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Characterization of Chimeric Plasmid Cloning Vehicles in *Bacillus subtilis*

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Restriction endonuclease cleavage maps of seven chimeric plasmids that may be used for molecular cloning in *Bacillus subtilis* are presented. These plasmids all carry multiple antibiotic resistance markers and were constructed by in vitro molecular cloning techniques. Several of the antibiotic resistance markers were shown to undergo insertional inactivation at specific restriction endonuclease sites. Kanamycin inactivation occurred at the *BglII* site of pUB110 derivatives, erythromycin inactivation occurred at the *HpaI* and *BclI* sites of pE194 derivatives, and streptomycin inactivation occurred at the *HindIII* site of pSA0501 derivatives. A stable mini-derivative of pBD12 was isolated and characterized. By using these plasmids, we identified proteins involved in plasmid-coded kanamycin and erythromycin resistance. The properties and uses of these chimeric plasmids in the further development of recombinant deoxyribonucleic acid technology in *B. subtilis* are discussed.

The use of *Bacillus subtilis* as a system for recombinant DNA technology has been advanced by the observation by Ehrlich (5) that several antibiotic resistance plasmids from *Staphylococcus aureus* can be transformed into *B. subtilis* and are stably maintained and expressed in this host. Subsequent studies have demonstrated the use of these and other *S. aureus* plasmids as cloning vehicles in *B. subtilis* (6, 9, 11, 12, 15).

This laboratory has previously described a number of useful plasmid cloning vehicles and a series of multiple antibiotic resistance chimeric plasmids constructed by in vitro manipulation (9). Inactivation of plasmid markers by the cloning of foreign DNA into a given restriction endonuclease cleavage site (insertional inactivation) is a useful property since it permits the detection of recombinant plasmid molecules. This report presents restriction endonuclease cleavage site maps of the *B. subtilis* chimeric plasmids previously described (9) and demonstrates several plasmid antibiotic resistance markers which undergo insertional inactivation, making these plasmids useful for molecular cloning experiments in *B. subtilis*.

MATERIALS AND METHODS

Bacterial strains. For the experiments, we used the *B. subtilis* strain BD170 (*trpC2 thr-5*) and the minicell producer BD474 (*thyA thyB metB divIVB1*).

Bacterial plasmids. The parental plasmids are listed in Table 1, and a catalog of chimeric plasmid derivatives is presented in Table 2. The derivations of the latter plasmids are described in a previous publication (9), except for pBD64 which is described in this report. Physical maps of the chimeras are shown in Fig. 1. Isolation of plasmid DNA was previously described (8).

Restriction endonuclease digestions. Restriction endonuclease digestions were performed as previously described (8) with the addition of digestions with *MboI* (7), *PvuII* (16) (New England Biolabs), and *TaqI* (13) (Bethesda Research Laboratories) which were according to manufacturer instructions. *BclI* (1) (New England Biolabs) digestions were performed at 50°C for 30 min (A. G. Bingham, personal communication). Multiple restriction endonuclease digestions were as previously described (8).

Restriction fragment mapping. Agarose gels were run as previously described (8). Polyacrylamide gel electrophoresis was performed by using a modification of the procedures of Bolivar et al. (2). Acrylamide gels (7.5%) were made by mixing 2.4 ml of 10× Tris-borate buffer, 15.6 ml of water, 6 ml of 30% acrylamide (Sigma Chemical Co.), 0.8% *N,N'*-methylenebisacrylamide (Eastman Organic Chemicals) and 12 μl of *N,N,N',N'*-tetramethylethylenediamine (Eastman Organic Chemicals). After degassing, 120 μl of 10% ammonium persulfate was added, and the mixture was cast into a standard vertical gel apparatus (20). Gels were electrophoresed at 120 V for 2 h, stained with ethidium bromide (2 μg/ml) for 15 min, destained in water for 5 to 10 min, and photographed. The fragments were sized by comparison with *HindIII* digests of λ (14) and PM2 (2) DNA. Multiple digestions and partial restriction digests were analyzed to determine restriction maps.

Ligation conditions. Conditions for plasmid-plasmid and plasmid-chromosome ligations were described previously (9).

TABLE 1. *Parental plasmids*

Plasmid	Mol wt	Resistance conferred	Single restriction sites	Other useful properties	
				Segregates into minicells	Temp sensitivity
pUB110	3.0×10^6	Km^r	*Eco*RI *Bam*HI *Xba*I *Tac*I *Bgl*II *Pvu*II	Yes	Resistant
pE194	2.4×10^6	Em^r	*Hpa*I *Bcl*I *Pst*I *Xba*I *Hae*III	Yes	Sensitive
pC194	2.0×10^6	Cm^r	*Hae*III *Hin*dIII *Hpa*II *Hha*I	Yes	Resistant
pSA0501	2.7×10^6	Sm^r	*Eco*RI *Hin*dIII *Hin*dII *Xba*I	NT[a]	Sensitive

[a] NT, Not tested.

Plasmid transformation. Transformation of competent cells and minicells was performed by using previously described procedures (3, 4).

Protein analysis. Procedures for the analysis of plasmid-encoded proteins in *B. subtilis* minicells by sodium dodecyl sulfate-polyacrylamide gel electrophoresis were described previously (18).

RESULTS

Inactivation of kanamycin resistance. Construction and partial characterization of the chimeric plasmid pBD9 has been previously described (9). This plasmid, which confers resistance to kanamycin (Km^r) and erythromycin (Em^r), has single cleavage sites for the restriction endonucleases *Eco*RI, *Bam*HI, *Bgl*II (from the pUB110 parent), *Hpa*I, *Pst*I, and *Bcl*I (from the pE194 parent). A restriction endonuclease map of this plasmid is shown in Fig. 1. Previous studies have shown that the single *Xba*I site on either parental plasmid does not inactivate resistance to kanamycin and erythromycin, since ligation at these sites was used to construct the composite double resistance plasmid (9). Similarly, cloning of foreign DNA into the *Eco*RI site of pUB110 does not inactivate kanamycin resistance (9, 11). Cloning and deletion analysis (Gryczan and Dubnau, unpublished data) have shown that the *Bam*HI site on this plasmid is not located in the Km^r gene.

Since the *Bgl*II site on pBD9 is relatively distant from the *Eco*RI and *Bam*HI sites, it was desirable to test whether cloning into the *Bgl*II site will inactivate kanamycin resistance. The plasmid DNA was cut with *Bgl*II and ligated to a fivefold excess of *Bacillus licheniformis* chromosomal DNA. The ligation mixture was used to transform *B. subtilis* competent cells with selection for erythromycin resistance. Ten Em^r transformants were screened for kanamycin resistance, and two transformants with the phenotype Em^r Km^s were isolated. Analysis of the plasmid carried by one of these transformants indicated the presence of a new plasmid, larger than the original pBD9 plasmid (Fig. 2). Digestion of this new plasmid with *Bgl*II yielded two restriction fragments on agarose gels, one corresponding to linear pBD9 and a second fragment with a mass of ~0.62 megadaltons (Mdal). The inactivation of Km^r by insertion into this *Bgl*II site has been confirmed by the use of pBD9, pBD12, and pBD64 for several cloning experiments in our laboratory (Gryczan and Dubnau, unpublished data; T. Gryczan et al., Mol. Gen. Genet., in press).

Inactivation of erythromycin resistance. The effect of interference with the three single restriction enzyme sites on the pE194 moiety of pBD9 was tested for the effect on erythromycin resistance. Plasmids pBD10 and pBD11 (9) lack the *Pst*I site of pE194 but express erythromycin resistance, indicating that loss of sequences including the *Pst*I site has no effect on erythromycin resistance. Cloning of foreign *B. licheniformis* chromosomal DNA into the *Bcl*I site on pBD9 results in the formation of Km^r Em^s transformants, one of which was tested and found to contain a new larger plasmid. *Bcl*I digestion of this plasmid revealed two *Bcl*I fragments, one the size of linear pBD9 and a second fragment of ~0.3 Mdal (Fig. 3A).

Since the *Hpa*I site on pBD9 is located only about 0.25 Mdal from the *Bcl*I site, it was reasonable to test whether the *Hpa*I site is situated in an essential erythromycin resistance gene. pBD9 was digested with *Hpa*I, and the linearized plasmid was rejoined with a T4 DNA ligase preparation which we knew contained contaminating exonuclease. Km^r Em^s transformants were isolated, and nine of these were screened for their plasmid DNA content. Four of these plasmids were indistinguishable in size from pBD9, whereas the other five plasmids were missing from 0.9 to 2.3 Mdal of DNA. All of the four plasmids which appeared similar in size to pBD9 were found to have lost their *Hpa*I sites.

TABLE 2. *Properties of chimeric plasmids*

Plasmid	Source (reference)	Mol wt	Resistance conferred	Single sites[a]	Inactivated resistance marker	Other useful properties		
						Amplifiable[b]	Segregates into minicells	Temp sensitivity
pBD6	9	5.8×10^6	Km^r Sm^r	*Bam*HI	None	NT	NT	Sensitive
				*Tac*I	None			
				*Bgl*II	Km^r			
				*Hin*dIII	Sm^r			
pBD8	9	6.0×10^6	Km^r Sm^r Cm^r	*Eco*RI	Sm^r	NT	NT	Sensitive
				*Hin*dIII	Sm^r			
				*Bgl*II	Km^r			
				*Bam*HI	None			
				*Xba*I	None			
pBD9	9	5.4×10^6	Km^r Em^r	*Eco*RI	None	Yes	Yes	Resistant
				*Bam*HI	None			
				*Tac*I	None			
				*Bgl*II	Km^r			
				*Bcl*I	Em^r			
				*Hpa*I	Em^r			
				*Pst*I	None			
pBD10	9	4.4×10^6	Km^r Cm^r Em^r	*Bgl*II	Km^r	NT	Yes	Resistant
				*Bam*HI	None			
				*Xba*I	None			
				*Hpa*I	Em^r			
				*Bcl*I	Em^r			
pBD11	9	4.4×10^6	Km^r Em^r	*Xba*I	None	NT	Yes	Resistant
				*Bam*HI	None			
				*Bgl*II	Km^r			
				*Hpa*I	Em^r			
				*Bcl*I	Em^r			
pBD12	9	4.5×10^6	Km^r Cm^r	*Eco*RI	None	Yes	Yes	Resistant
				*Xba*I	None			
				*Bam*HI	None			
				*Tac*I	None			
				*Bgl*II	Km^r			
				*Hin*dIII	None			
pBD64	This report	3.2×10^6	Km^r Cm^r	*Eco*RI	None	NT	NT	Resistant
				*Xba*I	None			
				*Bam*HI	None			
				*Tac*I	None			
				*Bgl*II	Km^r			

[a] These unique restriction sites are available for cloning since their removal by insertion of foreign DNA or by deletion does not interfere with replication of the chimeric plasmid (Gryczan and Dubnau, unpublished data).

[b] This refers to amplification of plasmid copy number in a Ts *dnaC30* host strain during incubation at the nonpermissive temperature (Shivakumar and Dubnau, unpublished data). pBD6 and pBD8 were not tested (NT) because they are cured at elevated temperature. Although pBD10, pBD11, and pBD64 were also not tested, they are most likely amplifiable since their parental plasmids are.

The results with one of them (pBD40) are shown in Fig. 3B. pBD40 may have been generated by a small deletion or even by a point mutation, altering the *Hpa*I site with simultaneous inactivation of erythromycin resistance. Acrylamide gel analysis indicates that if the alteration in pBD40 causing erythromycin sensitivity is a deletion, it must be composed of less than 20 base pairs (Gryczan and Dubnau, unpublished data). These results strongly suggest that the

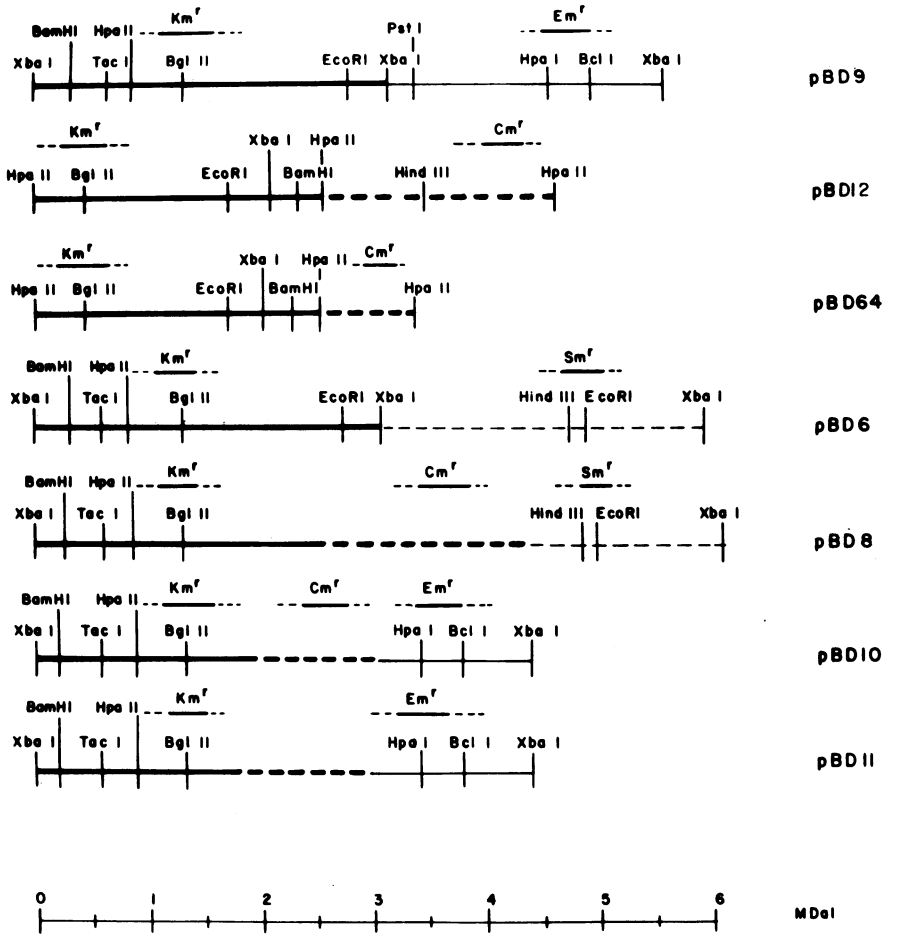

FIG. 1. *Restriction endonuclease cleavage maps. Maps of the chimeric plasmids used in this study are shown in a linear form cleaved at a specific restriction enzyme site. Derivation of chimeric plasmid DNA is as follows: ——, pUB110; ——, PE194; -----, pC194; -----, pSA0501. In pBD8, pBD10, and pBD11, boundaries between parental plasmid DNAs are uncertain and are given as approximate values. Locations of specific antibiotic resistance markers are listed above the linear restriction enzyme cleavage maps.*

*Hpa*I site is located in an erythromycin-resistant gene of pBD9 and that insertional inactivation of erythromycin resistance at this site could be employed for molecular cloning.

Inactivation of streptomycin resistance. It has been previously shown that cloning of DNA into the single *Eco*RI site of pSA2100 results in the inactivation of streptomycin resistance (Sm^r) (12). Restriction mapping data indicate that there is a *Hin*dIII site about 0.1 Mdal away from the *Eco*RI site on all of the Sm^r plasmids derived from pSA0501 including pSA2100 (Fig. 1) (8). To test the effect of cloning into this *Hin*dIII site, pBD6, a chimeric plasmid which confers resistance to kanamycin and streptomycin, was used. A 1:1 mixture of *Hin*dIII-cleaved pBD6 and *Hin*dIII-cleaved pHV11 (6) was ligated and used for transformation. pHV11 confers resistance to chloramphenicol (Cm^r) and tetracycline (Tc^r) and is cleaved by *Hin*dIII into two fragments (6). Transformants with the phenotype Km^r Sm^s were isolated, and when plasmid DNA was analyzed by agarose gel electrophoresis, new plasmids larger than pBD6 were obtained. *Hin*dIII digestion of one of these plasmids produced two *Hin*dIII fragments, one corresponding to linear pBD6 (5.8 Mdal) and a second containing 1.5 Mdal, corresponding to the smaller *Hin*dIII fragment of pHV11 (Fig. 4). This new plasmid (pBD63) also expresses the tetracycline resistance conferred by the smaller *Hin*dIII fragment of pHV11 (6).

Derivation of plasmid pBD64. A physical map of pBD12 (9) is presented in Fig. 1. Although the *Bgl*II site on this plasmid has been utilized to clone a number of plasmid and chro-

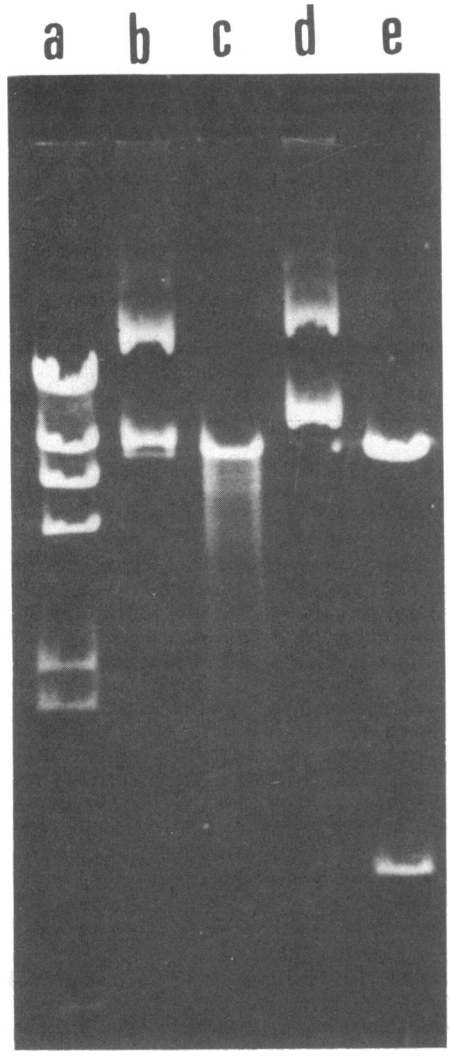

FIG. 2. *Insertional inactivation of Km^r in pBD9 at the BglII site. Plasmids and restriction digests were electrophoresed on an 0.8% agarose gel at 50 V for 3 h. (a) λ + HindIII; (b) pBD9; (c) pBD9 + BglII; (d) Km^s Em^r insert plasmid; (e) Km^s Em^r insert plasmid + BglII.*

mosome-derived restriction fragments with screening for loss of kanamycin resistance (Gryczan et al., in press), its usefulness as a cloning vehicle was limited by the frequent spontaneous appearance of smaller deletion plasmids. Transformation of a plasmid-negative recipient with a pBD12 DNA preparation which contained such a smaller derivative resulted in the isolation of Km^r Cm^r transformants that contained only the smaller pBD12 derivative plasmid. This new plasmid, pBD64, has a mass of 3.1 to 3.2 Mdal, indicating that a deletion of 1.2 to 1.3 Mdal of pBD12 DNA occurred. pBD64 appeared to be quite stable. Growth on media containing either chloramphenicol or kanamycin and replication onto media with both drugs indicated no loss of either resistance character. Repeated isolation of pBD64 DNA has not revealed the presence of smaller derivatives. Restriction enzyme analysis indicates that the deletion event which generated pBD64 occurred entirely within the pC194 portion of pBD12. A physical map of pBD64 is shown in Fig. 1. This plasmid exhibits inactivation of kanamycin resistance at the *Bgl*II site as expected, and has been employed as a cloning vehicle for a variety of plasmid and chromosomal fragments (Gryczan et al., in press). No cleavage site which is located within the Cm^r determinant has been identified.

Temperature sensitivity of chimeric plasmids. Since the pE194 (21) and pSA0501 (10) plasmids have been shown to be temperature sensitive (Ts) for replication, it was desirable to know whether any pE194 or pSA0501-derived plasmid chimeras would also be temperature sensitive for plasmid replication. Strain BD170 derivatives containing these chimeric plasmids were grown on tryptose blood agar base plates

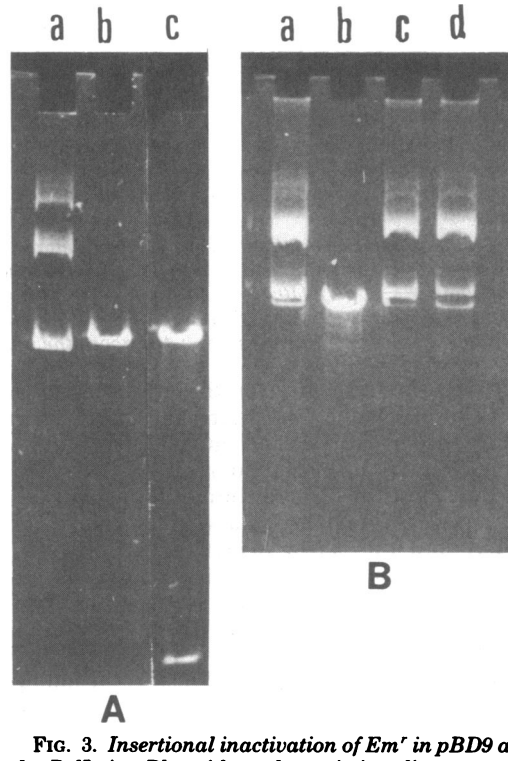

FIG. 3. *Insertional inactivation of Em^r in pBD9 at the BclI site. Plasmids and restriction digests were electrophoresed on an 0.8% agarose gel at 50 V for 3 h. A: (a) pBD9; (b) pBD9 + BclI; (c) Km^r Em^s insert plasmid + BclI. B: (a) pBD9; (b) pBD9 + HpaI; (c) pBD40; (d) pBD40 + HpaI.*

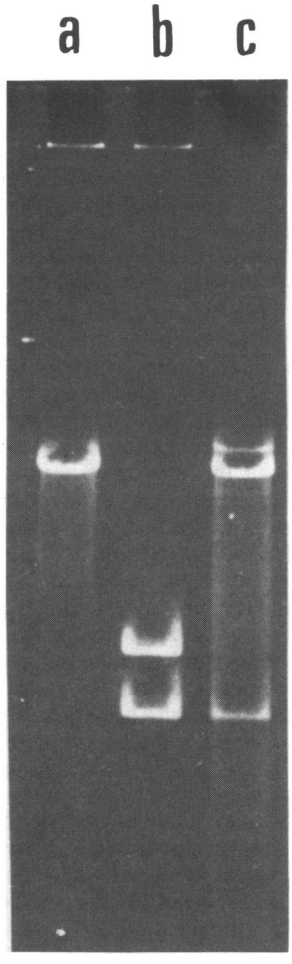

FIG. 4. *Insertional inactivation of Sm^r in pBD6 at the HindIII site. Plasmids and restriction digests were electrophoresed on an 0.8% agarose gel at 50 V for 3 h. (a) pBD6 + HindIII; (b) pHV11 + HindIII; (c) pBD63 + HindIII.*

at 48°C, and the colonies were either replicated or patched onto tryptose blood agar base plates containing the appropriate antibiotics and incubated at 32°C. Results indicate that pBD6 and pBD8 are both temperature sensitive for plasmid replication since all colonies grown at 48°C were found to be antibiotic sensitive and to have been cured of their plasmids. These two plasmids thus appear to replicate under the control of the Ts Sm^r parental plasmid. pBD9, which contains all of the plasmid pE194, is temperature resistant, indicating that the replication system of the pUB110 parent is still functional. pBD10 and 11, both of which have deletions which eliminate about 50% of the pE194 derived sequences, are also both temperature resistant.

Chimeric plasmid protein alterations. pBD9 has been previously shown to specify all of the proteins coded by the parental plasmids (pUB110 and pE194), with the exception of two (K1 and E1) (18). A pBD9 derivative which contains a *B. licheniformis* fragment inserted in the *Bgl*II site was transformed into the *B. subtilis* minicell strain, and the plasmid-specific proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Results (Fig. 5) indicate that the plasmid showing kanamycin resistance inactivation produces an altered form of a protein which normally has a molecular weight of 38,000. Y. Sadaie (personal communication) has identified this 38K protein as a kan-

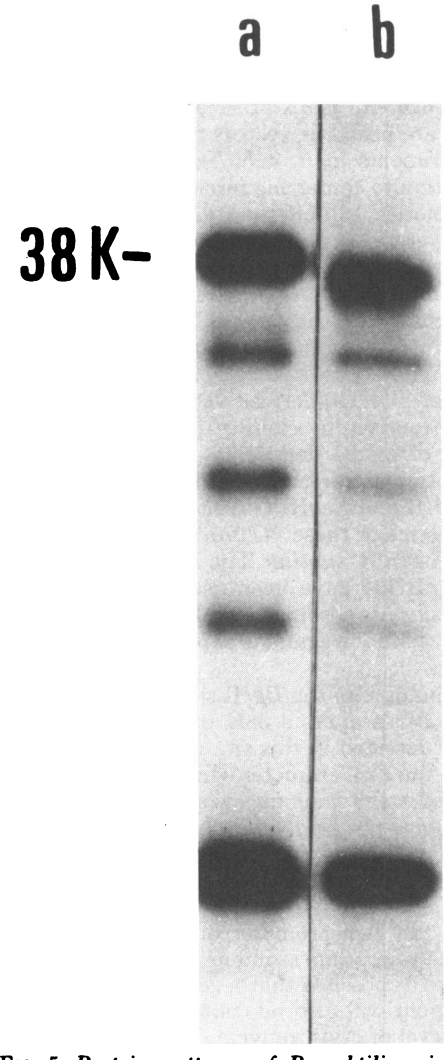

FIG. 5. *Protein patterns of B. subtilis minicell strain carrying pBD9 and Km^s Em^r insert plasmid. Minicells were labeled, and the protein products were analyzed on sodium dodecyl sulfate-polyacrylamide gels, as previously described (18). (a) pBD9; (b) Km^s Em^r insert plasmid.*

amycin nucleotidyltransferase. We have shown that a 29K protein specified by pE194 is responsible for erythromycin resistance (18). The determinant for this protein contains the *Hpa*I site of pE194 and of its derivatives, since mutations affecting the *Hpa*I site of pBD9, including the one in pBD40 described above (Fig. 3), result in the production of an altered 29K protein (Shivakumar, Gryczan, and Dubnau, unpublished data). These findings thus identify proteins involved in resistance to kanamycin and erythromycin, demonstrating that insertional inactivation of these two drug resistance markers alters proteins coded by the resistance components of the plasmid chimera.

DISCUSSION

This report presents restriction endonuclease cleavage site maps of seven chimeric plasmids that are useful as vectors for molecular cloning experiments in *B. subtilis*. These plasmids, in addition to conferring resistance to two or more antibiotics, exhibit insertional inactivation (19) of several of these drug resistance markers. This phenomenon facilitates the construction of plasmid-foreign DNA recombinant molecules by screening for the loss of one of the plasmid antibiotic resistance markers. Thus, inactivation of kanamycin resistance by insertion in the *Bgl*II site of pUB110-derived vectors, inactivation of erythromycin by cloning in the *Hpa*I and *Bcl*I sites of pE194 derivatives, and inactivation of streptomycin resistance by cloning into the *Hin*dIII and *Eco*RI (12) sites of pSA0501 derivatives make these vectors useful for molecular cloning in *B. subtilis*. The *Bgl*II sites of pBD12 and pBD64 have been used for cloning *B. licheniformis* chromosomal DNA and for cloning all five *Mbo*I fragments of pE194 (Gryczan et al., in press).

Cloning into the *Bgl*II site of pUB110 and its derivatives alters a 38K protein. Thus, a fragment inserted in this site might be transcribed from the Km^r promoter which is required for the expression of kanamycin resistance. Since elimination of the *Hpa*I site which is in the Em^r gene of pE194 alters a 29K polypeptide which is involved with erythromycin resistance, it is likely that cloning in the HpaI site may fuse the insert with the Em^r promoter of this plasmid. Since the 29K protein is inducible by erythromycin (18), it is possible that a gene carried on such a fragment will also be controlled (inducible) by the level of erythromycin. This might be useful for a variety of studies, including investigation of the mechanism of induction of erythromycin resistance (21). Mutants have been isolated which produce the 29K protein constitutively (21). These might be advantageous in some cloning experiments. This *Bcl*I site, on the other hand, appears to be within the Em^r promoter, and interference with this site eliminates synthesis of the 29K protein (Y. Kozlov, T. Gryczan, A. G. Shivakumar, and D. Dubnau, unpublished data). Thus, cloning into the *Bcl*I site may be a useful way to insure that a foreign gene is under the control of its own promoter. The expression of genes cloned into the *Hpa*I and *Bcl*I sites is under investigation.

The temperature sensitive property of plasmids pBD6 and pBD8 may give these vectors added utility in molecular cloning experiments. For example, if a putative clone carrying a given DNA fragment has been isolated and it is desirable to know if the fragment is plasmid borne or has integrated into the host chromosome, the temperature-sensitive property of these vectors could be a decided advantage. If the fragment were plasmid borne, growth at the nonpermissive temperature would result in the loss of the fragment and its associated phenotype, whereas a fragment which has integrated into the chromosome and is under its replication control would not be lost at the high temperature. Thus, any plasmid vector which is temperature sensitive may have additional usefulness in the analysis of the replication and expression of cloned DNA fragments.

In addition to analysis of cloned fragment expression, the temperature-sensitive property of plasmid vectors may give added insight into the replication control of these plasmids. Plasmids pBD9, pBD10, and pBD11 are derived from a temperature-sensitive parental plasmid (pE194) and are temperature resistant, whereas pBD6 and pBD8 retain the temperature-sensitive property of one of their parental plasmids (pSA0501). Since some of our chimeric plasmids are additive combinations of two parental plasmids, they may be dual replicons, potentially containing intact replication genes of both plasmids. As a result, replication may be controlled by either or both replicons, depending on the growth temperature and the restriction sites used to form the chimera.

We have previously shown that pUB110 continues to replicate in several *dna*(Ts) hosts when chromosomal replication is restricted by incubation at a nonpermissive temperature. In this way copy numbers of about 1,000 were reached (17). This observation has been extended to pC194 and to several chimeric derivatives of pUB110 and pC194 (Table 2). In addition to providing a useful aid for the isolation of plasmid DNA, this amplification system permits continued protein synthesis under conditions of ele-

vated plasmid-gene dosage. In this way we have been able to detect and study plasmid-specific proteins without recourse to minicells (Shivakumar and Dubnau, unpublished data). We anticipate that this method will be useful for the study of both plasmid-specific proteins and the expression of cloned DNA fragments.

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