NOTES

Localization of the Replication Origin of Plasmid pE194

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The pE194 replication origin was localized to a 265-base-pair interval by analyzing the ability of purified pE194 restriction fragments to direct replication of heterologous plasmids. Replication was dependent upon RepF protein supplied in *trans*. The origin region contained a GC-rich dyad symmetry which may serve as the RepF target.

pE194 is a 3.7-kilobase staphylococcal plasmid which confers resistance to the macrolide-lincosamide-streptogramin B group of antibiotics. Replication of pE194 is known to be naturally temperature sensitive (12) and requires the plasmid-encoded *trans*-acting RepF protein for initiation (14). Recent data have suggested that pE194 replicates asymmetrically by producing covalently closed single-stranded circular DNA as a replication intermediate (13). Earlier studies demonstrated that autonomous pE194 replication activity resided within the two largest *Mbo*I restriction fragments (positions 1 to 2516 [6]) and within the smaller *Cfo*I restriction fragment (positions 762 to 1925 [14]). This interval, in addition to containing the origin of replication, encodes the RepF initiator protein and a negative control element, *cop* (6, 14). In this report, we localize the pE194 replication origin to a 265-base-pair (bp) interval located between nucleotide positions 864 and 1129 by taking advantage of the ability of the origin to be *trans* complemented by RepF initiator activity.

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pE194 derivatives were constructed in vitro (Fig. 1) and analyzed for their ability to replicate in the gram-positive *Bacillus subtilis* host either in the presence or in the absence of a helper plasmid supplying the pE194 RepF initiator protein. Strains and plasmids used are listed in Table 1. Various gel-purified pE194 restriction fragments were inserted into the vector pCP115, which is devoid of a gram-positive replicon but contains the chloramphenicol resistance determinant of pC194 (10). Plasmid pBD406 contains the 542-bp *Taq*I-*Hpa*II restriction fragment (positions 587 to 1129) of pE194 inserted into the *Cla*I site of pCP115 (Fig. 1). pBD232, a spontaneous excision plasmid isolated from integrated pE194 strain BD892 (7), was used to generate pBD408. pBD232 consists of pE194 carrying a 1.3-kilobase fragment derived from the *B. subtilis* chromosome. A unique *Sma*I recognition site within the pBD232 plasmid was fortuitously created at the crossover junction derived from in vivo recombination with the *B. subtilis* chromosome (2) so that a

342-bp *Sma*I-*Sph*I restriction fragment obtained from pBD232 contained only pE194 DNA sequences (positions 864 to 1205). This fragment was inserted into *Eco*RV-*Sph*I-cleaved pCP115 (Fig. 1). pLD7 was obtained by cloning the recombinant plasmid chromosome junctions of a pE194 integrant, BD889, by replacement recombination with a pCP115 derivative, pBD329 (Dempsey and Dubnau, submitted). This plasmid contains pE194 sequences from positions 930 (crossover site) to the *Cla*I site at 1939, as well as 1.7 kilobases of chromosomal DNA, in addition to the pCP115 vector sequences.

The plasmid constructions were initially used to transform *Escherichia coli* to ampicillin resistance, and the predicted structures of pBD406 and pBD408 were verified by restriction mapping and Southern hybridizations (data not shown). These constructs were subsequently used to transform *B. subtilis recE4* strains to chloramphenicol resistance. The chimeric plasmid pBD329 (pCP115 and pE194 fused at their *Cla*I sites) was used as a control. The vector pCP115 failed to successfully transform any of the *B. subtilis* hosts, confirming its lack of a gram-positive replicon. However, pBD329 was able to transform both plasmidless BD224 and the pE194-containing strains BD432 and BD433 (Table 2). The pE194 derivatives (pBD406 and pBD408) which lacked the *repE* coding region (positions 1244 to 1856 [14]) failed to transform BD224. In contrast, both pBD406 and pBD408 were able to transform *B. subtilis* strains carrying either pE194 or pBD15 (a high-copy-number derivative of pE194 carrying the *cop-6* marker) to chloramphenicol resistance (Table 2). This effect was not due to homologous recombination between the pE194 moieties of the donor and resident plasmids, as the recipient strains were *recE4* mutants, which are defective in homologous recombination (3). Moreover, analysis of plasmid DNA from the chloramphenicol-resistant transformants by agarose gel electrophoresis revealed a plasmid species which comigrated with the donor plasmid and no evidence for in vivo-derived recombinant forms (data not shown). On the other hand, pLD7 failed to transform BD224, although this plasmid contains the entire *repF* gene. pLD7 also failed to transform strains which carried a pE194 helper plasmid, which suggests that this plasmid lacks or is disrupted in the pE194 origin region. pLD7 DNA was able to transform *E. coli* to ampicillin resistance (not shown).

pBD406 and pBD408 were shown to be dependent on pE194-specific replication and probably on the resident

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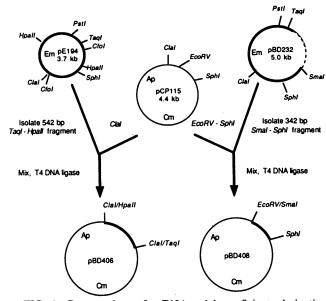
FIG. 1. Construction of pE194 origin-proficient derivatives pBD406 and pBD408. pBD406 was constructed by inserting the gel-purified 542-bp TaqI-HpaII fragment of pE194 (positions 587 to 1129) into the ClaI site of pCP115. pBD408 was constructed by inserting the gel-purified 342-bp SmaI-SphI fragment of pBD232 (positions 864 to 1205) into the larger compatible EcoRV-SphI fragment of pCP115. Light lines depict vector pCP115 sequences, heavy lines depict pE194-derived sequences, and the dashed line in pBD232 depicts B. subtilis chromosomal sequences.

TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Genotype	Source or reference
B. subtilis		
BD224	trpC2 thr-5 recE4	This laboratory
BD432	trpC2 thr-5 recE4(pE194)	15
BD433	trpC2 thr-5 recE4(pBD15)	15
E. coli HB101	F⁻ hsd20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44	1
Plasmid		
pE194	Emʳ	9
pBD15	Emʳ	15
pCP115	Apʳ Cmʳ	10
pBD232	Emʳ, spontaneous excision product from pE194 integrant BD892	7
pBD318	Kmʳ Cmʳ containing the CfoIB fragment of pE194 replacing CfoIB of pBD64	14
pBD329	Emʳ Apʳ Cmʳ chimera of pE194 and pCP115	2
pBD347	Cmʳ, pIM13 replicon	11
pBD406	pCP115 containing the 542-bp TaqI-HpaII fragment of pE194	This work
pBD408	pCP115 containing the 342-bp SmaI-SphI fragment of pBD232	This work
pLD7	pCP115 containing the 2.7-kilobase pE194-B. subtilis DNA ClaI-HindIII junction fragment derived from the pE194 integrant BD889	2

pE194 plasmid to supply the initiator RepF by passaging the transformants at high growth temperatures (51°C) on non-selective agar. pE194 is unable to replicate at this temperature and is lost from the bacterial cell. Single colonies arising after overnight growth at 51°C were patched onto selective agar (5 μg of either erythromycin or chloramphenicol per ml). These colonies failed to grow at permissive growth

temperatures (32°C) on either selective medium, suggesting that both plasmids were lost. Gel electrophoresis performed on DNA lysates from the colonies obtained after growth at 51°C showed that this was so (data not shown).

A map of the basic replicon of pE194 is shown in Fig. 2. The locations of the repF and cop genes are depicted according to Villafane et al. (14). The segments of pE194
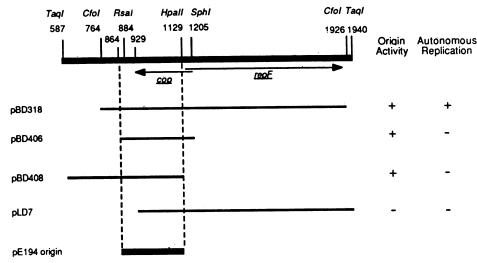

FIG. 2. The basic replicon of pE194. Origin activity indicates the ability to transform strains which harbored either pE194 or pBD15. Autonomous replication indicates the ability to transform a plasmidless B. subtilis strain. Relevant restriction endonuclease recognition sites of pE194 are shown with their corresponding nucleotide positions. The coding regions for the initiator repE and cop genes are indicated by the underlying arrows (14). In addition to the plasmids described in the text, pBD318 carries the CfoIB fragment of pE194, which contains the entire replication region (Table 1). The demonstration that pBD318 carries autonomous replication capacity was by Villafane et al. (14).

TABLE 2. Ability of pE194 derivatives to transform *B. subtilis*

Recipient strain	Donor plasmid	No. of Cm^r transformants/ μg of donor DNA
BD224	pCP115	0
	pBD329	2×10^3
	pBD347	5×10^5
	pBD406	0
	pBD408	0
	LD7	0
BD432(pE194)	pCP115	0
	pBD329	2.5×10^2
	pBD347	1×10^5
	pBD406	2.5×10^2
	pBD408	3×10^2
	pLD7	0
BD433(pBD15)	pCP115	0
	pBD329	3×10^2
	pBD347	1×10^5
	pBD406	1×10^2
	pBD408	1×10^2
	pLD7	0

which are present in the test plasmids are shown by the bars placed below the replicon map. Origin activity is localized to a 265-bp interval located between position 864 (*Sma*I recognition site of pBD232) and the *Hpa*II recognition site at 1129, although the actual site of the origin, presumably the site of interaction with the RepF product, is possibly much smaller. It is likely that pLD7, which lacks pE194 sequences from 864 to 929 and cannot support plasmid replication, is missing sequences critical for origin activity. Deletion of an *Rsa*I fragment delimited by positions 634 and 881 inactivates replication activity, whereas removal of a *Dde*I fragment (684 to 840) does not (S. Projan, personal communication). These data are consistent with the *ori* location depicted in Fig. 2. We have previously suggested, on the basis of

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860       870       880       890       900       910
 |         |         |         |         |         |
TTTAGGGGAGAAAACATAGGGGGGGTACTACGACCTCCCCCCTAGGTGTCCATTGTCCATT
AAATCCCCTCTTTTGTATCCCCCCATGATGCTGGAGGGGGGATCCACAGGTAACAGGTAA
               ------->          <-------
                     Rsal

920       930       940       950       960       970
 |         |         |         |         |         |
GTCCAAACAAATAAATAAATATTGGGTTTTTAATGTTAAAAGGTTGTTTTTTATGTTAAA
CAGGTTTGTTTATTTATTTATAACCCAAAAATTACAATTTTCCAACAAAAAATACAATTT

980       990      1000      1010      1020      1030
 |         |         |         |         |         |
GTGAAAAAAACAGATGTTGGGAGGTACAGTGATGGTTGTAGATAGAAAAGAAGAGAAAAA
CACTTTTTTTGTCTACAACCCTCCATGTCACTACCAACATCTATCTTTTCTTCTCTTTTT

1040      1050      1060      1070      1080      1090
 |         |         |         |         |         |
ATTGCTGTTACTTTAAGACTTACAACAGAAGAAAATGAGATATTAAATAGAATCAAAGAA
TAACGACAATGAAATTCTGAATGTTGTCTTCTTTTACTCTATAATTTATCTTAGTTTCTT
                                             HinII

1100      1110      1120      1130
 |         |         |         |
AAATATAATATTGGCAAATCAGATGCAACCGG
TTTATATTATAACCGTTTAGTCTACGTTGGCC
                        HpaII
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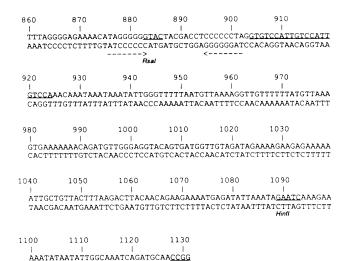
FIG. 3. Nucleotide sequence of the pE194 region containing the replication origin. The location of the GC-rich dyad symmetry element is shown by the arrows. The nucleotide sequence is from Horinouchi and Weisblum (8) as corrected by Villafane et al. (14).

deletion analysis, that the pE194 *ori* is located within the segment 1389 to 1588 (14). We have now concluded that this was an error which was based on faulty mapping data for one terminus of a deletion mutation. However, since this deletion (Δ35) removes the *Hpa*II site at 1129 (Fig. 2) and plasmids carrying this deletion are capable of replication when provided with *repF* in *trans*, we conclude that *ori* does not include the *Hpa*II site. The nucleotide sequence of the origin region, shown in Fig. 3, also contains a GC-rich dyad symmetry element (positions 876 to 902), and an adjacent 7-bp tandem repeat [5'-T(G)TGTCCA'-3] is present in three copies (positions 905 to 924). This interval in pE194 (860 to 930) is also a major recombination site for the low-frequency RecE-independent integration of pE194 into the *B. subtilis* chromosome (2, 7). We hypothesize that the recombination site may be part of the origin of pE194 replication, possibly the site of RepF interaction.

Horinouchi and Weisblum (8) suggested that the dyad symmetry element was involved in pE194 replication by analogy to the replication region of another staphylococcal plasmid, pC194. The minimal origin of pC194 has been reported recently (4) to be contained within a 65-bp interval, which also contains a GC-rich dyad symmetry. It is interesting that both pE194 and pC194, as well as plasmid pT181, have relatively high GC contents (40 to 50%) in the regions necessary for origin activity, although the overall GC content of these plasmids is low (29 to 32%). All three plasmids are hypothesized to replicate by a rolling-circle mechanism (5, 13), and the similarity in the origin regions may reflect common functional requirements.

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