JOURNAL OF BACTERIOLOGY, Nov. 1982, p. 722–735 Vol. 152, No. 2
0021-9193/82/110722-14$02.00/0
Copyright © 1982, American Society for Microbiology

Replication and Incompatibility Properties of Plasmid pE194 in *Bacillus subtilis*

T. J. GRYCZAN, J. HAHN, S. CONTENTE,† AND D. DUBNAU*

Department of Microbiology, The Public Health Research Institute of the City of New York, Inc., New York, New York 10016

Received 7 April 1982/Accepted 6 August 1982

pE194, a 3.5-kilobase multicopy plasmid, confers resistance to the macrolide-lincosamide-streptogramin B antibiotics in *Bacillus subtilis*. By molecular cloning and deletion analysis we have identified a replication segment on the physical map of this plasmid, which consists of about 900 to 1,000 base pairs. This segment contains the replication origin. It also specifies a *trans*-acting function (*rep*) required for the stable replication of pE194 and a negatively acting copy control function which is the product of the *cop* gene. The target sites for the *rep* and *cop* gene products are also within this region. Two incompatibility determinants have been mapped on the pE194 genome and their properties are described. One (*incA*) resides within the replication region and may be identical to *cop*. *incB*, not located in the replication region, expresses incompatibility toward a copy control mutant (*cop-6*) but not toward the wild-type replicon.

The replication properties of plasmids, often regarded as models for the analysis of larger, presumably more complex replicons, have received much attention. Although differences in complexity and in the detailed architecture of replication control circuits are apparent, certain features common to most plasmid systems seem to be emerging. Plasmids replicate uni- or bidirectionally from a fixed origin (22, 23). In many systems negatively acting control elements have been identified, which are also responsible for at least one manifestation of incompatibility (30, 32, 36). An RNA replication primer has been implicated, notably in the case of ColE1 and the closely related CloDF13 plasmids, and a second, overlapping transcript has been identified which appears to be a negative control element in these systems (19, 26, 38). Although in several cases plasmid-determined proteins have been implicated in replication or its control or both (16, 20, 24a), in other systems no evidence for such protein components has been presented. In fact, support has been adduced for the notion that no plasmid protein is necessary for ColE1 replication (6).

pE194 is a small, multicopy plasmid which confers resistance to the macrolide-lincosamide-streptogramin B group of antibiotics. Isolated from *Staphylococcus aureus* (17), pE194 was transferred in our laboratory to *Bacillus subtilis*. In the latter organism, pE194 has been used for the study of macrolide-lincosamide-streptogramin B resistance and its expression (8, 11, 13, 14, 33–35) and replication (31, 39) and for the construction of molecular cloning vectors (12). In view of the considerable knowledge accumulated concerning pE194 (including DNA sequence data), the ease of isolation of *cop* mutants, and the relative paucity of information concerning the replication of plasmids indigenous to gram-positive bacteria, we have analyzed its replication and incompatibility properties.

MATERIALS AND METHODS

Strains and plasmids. All bacterial strains used were derivatives of *B. subtilis* 168. The strains are listed in Table 1 and the plasmids used are given in Table 2.

Media, transformation, and transduction. The liquid medium used in all experiments (unless otherwise indicated) was VY (24). Solid medium was tryptose blood agar base (Difco Laboratories). Competent cells were prepared, stored at −70°C, and transformed as described previously (7). Transduction was carried out with phage AR9 (1). In all cases selection for antibiotic resistance was by overlay after a 90-min delay for expression. Antibiotic concentrations used for erythromycin (Em), chloramphenicol (Cm), and kanamycin (Km) were 5 μg/ml. Tetracycline (Tc) resistance was selected at 15 μg/ml.

Isolation and in vitro manipulation of plasmid DNA. Plasmid DNA was isolated by the CsCl-ethidium bromide centrifugation method (29), as described before (9). Restriction enzymes, DNA polymerase, T4 DNA ligase, and *Bal*31 exonuclease were obtained from Bethesda Research Laboratories or New England Bio-

† Present address: Laboratory of Molecular Genetics, National Institute of Child Health, National Institutes of Health, Bethesda, MD 20205.

TABLE 1. Bacterial strains

Strains	Genotype
BD170	*trpC2 thr-5*
BD224	*trpC2 thr-5 recE4*
BD630	*his leu met*
BD842[a]	*his leu met* [pE194][b]

[a] Source, E. Dubnau.

[b] [pE194] indicates that pE194 has been integrated into the chromosome. A pE194 derivative carrying a fragment of *B. subtilis* chromosomal DNA was isolated. A strain carrying this plasmid was grown at high temperature (51°C) and plated with selection for Em^r. The resulting strain carried pE194 in integrated form. The site of integration was mapped between *lys* and *aroD* (E. Dubnau et al., personal communication).

labs and were used according to the producers' recommendations. *Bal*31 incubations were varied as to time and temperature to achieve the desired extents of digestion (see text).

Copy number determinations. Copy numbers were determined as described previously (39). Small (1.5 ml) cultures were grown from densities of about 10^5 to about 2×10^8/ml in the presence of 25 μCi of [^{3}H]thymidine and 250 μg of deoxyadenosine per ml. The cultures were then harvested and lysed, and total DNA was extracted and resolved on vertical 0.8% agarose (Seakem, type LE) slab gels. The gels were stained with ethidium bromide, and the chromosomal and plasmid bands, visualized with UV light, were excised separately. These samples were covered with 2 ml of water in scintillation vials and placed in a boiling water bath until the agarose had melted. Each sample received 15 ml of Liquiscint (National Diagnostics). Radioactivity was determined and copy numbers were calculated by using known values for plasmid molecular weights and for that of the chromosome (2.5×10^9). Alternatively, samples were prepared as above but without the addition of [^{3}H]thymidine and deoxyadenosine, the gels were directly traced, and peaks were integrated by using a Shimadzu fluorescence scanner. In the latter case, a correction factor of 1.36 was used for the differential fluorescence of covalently closed circular and non-covalently closed circular DNA (S. Projan, personal communication), since covalently closed circular DNA binds less ethidium bromide than does relaxed DNA. In general, copy numbers obtained with the scanner were not distinguishable from those obtained from radioactive counting.

TABLE 2. Plasmids

Plasmid	Mol wt (×10^6)	Relevant properties	Source
pE194	2.4	Em^r	17
pBD15	2.4	Em^r *cop-6*	39
pBD12	4.4	Km^r Cm^r	10
pBD64	3.1	Km^r Cm^r	12
pBD9	5.4	Km^r Em^r	10
pBD35	5.4	Km^r Em^r *cop-6*	31
pBD9Δ1	3.1	Km^r Em^s	34
pBD9Δ2	5.4	Km^r Em^s	34
pBD9Δ3	4.3	Km^r Em^s	34
pBD9Δ7	4.0	Km^r Em^s	34
pBD9Δ8	3.8	Km^r Em^s	34
pBD9Δ9	4.5	Km^r Em^s	34
pE194(Ts)	2.4	Em^r	S. Gruss

RESULTS

A *trans*-acting replication product is produced by pE194. The replication origin (*ori*) of pE194 has been mapped by electron microscopy, and replication has been shown to proceed unidirectionally by a θ mechanism (31). The location of *ori* and the direction of replication are indicated on the maps shown in Fig. 1, 2, 3, and 4. *Mbo*I generates five fragments from pE194 DNA. To determine which of these fragments is required for autonomous replication of pE194, we used a nonreplicating Cm resistance fragment derived by cutting pBD64 DNA with *Bam*HI and *Bgl*II (Fig. 1). pBD64 had been constructed by inserting a portion of the Km^r plasmid pUB110, carrying the pUB110 *ori*, into the single *Hpa*II site of pC194 (10, 12). Insertion into this *Hpa*II site has been shown to inactivate pC194-specific replication (3). Since the Cm^r *Bgl*II-*Bam*HI fragment of pBD64 does not contain the pUB110 *ori*, we were confident that this fragment, which confers Cm resistance, lacks the information required for autonomous replication. In fact, transformation with self-ligated pBD64 DNA, cut with *Bgl*II and *Bam*HI, repeatedly failed to yield Cm^r plasmids consisting only of the *Bgl*II-*Bam*HI fragment (not shown).

pBD64 DNA, cut with *Bgl*II and *Bam*HI, was ligated to a partial digest of pBD15, a *cop-6* mutant of pE194, cut partially with *Mbo*I. The ligated DNA was used to transform BD630 with selection for Cm^r. Before transformation, the ligated DNA was cleaved with *Eco*RI to minimize inclusion of the pBD64 replication fragment (Fig. 1). (Restriction enzyme-cleaved plasmid DNA will not transform *B. subtilis* [5].) A total of 197 Cm^r transformants were analyzed by digestion with *Mbo*I, and all were found to carry the *Mbo*I-A and -B fragments occurring in the same relative juxtaposition as in pE194. The A + B segment was found in both orientations with respect to the Cm^r fragment (Fig. 1). This segment corresponds to the minor arc between coordinates 7 and 74 on the restriction maps of pE194 (Fig. 1 and 3). We conclude from the structure of the 2.8-megadalton plasmids carrying the *Mbo*I-A and -B fragments of pBD15 (*cop-6*) (designated pBD89 and -94, corresponding to orientations 1 and 2, respectively) that these fragments are necessary and sufficient for stable autonomous replication and inheritance.

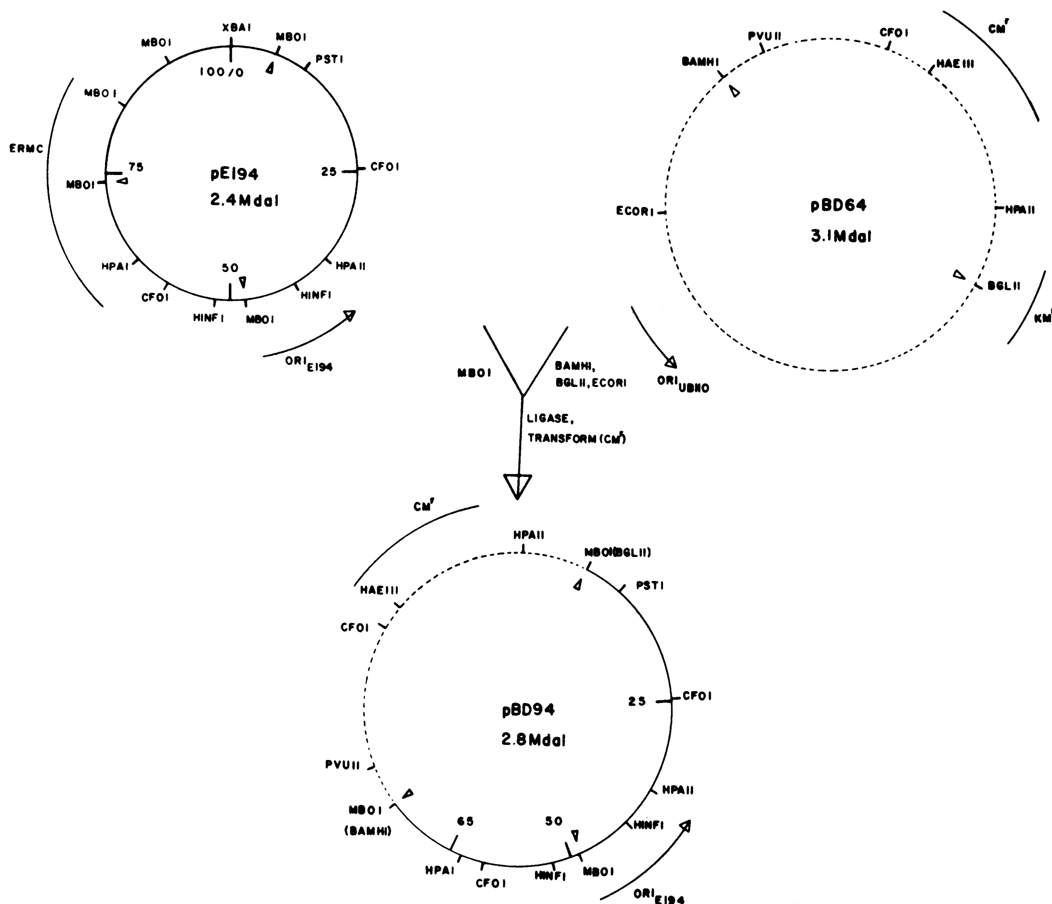

FIG. 1. Identification of pE194 *Mbo*I fragments required for replication. pE194 was cut with *Mbo*I to generate five fragments. pBD64 was cut with *Bam*HI, *Bgl*II, and *Eco*RI, producing three fragments, one of which is a nonreplicating Cm resistance fragment. The cleaved samples were mixed, ligated, and used to transform a competent recipient for Cm^r. The transformants contained a new plasmid consisting of the Cm^r fragment from pBD64, plus the *Mbo*I-A and -B fragments of pE194. The latter were always connected in their native configuration. The derivative shown (pBD94) carries orientation 2. The related plasmid with the reverse orientation of *Mbo*I-A and -B with respect to the Cm^r fragment (orientation 1) is called pBD89. The locations of *ermC*, Km^r and Cm^r determinants, and Ori_{E194} Ori_{UB110} (on pBD64) are shown. The open triangles indicate the *Mbo*I sites on pE194 which delimit the A and B fragments and the *Bam*HI and *Bgl*II sites on pBD64. pE194 and pBD64 segments are shown by solid and dashed lines, respectively. The *Mbo*I sites on pBD94 which consist of half-*Bam*HI and half-*Bgl*II recognition sequences are denoted *Mbo*I (*Bam*HI) and *Mbo*I (*Bgl*II). The coordinates in all cases are those of the pE194 physical map, as defined in Fig. 3.

The dependence of replication on the *Mbo*I-A and -B fragments was verified by an additional experiment which also suggested the existence of a *trans*-acting replication product. pBD89 was cut with *Mbo*I (yielding three fragments, *Mbo*I-A and -B from pE194 and the nonreplicating Cm^r fragment from pBD64). The digest was self-ligated and used to transform BD170 as well as BD433. The latter is a *recE4* strain isogenic with BD170 and also carrying pBD15, the *cop-6* mutant derivative of pE194. Selection was for Cm^r. Eleven colonies resulting from transformation of BD170 were analyzed for plasmid content and by restriction site mapping of their plasmids. Nine carried plasmids identical to pBD89, and two were identical to pBD94 (orientations 1 and 2). Eleven transformant derivatives of BD433 were likewise analyzed. All carried pBD15 in addition to a new plasmid, and all were found to be resistant to both Cm and Em. Restriction endonuclease cleavage demonstrated that the new plasmid contained only two *Mbo*I fragments, the Cm^r fragment and *Mbo*I-A. These fragments were present in the same relative

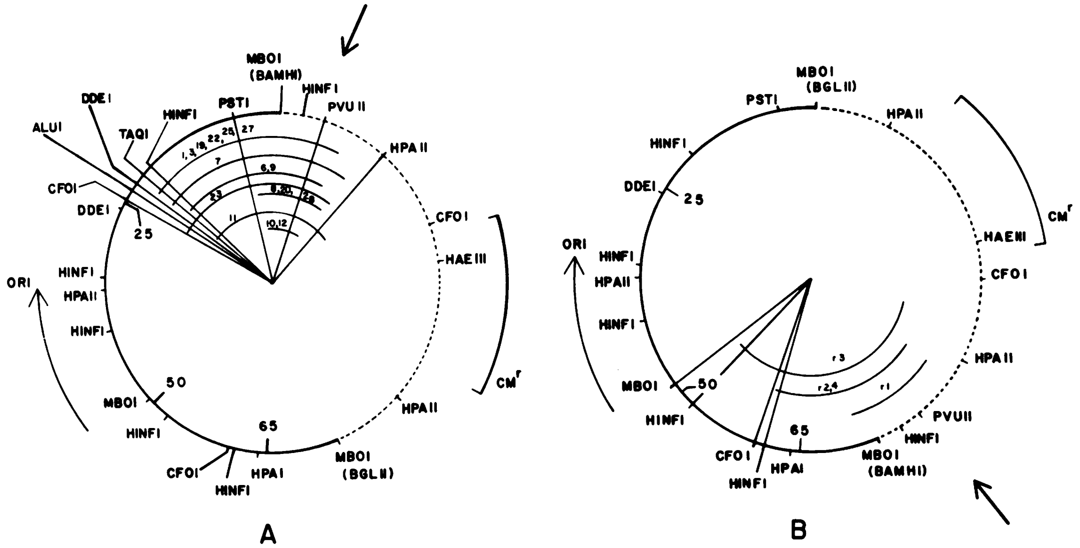

FIG. 2. Deletion map of the pE194 replication region. pBD89 (A) and its orientation 2 equivalent pBD94 (B) were cut at their single *Pvu*II sites and treated with *Bal31* as described in the text. The *Pvu*II sites are indicated by the heavy arrows. Deletion derivatives were obtained and their restriction site contents were determined. The coordinate numbers refer to the map of pE194 (Fig. 3). pE194 and pBD64 sequences are shown by solid and dashed lines, respectively. Half-*Bam*HI and half-*Bgl*II sites are denoted by *Mbo*I (*Bam*HI) and *Mbo*I (*Bgl*II). The locations of Ori_{E194} and of the Cm^r determinant are shown. Since the extents of the deletions were determined by the presence or absence of restriction sites, their termini can only be specified as occurring within the corresponding intervals.

orientation as in pBD89 in nine cases (orientation 1) and in the reverse orientation in two of the clones (orientation 2). The new plasmids were designated pBD93 and -96, respectively. It appears that, in the absence of *Mbo*I-B, stable replication requires the presence, in trans, of a pE194 replicon. The *Mbo*I-A fragment, which alone can support such "dependent" replication, is defined by coordinates 7 and 48 on the pE194 map (Fig. 1 and 3). This fragment carries the pE194 replication origin (31).

To confirm this conclusion, pBD93 and pBD89 were used to transform BD224 and BD432 with selection for Cm^r (Table 3). The latter is a *recE4* strain isogenic with BD170 and carrying pE194. BD224 is a *recE4* plasmid-free strain. pBD89 DNA, which contains the *Mbo*I-A and -B fragments, was capable of transforming the *recE4* plasmid-free recipient to Cm^r. The DNA from pBD93, which carries *Mbo*I-A but lacks *Mbo*I-B, yielded Cm^r transformants only if the recipient strains carried either pE194 (Table 3) or pBD15 (not shown). Similar results were observed with pBD94 and pBD96 (orientation 2). We ascribe the low yield of Cm^r transformants (using pBD93 and pBD96) to an incompatibility effect (see below). This incompatibility is not observed with pBD89 as a donor, since selection for Cm^r causes the resident plasmid to be lost (not shown). This cannot happen with pBD93, since its replication is dependent on the presence of the resident plasmid.

We conclude from this series of experiments that a *trans*-acting replication substance is required for the stable replication or inheritance or both of pE194. This substance appears to be encoded by a gene that spans the *Mbo*I site joining the *Mbo*I-A and -B fragments (coordinate 48, Fig. 1 and 3), since all of the self-replicating *Mbo*I-A- and -B-containing plasmids analyzed carry these fragments in their "native" configuration.

pE194 specifies a positive-acting replication effector. We have shown above that a *trans*-acting substance is specified by pE194 and pBD15 and

TABLE 3. Transformation by pBD89 and pBD93 DNA

Donor DNA	Cm^r transformants per ml[a]	
	BD224	BD432
pBD89	3.2×10^4	8.5×10^4
pBD93[b]	0	3.5×10^2

[a] BD224 and BD432 (recipients) are isogenic *recE4* strains. The latter carries pE194.

[b] This donor plasmid DNA was actually a mixture of pBD93 and pE194 "helper" DNA.

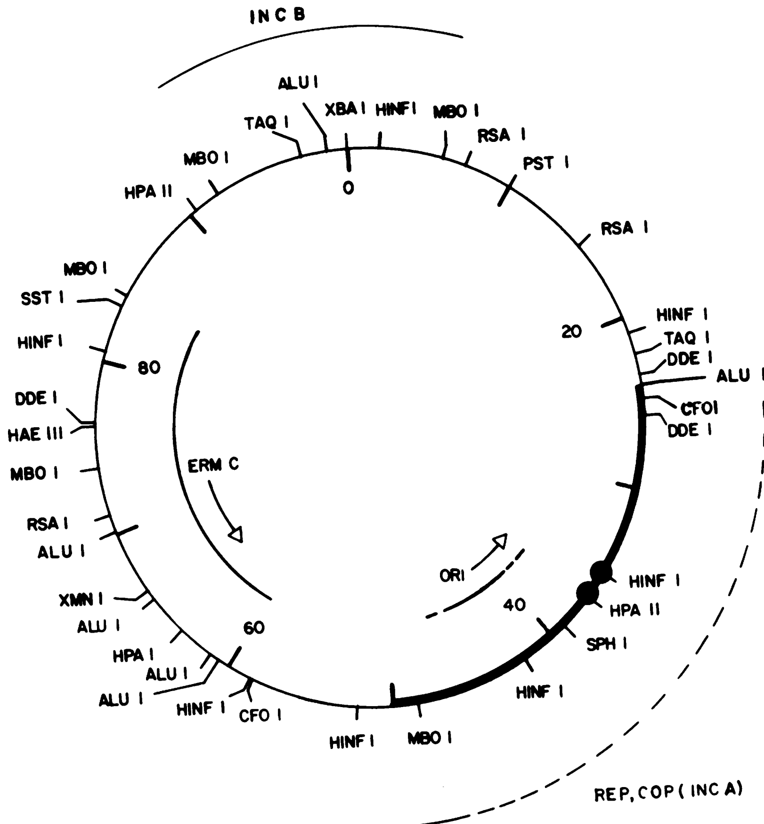

FIG. 3. Replication determinants and the physical map of pE194. The restriction map of pE194 (3.5 kilobases) is shown, with the unique *Xba*I site assigned to coordinate 100/0. The location of *ori* and the direction of replication and that of the *ermC* gene and its direction of transcription are shown. The heavy portion of the circle shows the limits of the required replication region, defined by deletion mapping. The *rep* determinant is within this segment and extends to the terminus of the replication region near coordinate 50. The *cop*(*incA*) determinant(s) is also within this region, but its position(s) within it have not been determined. *incB* is within the *Mbo*I-C fragment. The heavy dots indicate restriction sites within the replication region which are protected by RNA polymerase binding (34). The target sites for the *cop* and *rep* gene products are located within the *Mbo*I-A fragment.

is required to drive stable replication of derivatives carrying only *Mbo*I-A. It is not apparent from the experiments just described whether the *trans*-acting material is a positive-acting effector for replication or acts as a repressor, preventing runaway replication which may prove lethal to the cell.

To test the idea that the replication substance functions as a repressor, we have constructed a *recE4* strain carrying both pBD93 and pE194(Ts). The latter is a mutant of pE194 (isolated by S. Gruss) which cannot replicate above 37°C. pBD93 carries the *Mbo*I-A fragment of pE194 in orientation 1 and cannot replicate unless a helper pE194 replicon is present. As the growth temperature is increased, the replication rate of the pE194(Ts) helper will decrease. If the helper provides a replication effector, the replication rate of pBD93 will also decrease. If the helper provides a negatively acting control element, the pBD93 replication rate will increase. These changes in replication will be reflected by shifts in copy numbers. Figure 5 shows results of a typical experiment in which four *recE4* strains carrying pE194, pE194(Ts), pE194 + pBD93, and pE194(Ts) + pBD93 were grown for two doublings at 30, 34, 35, and 36°C after previous growth to the same cell density ($\sim 5 \times 10^7$ colony-forming units/ml) at 30°C. Equal portions were harvested and lysed, and equal volumes were analyzed by agarose gel electrophoresis. Fluorimetric scanning of the gels shown in Fig. 5 confirmed that equivalent amounts of chromosomal DNA were loaded in each well (within 10%). As the growth temperature was increased, the intensity of the pE194(Ts) band decreased, whereas that of the pE194 band was essentially invariant over the temperature range used. In

the pE194(Ts) + pBD93 strain, both plasmid bands decreased in intensity as a function of increasing temperature, whereas in the pE194 + pBD93 control, the intensities of both plasmids remained essentially constant. These results suggest that the helper plasmid drives pBD93 by providing a *trans*-acting replication effector, rather than by providing a repressor of runaway replication. The mutational lesion in pE194(Ts) may, in fact, affect this effector. We denote the gene encoding the replication effector as *rep*.

Mapping of the pE194 replication region. To further define the region required for pE194-specific replication, a series of experiments was performed with the exonuclease *Bal*31. These experiments utilized pBD89 (*cop-6*) and pBD94 (*cop-6*) DNA, which contain the replication region (within coordinates 7 and 74; Fig. 1 and 3) in orientations 1 and 2. The DNA samples were cleaved with *Pvu*II, which cuts asymmetrically within the Cm^r fragment (Fig. 2). The resulting linear DNA samples were incubated with *Bal*31 for various times. The trimmed DNA was self-ligated, recut with *Pvu*II, and used to transform BD170 with selection for Cm^r. Since plasmid-free *B. subtilis* competent cells cannot be transformed with cleaved plasmid DNA, we expected (and observed) that all of the Cm^r transformants obtained in this experiment would be deletants, lacking the *Pvu*II site. Since plasmid DNA with both fragment orientations was used, the deletions extended into the replication region (*Mbo*I-A and -B) from both ends.

When orientation 1 (pBD89) was used, 3.5 × 10^5 transformants per ml were obtained without *Bal*31 digestion. After *Bal*31 digestion for 10 min at 37°C, religation, and secondary cutting with *Pvu*II, 2.7 × 10^2 transformants per ml were observed. The low survival (~0.1%) suggested that any deletions obtained would define a boundary of the essential replication region. Sixteen of these transformants were analyzed by restriction mapping (Fig. 2A). Of these, eight carried deletions ending between the *Dde*I and *Taq*I sites within *Mbo*I-A. Two additional deletions ended within the adjacent *TaqI-Hin*fI segment, and five were shorter deletions ending within the *Mbo*I-*Pst*I segment. One additional deletion was found to extend beyond the *Alu*I site into the nearby *Alu*I-*Cfo*I segment. These data suggest strongly that one end of the replication region is located near the segment defined by the *Dde*I and *Cfo*I sites.

Similar experiments were conducted with pBD94 (orientation 2). In this case, transformation with DNA not digested with *Bal*31 yielded 2.1 × 10^5 Cm^r transformants per ml. *Bal*31 treatment (10 min, 37°C) followed by ligation and secondary *Pvu*II cleavage gave four Cm^r transformants in 0.8 ml plated. These survivors

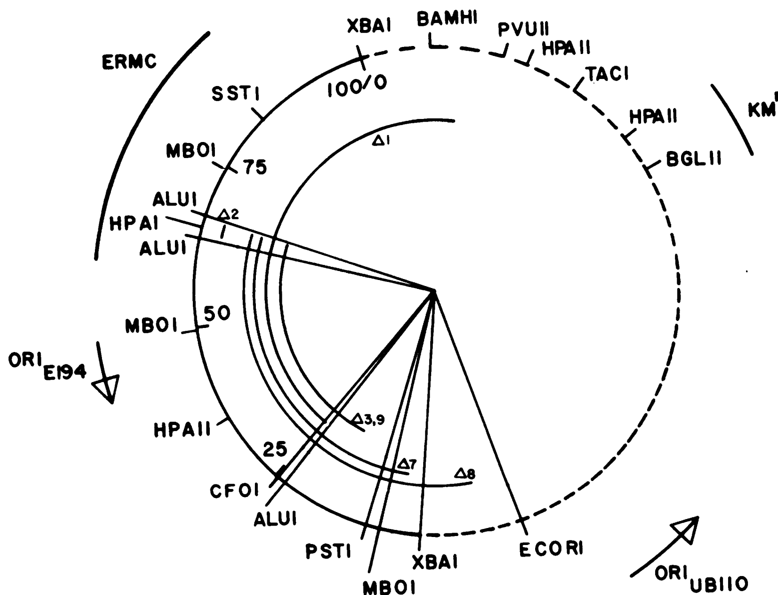

FIG. 4. Deletion map of pBD9. pBD9 is a cointegrate of pE194 (solid arc) and pUB110 (dashed arc) joined at their unique *Xba*I sites. The locations and replication directions of *ori* (E194) and *ori* (UB110) are shown. Both origins are active in this cointegrate (31). The positions of *ermC* and the pUB110 Km^r determinants are also shown. The extents of the various deletions discussed in the text are shown on the physical map of pBD9. All of the deletants but pBD9Δ2 necessarily replicate with *ori* (UB110). The isolation of these deletants was described previously (34). The coordinates refer to the map of pE194 (Fig. 3).

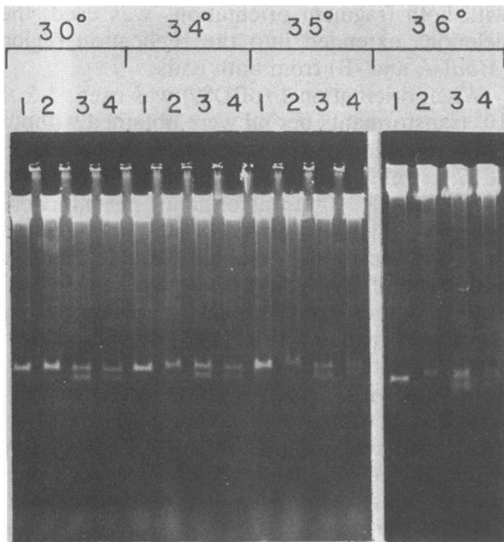

FIG. 5. Temperature effect on replication driven by pE194 and pE194ts. *recE4* strains carrying pE194 (1), pE194ts (2), pE194 + pBD96 (3), and pE194ts + pBD96 (4) were grown at 30°C and then at 30, 34, 35, and 36°C for two generations. DNA was isolated and analyzed on 0.8% agarose slab gels. Equal amounts of chromosomal DNA were loaded in each well. The lower (faster migrating) fluorescent bands in lanes 3 and 4 represent the covalently closed circular (CCC) monomer of pBD96. The top bands and the bands in lanes 1 and 2 are the CCC monomer of pE194 or pE194ts.

were analyzed for plasmid DNA content (Fig. 2B). Δr3 extended into the *Hin*fI-*Mbo*I interval near the *Mbo*I-A and -B junction, but did not cross this junction. The remaining three deletions were shorter. Since the *Mbo*I-A fragment alone is incapable of supporting stable autonomous replication in the absence of a second pE194 replicon, we conclude from Δr3 that only a small portion of the *Mbo*I-B fragment, adjacent to the junction with *Mbo*I-A, is required for synthesis of the *rep* product. It is likely that either the *rep* promoter or the *rep* terminator lies within the 130-base pair *Hin*fI-*Mbo*I interval thus defined.

The heavy line in Fig. 3 depicts our current estimate of the extent of the pE194 genome required for stable replication. This region includes about 900 base pairs.

Copy numbers of pE194 and its derivatives. For comparison purposes, it was desirable to construct a low-copy (wild-type) derivative of pBD89 (*cop-6*). This derivative was obtained by recombination. pBD89 DNA was linearized by cleavage at its unique *Hpa*I site and used to transform a recipient culture carrying pE194. Selection for Cm^r yielded low-copy-number Em^s recombinants. The electrophoretic behavior and restriction digests of plasmid DNA from these recombinants were identical to those of pBD89. We conclude that the linearized donor DNA was repaired by recombination with the recipient and thus became Cop^+ (4). This new plasmid was named pBD95.

The copy numbers of pE194, pBD15 (the *cop-6* mutant of pE194), and several of the recombinant plasmids and *Bal*31 deletants described above are presented in Table 4. These data show clearly that pBD89 (*cop-6*) is indistinguishable in copy number from the pBD15 (*cop-6*) parent, whereas the cop^+ derivative (pBD95) has a copy number similar to that of pE194. pBD94 (*cop-6*), the orientation 2 version of pBD89 (*cop-6*), also has a copy number similar to that of pBD15 (not shown). We conclude that the *cop-6* mutation (and hence the *cop* gene) is located within the *Mbo*I-A or -B fragment and that these fragments contain sufficient genetic information to specify copy control. Also shown in Table 4 (experiment 2) are copy numbers of several deletion derivatives of pBD89 (*cop-6*) (orientation 1) and pBD94 (*cop-6*) (orientation 2). All are essentially indistinguishable in copy number from the *cop-6* parent, except for the plasmid carrying Δr3. This derivative has a lower copy number, suggesting that the deletion has impinged on the replication region of pE194. Indeed, Δr3 does extend further than the other deletions derived from pBD94 and may have affected *rep* (Fig. 2B).

Dominance relationships in copy control. To investigate dominance relationships between *cop-6* and cop^+ plasmid derivatives, we determined copy numbers by using a *recE4* host (BD224) which carried pE194 or pBD15 together with one or another of the Cm^r derivatives (Table 4). The plasmid content was maintained during growth by the presence of both Cm and Em. In the absence of this selective pressure, rapid segregation occurred in many cases, due to incompatibility (see below). When pE194 and pBD95 (both Cop^+ plasmids) were maintained in the same cell, the copy numbers of both remained low. The data are not sufficiently precise to show whether the total number of copies is equivalent to that of either parent when present alone, although this seems likely. When pBD89 and pBD15 (both *cop-6* plasmids) were together, the total copy number did approximate that of either alone. This suggests the operation of a second (or residual) regulatory mechanism. The total plasmid mass, as well as total copy number, appears to be held constant when the *cop* gene is inactivated by mutation, and we cannot, strictly speaking, determine which parameter is determined by the regulatory system since the two plasmids in each case have similar molecular weights. We will nevertheless continue to use the term "copy control." The larger of the *cop-6*

TABLE 4. Copy numbers of pE194 derivatives and dominance relationships

Expt	Plasmid	Mol wt ($\times 10^6$)	*cop* genotype	% of total DNA as plasmid	Copy no.[a]
1	pE194	2.3	*cop*$^+$	0.79	8.6
	pBD15	2.3	*cop-6*	8.13	88.4
	pBD95	2.8	*cop*$^+$	0.97	8.7
	pBD89	2.8	*cop-6*	9.58	85.5
	pBD95 + pE194	2.8, 2.3	*cop*$^+$ *cop*$^+$	0.49 + 0.69	4.4 + 7.5
	pBD89 + pBD15	2.8, 2.3	*cop-6 cop-6*	3.80 + 4.85	33.9 + 52.7
	pBD95 + pBD15	2.8, 2.3	*cop*$^+$ *cop-6*	0.74 + 2.07	6.5 + 22.0
	pBD89 + pE194	2.8, 2.3	*cop-6 cop*$^+$	1.92 + 0.95	17.1 + 8.5
	pBD96 + pBD15	2.2, 2.3	—, *cop-6*	2.65 + 0.69	30.1 + 7.5
	pBD96 + pE194	2.2, 2.3	—, *cop*$^+$	0.44 + 0.47	5.1 + 5.1
2	pBD89	2.8		6.59	58.9
	pBD89Δ1	2.2		5.11	58.1
	pBD89Δ23	2.1		4.84	57.6
	pBD89Δr3	1.7		1.53	16.0
3	pBD89	2.3	*cop-6*	12.2	133
	pBD95	2.3	*cop*$^+$	1.00	11.9
	[pE194][b](pBD89)	2.3	*cop-6*	1.15	12.5
	[pE194][b](pBD95)	2.3	*cop*$^+$	1.45	15.7

[a] The copy numbers were determined by radioactive counting as described in the text. The strains were grown in the presence of antibiotic throughout (Cm or Cm + Em). We performed other experiments in the absence of antibiotic and the relative values were almost identical. The absolute copy numbers in these experiments were about 75% of those given above. We also calculated copy numbers by reading the gels on a direct tracing, and integrating Shimadzu fluorimeter. Similar results were obtained.

[b] [pE194] indicates the presence of pE194, inserted into the chromosome of *B. subtilis*.

plasmids (pBD89) has a somewhat lower copy number when present together with pBD15. Perhaps this bias reflects the longer elongation time for pBD89 replication. The reciprocal *cop*$^+$/*cop-6* situations were also analyzed. In these cases the copy number of the *cop*$^+$ plasmid (pE194 or pBD95) is similar to the values observed when these entities are alone, whereas the copy numbers of the *cop-6* plasmids (pBD15 or pBD89) are dramatically reduced. Both total copy number and total plasmid mass are intermediate between the usual *cop*$^+$ and *cop-6* values. The bias noted for the *cop-6*/*cop-6* situation is not observed in this case. The *cop-6* partner, not the smaller one, has a higher copy number. The total copy number and plasmid DNA mass values per bacterial genome in the reciprocal *cop*$^+$/*cop-6* situations are very similar (26 to 29 and 2.8 to 2.9% of the genome, respectively).

The dominance of *cop*$^+$ over *cop-6* can be demonstrated in a simpler system, using BD842. In this strain pE194 has been integrated into the chromosome, presumably as a single copy (E. Dubnau, K. Cabane, and I. Smith, personal communication). pBD89 (*cop-6*) and pBD95 (*cop*$^+$) were introduced and their copy numbers were compared with those in isogenic strains lacking integrated pE194 (Table 4, experiment 3). Clearly, the integrated copy of pE194 exerts a *trans*-dominant effect on the *cop-6* derivative (pBD89). It was necessary to exclude the possibility that recombination with the *cop*$^+$ chromosomal gene had converted the autonomous *cop-6* plasmid (pBD89) to *cop*$^+$. Plasmid DNA was isolated from the strain carrying both the *cop-6* plasmid and the chromosomally integrated pE194 and used to transform a plasmid-free recipient. Copy number determinations on several of the resulting Cm^r transformants clearly revealed that the plasmid present was *cop-6*. We interpret these experiments as indicating that pE194 elaborates a *trans*-acting inhibitor which regulates copy number. This inhibitor is altered in the *cop-6* mutant and therefore is most simply interpreted as being the product of the *cop* gene.

We have also examined the copy numbers of pBD96 and of pE194 or pBD15, when these are present in the same strain (Table 4, experiment 1). pBD96 was derived from pBD15, carries only the *Mbo*I-A fragment from the latter plasmid, and is incapable of stable replication unless driven by a pE194 replicon carrying a functional *rep* gene. When pBD15 is present with pBD96, the copy number of pBD15 is markedly reduced. In this case, the replication of total plasmid DNA mass may be limited by the concentration of available *trans*-acting *rep* product. Since pBD96 cannot produce this substance, its replication must be driven by pBD15. When pBD96 is in the presence of pE194 (*cop*$^+$), its copy

number is reduced nearly sixfold, presumably an effect of the *cop* inhibition product. Thus, receptivity toward the *cop* gene product resides within the *Mbo*I-A fragment (coordinates 7 and 48, Fig. 1 and 3). Unlike pBD15, the copy number of pE194 is not markedly lowered by the presence of pBD96, probably because in this case the concentration of replication substance is not limiting.

Incompatibility. To examine the incompatibility behavior of pE194 and its relationship to replication control, we have used a variety of derivatives, all previously described. pBD9 is a cointegrate of pUB110 and pE194, joined at their single *Xba*I sites (10). pBD9 can use either the pE194- or the pUB110-specific replication origins (31). We have constructed a series of derivatives of pBD9 in which various portions of the pE194 moiety, including the *ermC* gene, are deleted (34). The extents of these deletions are shown in Fig. 4.

To test the incompatibility of the pBD9 (cop^+) deletants, we performed reciprocal transduction experiments in which the Em^s derivatives were crossed with pE194 (cop^+) and pBD15 (*cop-6*) (Table 5). Bacteriophage AR9 was grown on strains carrying the pE194 derivatives listed in Table 5 and used to transduce strains carrying other derivatives, with selection for either a donor marker alone or donor and recipient markers simultaneously. Similar experiments were performed by transformation, with virtually identical results. pBD9Δ2, which carries a small deletion (<10 base pairs) within *ermC*, exhibits strong incompatibility with both pE194 (cop^+) and pBD15 (*cop-6*) when used as either a donor or a recipient, although the manifestations of incompatibility differ in the reciprocal crosses. When pBD9Δ2 is used as a donor, transduction for the donor marker alone (Km^r) is efficient, presumably since this cointegrate plasmid can utilize pUB110-specific replication. Coinheritance of pBD9Δ2 and pE194 (cop^+) or pBD15 (*cop-6*), however, does not occur. In the reciprocal situation (pBD9Δ2 as a resident plasmid) neither pE194 (cop^+) nor pBD15 (*cop-6*) transduction is observed. We interpret this behavior as indicating the presence of a *trans*-acting, pE194-specific incompatibility substance produced by pBD9Δ2. Note that the design of this particular experiment does not permit us to determine whether pE194 (cop^+) and pBD15 (*cop-6*) also produce this substance, since pBD9Δ2 is a cointegrate with dual replication

TABLE 5. Compatibility of pBD9 derivatives with pE194 and the pE194 *cop-6* mutant[a]

Donor plasmid	Selected marker(s)	Recipient plasmid (transductants per ml)						
		pBD9Δ1	pBD9Δ2	pBD9Δ3	pBD9Δ7	pBD9Δ8	pBD9Δ9	None[b]
pBD9 derivatives as recipients								
pE194	Em^r	840	20	605	755	860	485	930
	$Em^r + Km^r$	825	<10	580	905	810	470	
pBD15	Em^r	2,005	25	390	675	725	210	1,355
	$Em^r + Km^r$	2,590	<10	<10	150	100	<10	
pSH1[c]	Tc^r	505	690					480
pBD9 derivatives as donors			pE194			pBD15		
pBD9Δ1	Km^r		270			186		227
	$Km^r + Em^r$		240			190		
pBD9Δ2	Km^r		1,543			520		863
	$Km^r + Em^r$		<10			<10		
pBD9Δ3	Km^r		425			120		380
	$Km^r + Em^r$		500			<10		
pBD9Δ7	Km^r		265			130		495
	$Km^r + Em^r$		280			<10		
pBD9Δ8	Km^r		1,020			550		1,620
	$Km^r + Em^r$		1,055			<10		
pPB9Δ9	Km^r		340			110		470
	$Km^r + Em^r$		455			<10		
pUB110	Km^r		415			670		440
	$Km^r + Em^r$		350			455		

[a] The pBD9 derivatives (Δ1 to Δ9) were all Km^r Em^s; pE194 and the *cop-6* mutant were Km^s Em^r. The transducing phage was AR-9. All of the recipient strains were *recE4*.

[b] The plasmid-free control strain was BD224 (*trpC2 thr-5 recE4*), which is isogenic with the other recipient strains.

[c] AR-9 lysates of a strain carrying the tetracycline (Tc) resistance plasmid pSH1 were used as a control for the transducibility of the pBD9Δ2 recipient.

capability. However, some type of incompatibility is expressed by both pE194 and pBD15, since coinheritance of these plasmids with pBD9Δ2 does not occur. Table 5 reveals that pBD9Δ1, which carries the most extensive deletion and lacks the pE194 replication region (Fig. 3 and 4), expresses no detectable incompatibility toward pE194 or pBD15 when present as a recipient or donor plasmid. The remaining deletants, which are also missing the replication region but retain other portions of pE194, exhibit altered incompatibility behavior. They appear to be compatible with pE194 (cop^+) when used as either donors or recipients. However, they retain an expression of incompatibility toward pBD15 (*cop-6*). When these deletants are resident plasmids, they do not prevent the introduction of pBD15 (*cop-6*), but coinheritance of donor and resident plasmid is decreased. When the deletants are used as donors, analogous behavior is observed; only coinheritance is affected. This pattern suggests several conclusions: there seem to be two pE194-specific incompatibility determinants on pBD9 (cop^+) and, by reasonable assumption, on pE194. One (*incA*) is located within the replication region and appears to specify a *trans*-acting incompatibility material. A second (*incB*) expresses incompatibility only toward pBD15, the *cop-6* mutant of pE194, interfering with coinheritance of pBD15 and an $incB^+$ plasmid. This determinant is located in the region common to pBD9Δ3, -7, -8, and -9, which corresponds to the region of the pE194 maps shown in Fig. 3. The mode of action of *incB* is different from that of *incA*. It behaves symmetrically (in reciprocal crosses). It does not prevent introduction of an incompatible plasmid, but prevents stable coinheritance.

The five *Mbo*I fragments of pE194 have been cloned into pBD12, a Km^r Cm^r vector constructed from pC194 and pUB110 (34). All but the *Mbo*I-C fragment were cloned in both orientations. This series will be referred to as pBD12 (A_1, A_2, B_1, B_2, C, etc.). The incompatibility behavior of these recombinant plasmids is shown in Table 6. In these experiments, DNA from the recombinant plasmids was used as donor, with recipient strains carrying pE194 (cop^+) or pBD15 (*cop-6*). Donor (Cm^r) and donor plus recipient (Cm^r Em^r) markers were scored in each case. The pBD12 (C) derivative exhibits incompatibility (failure to coinherit the donor and resident plasmid) toward pBD15 (*cop-6*), but not toward pE194 (cop^+). Thus, it behaves as expected for a plasmid carrying *incB*. We conclude that *incB* is contained within *Mbo*I-C. The pBD12 A_1 and A_2 derivatives exhibit failure of coinheritance when either pE194 (cop^+) or pBD15 (*cop-6*) is present as the resident plasmid, but selection for the donor marker (Cm^r) alone is efficient. The latter is to be expected, since the vector, pBD12, replicates by a pUB110-specific mechanism and pUB110 is compatible with pE194 (unpublished data). The failure of coinheritance of pE194 or pBD15 with the pBD12 (A_1, A_2) recombinant plasmids will be discussed below. We do not understand why pBD12 (B_1) exhibits some apparent incompatibility toward pE194 (but not toward pBD15). This was reproducibly observed.

Finally, we performed an experiment to determine the relationship between the *incA* and *cop* products, both of which act in *trans*. For this purpose we used pBD35, a cointegrate of pBD15 and pUB110 which is isogenic with pBD9, except that it carries the *cop-6* mutation (31). Competent strains carrying pBD9 (cop^+) and pBD35 (*cop-6*) were transformed with DNA from pBD89 (*cop-6*) (Table 7). The strain carrying pBD35 (*cop-6*) was readily transformed for the donor marker (Cm^r) and exhibited coinheritance of donor and recipient plasmids. pBD9

TABLE 6. Incompatibility of hybrid plasmids with pE194 and the *cop-6* mutant of pE194[a]

*Mbo*I fragment on hybrid donor[c]	Recipient plasmid (transformants per ml, corrected)[b]			
	pE194		pBD15	
	Cm^r	Cm^r Em^r	Cm^r	Cm^r Em^r
A_1	55,890	810	116,000	1,890
A_2	44,150	170	65,580	190
B_1	40,100	4,100	75,600	40,990
B_2	79,550	59,660	91,480	32,150
C	88,800	61,400	66,760	2,520
D_1	139,030	99,310	106,560	46,650
D_2	119,980	85,890	176,330	185,420
E_1	88,030	69,430	130,600	104,150
E_2	88,660	79,710	78,810	63,890

[a] Covalently closed circular DNA of each of the donor plasmids (at 1 μg/ml) was used to transform *recE4* strains carrying either pE194 or the *cop-6* mutant of pE194. Selection was for the donor marker (Cm^r) and for the donor and the recipient markers (Cm^r + Em^r).

[b] An isogenic plasmid-free *recE4* strain was also transformed to Cm^r with the same DNA preparations. In addition to the DNA preparations of hybrid plasmids carrying the *Mbo*I fragments of pE194 in orientations 1 and 2, vector (pBD12) DNA was used to transform the same three recipient cultures. Correction factors were thus derived for competence (relative to that of the plasmid-free strains) and for transforming activity of the DNA preparations (relative to that of the vector DNA).

[c] The hybrid plasmids carried the indicated *Mbo*I fragment of pE194 inserted in the *Bgl*II site of pBD12, a Cm^r Km^r vector. This insertion inactivates Km^r. In all cases but that of fragment C, both insert orientations were tested (i.e., A_1, A_2, etc.).

TABLE 7. Incompatibility of *cop-6* mutants

Donor plasmid	Donor genotype	Recipient plasmid	Recipient genotype	Transformants per ml[a]		
				Cm^r	Cm^r Em^r	Tc^r
pBD89	Cm^r *cop-6*	pBD35	Km^r Em^r *cop-6*	6.81×10^3	5.38×10^3	
pBD89	Cm^r *cop-6*	pBD9	Km^r Em^r *cop*$^+$	25	<10	
pSH1	Tc^r	pBD35	Km^r Em^r *cop-6*			1.33×10^3
pSH1	Tc^r	pBD9	Km^r Em^r *cop*$^+$			5.2×10^3

[a] Transformation was carried out with 0.5 μg of DNA per ml. The recipient strain carried the *recE4* mutation in addition to pBD9 or pBD35. Transformation with pSH1, an unrelated tetracycline (Tc) resistance plasmid, was to control for the transformability of the two recipients.

(*cop*$^+$), however, appeared to greatly reduce transformation by the Cm^r plasmid, a behavior typical of strains carrying *cop*$^+$ plasmids, when the latter are used as recipients (Table 5). It should be noted that pBD89 (*cop-6*) lacks the *Mbo*I-C fragment and that no *incB*-type behavior is exhibited in this experiment. It appears, from this observation, that expression of *incB* incompatibility requires that *Mbo*I-C be on both plasmids. Most important is our conclusion from the experiment in Table 7 that the expression of *incA* incompatibility is altered by the *cop-6* mutation. Thus, interpreted most economically, it appears that the *incA* and *cop* *trans*-acting products are the same.

This point was explored further by testing the segregation behavior of *cop*$^+$ and *cop-6* plasmids (Table 8). After about 36 generations of growth without selection, some loss of plasmids from the strains carrying pE194 (*cop*$^+$) or pBD15 (*cop-6*) alone was noted. The loss of pBD95 (*cop*$^+$) and pBD89 (*cop-6*) was comparable. The greatest segregation (lowest proportion of Cm^r Em^r clones) was observed from the strain carrying two *cop*$^+$ plasmids (pE194 and pBD95). The lowest rate of segregation was observed when two *cop-6* plasmids were together (pBD89 and pBD15). The reciprocal *cop*$^+$/*cop-6* situations exhibited intermediate rates of segregation. It is clear that *cop*$^+$ plasmids express a greater degree of incompatibility than *cop-6* plasmids when this is measured by segregation, a result in accord with the conclusion drawn above from the transformation experiment (Table 7).

DISCUSSION

The region of the ~3.5-kilobase plasmid pE194, which is both necessary and sufficient to drive autonomous replication, is contained within the 0.9-kilobase segment shown in Fig. 3 between coordinates 24 and 50. This segment contains *ori* (31), an incompatibility determinant (*incA*), and a copy control gene (*cop*) and specifies a *trans*-acting substance, the product of *rep*, which is required for the stable replication of pE194 derivatives. It also contains the target sites for the *rep* and *cop* (*incA*) products. The *cop* gene product appears to act as an inhibitor in regulating copy number, and it is reasonable to suppose that it is a repressor of the initiation of replication acting either directly or indirectly. Since the *cop-6* mutation not only inactivates this inhibition (Table 4) but also eliminates expression of *incA* (Tables 7 and 8), it is most economical to conclude that *incA* and *cop* are

TABLE 8. Segregation of *cop*$^+$ and *cop-6* plasmids

Plasmid	Segregation (%), with given phenotype[a]			
	Cm^r Em^s	Cm^s Em^r	Cm^r Em^r	Cm^s Em^s
pE194 (Em^r *cop*$^+$)		68		32
pBD15 (Em^r *cop-6*)		91		9
pBD95 (Cm^r *cop*$^+$)	66			34
pBD89 (Cm^r *cop-6*)	64			36
pBD95 (Cm^r *cop*$^+$) + pE194 (Em^r *cop*$^+$)	17	44	4	35
pBD95 (Cm^r *cop*$^+$) + pBD15 (Em^r *cop-6*)	47	2	35	16
pBD89 (Cm^r *cop-6*) + pE194 (Em^r *cop*$^+$)	9	53	12	26
pBD89 (Cm^r *cop-6*) + pBD15 (Em^r *cop-6*)	13	9	72	6

[a] Single colonies of each strain (in isogenic *recE4* backgrounds) growing on selective plates were inoculated into VY + relevant selective antibiotics and grown overnight at 32°C. The cultures were diluted 10^4-fold in VY containing no antibiotics and again grown for 20 h at 32°C. The cultures were diluted, spread on tryptose blood agar base plates without antibiotics, and incubated overnight at 32°C. Single colonies (100) from each were patched with toothpicks onto appropriate selective media. These plates were incubated at 32°C for 24 h before scoring. Some of the patches appeared "spotty." These were scored as resistant.

the same. The incompatibility behavior of recombinant and deletant plasmids in which the pE194 moiety is replicated either passively or actively is consistent with the conclusion that the *incA* product is a replication inhibitor (Tables 5 and 6). *cop* maps within *MboI*-A and -B, since derivatives in which only these fragments were derived from cop^+ or *cop-6* parents exhibit the expected copy numbers (Table 4). *incA* is mapped more precisely by analysis of deletions (Table 5) and is within the replication region of pE194. The control of copy number by the *cop* repressor is consistent with a variety of regulation models suggested originally by Pritchard et al. (27) for other plasmid systems. Some means of reversing the inhibitor action, perhaps by dilution due to cellular growth or by invoking inhibitor instability, must be posited (28). It appears that even in the *cop-6* mutants an alternative or residual copy control mechanism may be operative. pBD15 (*cop-6*) is maintained at a copy number of about 80. When two *cop-6* mutants are present in the same cell, a total average copy number of about 80 is also maintained (Table 4). It is possible that the *cop-6* mutation is somewhat leaky or that an entirely independent mechanism also operates.

A *trans*-acting substance is specified by the *rep* gene of pE194 and of pBD15 and is apparently required for replication of derivatives carrying only the *MboI*-A fragment (i.e., lacking the roughly 100-base pair segment required for production of this substance which is contained within the *MboI*-B fragment and adjoins one end of *MboI*-A). Transcription of the *trans*-acting *rep* substance presumably either terminates or initiates within this small segment. The low copy number of pBD89Δr3 (Table 4) is consistent with this conclusion. The experiment with pE194(ts) (Fig. 5) suggests that the *rep* product is a positive-acting effector of replication. It may nevertheless be identical to the *cop* (*incA*) product, which acts negatively to control replication. In this case, the *cop-incA-rep* gene product would have at least two functions. One observation might be interpreted as contradicting this simple unitary concept. The pBD12 recombinant plasmids carrying *MboI*-A derived from pE194 cannot stably coexist with either pE194 or pBD15 (Table 6). It is reasonable to suggest that this is a manifestation of *incA*. Since these recombinant plasmids lack the small portion of *MboI*-B which appears to be required for production of the *rep* product, this would be an argument that the *cop* (*incA*) product is specified in its entirety by *MboI*-A and is therefore not identical to the *rep* product. The pBD12(A_2) recombinant expresses this incompatibility effect nearly an order of magnitude more strongly than pBD12(A_1) (Table 6). Possibly the vector pBD12 provides a suitably positioned transcriptional terminator (or promoter) in both orientations, but more favorably in orientation 1. The *rep* and *incA* (*cop*) functions might therefore be encoded in overlapping fashion or might represent different functional domains of a single product. The likely identity of the *incA* product with a copy control repressor is consistent with findings in other systems (2, 21, 30, 36, 37), as well as with various theoretical formulations (18, 25).

The data in Table 4 demonstrate that the Cm^r derivative carrying only *MboI*-A (pBD96) is subject to *cop* control. We have repeatedly observed that, when pBD96 DNA is used to transform cultures carrying pE194, low yields of Cm^r transformants are obtained (Table 3), a further indication that this plasmid is a target for A-type incompatibility. It thus appears likely that the site of action of the *cop* (*incA*) product is within the *MboI*-A fragment, which also contains *ori*.

Novick and Hoppensteadt (25) and Ishii et al. (18) have pointed out that incompatibility between two related plasmids can be due to competition for a common partition mechanism as well as the use of a common copy control mechanism. We have shown that a second incompatibility determinant is located on *MboI*-C, and it is tempting to suggest that *incB* may be a component of such a partition mechanism. However, pE194 and pBD15 derivatives lacking *MboI*-C segregate plasmid-free daughters no more often than do their parents (unpublished data). The *MboI*-C fragment thus appears to be unnecessary for replication, maintenance, or partition of pE194. This fragment is contained within the gene for a 54,000-dalton pE194 polypeptide of unknown function. The coding sequence for this polypeptide neither initiates nor terminates within the *MboI*-C fragment (34).

The replication region of pE194 is actively transcribed and contains an RNA polymerase binding site at coordinates 35 and 37 (34) (Fig. 3). We have identified two major transcripts specified by this region, which are strikingly more abundant in extracts of cells carrying pBD15 than in those carrying pE194 (G. Grandi and D. Dubnau, unpublished data). None of the five major pE194 polypeptides so far identified in minicells are specified by the replication region (34). As is postulated in the case of ColE1 (26), it is possible that the two transcripts act as primer and regulatory element. Their superabundance in the *cop-6* derivative may reflect a direct effect of this mutation on transcription (i.e., a promoter or terminator mutation) or an indirect one (i.e., by inactivation of a repressor or autorepressor).

Horinouchi and Weisblum (15) have recently reported the complete DNA sequence of pE194.

They have also determined that the *cop-6* mutation is a guanine · cytosine→adenine · thymine transition occurring near coordinate 33 on our map (Fig. 1 and 3), within the essential replication region. Also reported by Horinouchi and Weisblum (15) is a striking series of six inverted complementary repeat sequences, which on our map occur between coordinates 23 and 25. These authors infer that these repeat sequences serve some function in plasmid replication. One of our deletants (pBD89Δ23) has lost most of the inverted repeats. Repeat segments 1 to 6, 6′, 5′, and 4′ are missing in this derivative, which has a copy number similar to that of its parent [pBD89 (*cop-6*)] (Table 4). Thus, although the inverted repeats may be involved in copy control or some other aspect of replication, they are not all required for replication.

ACKNOWLEDGMENTS

We thank R. Novick, E. Dubnau, G. Grandi, and I. Smith for valuable discussions. We thank S. Gruss and E. Dubnau for providing the pE194ts plasmid and the strain with integrated pE194. We gratefully acknowledge the help of S. Projan and E. Murphy with fluorescence scanning of agarose gels and the expert secretarial assistance of A. Howard.

This work was supported by Public Health Service research grants AI-17472 and AI-10311 from the National Institutes of Health and American Cancer Society research grant MV99, awarded to D.D.

LITERATURE CITED

1. **Belyaeva, N. N., and R. R. Azizbekyan.** 1968. Fine structure of new *Bacillus subtilis* phage AR9 with complex morphology. Virology 34:176–179.
2. **Cabello, F., K. Timmis, and S. N. Cohen.** 1976. Replication control in a composite plasmid constructed by *in vitro* linkage of two distinct replicons. Nature (London) 259:285–290.
3. **Chang, S., and S. N. Cohen.** 1979. High frequency transformation of *Bacillus subtilis* protoplasts by plasmid DNA. Mol. Gen. Genet. 168:111–115.
4. **Contente, S., and D. Dubnau.** 1979. Marker rescue transformation by linear plasmid DNA in *Bacillus subtilis*. Plasmid 2:555–571.
5. **Contente, S., and D. Dubnau.** 1979. Characterization of plasmid transformation in *Bacillus subtilis*: kinetic properties and the effect of DNA conformation. Mol. Gen. Genet. 167:251–258.
6. **Donoghue, D. J., and P. A. Sharp.** 1978. Replication of colicin E1 plasmid DNA in vivo requires no plasmid encoded proteins. J. Bacteriol. 133:1287–1294.
7. **Dubnau, D., and R. Davidoff-Abelson.** 1971. Fate of transforming DNA following uptake by competent *Bacillus subtilis*. I. Formation and properties of the donor-recipient complex. J. Mol. Biol. 56:209–221.
8. **Dubnau, D., G. Grandi, R. Grandi. T. J. Gryczan, J. Hahn, Y. Kozloff, and A. G. Shivakumar.** 1981. Regulation of plasmid specified MLS-resistance in *Bacillus subtilis* by conformational alteration of RNA structure, p. 157–167. *In* S. B. Levy, R. C. Clowes, and E. L. Koenig (ed.), Molecular biology, pathogenicity, and ecology of bacterial plasmids. Plenum Press, New York.
9. **Gryczan, T. J., S. Contente, and D. Dubnau.** 1978. Characterization of *Staphylococcus aureus* plasmids introduced by transformation into *Bacillus subtilis*. J. Bacteriol. 134:318–329.
10. **Gryczan, T. J., and D. Dubnau.** 1978. Construction and properties of chimeric plasmids in *Bacillus subtilis*. Proc. Natl. Acad. Sci. U.S.A. 75:1428–1432.
11. **Gryczan, T. J., G. Grandi, J. Hahn, R. Grandi, and D. Dubnau.** 1980. Conformational alteration of mRNA structure and the posttranscriptional regulation of erythromycin-induced drug resistance. Nucleic Acids Res. 8:6081–6097.
12. **Gryczan, T., A. G. Shivakumar, and D. Dubnau.** 1980. Characterization of chimeric plasmid cloning vehicles in *Bacillus subtilis*. J. Bacteriol. 141:246–253.
13. **Horinouchi, S., and B. Weisblum.** 1980. Posttranscriptional modification of mRNA conformation: mechanism that regulates erythromycin-induced resistance. Proc. Natl. Acad. Sci. U.S.A. 77:7079–7083.
14. **Horinouchi, S., and B. Weisblum.** 1981. The control region for erythromycin resistance: free energy changes related to induction and mutation to constitutive expression. Mol. Gen. Genet. 182:341–348.
15. **Horinouchi, S., and B. Weisblum.** 1982. Nucleotide sequence and functional map of pE194, a plasmid that specifies inducible resistance to macrolide, lincosamide, and streptogramin type B antibiotics. J. Bacteriol. 150:804–814.
16. **Inuzuka, M., and D. R. Helinski.** 1978. Requirement of a plasmid-encoded protein for replication *in vitro* of plasmid R6K. Proc. Natl. Acad. Sci. U.S.A. 75:5381–5385.
17. **Iordanescu, S.** 1976. Three distinct plasmids originating in the same *Staphylococcus aureus* strain. Arch. Roum. Pathol. Exp. Microbiol. 35:111–118.
18. **Ishii, K., T. Hashimoto-Gotoh, and K. Matsubara.** 1978. Random replication and random assortment model for plasmid incompatibility in bacteria. Plasmid 1:435–445.
19. **Itoh, T., and J.-I. Tomizawa.** 1980. Formation of an RNA primer for initiation of replication ColE1 DNA by ribonuclease H. Proc. Natl. Acad. Sci. U.S.A. 77:2450–2454.
20. **Khan, S. A., S. M. Carleton, and R. P. Novick.** 1981. Replication of plasmid pT181 DNA *in vitro*: requirement for a plasmid-encoded product. Proc. Natl. Acad. Sci. U.S.A. 78:4902–4906.
21. **Kline, B. C.** 1979. Incompatibility between F*lac*, R386 and F:pSC101 recombinant plasmids: the specificity of F incompatibility genes. Plasmid 2:437–445.
22. **Lovett, M. A., L. Katz, and D. R. Helinski.** 1974. Unidirectional replication of plasmid ColE1 DNA. Nature (London) 251:337–340.
23. **Lovett, M. A., R. B. Sparks, and D. R. Helinski.** 1975. Bidirectional replication of plasmid R6K DNA in *Escherichia coli*: correspondence between origin of replication and position of single-strand break in relaxed complex. Proc. Natl. Acad. Sci. U.S.A. 72:2905–2909.
24. **Morell, P., I. Smith, D. Dubnau, and J. Marmur.** 1967. Isolation and characterization of low molecular weight ribonucleic acid species from *Bacillus subtilis*. Biochemistry 6:258–265.

24a. **Novick, R. P., G. K. Adler, S. Majumder, S. A. Khan, S. Carleton, W. D. Rosenblum, and S. Iordanescu.** 1982. Coding sequence for the pT181 *rep*C product, plasmid-coded protein uniquely required for replication. Proc. Natl. Acad. Sci. U.S.A. 79:4108–4112.

25. **Novick, R. P., and F. C. Hoppensteadt.** 1978. On plasmid incompatibility. Plasmid 1:421–434.
26. **Polisky, B., M. Muesing, and J. Tamm.** 1981. Nucleotide sequence change in a ColE1 copy number mutant, p. 337–348. *In* S. B. Levy, R. C. Clowes, and E. L. Koenig (ed.), Molecular biology, pathogenicity, and ecology of bacterial plasmids. Plenum Press, New York.
27. **Pritchard, R. H., P. T. Barth, and J. Collins.** 1969. Control of DNA synthesis in bacteria. Symp. Soc. Gen. Microbiol. 19:263–298.
28. **Pritchard, R. H., and N. B. Grover.** 1981. Control of plasmid replication and its relationship to incompatibility, p. 271–278. *In* S. B. Levy, R. C. Clowes, and E. L. Koenig (ed.), Molecular biology, pathogenicity, and ecology of bacterial plasmids. Plenum Press, New York.
29. **Radloff, R., W. Bauer, and J. Vinograd.** 1967. A dye-

buoyant density method for the detection and isolation of closed circular duplex DNA: the closed circular DNA in HeLa cells. Proc. Natl. Acad. Sci. U.S.A. **57**:1514–1521.

30. **Rownd, R. H., A. M. Easton, and P. Sampathkumar.** 1981. Copy number control and incompatibility of *inc*FII R plasmids, p. 303–315. *In* S. B. Levy, R. C. Clowes, and E. L. Koenig (ed.), Molecular biology, pathogenicity, and ecology of bacterial plasmids. Plenum Press, New York.
31. **Scheer-Abramowitz, J., T. J. Gryczan, and D. Dubnau.** 1981. Origin and mode of replication of plasmids pE194 and pUB110. Plasmid **6**:67–77.
32. **Shepard, H. M., D. H. Gelfand, and B. Polisky.** 1979. Analysis of a recessive plasmid copy number mutant: evidence for negative control of Col E1 replication. Cell **18**:267–275.
33. **Shivakumar, A. G., and D. Dubnau.** 1981. Characterization of a plasmid-specified ribosome methylase associated with macrolide resistance. Nucleic Acids Res. **9**:2549–2562.
34. **Shivakumar, A. G., T. J. Gryczan, Y. I. Kozlov, and D. Dubnau.** 1980. Organization of the pE194 genome. Mol. Gen. Genet. **179**:241–252.
35. **Shivakumar, A. G., J. Hahn, G. Grandi, Y. Kozlov, and D. Dubnau.** 1980. Posttranscriptional regulation of an erythromycin resistance protein specified by plasmid pE194. Proc. Natl. Acad. Sci. U.S.A. **77**:3903–3907.
36. **Timmis, K. N., H. Danbara, G. Brady, and R. Lurz.** 1981. Inheritance functions of group IncFII transmissible antibiotic resistance plasmids. Plasmid **5**:53–75.
37. **Uhlin, B. E., and K. Norström.** 1975. Plasmid incompatibility and control of replication: copy mutants of the R-factor R1 in *Escherichia coli* K-12. J. Bacteriol. **124**:641–649.
38. **Veltkamp, E., and A. R. Stuitje.** 1981. Replication and structure of the bacteriocinogenic plasmids. Plasmid **5**:76–99.
39. **Weisblum, B., M. Y. Graham, T. Gryczan, and D. Dubnau.** 1979. Plasmid copy number control: isolation and characterization of high-copy-number mutants of plasmid pE194. J. Bacteriol. **137**:635–643.