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Analysis of Cloned DNA from *Leptospira biflexa* Serovar patoc Which Complements a Deletion of the *Escherichia coli trpE* Gene

DAVID B. YELTON* AND REUBEN A. COHEN

Department of Microbiology, West Virginia University School of Medicine, Morgantown, West Virginia 26506

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To analyze the cloned region of the chromosome of the spirochete *Leptospira biflexa* serovar patoc which complemented a defect in the *trpE* gene of *Escherichia coli*, we performed a series of experiments involving subcloning, transposon mutagenesis, and maxicells. By subcloning into pBR322 we were able to isolate the *Leptospira* genes on a 9.7-kilobase pair plasmid (pYC6). Transposon mutagenesis with Tn*5* identified a 2.8-kilobase pair region of this plasmid as being necessary to complement a *trpE* deletion mutation in *E. coli*. Transformation of plasmid pYC6 into *E. coli* cells deleted for *trpE* and the proximal end of *trpD* showed that the *Leptospira* DNA complemented both defects. A maxicell analysis of various transposon-induced mutations of the plasmid revealed that three proteins (53.5, 23.6, and 22 kilodaltons) were encoded by the 2.8-kilobase pair region of the *Leptospira* genome. Two different promoters controlled the production of these three proteins.

Leptospira spp. are thin, motile, spiral-shaped, aerobic bacteria which use long-chain fatty acids or alcohols as their sole carbon and energy sources (16, 19). Only a few studies have been done on the physiology of *Leptospira* (2, 6, 14, 19, 30). The results of these studies suggest that, with the exception of isoleucine, which can be made by either of two pathways, *Leptospira* synthesizes its amino acids by the usual pathways (6, 30). Phylogenetic studies based on analyses of 16S rRNA oligonucleotides have demonstrated that the spirochetes, including *Leptospira*, are an ancient, phylogenetically distinct group of eubacteria (13, 27). Therefore, one might expect that the organization and control of the genetic information of these bacteria could differ from the organization and control of the genetic information of other eubacteria.

The pathway for tryptophan biosynthesis is identical in all organisms investigated to date (8, 9, 33). The first enzyme in the pathway, anthranilate synthase (AS), is composed of two subunits, designated AS I and AS II (36). AS I is the product of the *trpE* gene and binds chorismic acid, NH_3, and anthranilic acid (28); AS II is the product of the *trpG* gene and binds glutamine (28). In cases such as *Escherichia coli*, in which the *trpG* and *trpD* genes have fused to form a single gene, the proximal end of the *trpD* gene product acts as AS II (26). AS converts chorismic acid to anthranilate, with glutamine serving as the NH_3^+ donor (28). The genes required for tryptophan biosynthesis in *E. coli* are organized as an operon under both repressor and attenuator control (32). In other bacterial genera *trp* genes are also organized into operons, although in some cases the operons are split (8, 31, 33). Little is known about the organization and control of the tryptophan biosynthetic genes in *Leptospira* (35).

Yelton and Charon cloned a 16 kilobase pair (kbp) fragment of *Leptospira* DNA which complemented a deletion of the *trpE* gene of *E. coli* (35). The cloned DNA was not able to complement point mutations in the *trpD*, *trpC*, *trpB*, and *trpA* genes of *E. coli*. The purpose of the present study was to more fully analyze the cloned DNA in order to (i) determine the position of the *Leptospira trpE* gene within the cloned piece of DNA, (ii) determine the amount of DNA needed to complement the defect in *E. coli*, (iii) determine whether a *trpG* gene was present on the cloned *Leptospira* DNA, and (iv) determine the number of proteins encoded by that region of the *Leptospira* chromosome.

(Portions of this work have been presented in preliminary form previously [Yelton, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, H61, p. 101; Cohen and Yelton, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, H40, p. 114].)

MATERIALS AND METHODS

Bacterial strains. *E. coli* JA221 Δ*trpE5 leuB6 hsdR* (*hsdR*$^-$ *hsdM*$^+$) was used for cloning experiments and for screening plasmids for expression of the *Leptospira trpE* gene (7). *E. coli* HB101 was used as the host for transposon mutagenesis (12). *E. coli* LE392 was used to prepare stocks of lambda 467::Tn*5* (12). *E. coli* CSR603 was used for the maxicell experiments (29). *E. coli* strains W3110 *his cysB* Δ *trpED5* and W3110 *his cysB* Δ *trpED23* were used to test for the presence of a *trpG* gene within the cloned *Leptospira* DNA (18).

Media. *E. coli* strains were grown in LB broth (25) or on LB agar (1.5%). M9 medium (25) supplemented with the appropriate amino acids and 0.2% glucose was used to characterize transformants. M9 medium supplemented with 1% Casamino Acids and 0.2% glucose was used to determine the phenotypes of transposon-induced mutants. K medium (24) was used to grow cells for the maxicell analysis.

Chemicals and enzymes. All amino acids and antibiotics were obtained from Sigma Chemical Co. Restriction endonucleases were obtained from Bethesda Research Laboratories, Inc., and were used as recommended by the supplier. L-[^{35}S]methionine was obtained from New England Nuclear Corp.

Electrophoresis. Agarose gel electrophoresis was done in the submerged mode by using 0.8% high gelling temperature agarose (Bio-Rad Laboratories) and 89 mM Tris–8.9 mM borate–2 mM EDTA buffer (pH 8.3). Gels were stained with ethidium bromide and photographed under UV light. Poly-

* Corresponding author.

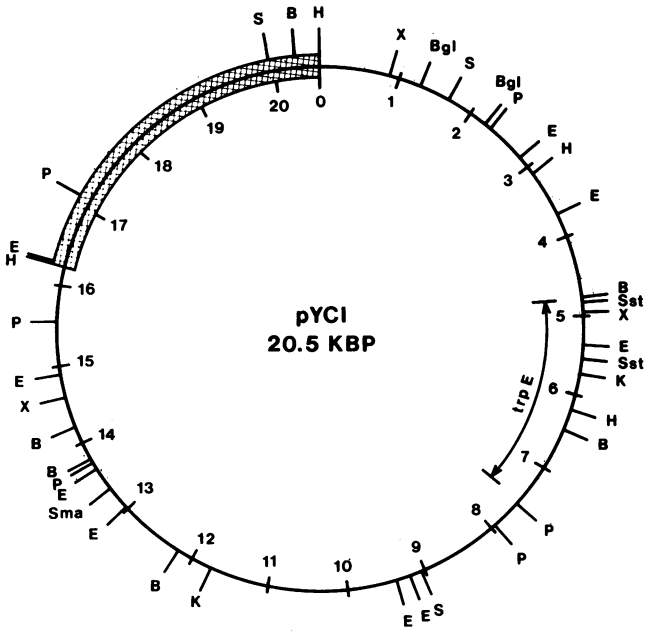

FIG. 1. Map of restriction endonuclease sites on pYC1. The map was constructed by using data from both single and double digestions of pYC1 with restriction endonucleases. The cross-hatched area represents pBR322; the single line represents *L. biflexa* serovar patoc DNA. H, *Hin*dIII; S, *Sal*I; X, *Xba*I; K, *Kpn*I; B, *Bam*HI; E, *Eco*RI; P, *Pst*I; Bgl, *Bgl*II; Sma, *Sma*I; Sst, *Sst*I.

acrylamide gel electrophoresis was performed by the method of Laemmli (22), using 12.5% gels and a discontinuous Tris-Glycine-sodium dodecyl sulfate buffer system at pH 8.9.

Plasmid DNA. Large batches of plasmid DNA were prepared as described by Davis et al. (10). The extracted plasmid DNA was subsequently purified through two cycles of equilibrium centrifugation in CsCl-ethidium bromide gradients. Small-scale plasmid isolation was performed by boiling lysozyme-treated cells in the presence of Triton X-100 (15). The plasmid DNA was precipitated with isopropanol, and the pellet was washed with 95% ethanol before being dissolved in 10 mM Tris–1 mM EDTA buffer (pH 7.5).

Restriction mapping. A map of restriction endonuclease recognition sites was constructed from the data obtained by determining the sizes of the DNA fragments produced after digestion of plasmid DNA with restriction endonucleases. Data from both single and double enzyme digests were used (23).

Transformation. *E. coli* strains were transformed by using the method described by Kushner (21). After transformation, drug-resistant colonies were selected for at 30°C.

Maxicells. *E. coli* CSR603 containing various plasmids was used in the maxicell procedure of Sancar et al. (29), except that all manipulations were done at 30°C. Protein was labeled with [^{35}S]methionine for 1 h at 30°C. For analysis 100,000 cpm was loaded into each well of the gel. After electrophoresis, the gels were analyzed by fluorography.

Transposon mutagenesis. Mutagenesis was performed with Tn*5* contained in lambda 467 (*b*221 *c*I857 *O*am29 *P*am80 *rex*::Tn*5*) by using the procedure described by de Bruijn and Lupski (12). pYC6 was introduced into *E. coli* HB101 before transposon mutagenesis. After mutagenesis, kanamycin-resistant (Kan^r) colonies were washed from the plates, and the plasmid DNA was extracted from them. The plasmid DNA was then transformed into *E. coli* JA221. Kan^r colonies were selected and then screened for their Trp phenotypes. A correlated map of Tn*5* insertion sites and colonial phenotypes was made as described previously (11, 12).

RESULTS

Restriction mapping. Yelton and Charon cloned the *trpE* gene of *Leptospira biflexa* serovar patoc (35). This gene resided on a 16 kbp fragment of DNA cloned into pBR322 (pYC1). A restriction map of pYC1 was generated by using the method described by Lawn et al. (23). Ten restriction endonucleases, each recognizing a different hexanucleotide sequence, were used in the mapping experiments. Only fragments between 0.5 and 6.0 kbp long were used to construct the map shown in Fig. 1.

Subcloning of the *Leptospira trpE* gene. Subclones of pYC1 were produced by digestion with a restriction endonuclease which cut twice in the *Leptospira* DNA insertion. The resulting fragments were separated by agarose gel electrophoresis. The fragment containing pBR322 was isolated and self-ligated. The result was a derivative of pYC1 which had a fragment of *Leptospira* DNA removed (Fig. 2). Digestion with *Bgl*II removed a 900-base pair piece of DNA (pYC2); digestion with *Sst*I removed a 700-base pair piece (pYC3); and digestion with *Kpn*I removed a 6.4-kbp fragment (pYC4). Each of these subclones was then tested for its ability to complement a *trpE* defect in *E. coli*. Only pYC2 was able to do so (Fig. 2). Next, specific fragments of pYC1 were subcloned into the appropriate site on pBR322. The *Hin*dIII fragment (3.1 to 6.2 kbp), the *Bam*HI fragment (4.6 to 6.9 kbp), and the *Sal*I fragment (1.7 to 9.0 kbp) were each subcloned. After transformation into *E. coli* JA221, antibiotic-resistant cells were selected. The subclones were then tested for their ability to complement the *trpE* deletion in *E. coli* JA221. Only the *Sal*I subclone (pYC6) was able to do so (Fig. 2).

Restriction endonuclease digests of pYC6 were examined to confirm that its restriction site map was as expected (Fig.

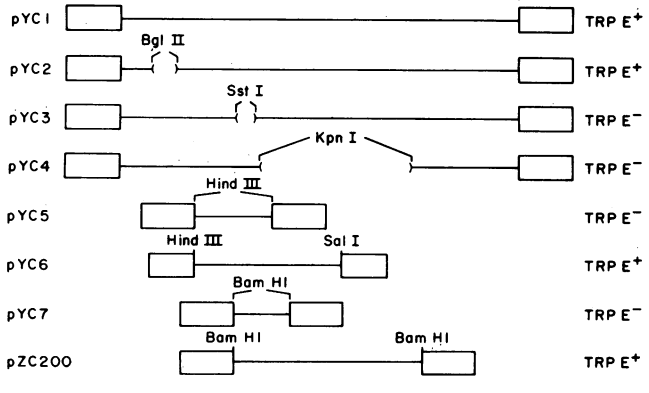

FIG. 2. Subclone analysis of pYC1. The various subclones of pYC1 which were analyzed are diagrammed. The single line represents *Leptospira* DNA; the boxes represent pBR322 DNA. The deleted regions are indicated by parentheses.

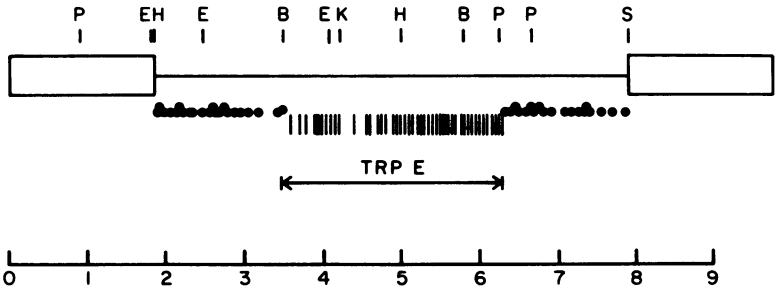

FIG. 3. Location of *trpE* as determined by transposon mutagenesis. The positions of the Tn*5* insertions are indicated by the markers. The dots indicate insertions having a Trp$^+$ phenotype; the vertical lines indicate insertions having a Trp$^-$ phenotype. Only a representative sample of the 200 independent insertions are diagrammed. The scale at the bottom is in kilobase pairs. P, *Pst*I; E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; K, *Kpn*I; S, *Sal*I.

3). More than 100 independently derived *Sal*I subclones from three different ligation reactions were examined, and they were all found to have been cloned into pBR322 in the same orientation. The orientation of the cloned *Leptospira* DNA in pYC6 was opposite to its orientation in pYC1 (Fig. 1 and 3). In addition, all of the *Sal*I subclones had undergone a spontaneous deletion. Digestion of the ligated vector and insertion DNAs with *Sal*I showed that neither had suffered deletions or rearrangements during ligation. Thus, the deletion seemed to have occurred after transformation into *E. coli*. This deletion removed the DNA between 1.7 kbp and approximately 3.1 kbp on the map of pYC1. It also removed the small fragment of pBR322 between the *Sal*I and *Hin*dIII sites. While we do not understand the reason for this deletion, plasmid pYC6 was able to complement the *trpE* defect in *E. coli*. This plasmid was used for the remainder of the studies described below.

Plasmid pZC200 from a *L. biflexa* serovar patoc clone bank produced by Zuerner and Charon by using *Bam*HI also complemented the *trpE* deletion in *E. coli* strain JA221 (personal communication). The cloned *Leptospira* DNA in pZC200 corresponds to the piece of DNA located between 4.6 and 12.2 kbp on the map of pYC1. This result suggests that the map of pYC1 is an accurate reflection of the chromosomal situation and that no reassortment of restriction fragments occurred in vitro during the ligation process which generated pYC1. A summary of the data obtained with the various subclones is shown in Fig. 2. From these data we were able to locate the *trpE* gene of *L. biflexa* serovar patoc to a 4.4-kbp region of DNA defined by the *Bam*HI site (4.6 kbp) at one end and the *Sal*I site (9.0 kbp) at the other end.

Transposon mutagenesis. To determine precisely the region needed for expression of the *trpE* gene of *Leptospira*, transposon mutagenesis was used. pYC6 was mutagenized with Tn*5*. A total of 200 independently derived Kan^r colonies were screened for their Trp phenotypes; 101 of the colonies were tryptophan dependent for growth, and 99 were tryptophan independent. The plasmid DNA from each of these colonies was analyzed with restriction enzymes to locate the site of the Tn*5* insertion. Correlation of the phenotypic and genotypic information resulted in a map which defined the extent of the *trpE* region of *Leptospira* (Fig. 3). This function required 2.8 kbp of *Leptospira* DNA and extended from 4.6 to 7.4 kbp on the map of pYC1. This is approximately 1 kbp larger than the coding capacity needed for *trpE* in other microorganisms (1, 31, 33).

Transformation of *E. coli trpE-trpG* mutants. The large size of the region defined by transposon mutagenesis suggested that more than one protein might be encoded by this piece of DNA. Since AS is composed of two subunits in other bacterial systems, it was possible that both a *trpE* function and a *trpG* function were encoded by the cloned DNA. To test this possibility, two strains of *E. coli* deleted for both *trpE* and the proximal end of *trpD* were used (18). These two strains could grow on anthranilic acid, indicating that they contained a functional anthranilate phosphoribosyl transferase and the region from *trpC* through *trpA*. After transformation with pYC6, both of the *E. coli* strains were able to grow without added anthranilate. These results indicated that both a *trpE* function and a *trpG* function were present on pYC6, thus allowing conversion of chorismic acid to anthranilic acid.

Maxicell analysis. Further evidence that more than one protein was necessary for *trpEG* function was obtained by using maxicells (29). *E. coli* CSR603 was transformed with pYC6 and several derivatives of pYC6 containing Tn*5*-induced mutations. The resulting transformants were then tested in the maxicell system to determine the number of proteins related to tryptophan biosynthesis which were encoded by the plasmid (Fig. 4). Three proteins were encoded by this region of the plasmid. The molecular weights of these proteins were 53.5×10^3, 23.6×10^3, and 22×10^3. A map correlating the locations of the Tn*5* insertions and the three proteins is shown in Fig. 5. Assuming no overlapping of the genes, approximately 2.7 kbp was required to encode these proteins. As the experiments with Tn*5* defined a 2.8-kbp region that was essential for expression of this genetic information, there was sufficient capacity to encode these three proteins, as well as their genetic control elements (Fig. 5). Figure 5 shows only the approximate locations of the proteins; the positions of start and stop codons were not rigorously defined.

DISCUSSION

The genes required for tryptophan biosynthesis and the controlling elements associated with these genes have been investigated in a variety of microorganisms (1, 17, 20, 31, 33). These studies have led to a better understanding of genetic control and have given microbiologists insight into evolution of genetic systems (9, 33). Since the spirochetes represent a phylogenetically distinct group of eubacteria (13, 27), studies of their tryptophan genetic systems might give further insights into these questions.

Transposons have proven to be powerful tools for genetic analysis. We used Tn*5* in our studies to define the region of DNA required for *trpE* function in *L. biflexa*. As found with other bacteria, Tn*5* inserted into the leptospiral DNA, re-

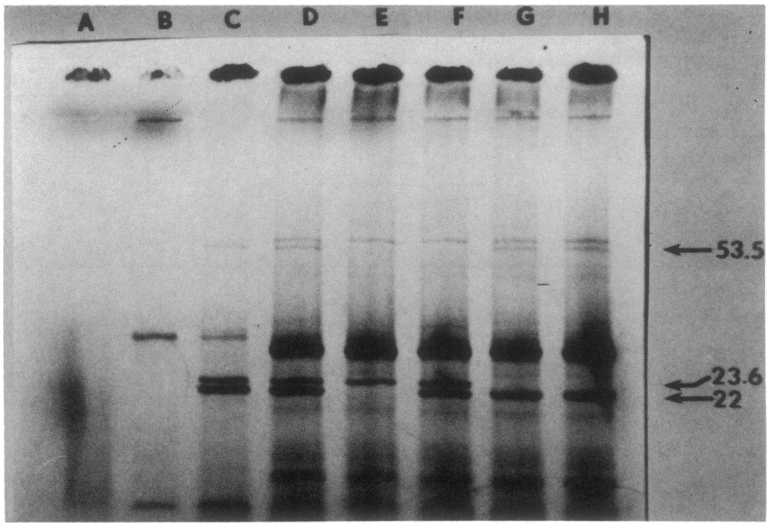

FIG. 4. Maxicell analysis of pYC6-encoded proteins. The labeled proteins were visualized by fluorography after electrophoresis. Lane A, CSR603; lane B, CSR603(pBR322); lane C, CSR603(pYC6); lane D, CSR603(pYC6::Tn*5*-71); lane E, CSR603(pYC6::Tn*5*-82); lane F, CSR603(pYC6::Tn*5*-158); lane G, CSR603(pYC6::Tn*5*-177); lane H CSR603(pYC6::Tn*5*-20). The bands at 53.5, 23.6, and 22 kDa are *Leptospira*-encoded proteins. The band at 28 kDa is the product of the kanamycin resistance gene; the band at 56 kDa is the product of the Tn*5* transposase regulation gene.

sulting in a transposition event approximately every 50 base pairs (3, 12). Since the leptospiral DNA could be cloned into pBR322 in either orientation (pYC1 and pYC6) and since insertions of Tn*5* on either side of the *trpE* region did not result in a loss of the ability to complement a *trpE* defect in *E. coli*, it is unlikely that expression of the tryptophan genes depends on pBR322 promoters. Expression from Tn*5* promoters also seems unlikely. Outward promoters are known for Tn*5* (4), but these are weak promoters and do not always result in expression of downstream genes (4). We found no evidence for weak expression of the cloned *trp* genes. In fact, the 22-kilodalton (kDa) protein was produced in very large amounts unless the gene for this protein was inactivated by a Tn*5* insertion. Thus, our results suggest that promoter regions may be located within the cloned *Leptospira* DNA. Whether these are native *Leptospira* promoters or merely sequences which fortuitously act as promoters has not been determined yet.

The maxicell experiments defined three proteins that are essential for expression of the TrpE$^+$ phenotype. Two of these proteins are clearly part of AS since Tn*5* insertion into the coding region for either the 53.5-kDa protein or the 23.6-kDa protein resulted in a loss of the ability to complement the *trpE* defect in *E. coli*. Since inactivation of the 22-kDa protein by the Tn*5* insertion also inhibited synthesis of the 53.5-kDa protein due to polar effects on the operon, we cannot be certain that the 22-kDa protein forms a part of AS.

The 53.5- and the 22-kDa proteins were apparently transcribed from the same promoter, whereas a separate promoter was used for the 23.6-kDa protein (Table 1). Tn*5* insertion 82 lost the ability to produce both the 22-kDa protein and the 53.5-kDa protein, whereas Tn*5* insertion 158 lost only the ability to produce the 53.5-kDa protein. Neither of these insertions lost the ability to produce the 23.6-kDa protein. As Tn*5* insertions are known to have strong polar effects, this suggests that there is a polycistronic message which encodes both the 22- and 53.5-kDa proteins and a separate message which encodes the 23.6-kDa protein. The map positions of Tn*5* insertions 82 and 158 are consistent with this interpretation. In addition, Tn*5* insertion 158 produces a truncated protein with a molecular weight of 34 ×

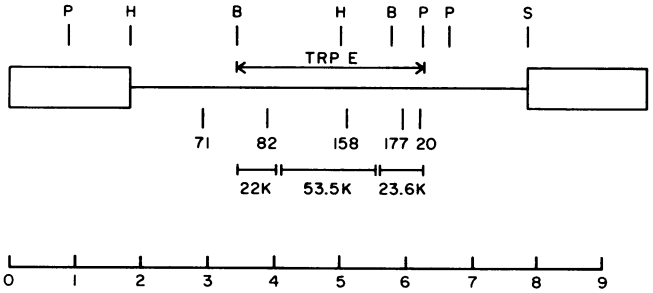

FIG. 5. Map of the *trpE* coding region of pYC6. The single line represents *Leptospira* DNA; the boxes represent pBR322 DNA. The positions of Tn*5* insertions 20, 71, 82, 158, and 177 are indicated. Abbreviations are as in the legend to Fig. 1. The scale at the bottom is in kilobase pairs. P, *Pst*I; H, *Hin*dIII; B, *Bam*HI; S, *Sal*I.

TABLE 1. pYC6-encoded proteins required for *trpE* function

Plasmid	Trp phenotype[a]	Presence of the following proteins:[b]		
		22 kDa	23.6 kDa	53.5 kDa
pYC6	+	+	+	+
pYC6::Tn*5*-71	+	+	+	+
pYC6::Tn*5*-82	−	−	+	−
pYC6::Tn*5*-158	−	+	+	−
pYC6::Tn*5*-177	−	+	−	+
pYC6::Tn*5*-20	−	+	−	+

[a] +, Able to grow on M9 medium supplemented with 1% Casamino Acids and 50 μg of ampicillin per ml; −, not able to grow on this medium.

[b] +, Protein present on the maxicell gel; protein not present on the maxicell gel.

10^3 (Fig. 4, lane F). Given the position of Tn*5* insertion 158, a peptide of this size would be expected if the message for the 53.6-kDa protein was transcribed rightward (Fig. 5). The 22-kDa protein was produced in such large amounts that it could be seen in Coomassie blue-stained gels. Neither the 53.5-kDa protein nor the 23.6-kDa protein could be seen on Coomassie blue-stained gels. Tn*5* insertions 20 and 177, which mapped distal to Tn*5* insertions 82 and 158, inactivated only the 23.6-kDa protein, suggesting that there is a separate promoter for this protein.

One of the proteins had a molecular weight of 53.5×10^3 and probably represented AS I. AS I from other bacterial systems has a molecular weight of about 58×10^3 (1, 5, 34). We were able to show by genetic complementation experiments that a *trpG* activity was also present on pYC6. In other systems the *trpG* protein has a molecular weight of about 20×10^3 (20, 33, 34). Thus, one of the small proteins defined by the maxicell experiments probably is the product of the *Leptospira trpG* gene. The role of the other small protein is unknown, but it could be a third subunit of AS or a regulatory protein. Cloning of the genes for the small proteins into expression vectors will allow us to determine which one is *trpG* and may help us define the function of the other protein as well.

It is clear from this study and our previous work (35) that the organization of the tryptophan operon in *Leptospira* is distinct from the organization of the tryptophan operon in other bacteria. The central position of the *Leptospira trpE* and the *trpG* genes on pYC6 and the inability of pYC6 or pYC1 (35) to complement defects in other genes of the *E. coli* tryptophan operon suggest that these genes are to be found elsewhere on the *Leptospira* chromosome. The AS system of *Leptospira* may be different from that found in other bacteria and fungi; it may consist of three distinct proteins, whereas all other known systems consist of only two proteins. Further study of this system should lead us to a better understanding of *Leptospira* genetics and may help in our understanding of bacterial evolution and evolution of the *trp* operon in particular.

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