Proc. Natl. Acad. Sci. USA
Vol. 75, No. 3, pp. 1428–1432, March 1978
Genetics

Construction and properties of chimeric plasmids in *Bacillus subtilis**

(molecular cloning/endonuclease *Xba* I/antibiotic resistance)

THOMAS J. GRYCZAN AND DAVID DUBNAU†

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

Communicated by George K. Hirst, December 12, 1977

ABSTRACT **Antibiotic resistance chimeric plasmids have been constructed by *in vitro* enzymatic manipulation and introduced into *Bacillus subtilis* by transformation. The parental plasmids used had been introduced into *B. subtilis* from *Staphylococcus aureus* by transformation. Of the seven recombinant plasmids constructed using restriction endonucleases, one was made using *Eco*RI, another using *Hpa* II, and five with *Xba* I (from *Xanthomonas badrii*), demonstrating the utility of the latter enzyme for molecular cloning experiments. Although all of the recombinant plasmids we have made replicate and express their antibiotic resistance characters, three of them have suffered a loss of DNA, either *in vitro* or, more likely, *in vivo*. The deletion event in all cases involved one of the two termini used to join the parental plasmids. The plasmid chimeras reported in this paper should prove useful for the study of plasmid replication, incompatibility, and recombination. In addition, the utility of the *B. subtilis* system for molecular cloning has been clearly illustrated.**

The ability to carry out molecular cloning in *Bacillus subtilis* would be useful for a variety of studies on sporulation, transformation, and gene expression. In addition, such a capability might be industrially significant, because *Bacillus* species are of considerable commercial importance. Ehrlich (1) has shown that several chloramphenicol and tetracycline resistance plasmids isolated from *Staphylococcus aureus* can be introduced by transformation into *B. subtilis*. This raised the possibility that *S. aureus* plasmids might be useful as vectors for molecular cloning in *B. subtilis*. We have transferred additional *S. aureus* plasmids to competent *B. subtilis* strains and have initiated a study of the molecular biology of these plasmids. This paper reports the construction of several plasmid chimeras by molecular cloning, thus demonstrating the utility of the *B. subtilis* system for recombinant DNA experiments and providing a collection of new plasmids for studies on replication, incompatibility, and transformation as well as for the engineering of better cloning vectors.

MATERIALS AND METHODS

The *B. subtilis* host strain used in this study was BD170 (*trpC2 thr-5*). The plasmids used were originally isolated from *S. aureus* and were introduced into *B. subtilis* by transformation. All plasmid DNA preparations used in this study were isolated from *B. subtilis*. The plasmids are listed in Table 1 together with some relevant restriction endonuclease sites and molecular weights. The documentation of this data will be published elsewhere. The isolation of covalently closed circular plasmid DNA was carried out by the sodium dodecyl sulfate/NaCl method of Guerry et al. (5) followed by dye–buoyant density centrifugation (6). The preparation of bacterial DNA and competent cells and the transformation procedure was as described previously (7), except that 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid was added to the plasmid transformation mixtures, because this raises the frequency of plasmid transformation (unpublished). Restriction endonucleases were purchased from New England BioLabs. Digestions of DNA mixtures were by standard procedures (8) and will be described in detail elsewhere. After heating at 65° for 10 min, the digested samples were incubated with phage T4 ligase (Miles), using the conditions of Tanaka and Weisblum (9). The final concentration of each restriction endonuclease-treated plasmid DNA in the reaction mixture was 10–15 μg/ml. Selection of antibiotic-resistant(r) clones following transformation was by the overlay method in tryptose blood agar base (Difco), allowing 90 min at 37° for expression before challenging with the drugs. Antibiotic concentrations used for selection were: chloramphenicol (Cm), 5 μg/ml; erythromycin (Em), 5 μg/ml; kanamycin (Km), 5 μg/ml; streptomycin (Sm), 50 μg/ml. Strains carrying presumptive chimeric plasmids were purified on selective media before isolation of plasmid DNA. Plasmids and restriction endonuclease fragments were analyzed on agarose gels [Seakem, LE (low electroendosmosis)] using Tris/borate buffer (10), and the gels were stained with ethidium bromide (1 μg/ml) for 30 min, destained for 30 min in H_2O, and photographed using UV light and Polaroid type 665 film. These studies involved only DNA that can "naturally" replicate in *B. subtilis* and were carried out under P1 conditions, in accordance with our Memorandum of Understanding and Agreement with the National Institutes of Health.

RESULTS

Table 2 presents the results of transformation experiments using plasmid DNA mixtures that had been digested with restriction endonuclease and ligated. Mixtures of plasmid DNAs that were neither endonuclease treated nor ligated gave no detectable transformation when selection was applied simultaneously for both parental resistance characters. In all cases, endonuclease-treated and unligated samples gave no detectable transformation, as expected from our previous observation that linearized plasmid DNA cannot transform *B. subtilis* (results not shown).

From each of these experiments, representative clones were screened for the presence of new plasmid species. Ten clones from exp. 1, one from exp. 2, eighteen from exp. 3, and five from exp. 5 were tested. All contained new plasmids that mi-

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: Cm, chloramphenicol; Em, erythromycin; Km, kanamycin; Sm, streptomycin; r, resistant; MDal, megadalton.

* In partial fulfillment of the requirements for the doctoral degree for T.J.G. in the Department of Microbiology, New York University School of Medicine.

† To whom reprint requests should be addressed.

Table 1. Properties of plasmids*

Plasmid†	Markers	Molecular weight $\times 10^{-6}$	Restriction endonuclease sites‡							Ref.
			Bgl II	*Eco*RI	*Hin*dIII	*Hpa* II	*Pst* I	*Sal* I	*Xba* I	
pCM194	Cm	2.0	0	0	1	1	0	0	0	2
pE194	Em	2.4	0	0	0	2	1	0	1	3
pSA0501	Sm	2.8	0	1	1	2	0	0	1	2
pSA2100	Sm, Cm	4.6	0	1	2	3	0	0	1	2
pUB110	Km	3.0	1	1	0	4	0	0	1	4

* The data concerning molecular weights and restriction sites were determined in our laboratory, except for the *Hin*dIII site on pCM194, which was discovered by Ehrlich (1).

† These *S. aureus* plasmids were introduced into *B. subtilis* by transformation. The data reported in this table were gathered using plasmid DNA isolated from *B. subtilis*.

‡ Because fragments of molecular weight less than about 0.1×10^6 would not be detected in our agarose gel experiments, these represent minimal estimates of the number of sites.

grated more slowly in agarose gels than the parent plasmids used in each case. All those new plasmids obtained from a given experiment migrated identically. From exp. 4, thirteen clones were tested. Nine, which were Km^r, Em^r, Cm^r, Sm^r, contained both parental plasmids (pBD8 and pE194) and were not studied further. One, which was Km^r, Em^r and had lost the Sm^r and Cm^r characters, contained a plasmid that migrated more slowly than the pE194 parent and more rapidly than pBD8. Three other clones, which were Km^r, Em^r, Cm^r and had lost Sm^r, contained indistinguishable plasmids that also migrated at an intermediate rate but more slowly than the Km^r Em^r plasmid. The electrophoretic mobility of each of the new plasmid types is shown in Fig. 1, along with that of their parents.

These covalently closed circular DNA preparations were then used in a second round of transformation and selection was applied for the single and multiple resistance characters. Table 3 shows that the number of transformants obtained was essentially the same for the single and multiple selections, providing strong evidence that the new plasmids carried drug resistance characters from each of their parent plasmids. Electrophoretic mobilities of plasmids isolated from this second round of transformations were identical to those shown in Fig. 1. The single recombinant plasmid isolated from experiment 6 was detected by screening 12 Km^r transformants electrophoretically for the presence of foreign DNA. One plasmid with increased size was obtained (Fig. 1*D*). This plasmid was used to transform a second recipient for Km^r, and the transformants were all found to contain this larger plasmid. Fig. 2 shows restriction endonuclease fragments obtained from the new plasmids, and Table 4 summarizes some of their properties. When linearized with *Eco*RI and *Hin*dIII, pBD6 and pBD9 have molecular weights of 5.8 and 5.4×10^6, respectively, which are indistinguishable from the sum of the parental fragments. When treated with *Xba* I (from *Xanthomonas badrii*), two fragments are obtained from each, corresponding to the linear forms of the parent plasmids (Fig. 2 *A* and *B*). The pBD6 *Xba* I products form a double band, as seen from comparison with the pBD6-*Hin*dIII linear product.

pBD7 has two fragments produced by *Eco*RI, one corresponding to linear pUB110 and the other having a size of 1 megadalton (MDal) (Fig. 2*D*). The new fragment, presumably derived from the *B. licheniformis* chromosome, contains a single *Sal* I site and two *Hin*dIII sites. The genetic composition of this fragment is unknown.

pBD8, 10, and 11 have molecular weights lower than expected from the sum of the parental plasmids. As seen in Fig. 2 *B* and *C*, an *Xba* I site is also missing from each, and resistance to Sm and Sm + Cm has been deleted from pBD10 and pBD11, respectively.

pBD12 (Figure 2 *E* and *F*) *Hpa* II digests display two bands

Table 2. Construction of plasmid chimeras

Exp.	Parental plasmids		Restriction endonuclease	Selection	Transformants/ml	
	Plasmid	Markers			After digestion and ligation	No enzyme treatment
1	pUB110	Km^r	*Xba* I	Km	47,100	105,000
	pE194	Em^r		Em	1,000	1,320
				Km + Em	490	<10
2	pUB110	Km^r	*Xba* I	Km	11,500	3,000
	pSA0501	Sm^r		Sm	9,000	10,300
				Km + Sm	2,510	<10
3	pUB110	Km^r	*Xba* I	Km	11,300	2,280
	pSA2100	Sm^r, Cm^r		Cm	43,500	7,900
				Km + Cm	8,200	<10
4	pBD8	Sm^r, Cm^r, Km^r	*Xba* I	Km	205,000	100,000
	pE194	Em^r		Em	27,800	11,000
				Km + Em	1,450	<10
5	pUB110	Km^r	*Hpa* II	Cm	5,700	200
	pCM194	Cm^r		Km	460	4,300
				Cm + Km	300	<10
6	pUB110	Km^r	*Eco*RI	Km	21,000	85,000
	B. licheni-formis 9945a					

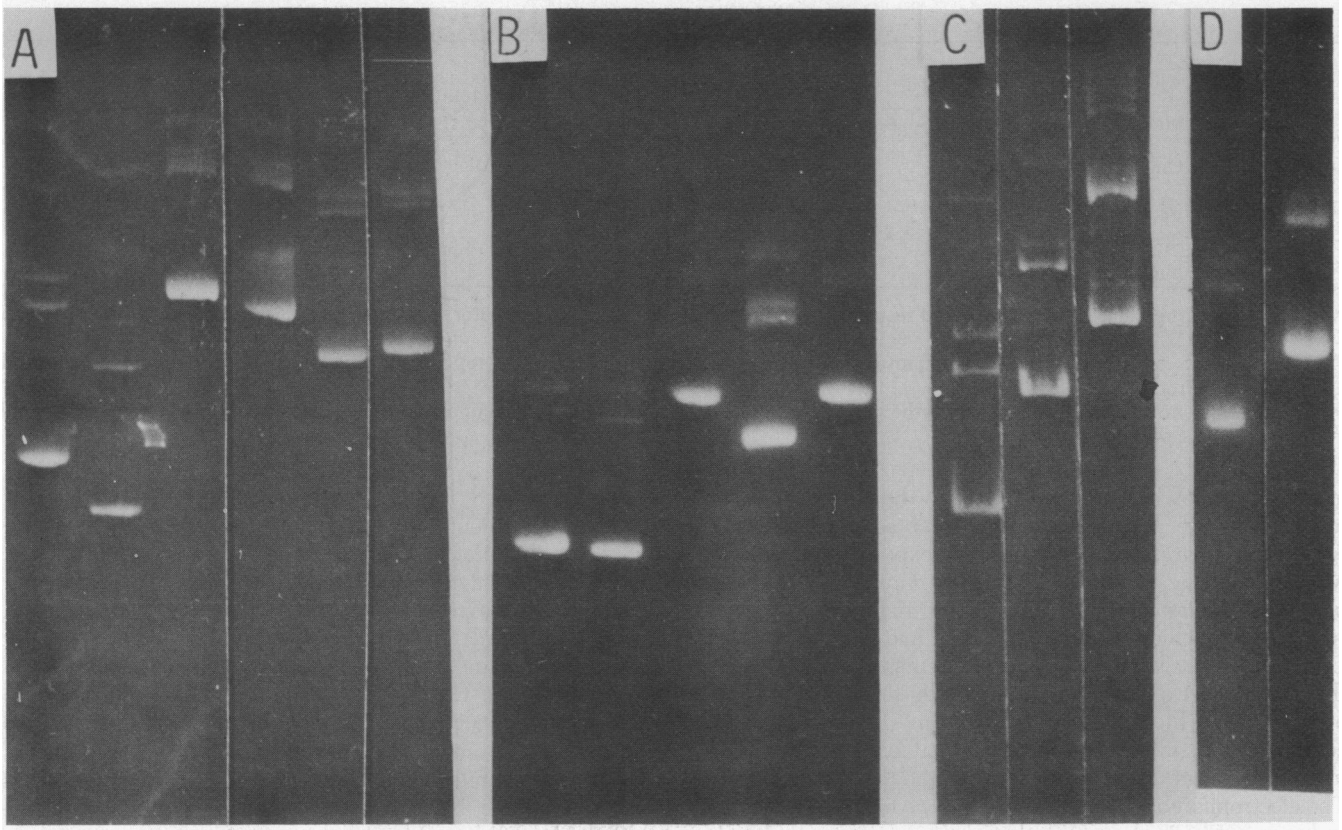

FIG. 1. Electrophoretic migration in 0.8% agarose of chimeric and parental plasmids. The chimeric plasmids are identified by resistance markers carried and by the experiment from which they originated (Table 2). From left to right the channels contain: (*A*) pub110, pE194, Km^r Cm^r Sm^r (exp. 3), Km^r Em^r (exp. 1), Km^r Em^r (exp. 4), Km^r Em^r Cm^r (exp. 4). (*B*) pUB110, pSA0501, Km^r Sm^r (exp. 2), pSA2100, Km^r Cm^r Sm^r (exp. 3). (*C*) pCM194, pUB110, Km^r Cm^r (exp. 5). (*D*) pUB110, Km^r *B. licheniformis* chimera (exp. 6).

when electrophoresed in agarose gels. The larger band (2.0 MDal) has anomalously high fluorescence and corresponds in size with both the *Hpa* II fragment A of pUB110 and with *Hpa* II-cleaved pCM194. The smaller band (0.5 MDal) corresponds in size to pUB110-*Hpa* II-B. No bands are seen corresponding to pUB110-*Hpa* II fragments C (0.45 MDal) and D (0.10 MDal).

Table 3. Transformation with chimeric plasmids

Exp.*	Selection	Transformants/ml
1	Km	124,000
	Em	99,000
	Km + Em	105,000
2	Km	340,000
	Sm	511,000
	Km + Sm	355,000
3	Km	381,000
	Km + Cm + Sm	582,000
4 (type 1)	Km	155,000
	Em	144,000
	Km + Em	167,000
4 (type 2)	Km	105,000
	Cm	67,500
	Em	116,000
	Km + Cm + Em	144,000
5	Km	47,100
	Cm	87,200
	Km + Cm	69,500

* These numbers indicate from which experiment in Table 2 the chimeric plasmids were derived.

Fig. 3 shows further analysis of the fragment composition of pBD12. When digested with *Hpa* II and *Eco*RI, a portion of the larger *Hpa* II band disappears and two new bands are formed, while the smaller band is unaffected. When digested with *Hpa* II and *Hin*dIII, the same change occurs in the larger band except that a single new band of anomalously high intensity is seen. This is to be expected if the large 2-MDal *Hpa* II band consists of *Hpa* II-cleaved pCM194 (2 MDal) + *Hpa* II-B of pUB110 (2 MDal), because pCM194 contains a single *Hin*dIII site and *Hpa* II-B has one *Eco*RI site. The sizes of the new fragments are precisely those expected from the restriction endonuclease maps of the two plasmids (unpublished). When *Hpa* II, *Eco*RI, and *Hin*dIII triple digestions are carried out, the entire large *Hpa* II band disappears, and all three expected new fragments are seen. Finally, *Bgl* II cleaves the smaller *Hpa* II fragment and does not affect the larger band, as expected from the fact that the *Bgl* II site in pUB110 is in *Hpa* II-B and pCM194 is not cleaved by this enzyme (unpublished). These facts confirm that pBD12 consists of pCM194 plus pUB110 *Hpa* II-A and B. Five independent Km^r Cm^r isolates from this experiment show the same endonuclease susceptibility pattern.

DISCUSSION

The results presented above demonstrate that several *S. aureus* antibiotic resistance plasmids are potentially useful as vectors for molecular cloning. The *Xba* I sites on pUB110, pE194, pSA0501, and pSA2100 have been successfully used for insertion of foreign DNA without loss of the Km^r, Em^r, Sm^r, and Cm^r characters of these plasmids. This enzyme, which is known to produce cohesive ends, is clearly useful for cloning (13).

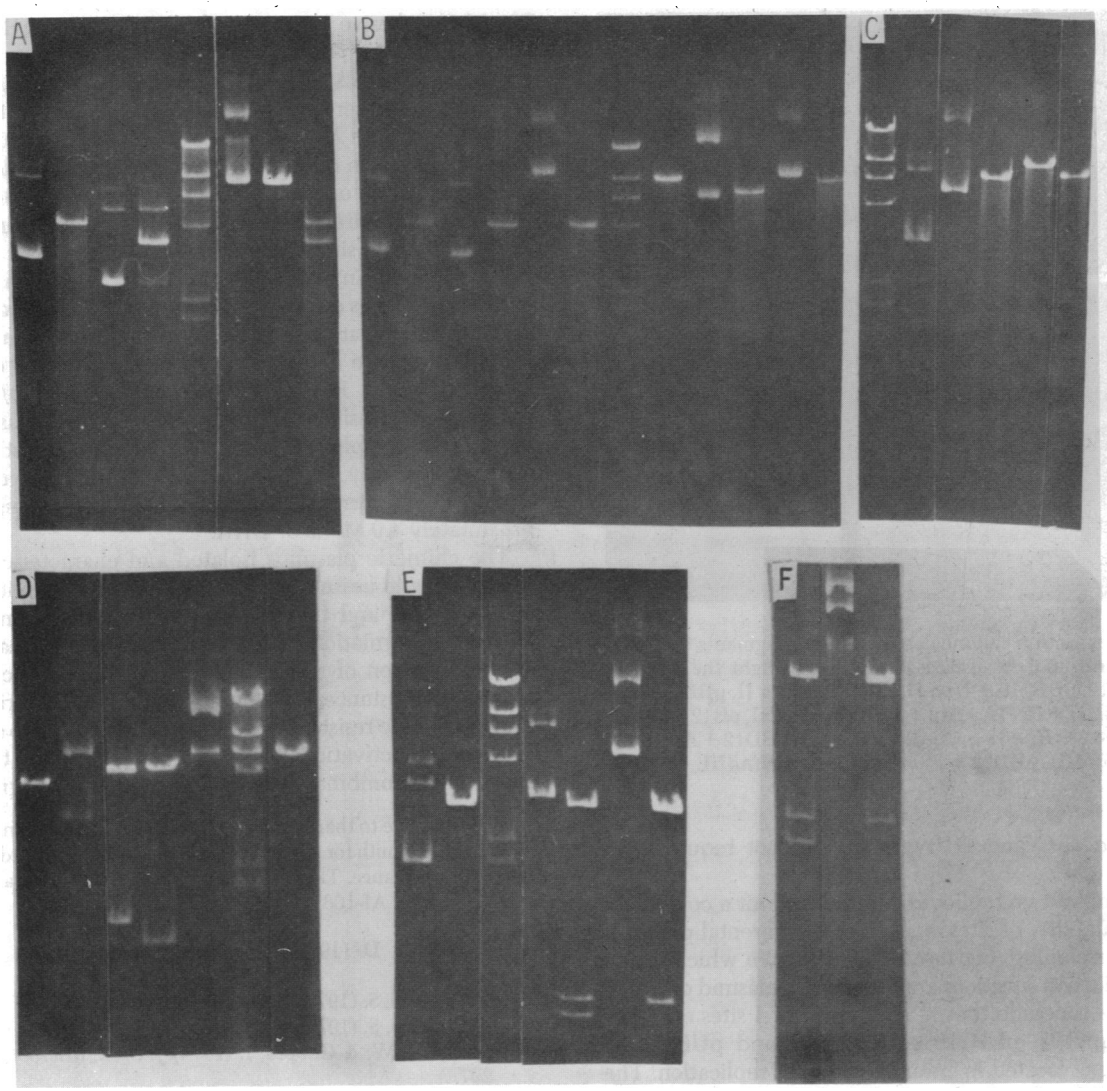

FIG. 2. Restriction endonuclease cleavage patterns of chimeric plasmids and parents. Gels *A* through *E* were run in 0.8% agarose and gel *F* was run in 1.5% agarose. For identification of chimeric plasmids, see Table 4. From left to right, the channels contain: (*A*) pUB110, pUB110 + *Xba* I, pE194, pE194 + *Xba*I, λ + *Hin*dIII, pBD9, pBD9 + *Eco*RI, pBD9 + *Xba* I. (*B*) pUB110, pUB110 + *Xba* I, pSA0501, pSA0501 + *Xba* I, pBD6, pBD6 + *Xba* I, λ + *Hin*dIII, pBD6 + *Hin*dIII, pSA2100, pSA2100 + *Xba* I, pBD8, pBD8 + *Xba* I. (*C*) λ + *Hin*dIII, pUB110, pBD11, pBD11 + *Xba* I, pBD8 + *Xba* I, pBD10 + *Xba* I. (*D*) pUB110 + *Eco*RI, pUB110 + *Hin*dIII, pBD7 + *Eco*RI, pBD7 + *Hin*dIII, pBD7, λ + *Hin*dIII, pBD7 + *Sal* I. (*E*) pCM194, pCM194 + *Hpa* II, λ + *Hin*dIII, pUB110, pUB110 + *Hpa* II, pBD12, pBD12 + *Hpa* II. (*F*) pUB110 + *Hpa* II, pBD12, pBD12 + *Hpa* II.

The pBD12 chimera was formed by using *Hpa* II. It contains the *Hpa* II-A and B fragments of pUB110, plus the entire pCM194 plasmid. Because several such independent isolates have the same structure, it appears that these three *Hpa* II fragments are needed to provide for replication, Km^r and Cm^r. Several possibilities exist for the assignment of these functions to particular fragments. Clearly, however, insertion into the single *Hpa* II site on pCM194 does not inactivate Cm^r, and the

Table 4. Characterization of recombinant plasmids

Plasmid	Origin*	Molecular weight × 10^{-6}			Resistance markers	Deleted markers
		Sum of parental molecular weights	Observed†	Deleted segment		
pBD9	1	5.4	5.4	—	Km, Em	—
pBD6	2	5.8	5.8	—	Km, Sm	—
pBD8	3	7.6	6.0	1.6	Km, Sm, Cm	—
pBD10	4	8.4	4.4	4.0	Km, Cm, Em	Sm
pBD11	4	8.4	4.0	4.4	Km, Em	Sm, Cm
pBD12	5	—	4.5	—	Km, Cm	—
pBD7	6	—	4.0	—	Km	—

* These numbers refer to the experiment in Table 2 in which the new plasmids were constructed.

† Determined by treatment with linearizing restriction endonucleases and comparison on agarose gels with bacteriophage λ DNA digested with *Hin*dIII (11, 12).

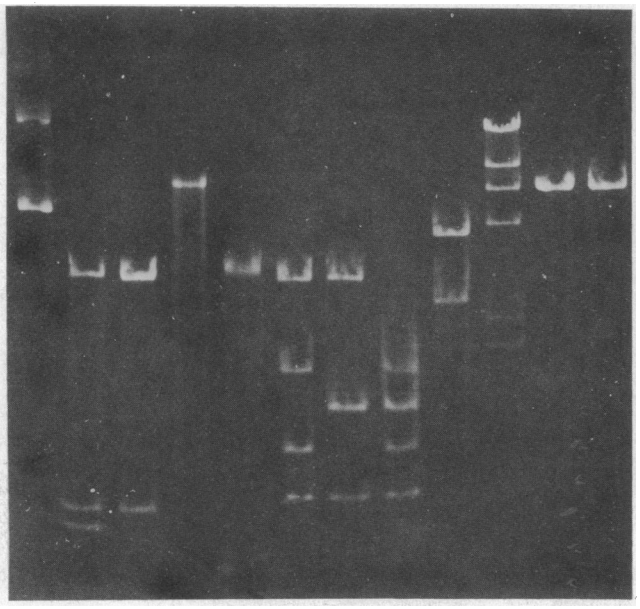

FIG. 3. Restriction endonuclease cleavage fragments of pBD12 and pUB110 run in 0.8% agarose. From left to right the channels contain: pBD12, pUB110 + *Hpa* II, pBD12 + *Hpa* II, pBD12 + *Bgl* II, pB12 + *Hpa* II + *Bgl* II, pB12 + *Hpa* II + *Eco*RI, pB12 + *Hpa* II + *Hin*dIII, pB12 + *Hpa* II + *Hin*dIII + *Eco*RI, pBD12 + *Hin*dIII + *Eco*RI, λ + *Hin*dIII, pBD12 + *Eco*RI, pBD12 + *Hin*dIII.

pUB110 *Hpa* II-C and D fragments are not required for Km^r.

The splicing of two replicons does not permit a conclusion as to the availability of sites on each of the parental plasmids for molecular cloning, because it is not known which of the parental replication functions are used by the plasmid chimera. However, we conclude that at least the *Xba* I sites of either pUB110 or pE194, pUB110 or pSA0501, and pUB110 or pSA2100 are not located in essential genes for replication. The isolation of pBD7, a recombinant derivative of pUB110 with a fragment of *B. licheniformis* DNA inserted in the *Eco*Ri site, demonstrates that this site is not in an essential plasmid gene. Keggans *et al.* (14) have also demonstrated the availability of this *Eco*RI site for cloning.

Several other properties make these plasmids attractive as molecular cloning vehicles. They are easily transferred by transduction between *B. subtilis* strains and they can all be transformed into *recE4*, a nonleaky recombination-deficient strain of *B. subtilis* that is fully competent, processes transforming DNA normally, but does not allow integration of transforming DNA (15). pUB110, at least, exists as a multicopy plasmid which can be amplified to a copy number of about 1,000 under conditions that permit transcription and translation of plasmid genes (unpublished). In addition, extensive restriction endonuclease maps of all the plasmids used in this study have been determined and will be published elsewhere. pUB110 seems particularly suited for use as a cloning vector because, in addition to the properties described above, it contains single cleavage sites for four endonucleases known to generate cohesive termini: *Eco*RI, *Xba* I, *Bam*HI, and *Bgl* II.

The loss of DNA suffered by three of the chimeras reported in this paper deserves discussion. All of these deletion events remove one of the *Xba* I sites used for cloning. This suggests that material has been removed either during endonuclease digestion and ligation or during uptake of an incompletely ligated (linear?) composite fragment during transformation. Alternatively, an *Xba* I site with accompanying material may have been lost due to an *in vivo* deletion event, for which precedent exists (16). This is our preferred interpretation because linear and nicked circular plasmid DNA does not transform *B. subtilis* (unpublished). In addition, the deletions seem to occur in a specific manner. For instance, in exp. 3 (Table 3), eighteen independent transformant clones contained new plasmids with identical electrophoretic mobilities which had lost 1.6 MDal of DNA. Similarly, in exp. 4 (Table 2), three independent Km^r Em^r Cm^r transformants lost the Sm^r character along with approximately 4.0 MDal of DNA.

The chimeric plasmids isolated and characterized in this report provide useful tools for the analysis of replication, incompatibility, and the uptake and recombination of DNA during transformation. They also provide the raw materials for the construction of second-generation cloning vectors in *B. subtilis*. For instance, the chimeric plasmids conferring two or more antibiotic resistance characters can be used to search for insertional inactivation (16) as a means to facilitate the identification of recombinant plasmids containing foreign DNA.

We would like to thank S. Contente, E. Dubnau, L. Mindich, R. P. Novick, and I. Smith for valuable discussions, and A. Howard for expert secretarial assistance. This work was supported by National Institutes of Health Grant AI-10311 awarded to D.D.

1. Ehrlich, S. D. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 1680–1682.
2. Iordanescu, S. (1975) *J. Bacteriol.* **124**, 597–601.
3. Iordanescu, S. (1977) *J. Bacteriol.* **129**, 71–75.
4. Lacey, R. W. & Chopra, I. (1974) *J. Med. Microbiol.* **7**, 285–297.
5. Guerry, P., LeBlanc, D. J. & Falkow, S. (1973) *J. Bacteriol.* **116**, 1064–1066.
6. Radloff, R., Bauer, W. & Vinograd, J. (1967) *Proc. Natl. Acad. Sci. USA* **57**, 1514–1521.
7. Dubnau, D. & Davidoff-Abelson, R. (1971) *J. Mol. Biol.* **56**, 209–221.
8. Roberts, R. J. (1976) *Crit. Revs. Biochem.* **4**, 123–163.
9. Tanaka, T. & Weisblum, B. (1975) *J. Bacteriol.* **121**, 354–362.
10. Greene, P. J., Betlach, M. C., Goodman, H. M. & Boyer, H. W. (1974) in *Methods in Molecular Biology*, ed. Wickner, R. B. (Dekker, New York), Vol. 7, pp. 87–111.
11. Murray, K. & Murray, N. E. (1975) *J. Mol. Biol.* **98**, 551–564.
12. Allet, B. & Bukhari, A. I. (1975) *J. Mol. Biol.* **92**, 529–549.
13. Zain, B. S. & Roberts, R. J. (1977) *J. Mol. Biol.* **115**, 249–255.
14. Keggans, K. M., Lovett, P. S. & Duvall, E. J. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 1423–1427.
15. Dubnau, D., Davidoff-Abelson, R., Scher, B. & Cirigliano, C. (1973) *J. Bacteriol.* **114**, 273–286.
16. Cohen, S. N., Cabello, F., Chang, A. C. Y. & Timmis, K. (1977) in *Recombinant Molecules: Impact on Science and Society*, 10th International Miles Symposium, eds. Beers, R. F., Jr. & Bassett, E. G. (Raven, New York), No. 10, pp. 91–105.