Characterization of Chimeric Plasmid Cloning Vehicles in *Bacillus subtilis*

THOMAS GRYCZAN, A. G. SHIVAKUMAR, AND DAVID DUBNAU*

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

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 dinIVB1).

**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 10X 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 digestes were analyzed to determine restriction maps.

**Ligation conditions.** Conditions for plasmid-plasmid and plasmid-chromosome ligations were described previously (9).
TABLE 1. Parental plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Mol wt</th>
<th>Resistance conferred</th>
<th>Single restriction sites</th>
<th>Other useful properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUB110</td>
<td>$3.0 \times 10^6$</td>
<td>Km'</td>
<td>EcoRI, BamHI, XbaI, Tci, BglII, PvuII</td>
<td>Yes, Resistant</td>
</tr>
<tr>
<td>pE194</td>
<td>$2.4 \times 10^6$</td>
<td>Em'</td>
<td>HpaI, BclI, PstI, XbaI, HaeIII</td>
<td>Yes, Sensitive</td>
</tr>
<tr>
<td>pC194</td>
<td>$2.0 \times 10^6$</td>
<td>Cm'</td>
<td>HaeIII, HindIII, HpaII, HhaI</td>
<td>Yes, Resistant</td>
</tr>
<tr>
<td>pSA0501</td>
<td>$2.7 \times 10^6$</td>
<td>Sm'</td>
<td>EcoRI, HindIII, XbaI</td>
<td>NT**, Sensitive</td>
</tr>
</tbody>
</table>

* 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') and erythromycin (Em'), has single cleavage sites for the restriction endonucleases EcoRI, BamHI, BglII (from the pUB110 parent), HpaI, PstI, and BclI (from the pE194 parent). A restriction endonuclease map of this plasmid is shown in Fig. 1. Previous studies have shown that the single XbaI 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 EcoRI site of pUB110 does not inactivate kanamycin resistance (9, 11). Cloning and deletion analysis (Gryczan and Dubnau, unpublished data) have shown that the BamHI site on this plasmid is not located in the Km' gene.

Since the BglII site on pBD9 is relatively distant from the EcoRI and BamHI sites, it was desirable to test whether cloning into the BglII site will inactivate kanamycin resistance. The plasmid DNA was cut with BglII 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' transformants were screened for kanamycin resistance, and two transformants with the phenotype Em' Km' 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 BglII 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' by insertion into this BglII 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 PstI site of pE194 but express erythromycin resistance, indicating that loss of sequences including the PstI site has no effect on erythromycin resistance. Cloning of foreign B. licheniformis chromosomal DNA into the BclI site on pBD9 results in the formation of Km' Em' transformants, one of which was tested and found to contain a new larger plasmid. BclI digestion of this plasmid revealed two BclI fragments, one the size of linear pBD9 and a second fragment of ~0.3 Mdal (Fig. 3A).

Since the HpaI site on pBD9 is located only about 0.25 Mdal from the BclI site, it was reasonable to test whether the HpaI site is situated in an essential erythromycin resistance gene. pBD9 was digested with HpaI, and the linearized plasmid was rejoined with a T4 DNA ligase preparation which we knew contained contaminating exonuclease. Km' Em' 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 HpaI sites.
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Source (reference)</th>
<th>Mol wt</th>
<th>Resistance conferred</th>
<th>Single sites*</th>
<th>Inactivated resistance marker</th>
<th>Other useful properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBD6</td>
<td>9</td>
<td>$5.8 \times 10^6$</td>
<td>Km' Sm'</td>
<td>BamHI TacI BglII HindIII</td>
<td>None None Km' Sm'</td>
<td>NT NT Sensitive</td>
</tr>
<tr>
<td>pBD8</td>
<td>9</td>
<td>$6.0 \times 10^6$</td>
<td>Km' Sm' Cm'</td>
<td>EcoRI HindIII BglII BamHI XbaI</td>
<td>Sm' Sm' Km' None None</td>
<td>NT NT Sensitive</td>
</tr>
<tr>
<td>pBD9</td>
<td>9</td>
<td>$5.4 \times 10^6$</td>
<td>Km' Em'</td>
<td>EcoRI BamHI TacI BglII BclI HpaI PstI</td>
<td>None None None Em' Em' None</td>
<td>Yes Yes Resistant</td>
</tr>
<tr>
<td>pBD10</td>
<td>9</td>
<td>$4.4 \times 10^6$</td>
<td>Km' Cm' Em'</td>
<td>BglII BamHI XbaI HpaI BclI</td>
<td>Km' None None Em' Em'</td>
<td>NT Yes Resistant</td>
</tr>
<tr>
<td>pBD11</td>
<td>9</td>
<td>$4.4 \times 10^6$</td>
<td>Km' Em'</td>
<td>XbaI BamHI BglII HpaI BclI</td>
<td>None None Km' Em' Em'</td>
<td>NT Yes Resistant</td>
</tr>
<tr>
<td>pBD12</td>
<td>9</td>
<td>$4.5 \times 10^6$</td>
<td>Km' Cm'</td>
<td>EcoRI XbaI BamHI TacI BglII HindIII</td>
<td>None None None None Km'</td>
<td>Yes Yes Resistant</td>
</tr>
<tr>
<td>pBD64</td>
<td>This report</td>
<td>$3.2 \times 10^6$</td>
<td>Km' Cm'</td>
<td>EcoRI XbaI BamHI TacI BglII</td>
<td>None None None None</td>
<td>NT NT Resistant</td>
</tr>
</tbody>
</table>

* 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).

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

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

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.
GRYCZAN, SHIVAKUMAR, AND DUBNAU

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 BglII 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' 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

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' Cm' 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

FIG. 2. Insertional inactivation of Km' 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' Em' insert plasmid; (e) Km' Em' insert plasmid + BglII.

FIG. 3. Insertional inactivation of Em' 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' Em' insert plasmid + BclI. B: (a) pBD9; (b) pBD9 + HpaI; (c) pBD40; (d) pBD40 + HpaI.
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 \textit{B. licheniformis} fragment inserted in the \textit{BglII} site was transformed into the \textit{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-

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{fig4.png}
\caption{Insertional inactivation of \textit{Sm} in pBD6 at the \textit{HindIII} site. Plasmids and restriction digests were electrophoresed on an 0.8\% agarose gel at 50 V for 3 h. (a) pBD6 + \textit{HindIII}; (b) pHVII + \textit{HindIII}; (c) pBD63 + \textit{HindIII}.}
\end{figure}

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 \textit{Ts Sm'} 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.

\textbf{Chimeric plasmid protein alterations.}

pBD9 has been previously shown to specify all

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig5.png}
\caption{Protein patterns of \textit{B. subtilis} minicell strain carrying pBD9 and \textit{Km'} \textit{Em'} insert plasmid. Minicells were labeled, and the protein products were analyzed on sodium dodecyl sulfate-polyacrylamide gels, as previously described (18). (a) pBD9; (b) \textit{Km'} \textit{Em'} insert plasmid.}
\end{figure}
amylic nucleotidytransferase. We have shown that a 29K protein specified by pE194 is responsible for erythromycin resistance (18). The determinant for this protein contains the HpaI site of pE194 and of its derivatives, since mutations affecting the HpaI 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 BglII site of pUB110-derived vectors, inactivation of erythromycin by cloning in the HpaI and BclI sites of pE194 derivatives, and inactivation of streptomycin resistance by cloning into the HindIII and EcoRI (12) sites of pSA0501 derivatives make these vectors useful for molecular cloning in B. subtilis. The BglII sites of pBD12 and pBD64 have been used for cloning B. licheniformis chromosomal DNA and for cloning all five MboI fragments of pE194 (Gryczan et al., in press).

Cloning into the BglII site of pUB110 and its derivatives alters a 38K protein. Thus, a fragment inserted in this site might be transcribed from the Km' promoter which is required for the expression of kanamycin resistance. Since elimination of the HpaI site which is in the Em' 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' 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 BclI site, on the other hand, appears to be within the Em' 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 BclI 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 HpaI and BclI 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 dnaC(T's) 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.

ACKNOWLEDGMENTS

We acknowledge with gratitude continual discussions with I. Smith, E. Dubnau, and S. Contente, as well as the expert secretarial assistance of A. Howard.

This work was supported by National Institutes of Health grant AI-10311 and American Cancer Society grant VC300, both awarded to D.D.

LITERATURE CITED


