloned IS298, IS511, and IS125 elements are shown in Fig. 1B to D. These results strongly suggested that the IS511 and IS125 sequences are similar if not identical. This conclusion is suggested by the genomic Southern and sequence analyses discussed below.

Distribution and number of copies of IS298 and IS511. Hybrid plasmids pIS298 and pUCIS511 were nick translated and used as probes in Southern hybridizations to determine the number of copies of IS298 and IS511 in several *C. crescentus* strains and other *Caulobacter* species (21). Figure 2A shows the pattern of hybridization when genomic DNA was cleaved with *Pst*I and probed with nick-translated pIS298 DNA. *C. crescentus* CB2 and CB15 (Fig. 2A, lanes 3

and 5) showed four strongly hybridizing fragments of 6, 4.5, 4.3, and 2.5 kb, suggesting that these wild-type strains contained at least four copies of IS298. Weak hybridization of the 3.8-kb *Pst*I(a)-*Pst*I(b) fragment was due to the presence of genomic sequences that flank the IS298 element in the probe (Fig. 1A and B). *C. crescentus* CB13, by contrast, showed only one weakly hybridizing fragment of 4.3 kb which probably resulted from the hybridization of the 640-bp genomic sequence present in the probe (Fig. 2A, lane 4). The difference in the sizes of the *Pst*I-*Pst*I fragments, 3.8 kb in CB2 and CB15 and 4.3 kb in CB13, was presumably due to a restriction site polymorphism between the hook operon of strain CB13 and that of strains CB2 and CB15. Thus, strain CB13 does not appear to contain IS298. DNA from the original *fla* insertion mutant strain SC298 (Fig. 2A, lane 6) contained the same hybridizing fragments seen in the wild-type parent strain CB15 plus a strongly hybridizing fragment of 4.6 kb which corresponded to the 3.8-kb *Pst*I(a)-*Pst*I(b) fragment (Fig. 1A) plus the 800-bp IS298. An additional 5.1-kb fragment observed in Southern blots of genomic DNA from strain SC511 also resulted from hybridization of the 640-bp genomic sequence in the probe to the 3.8-kb *Pst*I(a)-*Pst*I(b) fragment (Fig. 1A), in which the 1.3-kb IS511 insertion occurred (Fig. 2A, lane 7).

A similar Southern hybridization study with *Pst*I-digested genomic DNA and labeled pUCIS511 as a probe (Fig. 2B) showed that IS511 was also present in four copies (10, 5.6, 4.4, and 4 kb) in strains CB2 (lane 3) and CB15 (lane 5), but at different chromosomal locations than IS298; i.e., a different set of *Pst*I digestion fragments hybridized to the two probes. The 3.8-kb fragment in CB15 and CB2, the 4.3-kb fragment in CB13 (Fig. 2B, lane 4), and the 4.6-kb fragment in SC298 (Fig. 2B, lane 6) hybridized to the pUCIS511 probe because of the flanking hook operon sequences cloned with IS511 (Fig. 1C). In SC511 DNA (Fig. 2B, lane 7), the 5.1-kb fragment containing IS511 hybridized strongly, as expected.

Figure 2C shows the results obtained when DNAs from strains CB15 and SC125 were probed with the internal *Sst*I-BamHI(b) sequence of IS25. The four *Pst*I bands from strains CB15 and SC125 that hybridized with the IS25 probe (Fig. 2C) were identical in size to those that hybridized with the IS511 probe (Fig. 2B). (The 10-kb band of SC125 in Fig. 2C, lane 2, is clearly visible at the longer exposure of the film.) In addition, SC125 contained a fifth band at 4.7 kb which was explained by the presence of the 1.3-kb insertion element in the 3.4-kb *Pst*I-*Pst*I fragment of the *ilvB* gene. The appearance of a new band is more clearly demonstrated by a comparison of lanes 3 and 4 of Fig. 2C, which shows the distribution of the elements in the *Bam*HI-digested DNA. Identical hybridization patterns were also observed when CB15 DNAs restricted with *Bam*HI, *Eco*RI, or *Sst*I were hybridized to either the IS511 or the IS25 probe (data not shown). These results and the identical restriction maps (Fig. 1C and D) strongly suggested that the two elements are identical, and we designate them IS511. Comparison of the Southern blots in Fig. 2A and B also showed that there was no detectable cross-hybridization between the IS298 and IS511 elements.

We conclude from these results that IS298 and IS511 are present in at least four copies in *C. crescentus* CB2 and CB15 and that IS511 and IS25 are identical (see below). These sequences are not found in the well-studied strain *C. crescentus* CB13 or in other, less-well-characterized *Caulobacter* strains, including *C. leidyia* CB37 (Fig. 2A and B, lanes 1), *C. vibrioides* CV115 (Fig. 2A and B, lanes 2), and *C. henricii* (data not shown).

FIG. 3. Southern analysis of IS511 sequences in *Dra*I fragments separated by PFGE. Genomic DNAs from CB15 (lane 1), SC125 (lane 2), and SC511 (lane 3) were digested with *Dra*I and separated by PFGE on 1% agarose, and the Southern blot was hybridized to nick-translated pUCIS511 DNA (see Materials and Methods). (A) Ethidium bromide staining. (B) Autoradiogram. Numbers between panels are in kilobases.

Location of IS511 on the genetic map. Since the IS511 element appeared to be the mutagenic agent that gave rise to the independent and spontaneously derived mutations in strains SC511 and SC125, we were interested in mapping the resident IS511 element. Southern analysis of the *Dra*I fragments separated by PFGE showed that the element occurs on four separate fragments in the wild-type strain CB15 (Fig. 3B, lane 1). An additional band at 400 kb was detected in SC511 (Fig. 3B, lane 3), consistent with the established assignment of the hook operon on this fragment (10). The pattern of hybridization of SC125 DNA was identical to that of CB15 DNA, since *ilvBN* genes are located on the 310-kb fragment (Fig. 3B, lane 2) (Tarleton and Ely, submitted).

Sequence analysis. The nucleotide sequences of IS298 and IS511 were determined on both strands by the dideoxynucleotide-chain termination method (24). Clones used to determine the sequences were obtained with *Bal*31 nuclease to generate a set of overlapping deletions (22). The sequencing strategy is summarized in Fig. 1B to D.

Nucleotide sequences of the junctions spanning genomic DNA and the left and right ends of IS298 were obtained on both strands with two M13 clones, 18298 and 19298, containing the *Sal*I(a)-*Bam*HI(c) fragment (Fig. 1B) in opposite orientations, and their *Bal*31 deletion derivatives, 19298/202 and 18298/125 (Fig. 1B). Comparison of these sequences with that of the wild-type hook operon (D. A. Mullin and A. Newton, unpublished data) showed that the element is inserted 34 bp from the *Sal*I(a) site and 84 bp from the *Bam*HI(c) site, placing the element 152 bp upstream from the ATG translation start codon of the hook protein gene, *flaK* (Fig. 1A). The sequences at the junctions of the element also

FIG. 4. Inverted repeats and duplicated target sites of IS298 and IS511 in the *C. crescentus* genome. (A) Genomic sequence at the site of insertion of IS298. The 4-bp direct repeat is boxed. (B) The 14-bp terminal inverted repeats at the ends of IS298 are underlined. (C) Sequence of the genomic sequence-IS511 junction. The 4-bp direct repeat is boxed. (D) The 32-bp terminal inverted repeats at the ends of IS511 are underlined. (E) Genomic sequence at the site of insertion of IS511 in SC125. The 4-bp insertion at the target site is boxed.

revealed that it is bounded by a direct repeat of 4 bp (5'-CTAG-3') that is duplicated from a genomic sequence at the site of insertion (Fig. 4A). The element contains 14-bp imperfect inverted repeats at the ends (Fig. 4B).

The IS511 element was sequenced on one strand (left to right in Fig. 1C, a and b) with a set of Bal31 deletions obtained from the M13 clone 18511-2. The right end of the element was also sequenced in the opposite direction starting from *SalI*(c) with another M13 clone, 18511-3. The left end of the element on the same strand was sequenced with a 17-mer synthetic oligonucleotide primer corresponding to nucleotides 261 to 277, numbered from the left end of the element. A comparison of these sequences with the wild-type hook operon sequence (Mullin and Newton, unpublished data) showed that the insertion occurred after the second base of codon 186 (Ala) of the hook protein gene (Fig. 1A). The element is bounded by a 4-bp direct duplication of the target-site DNA (Fig. 4C) and possesses imperfect inverted terminal repeats of 32 bp (Fig. 4D).

The ends of the IS125 element and the insertion-genomic sequence junction were also sequenced by the dideoxy-chain

termination method with M13 clones as well as by a double-stranded DNA sequencing procedure (5) with pUC19 clones and were compared with the wild-type sequence of the *ilvB* gene. The ends of the IS125 element are identical to those of the IS511 element (Fig. 4E). Thus, we also designate this insertion element IS511. The sequences at the insertion junction in *ilvB*, however, showed no duplication of the genomic sequence and instead contained a 4-bp insertion at the right end of the element (Fig. 4E) (see Discussion).

DISCUSSION

We have presented evidence to show that the *fla* mutations in *C. crescentus* SC298 and SC511 resulted from the insertion of the DNA elements IS298 and IS511, respectively, into the hook operon. Similarly, the *ilv* mutation in SC125 was shown to be caused by the insertion of IS511 into the *ilvB* gene. These mutations presumably arose by the transposition of endogenous, resident copies of IS298 or IS511 present in strain CB15. We were unable to detect any homology between IS298 and IS511.

IS298 and IS511 are present in at least four copies each in strains CB2 and CB15, but they are not present in strain CB13 or in several other species, including *C. vibrioides*, *C. leidyia*, and *C. henricii*. Poindexter (21) has observed that strains CB2 and CB15 have very similar morphologies and are likely to be closely related. Thus, these elements are relatively restricted in their distribution among the *Caulobacter* spp.

The sequence features at the ends of IS298 and IS511 examined are similar to those of many other insertion elements. The IS298 element has a 14-bp imperfect inverted terminal repeat and a 4-bp duplication of genomic DNA as a direct repeat at the site of insertion (Fig. 4A and B), while the IS511 element has a 32-bp imperfect inverted terminal repeat (Fig. 4D). A 4-bp duplication of genomic DNA was observed in strain SC511 at the insertion site (Fig. 4C), but there is no direct duplication of the target site in strain SC125. Instead, strain SC125 contains a 4-bp insertion at one side of the insertion element-genomic DNA junction (Fig. 4E). These extra base pairs have no counterpart at the site of insertion in the wild-type *ilvB* sequence or in the IS511 element isolated from strain SC511. It could be argued from the results that the IS511 insertion in strain SC511 also occurred via the addition of 4 bp that happened to be identical to the 4 bp in the genomic sequence to the left of the insertion site and not via a 4-bp duplication.

These sequence features suggest the possibility that IS298 and IS511 are transposable elements. This conclusion is supported by our identification of IS511 as the mutagenic agent in the two independently isolated spontaneous mutants SC511 and SC125. The two mutant strains behave differently, however, with respect to the rate of reversion. While the *ilv* mutation in SC125 is stable and reverts at a frequency of approximately 10^{-9} , the *fla* mutation in SC511 reverts at a relatively high frequency, as indicated by the appearance of a number of small flares around stabs of the mutant cells in motility agar after prolonged incubation (data not shown). Genomic DNA isolated from the *fla*⁺ revertants of SC511 had a distribution of IS511 identical to that found in wild-type strain CB15 (Fig. 2B, lane 8), indicating that the *fla*⁺ revertants arose by excision of the element. Preliminary sequence analysis of these elements revealed that each contains several possible open reading frames so that, like other transposable elements, each probably codes for an enzyme(s) required for transposition (Mullin and Newton, unpublished data).

Since IS511 in strain SC125 has extra base pairs of unknown origin instead of direct repeats at the insertion site, the insertion may have resulted from an imprecise transposition event. Such an event could occur if imprecise nicking of DNA by a transposase occurred at the site at which IS511 previously resided, followed by transposition of extra DNA. The extra 4 bp on the right end of IS511 in strain SC125 may represent sequences flanking an IS511 element elsewhere on the *C. crescentus* chromosome. Alternately, non-template-directed replication errors during transposition from the original site may have incorporated extra bases.

Only a few examples of transposable elements which do not duplicate DNA sequences upon insertion have been described. These include transposon Tn554 from *Staphylococcus aureus*, which does not have terminal repeat sequences and apparently transposes into a specific target DNA sequence (18), and IS91, initially isolated from a hemolysin plasmid of *E. coli* (9). In the latter, it is hypothesized that blunt cuts, rather than staggered cuts, are made during the transposition process (9). Another example of an

unusual transposition event has been observed with the conjugative transposon Tn916 (7), in which an extra 3 bp of nontarget, non-Tn916 DNA sequence were observed while the left end contained the normal wild-type sequence. The authors proposed a mechanism by which these extra 3 bp were brought into the site through homologous recombination between the transposon intermediate and the target site (7). Some DNA similarities were present between the flanking sequences in their examples so that homologous recombination could occur. By contrast, there was little similarity between the flanking sequences present at the insertion site in either SC511 or SC125.

Several features of the IS298 and IS511 sequences may be useful in the genetic analysis of *C. crescentus*. If the elements are shown to transpose at a reasonable frequency in *C. crescentus*, then either may be convenient for use as an alternative to transposon Tn5 (1) or as a second transposable element in strains that already contain a Tn5 element. We are currently testing these possibilities.

Another feature of IS298 which may be of use is that it acts as a strong transcription terminator in *C. crescentus*. We have observed previously that the presence of this insertion element in the polycistronic hook operon prevents the synthesis of hook gene mRNA in SC298 (6). This effect is in contrast to the effect of a Tn5 insertion at approximately the same position in the hook operon. Tn5 insertion mutants do not synthesize hook protein (20), but they do contain high levels of *flaK* mRNA, as determined by nuclease S1 assays (19).

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