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Restriction Enzyme Analysis of Lactose and Bacteriocin Plasmids from *Streptococcus lactis* subsp. *diacetylactis* WM_4 and Cloning of *BclI* Fragments Coding for Bacteriocin Production†

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The 131.1-kilobase (kb) bacteriocin production (Bac) plasmid pNP2 and the 63.6-kb lactose metabolism (Lac) plasmid pCS26, from *Streptococcus lactis* subsp. *diacetylactis* WM_4, as well as pWN8, a 116.7-kb recombinant plasmid from a Lac^+ transconjugant, were analyzed with restriction enzymes to determine the origin of pWN8. Plasmid pWN8 conferred a Lac^+ Bac^- phenotype, contained DNA derived from pCS26 and pNP2, and, like pNP2, exhibited self-transmissibility (Tra^+). In cloning attempts, Bac^+ transformant *S. lactis* KSH1 was isolated. The recombinant plasmid, pKSH1, contained three *BclI* fragments from pNP2. Bac^- transformants which individually contained each of the three fragments were also identified. Comparison of restriction maps of pKSH1 and pNP2 revealed an 18.4-kb region common to both plasmids, involving two of the three *BclI* fragments. *S. lactis* KSH1 also exhibited greater inhibitory activity against the indicator strain *S. diacetylactis* 18-16 than did a strain containing the 131.1-kb Bac plasmid.

Many strains of dairy streptococci are known to produce antagonistic substances (3). In some cases, plasmid DNA has now been implicated in their production (6, 15–17). For a substance that is active against potential spoilage or pathogenic organisms, it would be desirable to apply genetic engineering techniques to effect increased production of the inhibitor. Plasmid involvement in the production of some of these inhibitors will greatly facilitate the construction of strains capable of producing elevated levels of the antagonistic substances. This should lead to increased protection against undesirable organisms. Use of such a strain in food manufacture conceivably could extend product shelf life.

In our previous report (16), *S. diacetylactis* WM_4 was found to contain six plasmids ranging in size from 3.8 to 88 megadaltons. The 63.6-kilobase (kb) and the 131.1-kb plasmids (originally reported as 33 and 88 megadaltons, respectively) were sized by measuring restriction fragments (this study) and were linked to lactose metabolism (Lac) and to bacteriocin production (Bac) and self-transmissibility (Tra), respectively. The Lac^+ Bac^- tranconjugants examined in this study contained a 116.7-kb plasmid (originally reported as 65 megadaltons) and conjugated Lac at a higher rate than the parental strain or Lac^+ Bac^+ transconjugants containing the 63.6- and 131.1-kb plasmids. In the work described in this communication, we used restriction enzyme analysis of the 63.6-kb Lac and the 131.1-kb Bac Tra plasmids, as well as the 116.7-kb Lac^+ Bac^- recombinant plasmid, to determine the origin of the 116.7-kb plasmid and to locate the regions responsible for lactose metabolism, high-frequency conjugation, and bacteriocin production. Cloning techniques were also used to localize DNA segments carrying the *bac* genes.

All streptococcal strains used in this study were maintained in our stock culture collection and were transferred biweekly at 32°C in M17 broth (18) containing 0.5% glucose or 0.5% lactose. The strains and relevant properties are listed in Table 1. The method of Kekessy and Piquet (9) was used for detecting bacteriocin production. One drop of an overnight M17-lactose or M17-glucose culture was spot inoculated onto the surface of an Elliker agar (7) plate containing 0.5% glucose as the sole carbohydrate and 0.05% β-glycerophosphate, or individual colonies were transferred onto agar plates by using sterile toothpicks. When production of bacteriocin by different strains was compared, the cultures to be tested were grown to an optical density at 600_{nm} of 0.3, and 10 μl of each culture was spot inoculated onto the plate. *S. diacetylactis* 18-16 served as the sensitive indicator strain.

To cure the Bac plasmid, a Lac^+ Bac^+ streptomycin-resistant (Str^r) transconjugant isolated previously (16) was grown in the presence of novobiocin as described by McHugh and Swartz (12). After growth in M17-lactose broth containing 13 μg of novobiocin per ml, the culture was diluted and plated onto lactose indicator agar plates (14). Individual yellow (Lac^+) colonies were tested for their inability to produce bacteriocin as described above. Plasmid screening and isolation were done by the method of Anderson and McKay (1). Solid-surface matings between an *S. lactis* strain harboring only 63.6-kb Lac plasmid and plasmid-free Lac^- erythromycin-resistant (Ery^r) *S. lactis* LM2306 were performed as previously described (13). Lac^+ Ery^r transconjugants were selected on lactose indicator agar containing 15 μg of erythromycin per ml.

Restriction endonuclease digestions and fragment separation on a horizontal submerged 0.6% agarose gel in 40 mM Tris-acetate–2 mM EDTA buffer were performed by the method of Maniatis et al. (11). Restriction fragments generated by *Hin*dIII and *Xho*I digestions of bacteriophage lambda DNA served as DNA size standards. Plasmid pGB301, a streptococcal cloning vector encoding both erythromycin and chloramphenicol resistance (4, 5), and the Bac Tra plasmid pNP2 were digested with the enzyme *BclI*. Cut vector and passenger DNA were mixed in a concentration ratio of 1:3. Ligation was performed as outlined in the 1985 catalog of International Biotechnologies, Inc., New Haven, Conn. The ligated DNA preparation was used to transform

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TABLE 1. Bacterial strains and plasmids

S. lactis strain	Relevant plasmid(s)	Plasmid sizes (kb)	Plasmid-conferred phenotype	Description and reference
LM0230	Plasmid cured			Plasmid free, Lac$^-$ derivative of *S. lactis* C2 (13)
LM2306	Plasmid cured			Ery^r derivative of *S. lactis* LM0230
WW2	pNP2, pCS26	131.1, 63.6	Bac$^+$ Tra$^+$ Lac$^+$	Transconjugant of *S. lactis* subsp. *diacetylactis* WM_4 × *S. lactis* LM2301 (16)
CS26	pCS26	63.6	Lac$^+$	Novobiocin-plasmid-cured derivative of *S. lactis* WW2 (this study)
NP2	pNP2	131.1	Bac$^+$ Tra$^+$	Transconjugant of *S. lactis* subsp. *diacetylactis* WM_4 × *S. lactis* LM2301 (16)
WN8	pWN8	116.7	Lac$^+$ Tra$^+$	Transconjugant of *S. lactis* subsp. *diacetylactis* WM_4 × *S. lactis* LM2301 (16)
KSH1	pKSH1	44.8	Bac$^+$	Bac$^+$ transformant of *S. lactis* LM0230 (this study)

protoplasts of *S. lactis* LM0230, a plasmid-free derivative of *S. lactis* C2. Transformation was carried out by the protocol of Kondo and McKay (10). Erythromycin-resistant (Em^r) transformants were selected by using M17 soft agar (0.5% agar) overlay and basal agar (1.5% agar) plates, both containing 0.5 M sucrose and 3 μg of erythromycin per ml. Transformant colonies were tested for their ability to produce bacteriocin as previously described.

Three plasmids present in *S. diacetylactis* WM_4 or its transconjugants were investigated. The parental plasmids were a 63.6-kb Lac plasmid and a 131.1-kb Bac Tra plasmid, while the transconjugant plasmid was 116.7 kb and conferred a Lac$^+$ Tra$^+$ phenotype. The conjugal transfer of Lac by WM_4 and its transconjugants, during which Lac$^+$ Bac$^+$ transconjugants acquired both the 63.6- and 131.1-kb plasmids and Lac$^+$Bac$^-$ tranconjugants acquired a recombinant plasmid of about 100 or 116.7 kb, was previously reported (16). The Lac$^+$ Bac$^-$ transconjugants subsequently transferred Lac at an elevated frequency. To determine whether the 63.6- kb Lac plasmid possessed self-transfer capability, *S. lactis* WW2 (a Lac$^+$ Bac$^+$ tranconjugant containing both the 63.6-kb Lac plasmid and the 131.1-kb Bac Tra plasmid) (16) was cured of the Bac Tra plasmid by being grown in the presence of novobiocin. This strain, designated *S. lactis* CS26, contained only the 63.6-kb Lac plasmid pCS26 and was used as a donor in conjugal matings with *S. lactis* LM2306. No Lac$^+$ tranconjugants were observed, which suggests that the 63.6-kb Lac plasmid is not self-transmissible and that when transfer does occur, the DNA is transferred by the *tra* genes located on another resident plasmid in WM_4 or WW2, possibly pNP2, the 131.1-kb Bac Tra plasmid.

Restriction endonuclease mapping was performed to determine whether the 116.7-kb Lac$^+$ Bac$^-$ plasmid (pWN8) was derived from a recombination event between the 63.6-kb Lac (pCS26) and the 131.1-kb Bac Tra (pNP2) plasmids (Fig. 1). Plasmid pWN8 was found to have a 61.6-kb region of DNA with a restriction pattern the same as a portion of plasmid pNP2 and a 13-kb region with a restriction map the same as part of plasmid pCS26. Thus, pWN8 is a recombinant containing DNA from the Lac plasmid (pCS26) and from the Bac Tra plasmid (pNP2). pNP2 was previously shown to be self-transmissible (16). Plasmid pWN8 also appeared to contain DNA from an unidentified source (possibly chromosomal DNA), since it contained regions of DNA with restriction enzyme patterns not observed in pCS26 or pNP2. Our results indicate that the 13-kb segment of DNA common to both pWN8 and pCS26 harbors *lac* genes and that the segment of DNA common to both pWN8 and pNP2 contains *tra* genes. High-frequency conjugation and cell aggregation were previously associated with the formation of a recombinant plasmid in Lac$^+$ transconjugants derived from both *S. lactis* ML3 (2, 19) and *S. lactis* 712 (8) matings. Cell aggregation, however, was not apparent in the high-frequency recombinant plasmids observed in this study. Since pWN8 possessed a Lac$^+$ Bac$^-$ phenotype, the *bac* genes must have been deleted in the formation of the recombinant plasmid and thus must reside on the portion of pNP2 which does not have a restriction pattern similar to that of pWN8.

The Bac Tra plasmid (pNP2) contained at least 17 *BclI* restriction sites, ranging in size from about 1.4 kb to approximately 16 kb (data not shown). Cleavage of pNP2 with *BclI* and insertion of the fragments into the single *BclI* site of pGB301 provided an opportunity to determine whether the *bac* genes were located on any of these fragments. Upon screening 625 Em^r transformants for bacteriocin production, only one, designated *S. lactis* KSH1, was Bac$^+$. Analysis of plasmid DNA isolated from KSH1 indicated the presence of a plasmid larger than pGB301 (data not shown). To determine which *BclI* fragment(s) of pNP2 had been cloned into pGB301, the 131.1-kb Bac Tra plasmid and recombinant plasmid pKSH1 were digested with *BclI* and subjected to agarose gel electrophoresis (Fig. 2). In addition to the vector, there were three *BclI* fragments from pNP2 with sizes of 9.5, 12.1, and 13.4 kb. To determine which fragments present in pKSH1 contained the *bac* genes, we randomly examined individual Bac$^-$ Em^r transformants to determine whether any contained only the 9.5-, 12.1-, or 13.4-kb fragment. The presence of any one of these three fragments would eliminate the possibility that the *bac* genes were on that particular fragment. Among the Bac$^-$ Em^r transformants examined, three were found which each contained one of the three different pNP2 *BclI* fragments present in pKSH1 (data not shown). Our study indicates the presence of two 12.1-kb *BclI* fragments in pNP2 (data not shown). It is possible that the *bac* gene(s) are located on the 12.1-kb *BclI* fragment in the Bac$^+$ transformant and that a different 12.1-kb fragment, lacking the *bac* gene(s), is present in the Bac$^-$ transformant. Hence, the plasmids from KSH1 and a Bac$^-$ Em^r transformant were subjected to *BclI* and *SalI* restriction enzyme digestions. In both plasmids, *BclI-SalI* double digestions resulted in the disappearance of the 12.1-kb *BclI* fragment, and two new fragments of 4.5 and 7.6 kb were observed (data not shown). Thus, the same 12.1-kb *BclI* fragment exists in both the Bac$^+$ and Bac$^-$ transformants.

Plasmid pKSH1 was analyzed with two of the enzymes previously used to map pNP2, to compare the two plasmids (Fig. 3). A region of approximately 18.4 kb, including the 2.5-kb *XhoI-SalI* and the 15.9-kb *SalI-XhoI* fragments, had the same restriction pattern on both plasmids (Fig. 1 and 3).

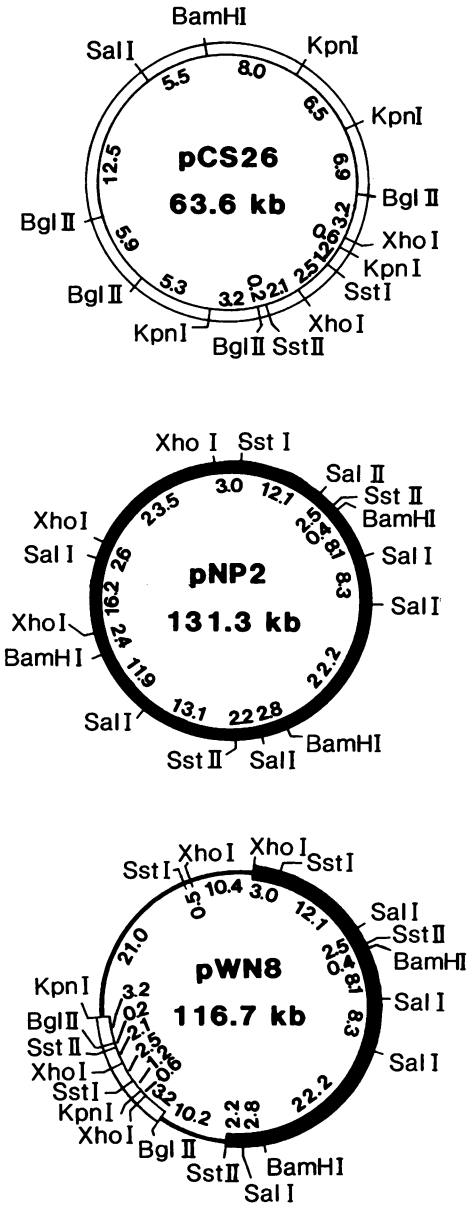

FIG. 1. Restriction enzyme maps of pCS26, the 63.6-kb Lac plasmid; pNP2, the 131.1-kb Bac Tra plasmid; and pWN8, the 116.7-kb recombinant Lac Tra plasmid. Sizes between enzyme recognition sites are expressed in kilobases. For pWN8, ▭, DNA derived from pCS26; ▬, DNA derived from pNP2. The single lines represent DNA from the junction segments and possibly additional DNA from an unidentified source.

These results suggest that the *bac* genes may contain a recognition site for *BclI* or that the *bac* genes and their promoter reside on separate *Bcl*I fragments, so that cloning of any one of the three individual fragments would result in a Bac$^-$ phenotype. In KSH1, however, the necessary fragments are present and are in the proper orientation, resulting in a complete, functional gene. The cloning of three *Bcl*I restriction fragments could be explained by incomplete digestion by *Bcl*I in the restriction digestion phase of the cloning experiment; alternatively, the fragments containing a portion of the *bac* genes could have reannealed in the proper

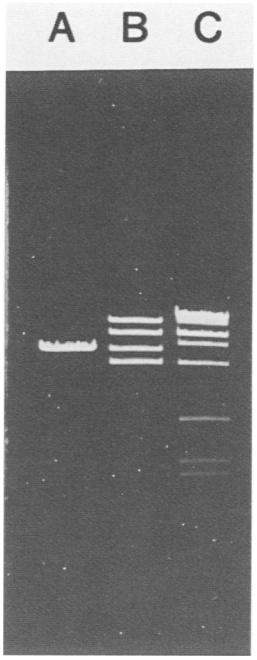

FIG. 2. Agarose gel electrophoresis of plasmid DNA digested with *Bcl*I. Lanes: A, vector plasmid (pGB301); B, digested pKSH1 DNA; C, *Bcl*I fragments from pNP2. The fragments present in lane B include, from bottom to top, the 9.5-kb fragment from pNP2, the 9.8-kb vector fragment, and the 12.1- and 13.4-kb fragments from pNP2.

orientation during the ligation step of the cloning experiment. The 18.4-kb segment common to both plasmids, including the 2.5-kb *Xho*I-*Sal*I and the 15.9-kb *Sal*I-*Xho*I fragments, encompassed the combined 9.5- and 13.4-kb *Bcl*I fragments. This region corresponds to the smallest *Xho*I fragment of pNP2 and presumably contains the *bac* genes. Since the cloned 12.1-kb *Bcl*I fragment contains a *Sal*I restriction site, and since the segment of DNA adjacent to the 18.4-kb *Xho*I fragment on pNP2 lacks a *Sal*I site, it was concluded that the 12.1-kb fragment is not involved in bacteriocin production and that it maps in another location

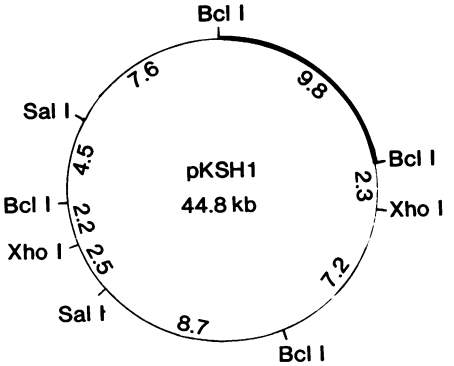

FIG. 3. Restriction enzyme map of pKSH1, the recombinant plasmid in the Bac$^+$ transformant *S. lactis* KSH1. The heavy portion of the plasmid represents DNA contributed by the vector plasmid, pGB301. The three *Bcl*I fragments derived from pNP2 are in the orientation shown. The 18.4-kb *Xho*I fragment appears to be the same as the smallest *Xho*I fragment of pNP2 (Fig. 1).

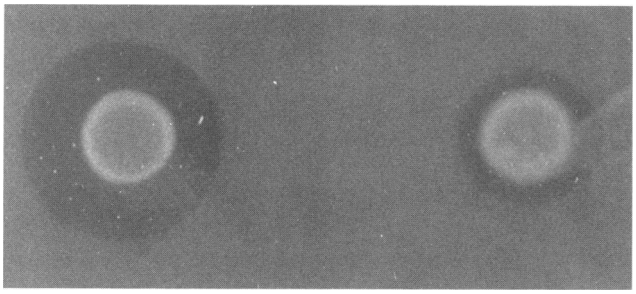

FIG. 4. Comparison of bacteriocin production by the Bac^+ transformant *S. lactis* KSH1 (left) and *S. lactis* NP2 (right). *S. diacetylactis* 18-16 was the indicator organism in the overlay.

on pNP2. It should also be noted that the 18.4-kb DNA segment on pNP2 is located on a portion of the plasmid which is not present in pWN8. This would be expected, since it involves the area not associated with Tra and therefore is more likely to contain the *bac* genes, as previously explained.

S. lactis KSH1 was found to have a greater inhibitory effect than *S. lactis* NP2 when *S. diacetylactis* 18-16 was used as the indicator strain. The NP2 culture produced a clear zone of about 1.4 cm in diameter, while KSH1 produced a larger inhibitory zone of 2.1 cm (Fig. 4). This may be due to an increased copy number for the plasmid containing the *bac* genes in KSH1. The copy number of pKSH1 is under the control of the vector pGB301 and is visibly higher than pNP2, so that more copies of the *bac* genes should be present. Because of the low copy number and large size of pNP2 (131.1 kb), attempts to isolate this plasmid were often unsuccessful. The increased copy number and decreased size of pKSH1 compared with pNP2 will facilitate its isolation and enhance further characterization of the genes responsible for bacteriocin production. Although the bacteriocin produced by WM_4 appears to be effective only against other lactic streptococci (unpublished data), our results indicate that with other inhibitory substance-producing organisms, it may be possible to construct dairy streptococci capable of enhanced production of antagonistic substances active against undesirable spoilage or pathogenic organisms.

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