Megacinogenic Plasmids of *Bacillus megaterium*

MICHAEL A. VON TERSCH AND BRUCE C. CARLTON*

Department of Molecular and Population Genetics, University of Georgia, Athens, Georgia 30602

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Megacins A-216 and A-19213 in *Bacillus megaterium* are plasmid encoded, as shown by analysis of cured, non-megacinogenic (Meg−) derivatives of strains 216 and ATCC 19213 and by polyethylene glycol-mediated protoplast transformation of Meg+ bacteria with plasmid DNA. The results of both techniques implicated a 31-megadalton plasmid, pBM309, in megacin A-216 production and a 29-megadalton plasmid, pBM113, in megacin A-19213 production.

A number of species of the genus *Bacillus* harbor plasmid DNA (2, 7, 16, 23, 24, 31). In some species plasmids are found in arrays of as many as 11 distinct size classes (7, 15). In a few cases, biological functions including antibiotic resistance (2, 27), bacteriocin production (2, 25), and insecticidal δ-endotoxin production (14) have been shown to be plasmid associated. Most *Bacillus* plasmids, however, remain functionally cryptic.

*Bacillus megaterium* strain 216 possesses a complex array of plasmids that has been characterized physically (4, 6, 8, 9). This strain also produces two bacteriocins, megacin A and megacin C (19, 20), which have been suggested to be plasmid associated (19, 21). *B. megaterium* ATCC 19213 produces an inducible bacteriocin, megacin A-19213, described in the accompanying paper (33), which is similar in some respects to megacin A-216. Three plasmids have also been reported in strain ATCC 19213 (18). We decided to determine the association, if any, between megacin production and specific plasmids of the array.

Our approach to the question of plasmid-associated megacin genes with both of these strains has been twofold: first, to isolate spontaneous non-megacinogenic (Meg−) variants and examine them for alterations in plasmid complement that correlate with the Meg− phenotype; second, to further demonstrate the association of megacin production with a specific plasmid via polyethylene glycol (PEG)-mediated protoplast transformation (10).

While these studies were in progress, Rostas et al. (28) demonstrated that a 31-megadalton (Md) plasmid, pBM309, encodes the genes for megacin A-216 production. In the present report, both curing studies and transformation results corroborate this association. We also present evidence that ATCC 19213 contains four distinct plasmids and that megacin A-19213 production is associated with a 29-Md plasmid, pBM113.

**MATERIALS AND METHODS**

**Reagents.** Bacterial growth media were obtained from Difco Laboratories, Detroit, Mich. Low electroendosmosis agarose was from Sigma Chemical Co., St. Louis, Mo. Megacins were purified as described previously (33).

**Bacterial strains.** Strains of *B. megaterium* used in this work are described in Table 1.

**Isolation of Meg− derivatives of *B. megaterium* strains 216 and ATCC 19213.** The lysis of megacin A-producing bacteria after induction of megacin production (21, 22) was exploited to enrich for spontaneously occurring MegA− bacteria. Early log-phase cultures of *B. megaterium* growing at 37°C in yeast extract-tryptone broth (32) were induced with 1 μg of mitomycin C per ml at an initial cell density corresponding to 15 to 30 Klett units (red filter). Incubation was continued for 60 min at 37°C. Samples were then plated onto nutrient agar at a sufficient dilution that Meg− cells could survive the lethal activity of released megacin A. Surviving colonies were scored in the presence and absence of mitomycin C for the ability to produce megacins (19). Meg− derivatives of ATCC 19213 were also readily isolated because they often appeared as delineated sectors within Meg− colonies on solid media.

**Plasmid pattern of B. megaterium ATCC 19213.** Plasmid DNA was initially prepared as a cleared lysate (16) and subsequently banded on CsCl-ethidium bromide gradients (6). Plasmid DNA was recovered and plasmid masses were determined by electron microscopy (11), using plasmid pBR322 as an internal size standard (molecular mass, 2.66 Md [30]).

**Slot-lysing electrophoresis.** For analytical purposes, the method of Eckhardt (12) was used for slot-lysing electrophoresis, with some modifications. Lysozyme and sodium dodecyl sulfate mixtures were prepared as described by Gonzalez et al. (16) except that the lysozyme concentration was decreased to 200 μg/ml. Test colonies were grown overnight at 30°C on nutrient agar plates. Approximately 2.5 mg of cells was resuspended in 50 μl of lysozyme mix and incubated for 15 min at room temperature. A 10-μl portion of the
TABLE 1. Strains of *B. megaterium*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant phenotype</th>
<th>Plasmids</th>
<th>Remarks</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>216</td>
<td>MegA⁺ MegC⁺ prototroph</td>
<td>pBM101, 102, 103, 309, 105, 106, 107, 108, 109, 110</td>
<td>Wild type</td>
<td>20, 21</td>
</tr>
<tr>
<td>VT100</td>
<td>MegA⁺ MegC⁺</td>
<td>pBM101, 102, 103, 309, 105, 107, 108, 109, 110</td>
<td>Spontaneously cured 216 derivative lacking pBM106</td>
<td>This study</td>
</tr>
<tr>
<td>VT131</td>
<td>MegA⁻ MegC⁺</td>
<td>pBM101, 102, 103, 107, 108, 109, 110</td>
<td>Cured VT100 derivative isolated after enrichment with mitomycin C</td>
<td>This study</td>
</tr>
<tr>
<td>VT145</td>
<td>MegA⁻ MegC⁺</td>
<td>pBM101, 102, 103, 107, 109, 110</td>
<td>Cured VT100 derivative isolated after enrichment with mitomycin C</td>
<td>This study</td>
</tr>
<tr>
<td>2165</td>
<td>MegA⁻ MegC⁻ Strr</td>
<td>pBM102, 103, 115, 116</td>
<td>Megacin indicator</td>
<td>3</td>
</tr>
<tr>
<td>ATCC 19213</td>
<td>MegA⁺ prototroph</td>
<td>pBM111, 112, 113, 114</td>
<td>Wild type</td>
<td>18</td>
</tr>
<tr>
<td>VT1007</td>
<td>MegA⁻</td>
<td>pBM111</td>
<td>Cured ATCC 19213 isolated after enrichment with mitomycin C</td>
<td>This study</td>
</tr>
<tr>
<td>VT1206</td>
<td>MegA⁻ Strr Arg⁻</td>
<td>pBM111</td>
<td>Sequential ethyl methane sulfonate mutagenesis of VT1007</td>
<td>This study</td>
</tr>
<tr>
<td>VT406</td>
<td>MegA⁺ MegC⁺ Tetr</td>
<td>pBC16, pBM101, 102, 103, 309, 105, 106, 107, 108, 109, 110</td>
<td>Transformant of 216 with pBC16</td>
<td>This study</td>
</tr>
<tr>
<td>VT1405</td>
<td>MegA⁺ Tetr</td>
<td>pBC16, pBM111, 112, 113, 114</td>
<td>Transformant of ATCC 19213 with pBC16</td>
<td>This study</td>
</tr>
<tr>
<td>VT1515</td>
<td>MegA⁺ Strr Arg⁻</td>
<td>pBM111, 113</td>
<td>Transformant of VT1206 with pBM113</td>
<td>This study</td>
</tr>
<tr>
<td>VT1517</td>
<td>MegA⁺ Strr Arg⁻</td>
<td>pBM111, 309</td>
<td>Transformant of VT1206 with pBM309</td>
<td>This study</td>
</tr>
</tbody>
</table>

resulting protoplast suspension was then layered under 10 μl of sodium dodecyl sulfate mixture directly in the wells of the agarose gel. Electrophoresis on 0.4 or 0.5% vertical slab gels was at 10 V for 1.5 h, followed by 120 V for 3 to 4 h.

**Mini-cleared lysates.** When appropriate, plasmid DNA was prepared by a modification of the cleared-lysate technique that eliminated the addition of NaCl. This modification obviated the necessity for dialysis or ethanol precipitation of cleared lysates before agarose gel electrophoresis. A 5.0 ml amount of cells actively growing in Spizizen minimal medium (29) supplemented with 0.1% yeast extract was pelleted by centrifugation. The pellets were resuspended in 0.5 ml of 20% sucrose in TES (Tris-hydrochloride, EDTA, NaCl) buffer (9) containing 1.0 mg of lysozyme and 80 μg RNase A per ml. After 60 min of incubation at room temperature, 0.5 ml of 2% sodium dodecyl sulfate in TES was added. The lysates were vortexed briefly and incubated overnight at 4°C. The chromosomal DNA-sodium dodecyl sulfate complexes were pelleted by centrifugation, and the mini-cleared lysate supernatant solution was recovered.

**PEG-mediated protoplast transformation.** For PEG-mediated protoplast transformation, the method of Chang and Cohen (10) was modified for use with strain VT1206. A minimal salts transformation medium (HAF broth; 13) was supplemented with 0.5% yeast extract and 0.5% tryptone (Difco) (RHAF broth). Solid media for cell wall regeneration were prepared by adding 1.0% agar to RHAF broth.

Strain VT1206 was grown in RHAF broth to a Klett reading of 20 (red filter). The cells were harvested by centrifugation and resuspended in 1/10 volume of HAF broth containing 200 μg of lysozyme per ml. After 15 min at room temperature, the resulting protoplasts were pelleted, washed with RHAF broth, and resuspended in RHAF in 1/10 the original culture volume. DNA was added and the protoplasts were diluted twofold with 30% PEG in HAF broth. The protoplasts were incubated for 4 min at 37°C and then diluted with 5 volumes of RHAF. The protoplasts were pelleted, resuspended in RHAF, and incubated at 30°C for 1.5 h before antibiotic selection. Selection for tetracycline resistance (Tetr) was conducted on RHAF plates containing 20 μg of tetracycline per ml. Selection for megacin immunity (Megimm) was conducted on nutrient agar replica plates prepared from lawns of regenerated protoplasts on RHAF plates. Generally, 100 U of megacin activity in soft-agar overlays was added per plate to select for megacin immunity.

**RESULTS**

**Plasmid DNA patterns in *B. megaterium.*** The molecular masses of the plasmids from strain 216 have been reported previously (8). During the course of the present studies, it became apparent that there were differences in the plasmid complement of strain 216 obtained from G. Ivanovics in 1975 (and used exclusively in the studies described in this report) and strain 216
originally obtained from I. B. Holland in 1968 and studied previously in this laboratory (3, 4, 6–9). These differences are confined to plasmids of >31 Md in mass. The agarose gel profile of plasmids isolated from strain 216 used in these studies is shown in Fig. 1. Molecular masses have been assigned based on mobility on agarose gels compared with the plasmids of strain ATCC 19213. We have chosen to name these plasmids sequentially by size beginning with the 4.0-Md plasmid pBM101. An exception to this convention is the 31-Md plasmid, which has already been designated pBM309 (28).

The agarose gel profile of plasmid DNA from B. megaterium ATCC 19213 is shown in Fig. 2, lane 4. Four plasmid size classes were evident in the wild-type strain. Molecular sizes of 6.4, 17, 29, and 60 Md have been assigned to these size classes, based on electron microscopic measurements with pBR322 as an internal size standard (30) (data not shown).

**Isolation of Meg^- mutants of B. megaterium.** Mitomycin C induction of Meg^+ cultures permitted the ready isolation of Meg^- mutants. As many as 58% of the bacteria surviving induction with mitomycin C were Meg^- (data not shown). The technique was equally successful for both megacin A-216 and megacin A-19213. Meg^- mutants of B. megaterium ATCC 19213 were also readily isolated as distinct sectors within Meg^+ colonies on solid media. Approximately 14% of the Meg^- mutants of strain VT100 (a Meg^- MegC^+ derivative of strain 216 cured for pBM106) were also Meg^- after mitomycin C enrichment. Mutants cured of the ability to produce either megacin A-216 or megacin A-19213 failed to show characteristic lysis (19, 22) upon mitomycin C induction (data not shown).

**Plasmid DNA analysis of cured derivatives.** A number of Meg^- mutants were examined for plasmid DNA by the slot-lysis method or by gel electrophoresis of mini-cleared lysates (Fig. 2). The results are summarized in Table 2. All Meg-A-216 mutants exhibited loss of a plasmid 31 Md in size (pBM309). A significant number of mutants had also lost a second plasmid, 33 Md in size (pBM105). In a few instances, a smaller plasmid, either pBM101 or pBM102, was lost as well. We interpret these data to indicate that megacin A-216 production is associated with pBM309.

Meg^- mutants of ATCC 19213 also showed an absolute correlation between the Meg^- phenotype and loss of a specific plasmid of the array. In each case a plasmid of 29 Md (pBM113) was lost. In many cases, additional alterations of the plasmid array were observed. The 60-Md plasmid pBM114 was frequently lost and, less frequently, the 17-Md plasmid pBM112 was lost. None of the mutants was cured of the 6.4-Md plasmid pBM111, however. An increase in the intensity of fluorescence of plasmid pBM112 was commonly observed when mutants were cured of pBM113 and pBM114. Such changes in intensity of bands in slot-lysates most likely reflect alterations in plasmid copy number.

**PEG-mediated protoplast transformation of B. megaterium VT1206.** PEG-mediated protoplast transformation (10) was used to demonstrate association of the megacin determinants with specific plasmids. Both Meg^- strains 216 and ATCC 19213 were initially transformed with pBC16, which encodes resistance to tetracycline (2). Strain VT406, containing a wild-type strain 216 plasmid complement and pBC16, and strain VT1405, containing an ATCC 19213 complement and pBC16, were used as sources of DNA for transformation to Tet' and Meg'. Strain VT1206, a cured derivative of ATCC 19213 which is Meg^- and contains only pBM111, was used as the recipient.

VT1206 was transformed at low frequencies

![FIG. 1. Agarose gel electrophoresis of plasmid DNA from B. megaterium 216. Plasmid DNA was purified as described in the text. Electrophoresis in 0.8% vertical agarose gels was conducted as described for slot-lysis.](http://jb.asm.org/)
for both Tet<sup>+</sup> and MegA<sup>+</sup> (Table 3). The frequency of transformation per microgram of pBC16 (Tet<sup>+</sup>) was about 10 times greater than that per microgram of pBM113 or pBM309 (both MegA<sup>-</sup>). Because both pBM113 and pBM309 are about 10 times larger in molecular mass than pBC16, the efficiency of transformation per plasmid molecule was therefore approximately the same for both Tet<sup>+</sup> and MegA<sup>+</sup>. Interestingly, Tet<sup>+</sup> transformants were found among cells selected for megacin A resistance at a frequency 2.0 × 10<sup>4</sup> times greater than the frequency of Tet<sup>+</sup> transformants among total regenerated protoplasts.

A number of MegA<sup>+</sup> transformants were examined for plasmid DNA by slot-lysis electrophoresis or by electrophoresis of mini-cleared lysates. All of the MegA-19213 transformants examined contained the resident plasmid pBM111 and had acquired pBM113. All of the megacin A-216 transformants examined contained pBM111 and had acquired pBM309 (Fig. 3).

**FIG. 2.** Slot-lysis electrophoresis of *B. megaterium* ATCC 19213 and MegA<sup>-</sup> derivatives. Electrophoresis was conducted as described in the text. Lane 1, MegA<sup>-</sup> VT1004; 2, MegA<sup>-</sup> VT1002; 3, MegA<sup>-</sup> VT1001; 4, MegA<sup>+</sup> ATCC 19213.

### TABLE 2. Alterations of plasmid complements in Meg<sup>-</sup> variants of *B. megaterium* VT100 and ATCC 19213 revealed by slot-lysis electrophoresis

<table>
<thead>
<tr>
<th>Strain</th>
<th>Cured plasmid(s)</th>
<th>MegA&lt;sup&gt;-&lt;/sup&gt; &lt;sup&gt;(n = 62)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MegA&lt;sup&gt;-&lt;/sup&gt; &lt;sup&gt;(n = 53)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MegA&lt;sup&gt;-&lt;/sup&gt;-MegC&lt;sup&gt;-&lt;/sup&gt; &lt;sup&gt;(n = 10)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MegA&lt;sup&gt;-&lt;/sup&gt; &lt;sup&gt;(n = 6)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>VT100</td>
<td>pBM309 309, 105</td>
<td>45</td>
<td>15</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>309, 105, 102</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>ATCC</td>
<td>pBM113 113, 114</td>
<td>.</td>
<td>8</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>19213</td>
<td>113, 114, 112</td>
<td>.</td>
<td>36</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>VT1517</td>
<td>VT1517</td>
<td>VT1517</td>
<td>VT1517</td>
<td>VT1517</td>
</tr>
</tbody>
</table>

<sup>a</sup> Meg<sup>-</sup> variants were isolated after enrichment with mitomycin C.

**TABLE 3. Protoplast transformation of VT1206 with plasmid DNA**

<table>
<thead>
<tr>
<th>Source of transforming DNA</th>
<th>Tet&lt;sup&gt;+&lt;/sup&gt; transformants/μg of pBC16</th>
<th>MegA&lt;sup&gt;-&lt;/sup&gt; transformants/μg of pBM309 or pBM113</th>
</tr>
</thead>
<tbody>
<tr>
<td>VT1405&lt;sup&gt;a&lt;/sup&gt;</td>
<td>700</td>
<td>80</td>
</tr>
<tr>
<td>VT406</td>
<td>1,300</td>
<td>140</td>
</tr>
</tbody>
</table>

<sup>a</sup> The frequency of Tet<sup>+</sup> transformants among MegA<sup>+</sup> transformants was 0.2.

**DISCUSSION**

Two different approaches have been used to examine the relationship between megacin A production and specific plasmids of the complex plasmid systems in *B. megaterium*. The analysis of cured Meg<sup>-</sup> derivatives and the results of PEG-mediated protoplast transformation have both indicated that the genes for two distinct
inducible megacinogenic plasmids, we found a significant increase in the frequency of Tet' transformants among MegA + transformants compared with that of Tet' transformants among regenerated protoplasts. Elevated levels of cotransformation have also been reported with B. subtilis (B. Michel, Rapport de DEA, Université Paris VI, 1979).

The identification and transmissibility of plasmids encoding type A megacins should facilitate comparative studies of the genes encoding these proteins, as well as the regulatory aspects of their induction. Megacins A-19213 and A-216 are clearly distinct proteins (33), yet the regulation of production of both megacins may involve common properties. Both of these proteins are produced in substantial quantities in response to specific induction and represent easily assayable phenotypes. For these reasons, further study of the molecular biology of these megacins may contribute significantly to our understanding of gene expression in bacilli.

ACKNOWLEDGMENTS

We thank José M. González, Jr., for helpful discussions. M.V.T. was supported by Public Health Service predoctoral training grant 5T32 GM 07103 from the National Institutes of Health.

LITERATURE CITED


FIG. 3. Agarose gel electrophoresis of plasmid DNA from MegA + transformants. Plasmid DNA was purified by CsCl-ethidium bromide equilibrium centrifugation. Agarose gel electrophoresis on a vertical 0.6% agarose gel was performed as described for slot-lysis electrophoresis. Plasmid DNA from: 1, the recipient VT1206; 2, VT1515, transformed for megacin A-19213; 3, VT1517, transformed for megacin A-216; 4, VT1405, used to transform for megacin A-19213; 5, VT406, used to transform for megacin A-216.


