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Replication Control Genes of Plasmid pE194†

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pE194, a 3.7-kilobase plasmid, confers resistance to macrolide, lincosamide, and streptogramin B antibiotics. The previously identified *cop* and *repF* genes of pE194 have been further localized by molecular cloning and mutational analysis together with DNA sequencing. The *Cfo*IB fragment of pE194 is capable of autonomous replication and contains both genes. Most of this region has been resequenced, and two errors reported in a previous study have been corrected. The corrected sequence indicates that the replication region contains a single large open reading frame, which we propose encodes the *repF* product. Northern blot (RNA blot) analysis of this region detected six transcripts, all transcribed in the same direction as one another and opposite to *repF*. A 350-base transcript is synthesized from the region containing *cop*. No in vivo transcript for the *repF* gene was detected, but a protein was observed in an in vitro transcription-translation system which appears to be its product. An ochre mutation was inserted in the putative *repF* open reading frame, and a nonsense fragment was detected in the in vitro system. When carried passively on a pUB110 replicon, this mutant product appears capable of inhibiting pE194 replicons in *trans*. The pE194 origin of replication has been localized to within 200 bases.

In most cases controlled plasmid replication requires a site from which replication initiates (the origin), a negatively acting element, a site upon which the latter acts, and a positively acting element (24). The regulation of plasmid replication often involves the interaction of a small RNA countertranscript (product of the *cop* gene) and a larger essential transcript which may be a primer (28) or the mRNA for a replication protein (14, 31). Maturation or translation of the larger transcript is inhibited by RNA-RNA interaction. Much of the work on plasmid replication control has been accomplished with plasmids of gram-negative bacteria. A notable exception has been the study of pT181, which is indigenous to the gram-positive bacterium *Staphylococcus aureus* (19).

The plasmid pE194 was originally isolated from *S. aureus* (13) and transferred to *Bacillus subtilis* (7), with which the work reported here was carried out. Previous studies have shown that pE194 replicates unidirectionally from a fixed point on the physical map (23). Plasmids carrying the copy number mutation *cop-6* were found to exhibit altered incompatibility properties, and the mutation was shown to be recessive to the wild-type allele (9). The *rep* gene product was characterized as a *trans*-acting element required for replication and was shown to be transcribed across an *Mbo*I site. We will refer to the pE194 *rep* gene as *repF*. Deletion analysis with BAL 31 localized the essential replication region of pE194 to a 900-base-pair (bp) segment. These studies thus established the presence and approximate locations in pE194 of the essential replication elements described above. The genome of pE194 has been completely sequenced (11).

To further characterize the replication region, a number of additional *cop* and *rep* mutations have been studied, transcription from the replication region has been examined by Northern blotting (RNA blotting), and translation of a likely *rep* protein has been studied in vitro.

MATERIALS AND METHODS

Strains and plasmids. All *B. subtilis* strains were derivatives of 168 and are listed in Table 1, together with the plasmids used. *Escherichia coli* JM109 and the M13 bacteriophage derivatives (mp18 and mp19) used for the sequencing studies were generous gifts of J. Messing (32).

Media. The liquid medium used for *B. subtilis* was either VY (5) or minimal Spizizen salts with supplements (1). When required, erythromycin, chloramphenicol, tylosin, or kanamycin was added to a final concentration of 5 μg/ml. Bacteria were plated on tryptose blood agar plates (Difco Laboratories).

Transformation. *B. subtilis* was made competent (4) and transformed with plasmid DNA (3) as described previously. *E. coli* was made competent and transformed as described previously (16).

In vitro manipulation of DNA. *B. subtilis* plasmid DNA was isolated and purified by a lysozyme-detergent method (8). The replicative forms (RF) of the M13 phage derivatives were isolated as described by Messing (16). DNA was analyzed by 0.8% agarose and 5% polyacrylamide gel electrophoresis. Restriction endonucleases were purchased from New England BioLabs, Inc., Bethesda Research Laboratories, Inc., and Boehringer Mannheim Biochemicals and used as suggested by the suppliers. DNA restriction fragments were isolated from polyacrylamide gels by electroelution at 150 V for 2 h in 0.1× Tris-borate buffer (6). T4 DNA ligase was purchased from Collaborative Research, Inc. Plasmid pBD212 was constructed by treatment of *Bcl*I-linearized pBD142 DNA with BAL 31 for 1 min at 37°C followed by self-ligation and transformation with selection for Cm^r.

Copy number determinations. Copy numbers were determined by measuring the ratio of radioactivity in plasmid and chromosomal DNA, after agarose gel electrophoresis of total lysates prepared from cells uniformly labeled with [^{3}H]thymidine (23).

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† Dedicated to the memory of Nathan Entner and J. Paul Youtz.

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TABLE 1. *B. subtilis* strains and plasmids

Strain or plasmid	Characteristics	Source or reference
Strains		
BD170	*trpC2 thr-5*	
BD224	*trpC2 thr-5 recE4*	
BD630	*hisH leu met*	
IS76	*hisH leu met sup-1*	20
Plasmids		
pBD9	Km^r Em^r, pUB110-pE194 cointegrate	8
pBD64	Km^r Cm^r, pUB110 replicon	9
pBD89	Cm^r, pE194*cop-6* replicon	9
pBD142	Cm^r Em^r, pE194*cop-6* replicon	9
pBD212	Cm^r Em^s, pE194*cop-6* replicon	This work
pBD318	Km^r Cm^s, pUB110 replicon	This work
pBD321	Km^s Cm^r, pUB110 replicon	This work
pBD350	Km^r Em^r, pUB110 and pE194 replicons	This work
pBD351	Km^r Em^r, pUB110 and mutant pE194 replicon	This work
pE194	Em^r	13
pE194ts	Em^r, temperature sensitive for replication	S. Gruss
pE194*cop-1*	Em^r, copy number mutant	This work
pE194*cop-6*	Em^r, copy number mutant	30
pE194*cop-45*	Em^r, copy number mutant	This work
pE194*cop-85*	Em^r, copy number mutant	This work
pE194*cop-93*	Em^r, copy number mutant	This work
pE194*cop-300*	Em^r, copy number mutant	S. Projan
pE194Δ-35	Em^r, replication-defective mutant	This work
pE194Δ-53	Em^r, replication-defective mutant	This work
pIM13	Em^r	17

Sequencing. Nucleotide sequences were determined by the dideoxynucleotide chain termination method of Sanger et al. (22) with the single-stranded phage vectors of Messing (32). Five primers were used in the sequencing: the universal primer (Amersham Corp.) and four custom-made primers specific for the replication region of pE194. The four primers, made by Michael Lonetto, Applied Microbiology Department, Public Health Research Institute, were 5′-CTTGTGAGATATATGCTT-3′ (1560 to 1577), 5′-AAAACATAGGGGGGTACT-3′ (870 to 887), 5′-GTAGTA CCCCCCTATGTTT-3′ (890 to 873), and 5′-TCTAGGATT TTTAATGG-3′ (740 to 756) (the positions in the pE194 sequence are given in parentheses). Primers were purified as described previously (15).

Northern blots. RNA was isolated as described previously (29) and used for blotting (2) after electrophoresis in formaldehyde-agarose gels. Single-stranded recombinant M13 phage containing *Hin*fI fragments from the replication region were labeled for use as DNA probes (12). Nick translation to prepare double-stranded probes was done as described previously (21).

In vitro translation. Coupled transcription and translation was carried out in *B. subtilis* S30 extracts as described by Narayanan and Dubnau (18). The extracts were supplied with circular or linear DNA templates as indicated.

Oligonucleotide mutagenesis. Oligonucleotide mutagenesis was carried out as described by Zoller and Smith (33) with single-stranded DNA derived from a suitable M13 derivative. The sequence of the mutagenic primer was 5′-AGG GTAATtAATCTTATG. The base in lowercase type was altered from the wild-type A.

RESULTS

Sequence of the replication region. pE194 contains two *Cfo*I sites (at positions 762 and 1922) (Fig. 1). The small *Cfo*IB fragment drives autonomous replication when ligated to a nonreplicating fragment containing a chloramphenicol resistance gene from pC194 (not shown). When the *Cfo*IB fragment was cloned into another replicon, pBD64 (9, 10), creating pBD318 (Fig. 2), it was found to contain both the *cop* and *rep* genes, since this new plasmid complemented mutations in these genes (not shown). These results confirmed the conclusions of our previous deletion analysis, which defined the limits of the replication region (9). Both strands of the replication region were sequenced by using single-stranded M13 phage derivatives made for this purpose, together with custom-made sequencing primers. All cloning sites were crossed in both directions, and the entire sequence was obtained on both strands. The sequence is shown in Fig. 3. Differences were found from the published sequence (11) at positions 1013 and 1509. At 1013 a G · C base pair was found in place of an A · T. An A · T base pair reported at 1509 was absent in our multiple sequencings of this region. As a consequence of the difference at 1509, our nucleotide numbering system differs from that of the published sequence. Another consequence of this correction is that the replication region contains two, rather than three, open reading frames (ORFs). The reading frames C and D of Horinouchi and Weisblum (11) have been merged into one ORF capable of encoding a 24,470-dalton protein.

Isolation of *cop* mutants. *cop* mutants were isolated as described previously (30). pE194 carries the inducible Em^r gene *ermC*. Erythromycin induces resistance to the noninducing macrolide antibiotic tylosin. Plasmids resistant to tylosin can result from mutations to either constitutive antibiotic resistance or high copy number, since the latter synthesize an elevated basal level of the *ermC* product. These two types of mutations can be readily distinguished by agarose gel electrophoresis of total crude DNA preparations. DNA from the presumptive *cop* mutants was used to transform plasmid-free recipients to ensure that the Ty^r was due

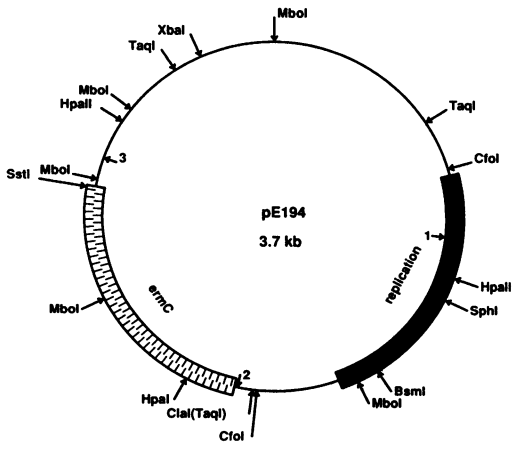

FIG. 1. Physical map of pE194. Several restriction enzyme cleavage sites and the locations of *ermC* and the essential replication region are indicated (9). The numbers and arrows on the inside of the map indicate positions in kilobases.

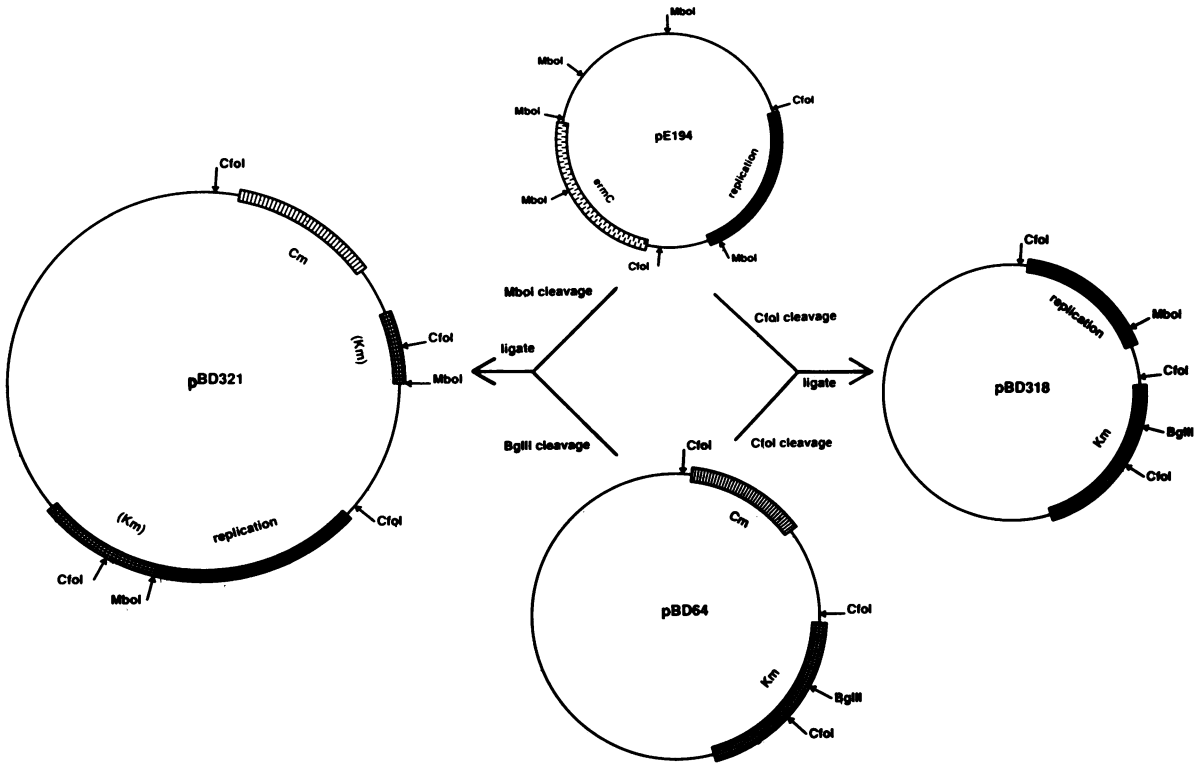

FIG. 2. Construction of pBD318 and pBD321. To construct pBD318, the minor *Cfo*I fragment of pE194 carrying the essential replication region was inserted in place of the *Cfo*I B fragment of pBD64. pBD321 was derived by insertion of the largest *Mbo*I fragment of pE194 into the single *Bgl*II site of pBD64. This *Mbo*I fragment carries a portion of the pE194 essential replication region. The interrupted Km^r determinant is indicated in parentheses.

to a plasmid mutation. The copy numbers of several plasmids selected for further study are shown in Table 2.

Dominance relationships of the *cop* mutations. Tests were performed to determine whether the *cop* mutations were dominant or recessive. Each mutant was transformed into a *recE4* recipient carrying pBD321. The latter plasmid carries the *Mbo*I A fragment of pE194 on a pUB110 replicon and is therefore incapable of pE194-driven replication, since the *repF* gene, which contains sequences from both the *Mbo*I A and B fragments, is incomplete (Fig. 2) (9). In the presence of pBD321 all the *cop* mutant copy numbers were restored essentially to the wild-type level (Table 2). We have shown previously that a strain carrying both pE194 (wild type) and a construct containing only the *Mbo*I A fragment of pE194 exhibits a copy number of the former plasmid that is not markedly different from that of pE194 alone (9). This suggests that the *trans* effect exerted by pBD321 is not due to general inhibition of replication by the truncated *repF* product (see below). We conclude that the *cop* mutations are recessive and that the *cop* gene is contained within *Mbo*I-A.

Sequencing of the *cop* mutations. Several of the *cop* mutations were characterized by DNA sequencing. The entire replication region of each was sequenced. All restriction sites were crossed, and the entire region was sequenced on both strands. Table 3 summarizes the mutational changes observed. The *cop-300* plasmid was isolated as a Ty^r mutant by S. J. Projan. It had a copy number of about 80 and was found to contain a deletion of 18 bp. Although all of the mutants were independent isolates, two of them, *cop-85* and *cop-93*, had identical changes (C · G → T · A) at the same nucleotide position. *cop-1* was located at the same nucleotide position, but consisted of a C · G → G · C transversion. The *cop-1* plasmid had a copy number of about 200, while *cop-85/93* conferred a copy number of about 80. *cop-6* had been previously sequenced by Horinouchi and Weisblum (11). Our results indicate that these authors had reversed the mutant and wild-type sequences, although we agree on the location of the change. The *cop-45* mutation consists of a 5-base tandem duplication.

The minimum length of the *cop* gene could be determined from the separation of the farthest recessive *cop* mutations, *cop-6* (position 1013) and *cop-85/93* (position 1258). The *cop* gene is thus at least 245 bases long. Although we have not tested the dominance of *cop-300*, if this mutation were considered in this length determination, the gene would be at least 283 bases long.

***repF* gene of pE194.** Several *repF* mutants have been characterized: a temperature-sensitive derivative, pE194ts, and two deletants, Δ53 and Δ35. Wild-type pE194 cannot replicate above about 43°C. However, pE194ts cannot replicate above 37°C. The mutational lesion in pE194ts affects the *rep* function (9). We have confirmed this by showing that complemention in *trans* of the temperature-sensitive defect requires the *Mbo*I-A/*Mbo*I-B junction (data not shown). Previous results had demonstrated that *repF* function requires the *Mbo*I A and B fragments to be joined in the proper orientation (9). pE194ts can be complemented at the nonpermissive temperature by plasmids such as pBD89, which carry *Mbo*I-A and *Mbo*I-B in the proper orientation, while pBD321, which contains only the pE194 *Mbo*I A fragment

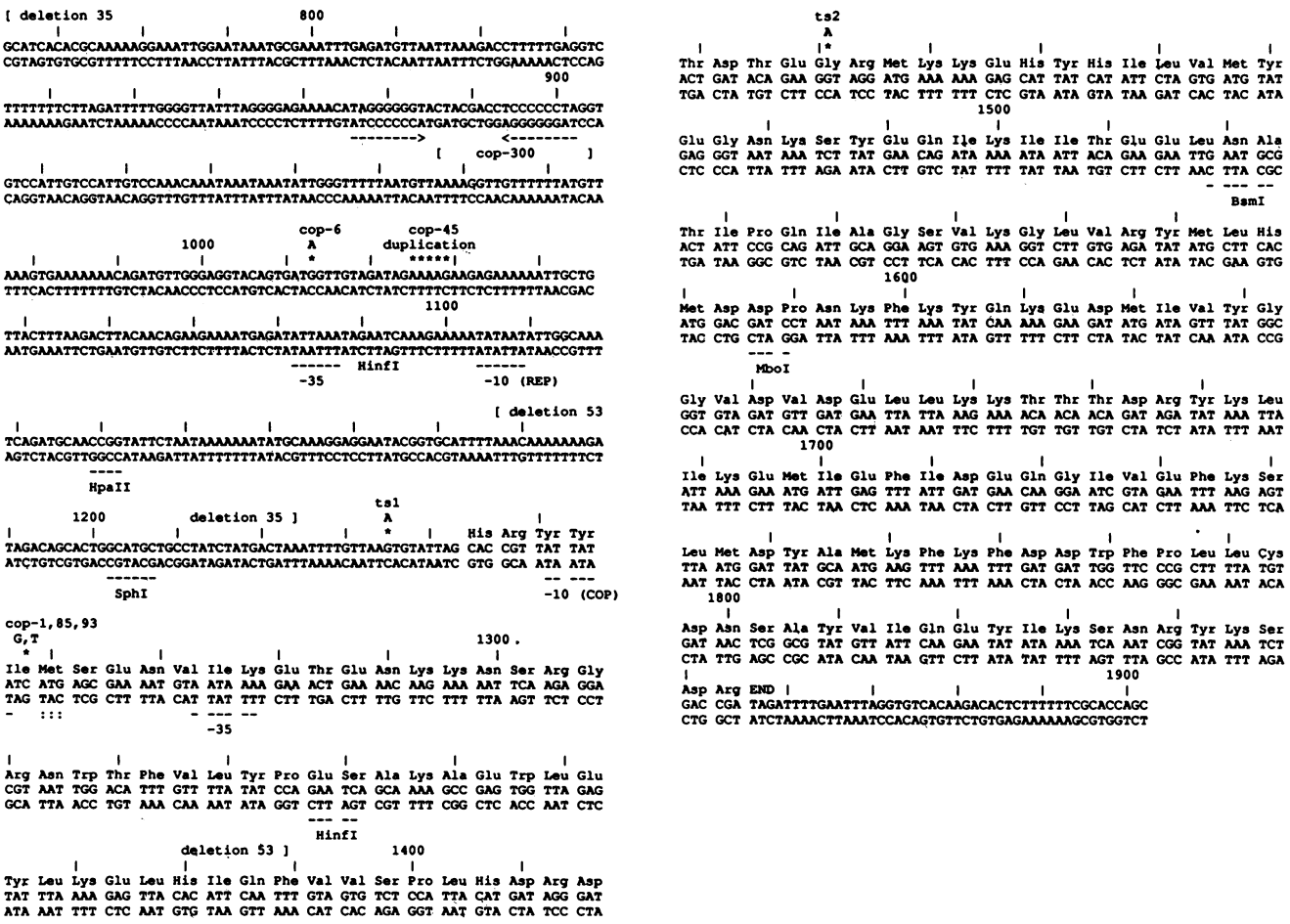

FIG. 3. Sequence of the pE194 replication region. The location of the *repF* reading frame and its inferred amino acid sequence is given. The likely *repF* start codon is underscored by three colons, and the suggested *repF* and *cop* promoters are marked. The positions of the *cop* and *rep* mutations discussed in the text are given. The location of the G+C-rich dyad mentioned in the text is indicated by arrows. The sequence presented in this figure is identical to that of Horinouchi and Weisblum (11), with the exceptions noted in the text.

(Fig. 2), fails to complement pE194ts at the nonpermissive temperature, as expected. pE194ts and pBD321 are compatible at the permissive temperature.

To test the independence of *repF* and *cop*, the pE194ts plasmid was placed in a *recE4* strain, together with pBD212, a Cm^r Em^s derivative of pE194 that carries the wild-type *rep* gene and the *cop-6* mutation (see Materials and Methods). At the nonpermissive temperature in the presence of pBD212, pE194ts has a normal copy number, confirming that the temperature-sensitive defect is complemented by pBD212 in *trans* (Table 4). At both the permissive and nonpermissive temperatures, the copy number of pBD212 in the presence of pE194ts was lowered, suggesting that pE194ts has a wild-type *cop* gene which complements the *cop-6* defect present in pBD212. This experiment therefore confirms that both the *cop* and *rep* genes function in *trans* and that *cop-6* and the *rep*(Ts) mutations are functionally independent.

The defect in pE194ts was localized by sequencing the

TABLE 2. Copy numbers and dominance relationships of *cop* mutants

Plasmid	Copy no.		
	Plasmid alone	Heteroplasmid strains	
		pBD321[a]	*cop* plasmid
pE194	14		
pBD321	30		
pE194*cop-1*	202	47	5
pE194*cop-45*	85	60	18
pE194*cop-85*	137	56	4
pE194*cop-93*	108	72	3
pE194*cop-6*	90		

[a] pBD321 carries the *Mbo*I A fragment from pE194. These measurements were carried out in the BD224 background.

TABLE 3. Mutations in the replication region of pE194

Mutation	Location	Alteration
cop-300	957–975	Deletion
cop-6	1013	G·C → A·T
cop-45	1025–1029	Duplication of AAAAG
cop-1	1258	C·G → G·C
cop-85/93	1258	C·G → T·A
repF(Ts)	1235, 1431	G·C → A·T G·C → A·T
Δ35[a]	764–1225	Deletion
Δ-53	1176–1389	Deletion

[a] The location of this deletion is approximate (see text).

entire replication region (Table 3). Two transition mutations were present, one at position 1431, which is within the large ORF, and the other at position 1235.

Isolation and analysis of deletion mutants. During the initial screening for *cop* mutants by electrophoresis, we found two lysates that contained a smaller plasmid in addition to the pE194-sized band. The deletions in these plasmids (Δ53 and Δ35) were mapped by restriction endonuclease digestion (data not shown) and were found to be located within the *Mbo*I A fragment. Both deletions removed the *Sph*I site, but only Δ35 removed the *Hpa*II site in the replication region (Fig. 1). Transformation studies have demonstrated that these deletions affect an essential function. First, we were unable to separate the small deletion plasmid from the wild-type-sized plasmid by transformation. Competent *B. subtilis* cells were transformed with plasmid DNA isolated from a strain carrying the Δ53 plasmid. Plasmid DNA lysates were prepared from the Em^r transformants. Of 50 such lysates, 48 contained only the larger plasmid, while 2 contained both wild-type and deleted plasmid DNAs. In a second experiment plasmid DNA preparations from the deletants were digested with *Sph*I to linearize the larger plasmid, leaving the deleted plasmid intact. Linear plasmid DNA molecules cannot transform competent *B. subtilis* (3). When transformation was carried out with these digested DNA samples, no Em^r transformants were obtained. Finally, when the *Cfo*I B fragment from pE194, pE194ts, or pE194*cop-1* is ligated to a nonreplicating Cm^r fragment and used to transform competent cells, Cm^r transformants are obtained. When a similar experiment was carried out with isolated *Cfo*I B fragments from the deletants, no Cm^r transformants were obtained. These results demonstrate that the deletants are incapable of independent replication and are dependent on the presence of a helper plasmid. We conclude that the Δ53 and Δ35 plasmids are *repF* defective and that the *repF* product is provided in *trans* by the helper. This, in turn, suggests that the site of action of the *repF* gene product, as well as the replication origin (*ori*), is present on both deleted plasmids.

Sequencing of Δ53 showed that 213 bp from positions 1176 to 1389 were missing, including several bases from within the large ORF (Fig. 3). The larger deletion (Δ35) was mapped by using a single dideoxynucleotide sequencing reaction and was found to be missing approximately 450 base pairs from about position 1225 to position 770. Δ35 eliminates sequences very close to, but not including, the *Cfo*I site (762) and up to about 19 bp from the large ORF (Fig. 3).

The origin of replication. Electron-micrographic analysis has indicated that the pE194 *ori* is located between the *Mbo*I site at position 1588 and the *Cfo*I site at position 762 (23). The deletion mutations enabled us to map *ori* more precisely. The combined sequence that is deleted from Δ53 and Δ35 extends from position 1389 to position 770. The origin must be located within the remaining portion of the essential replication region, namely in the 199-bp segment between positions 1588 and 1389.

TABLE 4. Independence of *cop* and *repF* in *trans*

Plasmid	Copy no. at:			
	32°C		37°C	
	pE194ts	pBD212	pE194ts	pBD212
pE194ts	10		1–2	
pBD212[a]		70		70
pE194ts/pBD212	13	3	13	4

[a] pBD212 carries a pE194*cop-6* replicon (see Materials and Methods).

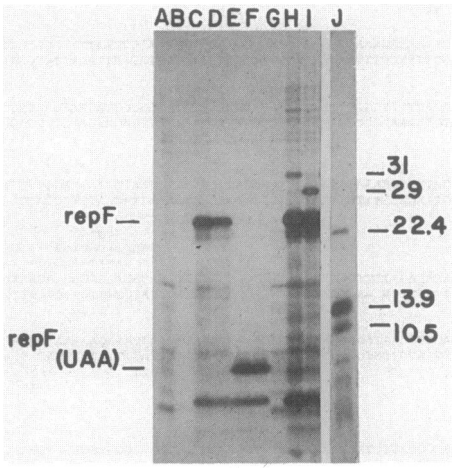

FIG. 4. In vitro translation of the wild-type and ochre mutant *repF* ORFs. The *B. subtilis* transcription-translation system was programmed by the following templates: none (lane A), 5 μg of M13mp18 RF DNA (lane B), 4 and 8 μg of M13mp18 RF DNA carrying the wild-type *Taq*I B fragment of pE194 (lanes C and D, respectively), 4 and 8 μg of M13mp18 carrying the ochre mutant *Taq*I B fragment of pE194 (lanes E and F, respectively), none (lane G), pE194 DNA (lane H), pE194 induced by 0.05 μg of erythromycin per ml (lane I), and bacteriophage φ29 DNA (to provide additional size markers) (lane J).

The large ORF encodes the *repF* product. Recently we described an in vitro transcription-translation system for *B. subtilis* (18). Circular plasmid DNA, as well as restriction endonuclease-cleaved DNA, can be used in this system to determine coding capacity. When pE194 or *cop-1* DNA was used as template, two major bands of 29 and 25 kilodaltons (kDa) were detected (Fig. 4). One of these bands (29 kDa) has been shown to be the erythromycin-inducible *ermC* product. Restriction endonuclease digestions which interrupt the *ermC* gene eliminate production of the *ermC* product in the in vitro system without affecting the 25-kDa protein (25K protein). Digestions with *Mbo*I or *Bsm*I, which cleave within the large ORF in the replication region, eliminate synthesis of the 25K protein. Cleavage by *Sph*I upstream from the large ORF also eliminates synthesis of the 25K protein. As noted above, the large ORF is capable of encoding a 24,470-Da protein.

We wished to test directly whether the large ORF corresponds to *repF*, the *trans*-acting replication function. Our strategy was to construct a plasmid with two different replicons, one of which consisted of the pE194 replication region containing a translational termination codon in the large ORF. If this ORF encoded the *repF* product, then we might be able to demonstrate complementation of pE194ts at restrictive temperatures in a suppressor host, but not in a wild-type background. The *Taq*I B fragment of pE194*cop-1* was cloned into the *Acc*I site of M13mp11. By oligonucleotide mutagenesis (33) codon 80 of the *repF* ORF was changed from AAA (Lys) to TAA (stop). The mutation was confirmed by dideoxynucleotide sequencing, and subsequent plasmid constructs were probed with ^{32}P-end-labeled mutagenic primer to confirm the presence of wild-type or mutant sequences. In the in vitro protein-synthesizing system, M13 RF DNA carrying the cloned *Taq*I B fragment specified a 25K protein identical in electrophoretic migration to the

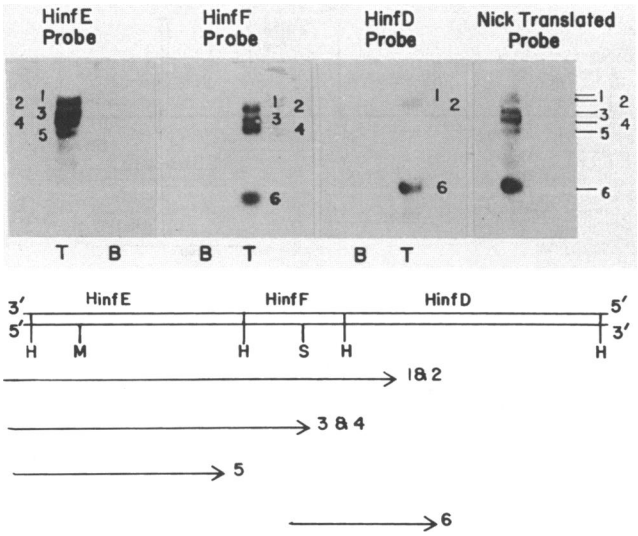

FIG. 5. Northern blot hybridization with replication region probes. Single-stranded DNA probes were prepared from the top (T) and bottom (B) strands of *Hin*fI fragments E, F, and D as described in Materials and Methods. H, S, and M indicate the locations of *Hin*fI, *Sph*I, and *Mbo*I cleavage sites. Nick-translated probe was also prepared from pBD318 DNA. The latter is homologous to the entire pE194 replication region. The results of Northern blotting to pE194 RNA are presented in the upper part of the figure. A map of the replication region and the inferred approximate transcription map are shown in the lower portion.

protein encoded by pE194 DNA (Fig. 4). When M13 RF DNA carrying the mutant *Taq*I B fragment was used as template, a much smaller ochre fragment (about 8 kDa) was detected (Fig. 4).

A *Pst*I-*Bam*HI fragment from the RF DNA containing either the wild-type or mutated *Taq*I B fragment was cloned into *Pst*I- and *Bam*HI-digested pBD9. Plasmid pBD9 is a cointegrate of pUB110 and pE194 joined at the unique *Xba*I sites. A *Pst*I-*Bam*HI digestion of pBD9 removes the entire replication region of pE194, leaving only about 500 bp of the pE194 moiety. The resultant Km^r Em^r plasmids, pBD350 (wild-type *rep* fragment) and pBD351 (mutant *rep* fragment), are maintained at a copy number characteristic of pUB110.

As noted above, our strategy was to demonstrate that pBD351, which contains the mutated *rep* sequence, could not complement pE194ts at the restrictive temperature in a wild-type host, but could do so in a Sup^+ host. However, we found that although a pBD350-containing strain can readily be transformed by both high- and low-copy pE194 derivatives, the pBD351-containing strain cannot be transformed by any pE194 replicons tested in either rec^+ or *rec* mutant backgrounds. For this purpose, pE194 derivatives carrying Cm^r were used. Control transformations with the unrelated pIM13 replicon (17), also with selection for Cm^r, showed no difference in transformation frequency betwen pBD350- and pBD351-containing strains. Since these two strains differ only in the single-base ochre mutation, we conclude that the mutant polypeptide fragment inhibits pE194 replication in *trans*.

In addition, we tested the ability of the wild-type and mutated *rep* regions to drive autonomous replication. Plasmids pBD350 and pBD351 were digested with *Taq*I, and the *rep*-containing fragments were isolated. These were ligated separately with the nonreplicating Cm^r *Taq*I fragment of pC194 and used to transform a plasmid-free strain (IS75). The ligation mix containing the wild-type fragment yielded 10^4 Cm^r transformants per ml. The mutant fragment mix yielded none, although the efficiency of ligation was similar for both fragments as judged from agarose gel electrophoresis. Similar results were obtained when the ochre suppressor strain IS76 (a *sup-1* strain which is otherwise isogenic with IS75) was transformed with the ligated DNAs. It seems that the level of suppression by *sup-1* (reported to be 25 to 30% [27]) is not sufficient to overcome the effect of the mutant polypeptide, or that the amino acid substituted by *sup-1* does not restore *repF* function.

We conclude that the single-base change in codon 80 of the putative *rep* coding sequence abolishes pE194 replication. Furthermore, the truncated polypeptide product shown to be encoded by the mutated *rep* region interferes with wild-type pE194 replication in *trans*. These data support the notion that the ORF we have designated as the *repF* coding sequence encodes a *trans*-acting replication function.

In vivo transcripts from the replication region. Three *Hin*fI fragments are present in the replication region of pE194 (Fig. 5). The *Hin*fI E, F, and D fragments were each cloned into M13 phage in both orientations, and the resulting DNA preparations were used to prepare probes for Northern blots with RNA isolated from cultures carrying either pE194 or the *cop-6* mutant. No differences were observed between the transcripts produced by the wild-type and *cop-6* plasmids (not shown). In addition, nick-translated pBD318 DNA was used as a probe. This plasmid contains the entire replication region (Fig. 2). In all, six transcripts were detected with the pBD318 probe (Fig. 5). All detectable transcripts were transcribed in the same direction, since they hybridized only with the top-strand probes. They ranged in size from about 350 to 1,100 bases. The two largest transcripts hybridized strongly to both the *Hin*fI E and F fragments and weakly to the *Hin*fI D fragment. The next two largest transcripts hybridized to the *Hin*fI E and F fragments. The fifth transcript showed a signal only with the *Hin*fI E fragments. The smallest transcript hybridized to the *Hin*fI F and D fragments, which are the fragments containing the *cop* mutations sequenced in this study (Table 3). This transcript is therefore a candidate for the product of the *cop* gene.

DISCUSSION

We have mapped the *cop* and *repF* genes of pE194. The replication origin has been localized to a 199-bp segment that lies within the coding region of *repF*. This segment also contains the site of action of the *repF* gene product, since it is sufficient for replication when driven in *trans* by the *repF* gene product. The dispositions of these elements are summarized in Fig. 6.

Location and properties of *cop*. A small transcript of about 350 bases was detected in Northern blot analyses of the replication region. This RNA spanned the *Hin*fI-F/*Hin*fI-D junction. Since these fragments contain all of the known recessive *cop* mutations, we suggest that this transcript may be the *cop* product. The small ORF noted in this study and previously by Horinouchi and Weisblum (11) overlaps the DNA segment that encodes *cop*. Three of the *cop* mutations that we sequenced are within this ORF. However one of these, *cop-6*, does not alter the predicted amino acid (Thr), as noted previously by Horinouchi and Weisblum (11). It is therefore likely that the active *cop* product is an RNA, as has been observed in other plasmid copy control systems.

The *cop* RNA may terminate at a G+C-rich dyad located at positions 876 to 902, which is followed by four T residues

(Fig. 3). If so, the initiation of transcription would occur near position 1250. There is a possible −10 sequence at 1251 to 1256 (TATAAT) and a possible −35 sequence at 1273 to 1278 (TTTATT), with a suboptimal spacing of 16 rather than the preferred 17 bp (Fig. 3). If this is the promoter used for the *cop* gene, then *cop-1* and *cop-85/93* (position 1258) may be promoter mutations.

Location and properties of *repF*. Previous cloning and deletion mutagenesis studies (9) have shown that the integrity of the *Mbo*I site at position 1696 is essential for plasmid replication, since a plasmid derivative interrupted at the *Mbo*I site can be complemented in *trans* by a *repF*$^+$ plasmid. In the present study, we have shown that the pE194ts plasmid cannot be complemented at the nonpermissive temperature by pBD321, which contains the *Mbo*I A fragment of pE194, but that it can be complemented by a plasmid containing both the *Mbo*I A and *Mbo*I B fragments (Table 4) or by a replicon that contains the entire *Cfo*I B fragment (not shown). These observations confirm the previous conclusion (9) that the temperature-sensitive lesion in pE194ts affects the *repF* function. One of the two sequence changes associated with the temperature-sensitive defect is located within the large ORF located between positions 1244 and 1856. The Δ53 mutation (Fig. 3) removes part of this ORF and prevents autonomous replication in the absence of a helper plasmid. These results suggest that this ORF may encode the *repF* gene product. The in vitro translation experiment demonstrates that this ORF encodes a 25K protein. Insertion of an in-frame stop codon renders the mutant plasmid incapable of replication and results in the production of a truncated protein fragment. The latter apparently can poison pE194 replication in *trans*. This effect may be due to competition with the wild-type *repF* product for binding to a target site or to formation of inactive mixed multimers of wild-type and mutant proteins. In any event, it appears very likely, despite our failure to demonstrate suppression of the ochre mutant, that the large ORF specifies a *repF* protein, required for pE194 replication. It is noteworthy that the in vitro system efficiently produces the 25K protein, but that this product is not detected in *B. subtilis* minicells (26). Furthermore, our Northern blots did not reveal a transcript corresponding to the large ORF. It appears that in vivo this product is under tight control and that this control is lost in vitro. Inspection of the promoter-proximal portion of the *repF* ORF did not reveal an obvious Shine-Dalgarno sequence associated with a start codon. This is puzzling, especially in view of the efficient translation of the *repF* product in vitro. The correspondence between the coding capacity of the entire *repF* ORF (24.5 kDa) and the size of the *repF* product determined by electrophoretic mobility (25 kDa) suggests that translation of *repF* initiates near the beginning of the ORF. Codon 6 of this ORF is AUG, and no other likely start codon occurs in this part of the ORF. This start would predict a product of 23.8 kDa, in excellent agreement with the observed value. The predicted size of the ochre fragment, assuming this start, is 9 kDa, also in accordance with the observed value of about 8 kDa. The next AUG, GUG, or UUG is a GUG at codon 51, which would yield *repF* and ochre fragment polypeptides of 18.3 and 3.6 kDa, respectively. We therefore tentatively conclude that the AUG at codon 6 of the ORF initiates *repF* translation, as indicated in Fig. 3.

The Δ35 mutation points to the importance of the region immediately upstream of the large ORF. This deletion removes the *Sph*I site (position 1202) and all the sequences up to but not including the *Cfo*I site at 762 and also confers a requirement for a helper plasmid. Interruption of the *Sph*I site was found to eliminate synthesis of the 25K protein in the in vitro translation system (data not shown). Thus the *repF* promoter is probably some distance upstream of the ORF, suggesting the presence of a substantial leader segment. In the *cop-300* mutant the sequence from 976 to 957 is deleted without compromising *repF* function. In *cop-45* the sequence between 1024 and 1028 is duplicated, again without any effect on autonomous replication. This places a limit on the extent of *repF*. Previous studies have shown that RNA polymerase binding protects the *Hpa*II (position 1129) and *Hin*fI (position 1090) sites (25). Protection disappears in the presence of all four ribonucleotides, suggesting that this binding is to a functional promoter. Inspection of the sequence reveals a possible promoter in this region with a consensus −10 sequence (TATAAT) at 1104 to 1109 and with a poor −35 sequence (ATTAAA) at 1082 to 1087 (Fig. 3). This would imply that *repF* mRNA possesses an untranslated leader of at least 130 bases. In addition it appears likely that the *cop* and *repF* mRNAs overlap by about 125 bases within this leader.

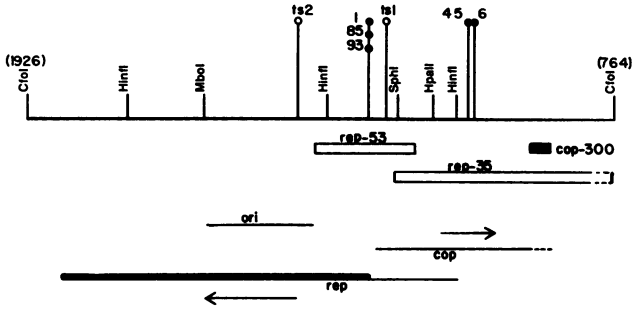

FIG. 6. Structure of the pE194 replication region. Symbols: ○, □, locations of the *repF* deletion mutations and the *repF*(Ts) mutations; ●, ■, *cop* mutations. The inferred locations of *ori*, *cop*, and *repF* are also shown, together with the directions of transcription of the last two determinants. The heavy bar indicates the extent of the probable *repF* coding sequence.

A previous study, involving the use of BAL 31-derived deletions to map the extent of the pE194 replication region, resulted in the isolation of a deletant that was missing the *Hin*fI site at 1722 but not the *Mbo*I site at 1696 (9). Although capable of replication, this deletant was shown to have a low copy number, suggesting that the replication region had been damaged. The present study reveals that position 1722 is within the C-terminal portion of the *repF* ORF. We conclude that the C-terminal moiety of the *repF* protein is not absolutely essential for function.

pE194 exhibits several features similar to those of other, better understood systems. These include a negatively acting copy control element, probably an RNA, that acts as an incompatibility determinant (9), a positively acting replication protein that is encoded by an mRNA with an untranslated leader, and apparent overlap of *cop* with this leader. The control of pE194 regulation may therefore be exerted by a mechanism that is also similar to control mechanisms of these other systems, perhaps most notably to the *S. aureus* plasmid pT181 (19). For instance, the *cop* countertranscript may interact with the *repF* mRNA and inhibit translation of the *repF* protein. Although the similar organization of the replication elements of these two plasmids is significant, we could find no homology between the replication regions of pE194 and that of any other *S. aureus* plasmid that has been sequenced, on either the DNA or protein levels.

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