Characterization of Lac\(^+\) Transductants of

*Streptococcus lactis*

T. A. MOLSKNESS, W. E. SANDINE, AND L. R. BROWN

Department of Microbiology, Oregon State University, Corvallis, Oregon 97331

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A phage-mediated transducing system was used in studying certain physiological characteristics of *S. lactis* C2 wild type, lactose-negative mutants, and lactose-positive transductants. Lac\(^-\) mutants, obtained by acriflavine treatment of the wild type, were similar to the wild type in all characteristics tested except they lacked \(\beta\)-d-phosphogalactoside galactohydrolase (\(\beta\)-Pgal) and could not transport \([^{14}C]\)lactose; they also had approximately 10% of the proteolytic ability than wild-type cells. The lactose-fermenting characteristic was transduced from the wild type to Lac\(^-\) mutants. The Lac\(^+\) transductants obtained were similar to the wild-type parent with respect to lactose fermentation and level of \(\beta\)-Pgal activity (0.186 U of protein per mg). These transductants, however, had not regained full proteolytic ability and were similar to the Lac\(^-\) mutant in this respect. Lactic acid production of the transductants in milk was approximately two-thirds that of the wild type. Data suggest that both the lactose-fermenting and proteolytic characters are carried on extrachromosomal particles (plasmids).

McKay et al. (11, 12, 13) have shown that the lactose-fermenting character of *Streptococcus lactis* C2 can be transduced from a wild-type (Lac\(^+\)) strain to a Lac\(^-\) mutant. The phage responsible for this transduction is a lysogenic phage carried in *S. lactis* C2 and is ultraviolet inducible.

In studying the properties of the wild-type and lactose-fermenting transductants, three characteristics were examined: enzyme activity of the lactose-hydrolyzing enzyme, \(\beta\)-d-phosphogalactoside galactohydrolase (\(\beta\)-Pgal); proteolytic ability; and the rate of lactic acid production.

**MATERIALS AND METHODS**

Microorganisms and media. Lactic streptococci used were obtained from the stock collection maintained by the Department of Microbiology, Oregon State University. Cultures were regularly maintained in sterile nonfat milk (NFM) containing 11% solids. Lactic broth (5) with lactose as the sole carbohydrate (LLB) served as the growth medium for the routine assay of \(\beta\)-Pgal. On occasion, glucose was substituted for lactose in this medium (GLB). The survey of organisms and determination of optimal assay conditions were carried out in this broth. For induction experiments, lactic broth was prepared with a reduced level of yeast extract (1 g/liter) and without carbohydrate. After sterilization of the medium, a filter-sterilized solution of the carbohydrate to be tested was added to a final concentration ranging from 0.001 to 0.005 g/ml. In all experiments involving broth, the organism was transferred three times over 36 h at 32 C in the appropriate broth before beginning the experiment.

Preparation of toluene-acetone-tested cells. Toluene-acetone (1:9)-treated cells were normally used when small numbers of cells were being assayed for enzyme activity. Cells were harvested, washed, and resuspended in 0.05 M sodium phosphate buffer at pH 7.0 to an optical density of 0.30 at 420 nm, and quantitated in terms of milligrams of cell (dry weight). This insured that a standard amount of cells would be solvent treated for each experiment. The procedure of Citti et al. (2) was followed when treating the cells with toluene-acetone, except cells were shaken vigorously for 10 min after addition of the solvent. The resultant suspension was assayed for enzyme activity.

\(\beta\)-Galactosidase and \(\beta\)-Pgal assays. Ortho-

nitrophenyl-\(\beta\)-d-galactopyranoside (ONPG) and ONPG-6-PO\(_4\) were obtained from Sigma Chemical Co. Modifications of the enzyme assay procedures of Citti et al. (2) and McKay et al. (15) were used. A solution containing 5.0 \(\times\) 10\(^{-4}\) M of either ONPG or ONPG-6-PO\(_4\) was prepared in the phosphate buffer. The assay mixture contained 0.5 ml of toluene-acetone-treated cells and 2.0 ml of the chromogenic substrate. Incubation was at 37 C for 10 min. The reaction was stopped by the addition of 2.5 ml of 0.4

1Technical Paper no. 3848, Oregon Agricultural Experiment Station.

2Present Address: Bureau of Water Works, 1800 S.W. 6th Ave., Portland, Ore. 97021.
M sodium carbonate. The release of ortho-nitrophenol (ONP) was measured colorimetrically at 420 nm, and micromoles of ONP released was determined from a standard curve. Cells were removed by centrifugation before absorbancy was measured.

One unit of enzyme was equivalent to 1 μmol of ONP liberated from ONPG, or ONPG-6-PO₄ per 10 min in the case of the solvent-treated cell assay. Specific activity was expressed as units per milligram of cell (dry weight).

Cells grown in milk. The method of Hull (8) was used to determine the degree of proteolysis that had occurred in 18-h-old cultures. Results were expressed as micrograms of tyrosine per milliliter released in the milk. Lactic acid production by Lac⁺ mutants and Lac⁺ transformants growing in milk was measured as follows: cultures (18-h) of organisms were inoculated into 50 ml of NF medium and incubated at 31 C for 6 h. Samples (10 ml) were then taken at 1-h intervals and the pH was determined with a Corning model 12 pH meter. Nearly equal numbers of cells were used as inoculum. β-gal activity in milk was assayed as previously described (16).

Isolation of lactose-negative mutants. Cultures of S. lactis C2 and 7962 were exposed to acriflavine using a modified procedure of McKay et al. (12). One drop of a 12-h-old culture of cells grown in LLB was added to fresh LLB containing 0, 1.0, 3.0, or 6.0 μg of filter-sterilized acriflavine per ml. Cultures were incubated at either 21 or 31 C for 24 h. After this period, 1-ml samples were removed, and the appropriate dilution was spread on indicator plates which were held for 24 to 48 h at 31 C and examined for the appearance of Lac⁻ mutants. Two types of indicator plates were used: lactic acid with lactose as the sole carbohydrate containing 0.004% of bromocresol purple, on which Lac⁺ colonies appeared yellow and Lac⁻ white, and a modification of an agar developed by Morse and Alire (17). It consisted of tryptone, 20.0 g; yeast extract, 5.0 g; gelatin, 2.5 g; lactose, 10.0 g; sodium chloride, 4.0 g; sodium acetate, 1.5 g; ascorbic acid, 0.5 g; tris(hydroxymethyl)aminomethane buffer (Sigma 7-9), 1.3 g; dihydrocholic acid, 1.5 g; neutral red, 0.075 g; agar, 15.0 g; and water to 1 liter. The final pH was 7.2 and Lac⁺ colonies appeared red on this medium, whereas Lac⁻ mutants were white or clear. Suspected Lac⁻ mutant colonies were picked, and inoculated into glucose broth and incubated at 31 C for 24 h. These cultures were then checked for their ability to grow in LLB and assayed for β-gal activity.

Induction of prophage in S. lactis C2. Cells of S. lactis C2, 7962, and S. cremoris HP were subjected to ultraviolet irradiation to induce phage lysis using the procedure of McKay and Baldwin (11).

Transduction. The lysates obtained from ultraviolet induction were filter-sterilized through a 0.45-μm membrane filter (Millipore Corp.). The recipient cells were Lac⁻ mutants obtained from the acriflavine treatment already described. The mutant was grown for approximately 4 h in GLB. The cells were then harvested and resuspended in 2 ml of lactic broth containing no sugar but with 5.0 × 10⁻⁴ M calcium carbonate added. This suspension contained approximately 6.8 × 10⁴ cells. The lysate was mixed (1:1) with the recipient and incubated at 31 C. Samples were removed at intervals and spread on LLB agar with 0.004% of bromocresol purple added. Plates were incubated at 31 C for 24 to 48 h and examined for the appearance of Lac⁺ transductants. Lac⁺ colonies were selected from these plates and examined further.

Characterization of Lac⁺ transductants and Lac⁻ mutants. Selected transductants and mutants were examined for drug resistance markers, carbohydrate fermentation patterns, β-gal and β-Pgal activity, lactic acid production in lactic broth and milk, and proteolytic activity in milk. Drug resistance was determined using Dispos-o-Disks (Difco) containing the antibiotic to be tested. Streptomycin, ampicillin, carbenicillin, gentamicin, kanamycin, chloromycetin, tetracycline, and furadantin were the antibiotics used. The culture to be tested consisted of log-phase cells suspended in overlay agar and spread on GLB agar plates. The disks were applied to the overlay agar after spreading.

Fermentation patterns were determined by using the API system for lactobacilli (Analytab Products, Inc., New York). β-gal and β-Pgal activity was determined as described above as well as lactic acid production and proteolytic activity.

## RESULTS

Effect of acriflavine treatment on S. lactis. Cells of S. lactis C2 and 7962 were treated with acriflavine (1.0 to 6.0 μg/ml) and examined for the appearance of Lac⁻ variants (Table 1). S. lactis C2 yielded Lac⁻ variants at a rate of approximately 3%; S. lactis 7962, however, did not yield any Lac⁻ variants, even though it was more sensitive to acriflavine than C2 with respect to the number of surviving cells. Lac⁻ variants were not observed to occur spontaneously in either C2 or 7962.

Comparison of S. lactis C2 wild type and Lac⁻ mutants. Lac⁻ mutants isolated from S. lactis C2 by acriflavine were compared with the parent strain for a number of characteristics. Table 2 lists the results of this comparison.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Acriflavine (µg/ml)</th>
<th>21 C</th>
<th>31 C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CFU/ml</td>
<td>No. of Lac⁻ variants</td>
<td>CFU/ml</td>
</tr>
<tr>
<td>C2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>6.8 × 10⁴</td>
<td>0</td>
<td>6.9 × 10⁴</td>
</tr>
<tr>
<td>1.0</td>
<td>1.9 × 10⁴</td>
<td>4 of 190</td>
<td>6.4 × 10⁴</td>
</tr>
<tr>
<td>3.0</td>
<td>7.0 × 10⁴</td>
<td>2 of 70</td>
<td>1.0 × 10⁴</td>
</tr>
<tr>
<td>6.0</td>
<td>1.0 × 10⁴</td>
<td>3 of 100</td>
<td>2.4 × 10⁴</td>
</tr>
<tr>
<td>7962</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>3.3 × 10⁴</td>
<td>0</td>
<td>3.0 × 10⁴</td>
</tr>
<tr>
<td>1.0</td>
<td>1.5 × 10⁴</td>
<td>0</td>
<td>1.0 × 10⁴</td>
</tr>
<tr>
<td>3.0</td>
<td>5.0 × 10⁴</td>
<td>0</td>
<td>1.2 × 10⁴</td>
</tr>
<tr>
<td>6.0</td>
<td>3.5 × 10⁴</td>
<td>0</td>
<td>No growth</td>
</tr>
</tbody>
</table>

*CFU, Colony-forming units.*
Lac" mutants were unable to transport and ferment lactose, but were only slightly impaired in galactose fermentation. The Lac" mutants also lacked detectable β-Pgal activity even when growing on galactose. The wild-type and mutant cells were the same in all other reactions; they were sensitive to ampicillin, carbenicillin, gentamicin, kanamycin, chloromycetin, tetracycline, and furadantin. The wild-type and the Lac" mutants were both resistant to streptomycin. Both cell types gave positive arginine hydrolysis reactions and did not produce diacetyl. Acriflavine treatment failed to cure the wild type with respect to lysogeny; ultraviolet treatment of both the Lac" and wild-type cells resulted in lysis of the cultures. Lysates of these Lac" cells, however, did not yield Lac" mutant, indicating that the lac marker had been irrevocably lost and that Lac" was not due to a point mutation.

Comparison of wild type, Lac" mutants, and Lac" transductants. In examining the Lac+ cells obtained by transduction, it was found that they had regained the ability to ferment lactose and also contained the same levels of β-Pgal as the wild type. These results are summarized in Table 3. The transductants were indistinguishable from the wild type in all characteristics examined except one; they were unable to produce lactic acid at the same rate as the wild type when growing in NFM. Figure 1 shows lactic acid production of the wild type, a Lac" mutant, and transductants (designated T8 and T9) when grown in milk. All initial inoculations were 1% and contained approximately 5.0 × 10⁶ organisms per ml. After 6 h at 30 C, the mutant had lowered the pH only about 0.15 U and had a cell concentration of 7.5 × 10⁷. The wild type lowered the pH about 1.1 U and had a final cell concentration of 3.5 × 10⁷. T9 lowered the pH about 0.9 of a unit and had a final cell concentration of approximately 1.0 × 10⁸. T8 lowered the pH 0.7 U. This difference in rate of acid production between the wild type and the transductants could not be demonstrated when the cells were growing in LLB as shown in Fig. 2. Here the Lac" mutant first lowered the pH slightly and then raised it again; final cell concentration was 9.0 × 10⁷. When the Lac" mutants were grown in GLB, the pH was lowered over 2.0 U and a final cell concentration of 6.9 × 10⁸ was achieved. The rates of acid production of the wild type and transductants T9 and T8 were essentially the same, the pH being lowered 2.0 U at final cell concentrations of approximately 4.0 × 10⁸. Because it appeared that the Lac" mutants could ferment glucose and produce lactic acid at a high rate when grown in broth, it was thought that the addition of glucose to NFM might restore the lactic acid production of the Lac" mutants in milk. Results of a typical experiment to test this idea are

Table 2. Comparison of physiological characteristics of S. lactis C2 wild type with a Lac" mutant

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Wild type</th>
<th>Lac&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferments</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactose</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Galactose</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fructose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mannose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Arginine hydrolysis</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>β-Pgal activity</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>β-gal activity</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lysogenic</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Uptake of [14C]lactose</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Proteolytic activity</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3. Comparison of S. lactis C2 wild type with S. lactis C2 Lac" transductants

<table>
<thead>
<tr>
<th>Character</th>
<th>Wild type</th>
<th>Transductants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T2</td>
</tr>
<tr>
<td>Ferments</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Galactose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mannose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>β-Pgal</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sp act x 10⁴</td>
<td>186</td>
<td>186</td>
</tr>
<tr>
<td>Lysogenic</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Fig. 1. Lactic acid production by S. lactis C2 growing to NFM at 31 C from a 1% inoculum: wild type (○), transductants T8 and T9 (Δ, ●), and Lac" mutant (■).

Fig. 2. Lactic acid production by S. lactis C2 growing to LLB, 30 C from a 1% inoculum: wild type (○), transductants T8 and T9 (Δ, ●), and Lac" mutant (■).
Escherichia in LLB: growing Lac- mutantations by eliminating mutants could with treatment acid that bonucleic 7962 ever. The mutants were proteolysis by increased; and the Lac+ mutants, even when glucose ty. The expressions between types were assayed of NFM for these organisms had been impaired (18, 20, 21). Proteolytic activity of the Lac- mutant, Lac+ transductant T8, and the wild type was, therefore, examined during growth in milk. Samples were assayed at 5 h when acid-producing differences between cell types was obvious. Table 4 summarizes the results. Wild-type cells growing in NFM exhibited considerable proteolytic activity as expressed by the liberated tyrosine. Both the Lac- mutant and Lac+ transductant, however, exhibited very little proteolytic activity. Even when glucose (1%) was added to the milk, the proteolytic ability of the Lac- mutant and the Lac+ transductant was not significantly increased; in fact, glucose appeared to inhibit proteolysis by the wild type.

**DISCUSSION**

Results given in Table 1 indicate that while S. lactis 7962 is much more sensitive to acriflavine treatment with respect to survivors than C2, no Lac- mutants could be isolated from 7962; Lac- mutants were readily obtained from C2, however. The lactose utilization system of S. lactis 7962 has been compared to that found in *Escherichia coli* (14). Acriflavine causes mutations by eliminating extrachromosomal deoxynucleic acid that may be present in the growing cell. Results presented here suggest that the lactose-hydrolyzing enzyme of *S. lactis* 7962 (β-gal) is coded for by the host chromosome as in *E. coli* and, therefore, not affected by acriflavine. In *S. lactis* C2, however, it would appear that the enzyme (β-Pgal) of this organism is carried extrachromosomally and thus subject to elimination by acriflavine treatment. In this regard, it is noteworthy that plasmids have been identified in *S. lactis* (3, 7, 19).

The Lac- mutants obtained by acriflavine treatment of *S. lactis* C2 were examined to see if other mutant characteristics could be identified in these organisms. This comparison was complicated by the fact that *S. lactis* C2 wild type is an extremely fastidious organism requiring a complex medium for growth. Thus, most mutations could be lethal. By identifying other mutations linked with the lactose-fermenting region, mapping of this region would be possible. Results given in Table 2 indicate that with the exception of the ability to ferment lactose and proteolytic activity (including lactose transport and β-Pgal activity) the mutants examined were indistinguishable from the wild type.

**TABLE 4. Proteolytic activity of S. lactis C2 wild type, Lac- mutant, and transductant sampled after growth in NFM for 5 h at 31 C**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Tyrosine (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>11% NFM</td>
</tr>
<tr>
<td><em>S. lactis</em> C2</td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>56.0</td>
</tr>
<tr>
<td>Lac- mutant</td>
<td>6.0</td>
</tr>
<tr>
<td>Lac+ transductant</td>
<td>6.0</td>
</tr>
<tr>
<td>Uninoculated control</td>
<td>0.0</td>
</tr>
</tbody>
</table>
Of special interest where the Lac- mutants were concerned was their ability to grow on galactose and yet not produce any β-Pgal. Since Molskness et al. (16) have shown that free galactose is a potent inducer of β-Pgal in lactic streptococci, the question arises as to what the role of this carbohydrate is during induction. Lactic streptococci have been shown by Lee et al. (9) to use free galactose by the Leloir Pathway, where the phosphorylated product is galactose-1-phosphate and not galactose-6-phosphate. In *S. aureus*, galactose-6-phosphate (a product of hydrolysis of lactose-6-phosphate) has been shown to play a role in the induction process (18). Studies to determine the actual inducer in *Streptococcus* organisms have been hampered by the fact that, unlike staphylococci, streptococci are impermeable to phosphorylated compounds. Our attempts to increase the permeability of the cell membrane and still leave the cell viable have been unsuccessful.

Upon initial examination, all the Lac+ transductants isolated from bromocresol indicator plates were indistinguishable from the parent *S. lactis* C2 wild type (Table 3). The transductants had regained full β-Pgal activity and once again could ferment lactose in LLB at a rate that was essentially the same as the wild type. When the transductants were examined for lactic acid production in milk, however, it was found that the transductants were somewhat slower in the rate at which they lowered the pH (lactic acid production). Slow lactic acid production in milk is usually an indication of poor proteolytic ability, though the cells are still able to ferment lactose (4, 22). Thus, cells of poor proteolytic ability for the purposes of discussion might be designated Lac+ Prt- to designate lactose fermentation but reduce proteolytic activity, whereas the wild type exhibits a Lac+ Prt+ phenotype. Upon examination of the Lac- mutants, it was found that these cells were indeed Prt-.

It is important to note that in the lactic streptococci, Prt- cells cannot be detected when grown in lactic broth. This is due to the large amount of small peptides already present in this medium. It is only when the cells are grown in milk and forced to hydrolyze casein to get their required amino nitrogen that the Prt- phenotype can be demonstrated.

In these experiments, Lac- was the only mutant phenotype selected. Prt- was not selected due to the lack of a screening method for this character. Yet as a result of this selection, all the Lac- mutants obtained were also Prt-; Lac- Prt+ types probably also exist, but were not found in this study. These data, along with a recent report by Pearce et al. (20), indicate that certain proteolytic enzymes of these organisms are carried on plasmids. These plasmids, like the lactose-fermenting character, are lost during acriflavine treatment. Westhoff et al. (21, 22, 23) have characterized both intra- and extracellular proteases in lactic streptococci. The nature of the lost protease responsible for the Prt- phenotype found in this study is not known but presumably it is the surface-bound activity described by Pearce et al. (20). It is well known that lactic streptococci are unstable with respect to proteolytic activity. Strains become slow lactic acid producers, as a result of losing their proteolytic ability, at a rate of about 1% based on colony isolation of plated cultures. The Prt- strains obtained by this type of spontaneous mutation, however, are still Lac+. On the other hand, spontaneous mutation to Lac- has never been reported or observed in this laboratory, indicating that it occurs at a very low frequency. The difference in stability of the lactose and the proteolysis characters suggests that they are carried on different plasmids.

A hypothetical model of the lactose and proteolytic genes in *S. lactis* C2 might be constructed as follows: genes responsible for lactose fermentation are carried on one plasmid. The genes responsible for proteolytic activity are carried on a second plasmid and transduced independently of the lactose marker. Thus, when transductants are selected by the Lac+ phenotype alone, they do not necessarily regain their proteolytic ability. This would mean that when acriflavine treatment is used in obtaining Lac- mutants, Prt- mutants are formed concurrently. Indeed all the Lac- mutants examined up to this time have also been Prt-. The effect that acriflavine had on the Prt+ to Prt- mutation rate independent of the lac characteristic is yet to be determined.

Results of these studies indicate at least two areas where genetic manipulation might be used to improve lactic fermentation by lactic streptococci. One is the use of transduction to stabilize the Prt characteristic. Macrina and Balbinder (10) showed that when a mutant F' plasmid obtained from *Salmonella typhimurium* was transduced into *E. coli* it exhibited unique stability. If stability of this nature could be developed for the Prt characteristic in streptococci, the high rate of appearance of slow acid-producing mutants would be greatly reduced.

The second area where genetic manipulation could be used involves lactose fermentation itself. In results presented elsewhere (T. A.
Molskness, Ph.D. thesis, Oregon State University, 1974), it was shown that in S. cremoris HP lactic acid production in broth lags until β-Pgal reaches a certain level. This lag is not as long in cells inoculated into glucose broth, presumably because induction of β-Pgal is not critical in this case. From this it seems that it would be possible to increase acid production in an organism by creating stable merodiploids for β-Pgal. A higher level of this enzyme would decrease the lag time for growth and lactic acid production, and thus decrease the production time for fermented dairy products. Merodiploids have been constructed in E. coli K-12 (6) and the level of β-gal has been determined. It was found that the merodiploids contained approximately twice as much β-gal as found in the haploid cell; also Brenchley and Magasanik (1) have shown that a lac plasmid of Klebsiella aerogenes could be transferred to E. coli and Salmonella typhimurium, and that segregants losing the plasmid grew on lactose at only 50% the rate of the plasmid-containing strains and they contained only one-tenth to one-fifth as much β-gal. To create merodiploids in lactic streptococci, however, a method of selecting for the merodiploid cell must first be developed. Current methods of platting would not work since these methods only select for ability to ferment lactose and not the amount of β-Pgal present in the cell.

ACKNOWLEDGMENT

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LITERATURE CITED