Molecular Cloning of Structural and Immunity Genes for Megacins A-216 and A-19213 in Bacillus megaterium

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A host-vector system was developed for molecular cloning in Bacillus megaterium and used to clone the structural and immunity genes for megacins A-216 and A-19213. Recombinant clones that expressed immunity only or both immunity to and production of each megacin were obtained. Restriction mapping of native megacinogenic plasmids and recombinant clones was used to construct physical and genetic maps of megacinogenic plasmids pBM309 and pBM113. Limited sequence homology between pBM309 and pBM113 was detected by Southern blot hybridization and was mapped to, at most, a 6.4-kilobase-pair region of pBM309 and a 6.1-kilobase-pair region of pBM113.

MATERIALS AND METHODS

Reagents. Nutrient agar, tryptone, and yeast extract were obtained from Difco Laboratories, Detroit, Mich. Other chemicals for bacterial growth media and agarose gel electrophoresis were purchased from Mallinckrodt, Inc., or Sigma Chemical Co., St. Louis, Mo. Restriction endonucleases, E. coli DNA polymerase I, and T4 DNA ligase were obtained from Bethesda Research Laboratories, Rockville, Md., or New England Biolabs, Beverly, Mass.

Bacteria and plasmids. B. megaterium VT1517 is a derivative of B. megaterium ATCC 21923 that carries megacinogenic plasmid pBM309 and a cryptic plasmid pBM111 (19). An isolate of VT1517 carrying pBC16 was found to have spontaneously lost plasmids pBM309 and pBM111. From this isolate pBC16 was cured by growth in 0.5 μg of ethidium bromide per ml, generating the plasmid-free strain VT1660. VT1660 was the host strain for all the cloning experiments described in this report. VT1660 was transformed with plasmid DNA isolated from strains 216 and ATCC 19213 to generate strains harboring only the megacinogenic plasmids pBM309 and pBM113, respectively. Plasmids pBC16 (1), pUB110 (4), and pHV23 (10) were transformed into VT1660 and then isolated from VT1660, as described previously (19). Plasmid pBD64 (3) could not be introduced into VT1660. Plasmid pBD64 was isolated from B. subtilis BD170 (pBD64).

Restriction mapping. Restriction endonucleases were used by the method of Maniatis et al. (9). Restriction maps of the vector plasmid pBC16 and the megacinogenic plasmids pBM113 and pBM309 were constructed by analysis of single and multiple digestion patterns and by the DNA hybridization technique described by Palmer (13).

Cloning and transformation. T4 DNA ligase was used as described by Maniatis et al. (9). Polyethylene glycol-mediated transformation was performed as described previously (19), except that protoplasts were concentrated 50-fold rather than 10-fold before ligated DNA was added, and incubation for 1.5 h at 30°C was eliminated. Transformed protoplasts were allowed to regenerate as lawns on RHAF plates before selection for antibiotic resistance or megacin immunity. Selective levels in nutrient agar replica plates were as follows: tetracycline, 20 μg/ml; neomycin, 10 μg/ml; chloramphenicol, 20 μg/ml; megacins, 100 endpoint units per plate.

Megacin screening, induction, and titration. Colonies isolated as tetracycline resistant (Tc') or megacin immune

Megacins are bacteriocins produced by Bacillus megaterium that are categorized as types A, B, or C on the basis of mode of action, activity spectrum, and response to UV or mitomycin C induction (5). A-type megacins are a heterogeneous class of bacteriocins in which synthesis can be specifically induced by low-level UV irradiation or mitomycin C treatment (5). Megacins A-216 and A-19213 are distinct bacteriocins that exert phospholipase activity (12, 18) and are produced in much greater amounts than other A-type megacins after induction (6, 18). The structural genes and specific immunity genes for megacin A-216 and megacin A-19213 are encoded on plasmids pBM309 (48 kilobase pairs [kb]) and pBM113 (44 kb), respectively (17, 19).

In an effort to study the genetic organization and regulation of megacin genes, we undertook the molecular cloning of the structural and immunity genes and related regulatory sequences for both megacins A-216 and A-19213. For several reasons, we chose to conduct the initial cloning in B. megaterium. First, we reasoned that expression of megacin genes was most likely in a homologous host known to express megacin structural and immunity genes carried on native megacinogenic plasmids (19). If other, as yet unidentified, nonplasmid genes affect megacin regulation, the choice of a homologous host could be critical to ensure megacin gene expression as it naturally occurs. Second, selection for immunity for desired recombinants was available in B. megaterium. We have shown that megacin immunity can be used to isolate transformants for native megacinogenic plasmids (19) and we reasoned that this property could be used to select recombinant clones carrying either immunity genes alone or both immunity and structural genes. Neither Bacillus subtilis nor Escherichia coli is naturally sensitive to megacin A (11), so that selection for immunity is not possible with either of these host systems. Third, B. megaterium protoplasts can be transformed at low efficiencies (2, 7, 19, 20); however, we have achieved sufficiently high transformation efficiencies for some plasmids to permit their direct use as cloning vehicles (see below).

In this report we describe the development of a host-vector system for cloning in B. megaterium and report the molecular cloning of both structural and immunity genes for megacins A-216 and A-19213.

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(Meg\textsuperscript{mm}) were tested on agar plates for production of A-type megacins by overlaying with soft agar containing mitomycin C (1 \(\mu\)g/ml) and cells of the indicator strain 2165 (19). Induction and titration of megacins from broth cultures were conducted as described previously (18).

**Southern hybridization.** Plasmid DNA was \(^{32}\)P-labeled by nick-translation as described by Rigby et al. (16). Restriction fragments were resolved by horizontal 0.7% agarose gel electrophoresis. DNA was blotted onto nitrocellulose and hybridized under high stringency as described by Maniatis et al. (9). In some cases, reduced stringency of hybridization was achieved by increasing the buffer concentration of the final wash solution from 0.1 \(\times\) SSC (1 \(\times\) SSC is 0.15 M NaCl plus 0.015 M sodium citrate) to 3 \(\times\) SSC.

**RESULTS**

**Cloning strategy for** \textit{B. megaterium}. Several plasmids carrying drug resistance determinants were transformed into \textit{B. megaterium} VT1660 to assess their potential as cloning vectors. The Tc\textsuperscript{c} plasmid pBC16 (1) yielded more than 10\textsuperscript{5} transformants per \(\mu\)g of DNA and was approximately 100-fold more transformation efficient than pUB110 (4). A. \textit{subtilis} cloning vector pBD64 (3) and \textit{B. subtilis}-\textit{E. coli} shuttle vector pHV23 (10) transformed VT1660 very poorly or not at all. Because of its greater transformation efficiency and the availability of an immunity selection that obviated the need for insertional inactivation, pBC16 was chosen as a cloning vector for \textit{B. megaterium}. We constructed a restriction map of pBC16 for restriction endonucleases that cleaved the plasmid at one or two sites (Fig. 1). EcoRI cleaves pBC16 twice, generating a 3.0-kb A fragment and a 1.6-kb B fragment. Polak and Novick (14) have shown that the A fragment alone can be stably maintained and expresses tetracycline resistance in \textit{B. subtilis}. We therefore considered either or both EcoRI sites as well as the unique \textit{BamHI} and \textit{XbaI} sites located within the EcoRI B fragment as potential cloning sites that would maintain the integrity of both the replicon and the Tc\textsuperscript{c} gene.

**Cloning megacin structural and immunity genes.** Megacinogenic plasmid pBM309 or pBM113 and the vector plasmid pBC16 were restricted, mixed, ligated, and transformed into \textit{B. megaterium} VT1660. Transformants were selected for megacin immunity to obtain desired clones and for tetracycline resistance to assess the frequency of recombinant molecules. Some Tc\textsuperscript{c} transformants were randomly screened for recombinant plasmids by modified Eckhardt gel electrophoresis (19) (data not shown). In a cloning experiment using EcoRI, we found that 35% of the Tc\textsuperscript{c} transformants contained recombinant plasmids larger than pBC16. Another experiment with XbaI yielded 6% recombinants. Some recombinant plasmids contained inserts greater than 20 kb.

A restriction map for pBM113 and several clones obtained by immune selection that expressed either immunity alone or both immunity to and production of megacin A-19213 is shown in Fig. 2. Plasmid pVT279 consisted of the EcoRI F fragment (2.0 kb) of pBM113 ligated to the EcoRI A fragment of pBC16. Plasmid pVT54 consisted of the \textit{ClaI} D fragment of pBM113 ligated to the unique \textit{ClaI} site of pBC16. Insertion at the \textit{ClaI} site of pBC16 inactivated tetracycline resistance. Plasmids pVT211 and pVT214 were constructed with XbaI and included two and three \textit{XbaI} fragments of pBM113, respectively (Fig. 2). All four of these plasmids expressed immunity to megacin A-19213. No clones were obtained with EcoRI, XbaI, or \textit{ClaI} that expressed production of megacin A-19213. To obtain such clones, we ligated \textit{BglII} fragments of pBM113 to the unique \textit{BamHI} site in pBC16. Plasmid pVT525 contained the \textit{BglII} A fragment (18.5 kb) inserted at the \textit{BamHI} site. Plasmid pVT525 encoded both immunity to and production of megacin A-19213. Some Meg\textsuperscript{mm} transformants were tetracycline susceptible. Rather than recombinant derivatives of pBC16, these transformants contained derivative forms of pBM113 deleted for specific \textit{XbaI} or \textit{ClaI} fragments. Deleted derivative plasmid pBM113A1 encoded both immunity and production and included \textit{XbaI} fragments of pBM113 totaling 25.6 kb. Plasmid pBM113A2 encoded immunity only and is composed of the \textit{ClaI} C and D fragments (18.3-kb total) of

![FIG. 1. Restriction map of pBC16. The EcoRI A fragment is indicated by the heavy line.](http://jb.asm.org/)

![FIG. 2. Physical map of pBM113 and several derivative plasmids. The XbaI E, G, H, and J fragments of pBM113 are not ordered. Sequences represented in recombinant pBC16 plasmids and deleted derivatives of pBM113 are indicated by concentric arcs. The approximate locations of the megacin A activity (\textit{maa}) and immunity (\textit{maI}) genes are indicated. Sequence homology between pBM309 and pBM113 is indicated by the cross-hatched arc. Restriction endonuclease abbreviations are as follows: Bm, \textit{BamHI}; B, \textit{BsrEI}; Ss, \textit{SstI}; S, \textit{SalI}; K, \textit{KpnI}; St, \textit{Stul}; X, \textit{XhoI}; P, \textit{PstI}.)](http://jb.asm.org/)
FIG. 3. Physical map of pBM309 and several derivative plasmids. Restriction sites for SalI, BglII, and PstI are taken from Rosta et al. (17). Sequences present in recombinant pBC16 plasmids are indicated by concentric solid arcs. The XbaI B fragment is inverted in pVT27 relative to its orientation in pBM309. The approximate locations of the megacin A activity (maa) and immunity (maii) genes are indicated. Sequence homology between pBM309 and pBM113 is indicated by the cross-hatched arc. Restriction endonuclease abbreviations are as follows: Bm, BamHI; X, XhoI; K, KpnI; S, SmaI; B, BstEII.

pBM113 (Fig. 2). The Clal C and D fragments are oriented similarly in pBM113 and pBM113Δ2; thus, the Clal E fragment is critical for structural gene expression.

Cloning of the immunity and structural genes for megacin A-216 is summarized in Fig. 3. Plasmids pVT210 and pVT28 encoded immunity only and contained the XbaI G fragment and G plus H fragments of pBM309, respectively. Plasmids pVT26 and pVT27 encoded both immunity to and production of megacin A-216. These plasmids contained five and four XbaI fragments of pBM309. The unique PstI site in pVT279, a megacin A-19213 immunity plasmid, was used to clone the PstI B fragment of pBM309, yielding pVT65 which encoded both immunity to and production of megacin A-216. Plasmids pVT26, pVT27, and pVT65 contain the XbaI G, H, and E fragments (8.1-kb total) in common (Fig. 3). Thus, sequences involved in megacin A-216 immunity and production appear to be limited to, at most, about 17% of pBM309.

Properties of megacin-immune and producer bacteria. Plasmids constructed in vitro that expressed either immunity alone or both immunity to and production of each megacin were tested quantitatively for megacin production, megacin sensitivity, and lysis after induction with mitomycin C (Table 1). Megacin titers of VT1660 carrying pVT26, pVT27, or pVT65 were three times greater than for wild-type 216. Megacin titers of VT1660(pVT525) were equal to wild-type ATCC 19213. These findings may be related to plasmid copy number effects. Based on the intensities of plasmid bands in agarose gels of Eckhardt lysates (19) (data not shown), plasmids pVT26, pVT27, and pVT65 appeared to be maintained in higher copy numbers than pBM309 in strain 216. The relative intensities of these recombinant plasmids compared with pBC16 appear to reflect the amount of DNA represented in the insert. Eckhardt lysates of pVT525 did not fluoresce as intensely as pVT27 (of comparable size); rather, lysates of pVT525 fluoresced about as intensely as lysates of pBM113 or pBM113. This suggests that pVT525 may be limited in copy number by DNA derived from pBM113. Copy numbers of other recombinant plasmids appear to be controlled by the pBC16 replicon.

Endpoint sensitivity testing (18) revealed that presumed high-copy-number megacin A-19213 immunity plasmids (e.g., pVT211, pVT279) conferred greater immunity to megacin A-19213 than does native plasmid pBM113 (Table 1). This immunity was specific for megacin A-19213. Plasmids that confer immunity to megacin A-216 (e.g., pVT26, pVT28, pVT210) were specific for megacin A-216 and conferred immunity levels at least equal to that of native plasmid pBM309 in 216. Strain 216 is less sensitive to megacin A-216 than ATCC 19213 is sensitive to megacin A-19213 (Table 1) (18). Plasmid pVT65 expressed low sensitivity to both megacin A-216 and megacin A-2123; thus, cloning into the unique PstI site in pVT279 did not insertionally inactivate the megacin A-19213 immunity gene in pVT279.

Lysis after induction with mitomycin C was examined for a number of strains carrying recombinant plasmids. Only those strains that produced one of the megacins showed the characteristic lysis associated with megacin production. The proteins produced by megacinogenic recombinants after induction were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. VT1660 clones containing pVT65 or pVT27 produce megacin A-216; additionally, the increased megacin titer of clones containing pVT27 or

<table>
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<th>Strain</th>
<th>Phenotype</th>
<th>Endpoint sensitivity to megacin A-216</th>
<th>Megacin titer</th>
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<tr>
<td>VT1660(pBC16)</td>
<td>Meg A *</td>
<td>5.0 x 10^4</td>
<td>100</td>
</tr>
<tr>
<td>216</td>
<td>Meg A *</td>
<td>3.0 x 10^4</td>
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<td>Meg A *</td>
<td>&lt;10</td>
<td>300</td>
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* Endpoint sensitivity to megacin A-216 and megacin A-19213 was determined as described previously (18).

* Expressed as a percentage of wild-type levels.
pVT65 is reflected in the amount of α and β subunits (Fig. 4). A substantial cryptic peptide from 216 is also encoded by pVT27 and pVT65 (Fig. 4, arrow). The presence of α and β subunits for megacin A-19213 in induced lysates of VT1660(pVT525) is demonstrated in Fig. 4 (lane 2).

Sequence homology between megacinogenic plasmids pBM113 and pBM309. Plasmid pBM113 was nick-translated and hybridized against restriction fragments of pBM309 (Fig. 5). Plasmid pBM113 hybridized to the XhoI B (lane 1), SalI C (lane 3), and Stul A and B (lane 6) fragments of pBM309, as well as to double-digestion fragments generated by digestion with SalI-XhoI and SalI-BglII, but failed to hybridize to either pBR322 or λ DNA (lane 8). Hybridization to bands other than the BglII B fragment (lane 5) reflects incomplete digestion of pBM309 with BglII. The hybridizing fragments all overlap on the physical map of pBM309, and the homology is limited to, at most, 6.4 kb of pBM309 (Fig. 3).

Plasmid pBM309 was also nick-translated and probed against restriction fragments of pBM113 (Fig. 6). Specific hybridization to the BglII-B (lane 2) and a 6.1-kb Stul-XhoI double-digestion fragment (lane 3) of pBM113 was clearly observed only under relaxed stringency. No hybridization with pBR322 or λ DNA was seen. The region of pBM113 homologous with sequences of pBM309 is indicated on the physical map of pBM113 (Fig. 2).

**DISCUSSION**

A host-vector system for molecular cloning in *B. megaterium* has been developed and used to clone both immunity and structural genes for megacin A-216 and megacin A-19213. Recombinant derivatives were constructed with the XhoI, BamHI, EcoRI, and ClaI sites of pBC16. Megacin immunity facilitated this work by permitting the direct selection of desired clones. In the absence of such a selection, however, one could expect to find desired recombinants only if a reasonable fraction of transformants contained recombinant molecules. Our results indicate that recombinant plasmids can be readily obtained in *B. megaterium* and that such recombinant plasmids can contain quite large inserts; pVT27 contained an 18.1-kb insert, pVT525 contained an 18.5-kb insert, and other cryptic plasmids contained inserts greater than 20 kb. In at least some cases, the copy number of recombinant plasmids appears to be subject to copy number control of pBC16. These attributes may prove useful for the cloning of other gene products of *Bacillus* species. This system has been recently used to clone the insecticidal toxin gene of *Bacillus thuringiensis var. israelensis* (V. Sekar and B. C. Carlton, Gene, in press).

Analysis of a number of recombinant plasmids permitted the genetic mapping of native megacinogenic plasmids pBM113 and pBM309. All megacin A-19213-immune clones contained common DNA sequences from pBM113 (Fig. 2). The smallest clone, pVT279, includes the EcoRI F fragment of pBM113, whereas other immunity clones include part of the F fragment, extending to the ClaI site within the F fragment. Because cloning at the PstI site did not insertionally inactivate the immunity gene, it appears likely that this gene is located within the 1.7 kb of DNA between the ClaI and PstI sites of the EcoRI F fragment. Although endpoint sensitivity as a measure of immunity to megacins is, at best, semiquantitative, strains carrying pVT279 or pVT211 are nonetheless clearly more resistant than are wild types. Most likely this is related to gene dosage effects, although other explanations cannot be excluded until the immunity gene

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**FIG. 4.** Sodium dodecyl sulfate-15% polyacrylamide gel electrophoresis. Lysates of VT1660 harboring pVT27, pVT65, and pVT525, as well as wild-types 216 and ATCC 19213, were prepared, concentrated 100-fold by precipitation at 80% saturation with ammonium sulfate, and dialyzed against 20 mM Na2PO4 buffer (pH 7.2), and equivalent amounts were electrophoresed on sodium dodecyl sulfate-15% polyacrylamide gels (8) at 20 mA until the bromphenol blue tracking dye reached the bottom of the resolving gel. Lane 1, ATCC 19213; lane 2, VT1660(pVT525); lane 3, VT1660(pVT27); lane 4, VT1660(pVT65); lane 5, 216. The α and β subunits of megacin A-216 and megacin A-19213 and a cryptic peptide associated with megacin A-216 production (arrow) are indicated. The β subunits are poorly stacked and thus run as diffuse bands.

**FIG. 5.** Southern blot hybridization at high stringency between 32P-labeled pBM113 and restriction digests of pBM309. Plasmid pBM309 was digested with XhoI (lane 1), SalI and XhoI (lane 2), SalI (lane 3), SalI and BglII (lane 4), BglII (lane 5), Stul (lane 6; BglII-digested pBM113 (lane 7); pooled marker fragments of EcoRI-digested λ DNA and HaeIII-digested pBR322 (lane 8). (A) Agarose gel electrophoresis (6.7%). (B) Autoradiography after transfer and hybridization. The sizes (in kb) of marker fragments in lane 8 are indicated.
product has been identified and characterized. The structural genes for megacin A-19213 are less well mapped; the single recombinant plasmid that encodes megacin production (pVT525) contains a very large insert. However, the analysis of pBM113Δ2, a deleted variant of the native pBM113 plasmid, revealed that sequences contained in the ClaI E fragment are required for megacin A-19213 production. Derivative forms of pBM113 deleted for specific restriction fragments also revealed that large segments of pBM113 are unnecessary for maintenance and replication of pBM113. For example, pBM113A1 has lost 19.4 kb, and pBM113Δ2 has lost 26.7 kb, of pBM113.

The immunity gene for megacin A-216 has been mapped to the 3.0-kb Xbal G fragment of pBM309. Three recombinant plasmids that produce megacin A-216 suggest that the structural and immunity genes are contained within the Xbal G, H, and E fragments. Because pVT28 and pVT210 encode only immunity to megacin A-216 and not megacin production, we conclude that the Xbal E fragment must be involved in structural gene expression. Riabchenko and Rostás (15) have recently mapped the structural and immunity genes for megacin A-216 by analysis of deletion derivatives of pBM309. Their results and the results reported here are in full agreement concerning the localization and orientation of the structural and immunity genes for megacin A-216. Interestingly, Riabchenko and Rostás have found that the PstI B fragment cloned in pBD9 failed to render cells inducible by mitomycin C; whereas we found that cells carrying the PstI B fragment cloned in pVT279 (i.e., pVT65) are fully inducible. Possibly, pBM113 DNA in pVT279 regulates the induction of megacin A-216 in cells carrying pVT65.

Limited sequence homology was detected between megacinogenic plasmids pBM113 and pBM309 and mapped to a 6.4-kb region of pBM309 and a 6.1-kb region of pBM113. No homology was found between sequences coding for either megacin production or immunity. This was not surprising since megacins A-216 and A-19213 are immunologically and structurally distinct proteins, and their respective immunity genes are specific for the homologous megacin only (18). The deletion derivatives of pBM309 reported by Riabchenko and Rostás (15) have lost the sequences homologous with pBM113, and both pBM113A1 and pBM113Δ2 have lost the sequences homologous with pBM309, so that the homologous region is not necessary for the maintenance and replication of either native megacinogenic plasmid. The homology between pBM309 and pBM113 remains of unknown significance.

The construction of recombinant plasmids employing a replicon known to be stable in *B. subtilis* (1, 14) should permit the introduction of megacin genes into *B. subtilis*. In this heterologous host, we should be able to assess whether pVT26, pVT27, and pVT525 are sufficient to specify megacin synthesis or whether other currently unidentified *B. megaterium* gene products are necessary for, or affect, megacin gene expression. Experiments to address these questions are currently in progress.

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LITERATURE CITED


