Molecular and Genetic Characterization of Lactose-Metabolic Genes of *Streptococcus cremoris*

JULIA M. INAMINE,*† LINDA N. LEE, AND DONALD J. LeBLANC

Bacterial Virulence Section, Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, Fort Detrick, Frederick, Maryland 21701-1013

Received 24 February 1986/Accepted 27 May 1986

Lac⁺ plasmid DNA from *Streptococcus cremoris* H2 was subcloned with an *Escherichia coli* vector on a 3.5-kilobase-pair *Pst*I-*Ava*I fragment. Genetic analysis of the cloned DNA was possible because linear Lac⁺ DNA fragments were productive in the *S. sanguis* transformation system. Complementation of *S. sanguis* Lac⁻ mutants showed that the 3.5-kilobase-pair fragment included the structural gene for 6-phospho-β-D-galactosidase and either enzyme II-lac or factor III-lac of the lactose-specific phosphoenolpyruvate-dependent phosphotransferase system. Expression of the *S. cremoris*-like 40,000-dalton 6-phospho-β-D-galactosidase in *S. sanguis* Lac⁺ transformants, rather than the 52,000-dalton wild-type *S. sanguis* enzyme, demonstrated the occurrence of gene replacement and not gene repair. The evidence supports chromosomal integration as the mechanism by which *S. sanguis* Lac⁻ recipients are converted to a Lac⁺ phenotype after transformation with Lac⁺ DNA. Southern blot data suggest that the Lac⁺ DNA does not reside on a transposon, but that integration always occurs within a specific *Hinc*II fragment of the recipient chromosome. Hybridization experiments demonstrate homology between the *S. cremoris* Lac⁺ DNA and cellular DNA from Lac⁺ strains of *Streptococcus lactis*, *S. mutans*, *S. faecalis*, and *S. sanguis*.

The same pathway of lactose fermentation is utilized by many streptococci (reviewed in reference 11). Briefly, lactose is phosphorylated and transported into the cell as lactose phosphate by the phosphoenolpyruvate-dependent lactose-specific phosphotransferase system (lac-PTS). 6-Phospho-β-D-galactosidase (p-β-gal; EC 3.2.1.85) hydrolyzes lactose phosphate to glucose and galactose 6-phosphate. Galactose 6-phosphate is further metabolized by the enzymes of the tagatose 6-phosphate pathway to the triose phosphates. Streptococci which metabolize lactose by this pathway include *Streptococcus cremoris* (21), *S. faecalis* (10), *S. lactis* (21), *S. mutans* (7), *S. salivarius* (7), and *S. sanguis* (7).

The genetic determinants for lactose metabolism can be either chromosomal, as in *S. sanguis* (25), or plasmid borne, as in *S. cremoris* and *S. lactis* (21). These species also differ in the molecular size and regulation of expression of the p-β-gal enzyme. For example, the 40,000-dalton p-β-gal (25) of *S. lactis* is synthesized constitutively; the enzyme levels found in cells grown on ribose are the same as in those grown on lactose (12, 13). The synthesis of the 52,000-dalton p-β-gal (25) of *S. sanguis* is inducible; full expression occurs when cells are grown on lactose but not when cells are grown on galactose (LeBlanc et al., unpublished observations). In addition, the substrate specificities of the lac-PTS of *S. sanguis* and *S. lactis* are distinct, with characteristic galactose/lactose PTS activity ratios of 0.05 and 0.30 (25), respectively.

The differential aspects of the lactose-metabolic genes and enzymes have been used to initiate the genetic and molecular analysis of *S. lactis* lactose metabolism in *S. sanguis*. The *S. sanguis* host is useful because it is naturally transformable and Lac⁻ mutants are available. The *S. sanguis* lac-8 strain has lac-PTS activity but is missing p-β-gal activity (8; E. St.

Martin, unpublished observation). The lac-83 strain is a lactose-insensitive derivative of the lac-8 strain that is Lac⁻ due to mutations in both lac-PTS and p-β-gal (25). The lac-8 and lac-83 strains can be transformed to a Lac⁺ phenotype with *S. lactis* plasmid DNA (8, 25). The Lac⁺ transformants of the *S. sanguis* lac-83 strain express the *S. lactis* genes as judged by the presence of the 40,000-dalton *S. lactis* plasmid-encoded p-β-gal and by the characteristic galactose/lactose lac-PTS activity ratio (8, 25). Plasmid DNA has not been recovered from any of these transformants (8, 25). *S. sanguis* Lac⁺ transformants can also be obtained with restriction endonuclease-digested *S. lactis* plasmid DNA or with purified fragments thereof (9; LeBlanc et al., unpublished observations), suggesting that these transformants have resulted from the integration of *S. lactis* lac sequences into the *S. sanguis* chromosome. Integration is not the only mechanism for successful transformation of *S. sanguis* by *S. lactis* DNA, because a 23-kilobase-pair (kbp) *S. lactis* Lac⁺-encoding plasmid fragment has been subcloned in *S. sanguis* on a plasmid vector (9).

In this laboratory we have studied the lactose-metabolic genes of *S. cremoris* by transforming *S. sanguis* Lac⁻ mutants with both whole and restriction endonuclease-digested *S. cremoris* lactose plasmid DNA. As in the *S. lactis* plasmid studies, *S. cremoris* plasmid DNA could not be isolated from the Lac⁺ *S. sanguis* transformants. Attempts were made to clone the lactose-metabolic *S. cremoris* DNA in *S. sanguis* with streptococcal plasmid vectors. Although stable Lac⁺ *S. sanguis* transformants were obtained, recombinant plasmid DNA could not be recovered. These transformation results, together with those obtained with the *S. lactis* plasmids, suggest that chromosomal integration may be the major mechanism by which *S. sanguis* can stably maintain and express both *S. cremoris* and *S. lactis* lactose-metabolic genes. We sought to clarify this phenomenon by cloning *S. cremoris* Lac⁺ DNA in a heterologous system with *Escherichia coli* as the transformation recipient. The same approach was used by Maeda and

* Corresponding author.

† Present address: Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27514.

TABLE 1. Relevant properties of bacterial strains

Strain	p- β -gal			Source or reference
	Type of activity ^a	Sp act (U) ^b	Size ^c (daltons)	
<i>S. cremoris</i> H2	NT	1.938	40,000	G. Davey
<i>S. lactis</i> H1	NA	0.045	ND	G. Davey
J170	Constitutive	1.942	40,000	This work ^d
<i>S. sanguis</i> DL1	Inducible	1.142	52,000	(14)
<i>lac-8</i>	NA	0.006	ND	E. St. Martin
<i>lac-83</i>	NA	0.007	ND	(25)
J1131	Constitutive	1.017	40,000	This work ^e
<i>E. coli</i> TB1(pUC19)	NA	0.025	ND	BRL ^f

^a NT, Not tested; NA, not applicable.^b Specific activities after growth in the presence of 10 mM lactose.^c Polyacrylamide gradient gels were used for the molecular sizing of p- β -gal activity. ND, No activity band detected.^d Mating of *S. cremoris* H2 $\times$ *S. lactis* H1.^e Transformation of *lac-83* strain with 3.5-kbp *Pst*I-*Ava*I fragment of pJ1135.^f TB1 is an HsdR⁻ HsdM⁺ derivative of JM83 (28); IPTG is not required in media containing 5-bromo-4-chloro-3-indolyl- β -D-galactoside. BRL, Bethesda Research Laboratories.

Gasson (18) to clone the *S. lactis* NCDO 712 p- β -gal gene. In this report, the cloned *S. cremoris lac* DNA sequences were used to transform *S. sanguis* Lac⁻ mutants. Analysis of these Lac⁺ transformants permitted the molecular and genetic characterization of lac-PTS and p- β -gal genes of *S. cremoris*. The nature and distribution of *S. cremoris* Lac⁺ DNA sequences in these *S. sanguis* Lac⁺ transformants were explored by Southern blot analysis. In addition, DNA hybridization was used to examine the relationship between the Lac⁺ genes of *S. cremoris* and other gram-positive bacteria which metabolize lactose by this pathway.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this study are listed in Table 1. *Streptococcus sanguis* var. Challis *lac-8* (8; E. J. St. Martin, unpublished observation) and *lac-83* (25) were recipients in transformation experiments. *Escherichia coli* TB1 (Table 1) was the host for cloning procedures with the *E. coli* plasmid vectors pUC18 and pUC19 (28).

S. cremoris H2 was the donor and *S. lactis* H1 Lac⁻ was the recipient in a filter mating performed to isolate a lactose-specific plasmid. The multiple-plasmid-bearing donor, plasmid-free recipient, and transconjugants were provided by Graham Davey of the New Zealand Dairy Research Institute, Palmerston North, New Zealand, during his tenure as a visiting scientist in this laboratory. A transconjugant containing only the lactose-metabolic plasmid was identified by the plasmid screening method of Anderson and McKay (1) and is designated *S. lactis* J170.

Growth conditions. Streptococci were routinely grown in *Lactobacillus* carrying medium (LCM) modified from the basal medium of Eftymiou and Hansen (6). One liter of LCM contains 10 g of Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 5 g of yeast extract, 3 g of tryptose, 3 g of KH₂PO₄, 3 g of K₂HPO₄, 1.7 g of sodium acetate $\cdot$ 3H₂O, 1 ml of Tween 80, and 5 ml of salt solution. The salt solution contained 11.5 g of MgSO₄ $\cdot$ 7H₂O, 0.7 g of FeSO₄ $\cdot$ 7H₂O, 2.2 g of MnSO₄ $\cdot$ H₂O, and 1 ml of concentrated HCl per 100 ml. The pH of the complete medium was adjusted to 7. LCM or modified LCM (0.5 g of yeast extract

per liter) was supplemented with 20 mM glucose or 10 mM lactose. Carbohydrate solutions were filter sterilized and added to autoclaved media. Modified LCM without an added carbon source could not support the growth of the streptococcal strains; this characteristic was dependent on the lot of yeast extract used. *S. cremoris* and *S. lactis* were incubated at 30°C, and *S. sanguis* was grown at 37°C without aeration. *E. coli* was grown in LB broth (19) at 37°C with aeration. Solid media contained 1.5% agar (BBL).

Streptococci were grown under inducing and noninducing conditions to determine whether the level of p- β -gal activity increased with the addition of lactose to the growth medium. Noninducing medium was modified LCM broth containing 25 mM D-ribose for *S. lactis* and 20 mM galactose for *S. sanguis*; inducing medium contained 10 mM lactose. *S. cremoris* H2 could not grow on 25 mM D-ribose or 20 mM arginine (non-PTS growth substrates) and so could not be grown under noninducing conditions.

Isolation of DNA. Plasmid DNA was isolated from *S. cremoris* and *S. lactis* by the method of Anderson and McKay (1) and from *E. coli* by the alkaline lysis method (19), with minor modifications. Total cellular DNA was isolated by the method of LeBlanc and Lee (16). DNA was further purified by centrifugation in cesium chloride-ethidium bromide density gradients.

Restriction endonuclease digestion and gel electrophoresis. Restriction endonucleases were obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.) and were used according to the manufacturer's instructions. DNA fragments were separated by electrophoresis on horizontal 0.8% agarose gels in 1 $\times$ Tris-borate-EDTA buffer (19); small fragments were resolved on 10% polyacrylamide gels or 4% Nu-Sieve gels (FMC Corp., Rockland, Maine). DNA fragments were purified from agarose gel slices by electroelution into dialysis bags in 0.5 $\times$ Tris-borate-EDTA buffer followed by concentration and purification with an Elutip-d column (Schleicher & Schuell Co., Keene, N.H.) and ethanol precipitation.

Transformation of *S. sanguis*. Competent cultures of *S. sanguis* var. Challis were prepared as described by Rollins et al. (23), and DNA was added at a concentration of approximately 1 μ g/ml. After incubation for 4 h, samples were spread on modified LCM agar containing 10 mM lactose. The plates were incubated for 18 to 24 h. All incubations were at 37°C under anaerobic conditions (GasPak; BBL). The transformation assay is difficult to standardize because the state of maximum competence can be neither accurately predicted nor artificially prolonged. Valid comparisons of transforming DNAs can only be obtained from assays performed at the same time; therefore, the numbers of transformants are not averaged.

Cloning in *E. coli*. Recombinant plasmids were constructed in *E. coli* by ligation and transformation as described previously (19). Vector DNA was dephosphorylated as described by Ullrich et al. (27). Bacterial alkaline phosphatase and T4 DNA ligase were from Bethesda Research Laboratories. Transformants were selected on LB agar containing 50 μ g of ampicillin per ml and 40 μ g of 5-bromo-4-chloro-3-indolyl- β -D-galactoside (Sigma Chemical Co., St. Louis, Mo.) per ml.

DNA blotting and hybridization. DNA fragments were transferred from agarose gels to nitrocellulose paper (Schleicher & Schuell) by the method of Southern (24) as modified by LeBlanc and Lee (16). DNA to be used as a probe in hybridization reactions was labeled in vitro with ³²P-labeled dATP and dGTP (Amersham Corp., Arlington

Heights, Ill.) by nick translation (20) as described previously (16). Conditions for hybridization and the preparation of autoradiograms were those of Thayer (26) with minor modifications (16).

Enzyme assays. Cell extracts were prepared from 100-ml overnight cultures by the glass bead method of Ranhand (22) with modifications. Streptococci were grown in modified LCM broth supplemented with a carbon source as noted above. *E. coli* strains were grown in LB broth. The cells were washed and suspended in 1.0 ml of 20 mM Tris (pH 7.4) in a 30-ml Corex tube, and approximately 1.0 ml of glass beads (Arthur H. Thomas, Philadelphia, Pa.; 0.11- to 0.12-mm diameter, no. 5663R20) was added. The cells were broken by multiple cycles of high-speed vortexing (15 s) followed by cooling in an ice bath (15 s). After disruption, the glass beads and cellular debris were removed by centrifugation. The cell-free supernatant was used for enzyme assays. Protein concentration was determined with a microdetermination kit (no. 690-A; Sigma).

p- β -gal and β -galactosidase activities were assayed with the substrates *o*-nitrophenyl- β -D-galactoside (ONPG) 6-phosphate (ONPG-6-P; Sigma) and ONPG (Sigma) by the protocol of Citti et al. (2). Cell-free extracts (10 to 100 μ l) was added to 0.5 ml of 20 mM Tris (pH 6.5) containing 1 mM of substrate, and the reaction mixture was incubated at 37°C for 5 min. The reaction was stopped, and the color was developed by adding 0.5 ml of ice-cold 10% Na₂CO₃. Activity was measured at 420 nm by using an extinction coefficient of 0.001 optical density unit per 0.214 nmol of *o*-nitrophenol. p- β -gal activity was localized on 4 to 30% polyacrylamide gradient gels (Pharmacia Fine Chemicals, Piscataway, N.J.) after electrophoresis in Tris-borate buffer (7.89 g of Tris base and 1.09 g of boric acid per liter) by using Pharmacia gel apparatus GE-4. The gel was soaked in 10 ml of 0.2 M Tris (pH 6.5) containing 6 mg of ONPG-6-P at 40°C for 15 min and then placed in 10 ml of cold 10% Na₂CO₃. The yellow activity bands were marked with a needle dipped in India ink, and protein bands were visualized by staining with Coomassie blue G250 (5). The yellow activity bands are not amenable to photography and reproduction, and so the polyacrylamide gels are not shown.

E. coli transformants were screened for p- β -gal expression in microtiter tray wells. A 48-prong replica template was used to disperse cells in 25 μ l of 50 mM sodium phosphate buffer (pH 7.0). The cells were permeabilized by the addition of 1.5 μ l of toluene-acetone (1:9) and incubation at 37°C for 15 min. After the addition of 25 μ l of 24 mM ONPG-6-P, incubation was continued for 15 min before adding 50 μ l of 0.5 M Na₂CO₃. Wells with p- β -gal activity were visibly yellow.

RESULTS

Molecular cloning in *E. coli*. The molecular cloning of lactose-metabolic plasmid DNA from *S. cremoris* H2 was facilitated by isolating a Lac⁺ plasmid from this multiple-plasmid-containing strain. The Lac⁺ plasmid was identified by its ability to confer a Lac⁺ phenotype by filter mating *S. cremoris* H2 with a plasmid-free, Lac⁻ derivative of *S. lactis* H1. A Lac⁺ transconjugant, *S. lactis* JI70, received a single plasmid species from the *S. cremoris* donor. The 65-kbp Lac⁺ plasmid harbored by strain JI70 was designated pJI70.

*Pst*I and *Sst*I restriction endonuclease fragments of pJI70 were subcloned into an *E. coli* vector, pUC19 (27). Transformants of *E. coli* TB1 were selected on media containing ampicillin and 5-bromo-4-chloro-3-indolyl- β -D-galactoside.

TABLE 2. Transformation of *S. sanguis* Lac⁻ mutants with restriction endonuclease fragments of pJI70

Endonuclease used	No. of Lac ⁺ transformants of ^a :	
	<i>lac-8</i>	<i>lac-83</i>
<i>Ava</i> I	2,000	1,256
<i>Eco</i> RI	353	0
<i>Hinc</i> II	8	0
<i>Hind</i> III	49	14
<i>Pst</i> I	1,500	277
<i>Sst</i> I	988	0
None	34,700	15,900

^a Number of CFU per 100 ng of DNA on modified LCM containing 10 mM lactose. The control assay without DNA gave 2 CFU for the *lac-8* strain and none for the *lac-83* strain.

White colonies were screened for p- β -gal activity in microtiter wells with the substrate ONPG-6-P (β -galactosidase and p- β -gal activities showed no cross-reactivity on their respective substrates, ONPG and ONPG-6-P). Plasmid DNAs from the p- β -gal⁺ transformants were analyzed by restriction endonuclease digestion and agarose gel electrophoresis. p- β -gal⁺ plasmids contained either a 10.9-kbp *Pst*I fragment (pJI134) or a 10.1-kbp *Sst*I fragment (pJI133) of pJI70 DNA. pJI133 and pJI134 were found to be overlapping clones with a 9.9-kbp *Sst*I-*Pst*I fragment in common.

Genetic analysis by transformation of *S. sanguis* Lac⁻ mutants. Genetic analysis of the *S. cremoris* Lac⁺ plasmid genes was initiated by transforming *S. sanguis* Lac⁻ mutants with the pJI70 Lac⁺ plasmid. Lac⁺ transformants were selected for directly by plating the transformation mixture on modified LCM containing 10 mM lactose. An *S. sanguis* *lac-8* strain missing p- β -gal activity and a *lac-83* strain lacking both lac-PTS and p- β -gal activities could be transformed to a Lac⁺ phenotype with pJI70. The nature of the lac-PTS defect in the *lac-83* strain is not known; the lactose-specific mutation is in either factor III-lac or enzyme II-lac (4) of the lac-PTS. The *S. sanguis* Lac⁺ transformants were analyzed for extra chromosomal DNA by conventional procedures (1, 15), and no plasmids were detected. Restriction endonuclease-generated fragments of pJI70 could also be used as the source of Lac⁺ transforming DNA (Table 2). The number of transformants obtained with the digested DNAs was lower than with uncut pJI70, presumably because of the increased nuclease susceptibility of linear DNA fragments. The enzymes *Ava*I and *Pst*I cut pJI70 into fragments which transformed both the *lac-8* and *lac-83* strains to a Lac⁺ phenotype, whereas *Eco*RI and *Sst*I fragments could transform the *lac-8* strain but not the *lac-83* strain. *Hinc*II- and *Hind*III-digested pJI70 did not produce a significant number of Lac⁺ transformants of either the *lac-8* or *lac-83* strains. The transformation assay was also used to analyze the *S. cremoris* plasmid DNA cloned in *E. coli*. In agreement with the results obtained with pJI70, the 10.9-kbp *Pst*I fragment of *S. cremoris* DNA isolated from pJI134 could transform both *S. sanguis* *lac-8* and *lac-83* strains to Lac⁺, whereas the 10.1-kbp *Sst*I insert fragment of pJI133 could transform the *lac-8* strain but not the *lac-83* strain. Plasmid pJI134 was chosen for further study because it contained both lac-PTS and p- β -gal sequences as judged by the transformation assay. A partial restriction endonuclease map of pJI134 is shown in Fig. 1A.

Expression of *S. cremoris* p- β -gal in transformants. The transformation of the *S. sanguis* *lac-8* and *lac-83* strains to Lac⁺ with linear DNA fragments could be explained by gene repair as well as by gene replacement. It was important,

FIG. 1. Restriction endonuclease maps of plasmids composed of vector pUC18 or pUC19 (□) and fragments of pJ170 Lac⁺ DNA (—). Restriction endonuclease sites: A, *Ava*I; E, *Eco*RI; H, *Hind*III; P, *Pst*I; S, *Sst*I. A vector-derived promoter sequence, P_{lacZ'}, is indicated by an arrow which points 5' to 3' in the direction of transcription (28). The position of the promoter with respect to the insert is shown in both orientations (A versus B). The box to the right indicates the p-β-gal activity associated with each plasmid.

therefore, to determine whether an *S. cremoris* Lac⁺ gene was being expressed in the *S. sanguis* Lac⁺ transformants. Analysis of the expression of p-β-gal was chosen because the p-β-gal protein has been physically identified, whereas the lac-PTS components have not, and because the *S. sanguis* and *S. cremoris* p-β-gal proteins are distinctly different in size. Wild-type *S. sanguis* DL1 has a p-β-gal with a molecular size of 52,000 daltons (25). *S. cremoris* H2 has a p-β-gal which comigrates (this work) with the 40,000-dalton p-β-gal from *S. lactis* ATCC 11454 (25). The *S. lactis* transconjugant containing the *S. cremoris* Lac⁺ plasmid pJ170 also contains a p-β-gal of 40,000 daltons (data not shown). The *S. sanguis* Lac⁻ mutants, the *lac-8* and *lac-83* strains, contain no p-β-gal activity by standard assay or on activity-stained polyacrylamide gradient gels. Cell-free extracts from 11 independently isolated Lac⁺ transformants of the *S. sanguis lac-8* and *lac-83* strains (described in Fig. 2, bottom panel), as well as *E. coli* TB1 harboring pJI134, each contained a 40,000-dalton p-β-gal activity band (data not shown).

Deletion analysis of the cloned Lac⁺ DNA by transformation of *S. sanguis* Lac⁻ mutants. The *S. sanguis* transformation assay was used to further delineate the location of the Lac⁺ genes within the fragments cloned in *E. coli*. The 10.9-kbp *Pst*I insert fragment of pJI134 contained a single *Ava*I site which divided the insert into two *Pst*I-*Ava*I fragments of 3.5 and 7.4 kbp (Fig. 1). Since the *S. cremoris* Lac⁺ plasmid could be digested with the enzymes *Pst*I and *Ava*I and still transform the *S. sanguis lac-8* and *lac-83* strains to Lac⁺ (Table 2), it seemed likely that the lac-PTS and p-β-gal regions would be found on a single *Pst*I-*Ava*I fragment. The 3.5- and 7.4-kbp *Pst*I-*Ava*I fragments from pJI134 were used to transform the *S. sanguis* Lac⁻ mutants; only the 3.5-kbp fragment could transform the *S. sanguis lac-8* and *lac-83*

strains to Lac⁺. A Lac⁺ transformant of the *lac-83* strain was designated strain JI131. The 3.5-kbp *Pst*I-*Ava*I fragment was subcloned in *E. coli* with the pUC19 vector, and this derivative was designated pJI135 (Fig. 1A). Both *S. sanguis* JI131 and *E. coli* TB1(pJI135) contained the 40,000-dalton p-β-gal activity (data not shown).

The lac-PTS and p-β-gal sequences were more specifically located by transformation experiments with smaller fragments of the 3.5-kbp *Pst*I-*Ava*I fragment. Purified restriction endonuclease fragments of pJI134 were used to transform the *S. sanguis lac-8* and *lac-83* strains. Progressively smaller fragments, from the 3.5-kbp *Pst*I-*Ava*I fragment to the 2.5-kbp *Sst*I-*Ava*I and the 2.1-kbp *Eco*RI-*Ava*I fragments (Fig. 1), transformed the *lac-8* strain to a Lac⁺ phenotype (780, 200, and 90 CFU, respectively, per 100 ng of DNA on modified LCM containing 10 mM lactose; the control assay without added DNA gave 5 CFU). The *lac-83* strain, however, could be transformed to Lac⁺ only with the 3.5-kbp *Pst*I-*Ava*I fragment (154 CFU per 100 ng of DNA; no transformants were detected when either of the other two fragments or no DNA controls were assayed).

Expression of *S. cremoris* p-β-gal in *E. coli*. The *E. coli* plasmids pUC18 and pUC19 are vectors which contain the *lacZ'* promoter (P_{lacZ'}) juxtaposed to the multiple cloning sequence in each orientation (28). The effect of this promoter on the expression of the *S. cremoris* p-β-gal gene was examined. The 10.9-kbp *Pst*I fragment of Lac⁺ *S. cremoris* DNA was cloned in both orientations relative to P_{lacZ'} (indicated by arrows in Fig. 1; compare Fig. 1A with 1B). The 3.5-kbp *Pst*I-*Ava*I fragment was also inserted in both orientations by directional cloning in pUC19 (Fig. 1A) and pUC18 (Fig. 1B). Cell-free extracts of *E. coli* TB1 containing these recombinant plasmids expressed p-β-gal activity. A

FIG. 2. Southern blot analysis of Lac⁺ transformants. Chromosomal DNAs from *S. sanguis* lac-8 (A) and lac-83 strains (H), and Lac⁺ transformants derived from the lac-8 and lac-83 strains (see panel below blot for lanes and strain derivations) were digested with *Hinc*II. Controls were *Hinc*II-digested pJ170 (G and P) and pJ1135 (Q). The probe was the 3.5-kbp *Pst*I-*Ava*I Lac⁺ fragment from pJ1135. Hybridization was at 67°C for 18 h in a solution containing 3× SSC (1× is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.5 µg of probe (specific activity, 4.8 × 10⁷ cpm/µg). Washes were at 52°C for 15 min four times in 0.1× SSC plus 0.1% sodium dodecyl sulfate and 15 min four times in 0.1× SSC. The sizes of the *Hinc*II fragments of pJ1135 are given on the right.

reproducible difference was observed in the p-β-gal specific activities, which depended on the orientation of the fragment with respect to P_{lacZ'}; extracts from cells containing pJ1134 and pJ1135 (Fig. 1A) produced p-β-gal specific activities of 2.250 and 1.975 U (defined as nanomoles per minute per microgram of protein), respectively, whereas those with pDL134 and pDL135 (Fig. 1B) yielded 0.363 and 0.163 U, respectively. All activities were well above the 0.025 U of background activity obtained with either vector alone.

Analysis of the *S. sanguis* transformants by Southern blot hybridization. The *S. sanguis* Lac⁺ transformants were analyzed for the presence of *S. cremoris* Lac⁺ DNA by Southern blot hybridization. Chromosomal DNAs from the *S. sanguis* lac-8 and lac-83 strains, strain DL1, and Lac⁺ transformants of the lac-8 and lac-83 strains were digested with *Pst*I plus *Ava*I and hybridized with the 3.5-kbp *Pst*I-*Ava*I Lac⁺ fragment from pJ1135. All of the strains contained a single fragment of approximately 6.2 kbp which showed homology to the Lac⁺ probe (data not shown). However,

different hybridization patterns were detected when the DNAs were digested with *Hinc*II, *Acc*I, and *Hinc*II plus *Acc*I. The results obtained with the *Hinc*II-digested DNAs and the 3.5-kbp Lac⁺ probe are shown in Fig. 2. The derivations of the *S. sanguis* Lac⁺ transformants and a revertant are also given in Fig. 2. All of the Lac⁺ transformants contained one or more of the *Hinc*II fragments found within the 3.5-kbp *Pst*I-*Ava*I Lac⁺ fragment. The Lac⁻ lac-8 and lac-83 strains each contained a single *Hinc*II fragment of about 3,300 base pairs (bp) which showed homology to the probe. The differential banding patterns made it obvious that strain J1124, with DNA in lane D, was a Lac⁺ revertant of the lac-8 strain and not a transformant. The data from all the Southern blot hybridizations are summarized in schematic form in Fig. 3. The restriction enzyme site analysis (described in the legend) was used to infer that certain DNA regions were derived from the *S. cremoris* pJ170 Lac⁺ plasmid, whereas others must be the *S. sanguis* recipient chromosome.

FIG. 3. Summary of Southern blot hybridization data. A restriction endonuclease map of the 3.5-kbp *Pst*I-*Ava*I *Lac*⁺ probe was obtained by analysis of pJI135. Restriction endonuclease sites: Ac, *Acc*I; Av, *Ava*I; H2, *Hinc*II; H3, *Hind*III; P, *Pst*I. The probe can be divided into five *Hinc*II fragments with the sizes given in bp. The maps of the *Lac*⁺ regions of pJI70 and the transformants were deduced from Southern blots such as that shown in Fig. 2. Analysis of restriction sites found in pJI70 (+), sites present in pJI70 but missing in transformant DNA (○), and new sites appearing in transformant DNA which are not found in pJI70, was used to infer whether DNA regions in the transformants were derived from pJI70 (—) or the recipient chromosome (~~~~).

There was a correlation between the amount of *S. cremoris* DNA found in a transformant and the type of p- β -gal expression. Wild-type *S. sanguis* exhibited an inducible p- β -gal activity, whereas the *S. lactis* transconjugant containing the *S. cremoris* *Lac*⁺ plasmid demonstrated constitutive p- β -gal activity (Table 1). All of the *Lac*⁺ transformants of the *S. sanguis* *lac-8* and *lac-83* strains used in Fig. 3 contained the 40,000-dalton *S. cremoris* p- β -gal and not the 52,000-dalton *S. sanguis* enzyme, but p- β -gal activity was inducible in some and constitutive in others. For example, the p- β -gal activity of strains JI121 and JI122 was clearly inducible (0.100 and 0.038 U obtained from cells grown on galactose, and 0.525 and 0.438 U from cells grown on lactose, respectively), whereas the p- β -gal activity in strains JI131 and JI120 was constitutive (0.275 and 0.313 U from cells grown on galactose, and 0.388 and 0.363 U from cells grown on lactose, respectively). The strains containing the 400-bp *Hinc*II-to-*Acc*I region of pJI70 DNA (shown at the top of Fig. 3 to contain the 250-bp *Hinc*II fragment) had *S. cremoris*-like, constitutive p- β -gal expression, whereas strains without this DNA region expressed *S. sanguis*-like, inducible p- β -gal activity.

Homology of *S. cremoris* *Lac*⁺ sequences. The *S. cremoris* *Lac*⁺ DNA region was used as a probe to determine whether homologous sequences are found in other gram-positive bacteria which metabolize lactose by the same pathway. Southern blot hybridization was carried out with *Hinc*II-digested cellular DNAs and the 3.5-kbp *Pst*I-*Ava*I *Lac*⁺ fragment probe (Fig. 4). Homology was detected with DNA from *Lac*⁺ strains *S. lactis* ATCC 11454, *S. sanguis* DL1, and *S. faecalis* JH1. There was faint but detectable homol-

ogy to DNA from *S. mutans* 6715-10. There was no detectable hybridization to DNAs from *Lac*⁺ strains *S. salivarius* DL140, *Staphylococcus aureus* RN2425, *Lactobacillus casei* 64H (in agreement with Lee et al. [17]), and a *Lac*⁻ derivative of *S. lactis* ATCC 11454. The hybridization pattern obtained with DNA from the well-characterized strain *S. lactis* ATCC 11454 contained bands in common with that of the *S. cremoris* plasmid pJI70; this homology was absent in the *Lac*⁻ derivative of ATCC 11454.

DISCUSSION

Lac⁺ DNA from an *S. cremoris* plasmid, pJI70, was subjected to genetic analysis by transforming *Lac*⁻ mutants of *S. sanguis* and to molecular analysis by subcloning fragments in *E. coli* with plasmid vectors. The genetic integrity of the purified, cloned *S. cremoris* *Lac*⁺ DNA was confirmed because linear *Lac*⁺ DNA fragments were productive in the *S. sanguis* transformation system. Complementation of *S. sanguis* *Lac*⁻ *lac-8* and *lac-83* strains showed that the cloned *Lac*⁺ DNA includes the structural gene for p- β -gal and either enzyme II-lac or factor III-lac of the *lac*-PTS. The transformation, cloning, and hybridization data are summarized in the fine structure restriction enzyme map of the 3.5-kbp *Pst*I-*Ava*I *S. cremoris* *Lac*⁺ plasmid region shown in Fig. 5. The limits to which the *lac*-PTS and p- β -gal regions have been defined are indicated. The results show that the *lac*-PTS and p- β -gal sequences reside within a 3.5-kbp *Pst*I-*Ava*I fragment and suggest that the p- β -gal region is contained within a 2.1-kbp *Eco*RI-*Ava*I fragment. The latter possibility was explored by subcloning this

FIG. 4. Southern blot analysis of selected Lac⁺ gram-positive bacteria. Plasmids pJI70 (A) and pJI135 (J) and cellular DNAs from *S. lactis* ATCC 11454 (B), a spontaneously occurring Lac⁺ derivative of strain ATCC 11454 (C), *S. sanguis* DL1 (D), *S. mutans* 6715-10 (E), *S. salivarius* DL140 (F), *S. faecalis* JH1 (G), *Staphylococcus aureus* RN2425 (H), and *Lactobacillus casei* 64H (I) were digested with *Hinc*II. The probe was the 3.5-kbp *Pst*I-*Ava*I Lac⁺ fragment from pJI135 (specific activity, 3.2×10^7 cpm/ μ g). The hybridization and wash conditions were as described in the legend to Fig. 2. The sizes of the *Hinc*II fragments of pJI135 are given on the right.

2.1-kbp fragment in *E. coli* with the pUC18 and pUC19 vectors, but no p- β -gal activity was detected. The position of the *S. cremoris* p- β -gal gene with respect to the *Pst*I, *Cla*I, *Eco*RI, and *Hind*III sites appears to be the same as that determined by Maeda and Gasson (18) for a p- β -gal gene from *S. lactis*. They also found that deletion of DNA to the left of the *Eco*RI site, with respect to the map in Fig. 5, abolishes p- β -gal activity (18). In addition, our transformation results suggest that the lac-PTS region may be cleaved by *Eco*RI and *Sst*I. Alternatively, the inability of the smaller fragments to transform the *lac*-83 strain could reflect the interruption of DNA sequences necessary for integration into the *lac*-83 host chromosome. The region implicated in constitutive p- β -gal expression, a characteristic of the *S. cremoris* plasmid gene, mapped between 0.15 and 0.55 kbp, within the lac-PTS region which extended to the *Eco*RI site at 1.4 kbp. The absence of the *Acc*I site at map length 1.3

kbp in *S. sanguis* JI131 (Fig. 3) suggested that this site might provide a cut-off point for lac-PTS sequences. If the lac-PTS gene were flanked by a regulatory region and the *Acc*I site, it would span about 750 bp, enough DNA to encode a 28,000-dalton protein. The p- β -gal region, mapped between 1.3 and 2.9 kbp, contained more than enough DNA for the sequence of its 40,000-dalton protein product. The primary features of the *S. cremoris* plasmid Lac⁺ region are thus a putative p- β -gal-regulatory region followed by a lac-PTS gene and, finally, the p- β -gal structural gene. This Lac⁺ region may be organized as an operon. Crow et al. (3) calculate that a minimum of 6 to 7 megadaltons (approximately 9.1 to 10.6 kbp) would be required to encode for the six lactose-metabolic enzymes associated with Lac⁺ plasmids from *S. lactis*. Since plasmid pJI134 contains an insert of 10.9 kbp of *S. cremoris* Lac⁺ DNA, future experiments will determine whether any of the other enzymes required for lactose metabolism, specifically the enzymes of the tagatose 6-phosphate pathway, are expressed in *E. coli*.

Several lines of evidence suggested that chromosomal integration was the mechanism by which *S. sanguis* Lac⁻ *lac*-8 and *lac*-83 strains were converted to a Lac⁺ phenotype after transformation with Lac⁺ DNA. First, no plasmid DNA could be isolated from the *lac*-8 and *lac*-83 strains which had been transformed to Lac⁺ with whole *S. cremoris* plasmid pJI70 DNA. Second, the *S. sanguis* Lac⁻ mutants could be transformed to Lac⁺ with restriction endonuclease digests of pJI70 or with purified fragments of pJI70 isolated from recombinant plasmids propagated in *E. coli*. Finally, the expression of the *S. cremoris* 40,000-dalton p- β -gal protein in *S. sanguis* Lac⁺ transformants, rather than the 52,000-dalton p- β -gal found in wild-type *S. sanguis*, demonstrated that gene replacement and not gene repair was occurring. Further evidence for integration was obtained by Southern blot hybridization. The smallest amount of integrated *S. cremoris* DNA was found in *S. sanguis* JI121 and JI123 (Fig. 3). These transformants of strain *lac*-8 contained the 40,000-dalton *S. cremoris* p- β -gal, and so their Lac⁺ inserts defined the p- β -gal gene. Additional *S. cremoris* DNA was required to transform the *lac*-83 strain to Lac⁺, thus identifying the lac-PTS region. The minimum amount of DNA required to transform the *lac*-83 strain was demonstrated by strains JI79 and JI131 (the loss of the *Acc*I site in JI131 DNA may be due to a point mutation). In addition to the lac-PTS and p- β -gal regions, the 3.5-kbp *Pst*I-*Ava*I Lac⁺

FIG. 5. Fine structure restriction endonuclease map of the 3.5-kbp *Pst*I-*Ava*I map of *S. cremoris* Lac⁺ DNA. The limits to which the lac-PTS and p- β -gal regions have been defined are indicated by the lines below the map. No sites were detected for the enzymes *Bam*HI, *Bcl*II, *Bgl*II, *Eco*RV, *Hpa*I, *Kpn*I, *Nde*I, *Nru*I, *Sal*I, *Scal*, *Sma*I, *Sst*I, *Xba*I, and *Xho*I.

fragment contained all that was necessary and sufficient for integration into the *S. sanguis* chromosome. There was a considerable range in size of *S. cremoris* Lac⁺ DNA which could stably integrate. Among the 11 transformant DNAs examined, six different amounts of *S. cremoris* plasmid DNA were detected (Fig. 3). That six different insertion patterns were obtained suggested that the Lac⁺ DNA did not reside on a transposon, since a transposon is a discrete unit by definition. Although differences in recipient chromosomal sequences flanking the Lac⁺ inserts indicated that integration was not site specific, the *S. cremoris* Lac⁺ DNA always integrated into a specific 3,300-bp *HincII* fragment of *S. sanguis* recipient chromosome.

The finding that Lac⁺ *S. cremoris* DNA integrated into specific restriction endonuclease fragments of *S. sanguis* chromosomal DNA provides exciting possibilities for future research. Work is in progress to clone such a target fragment and thus provide a tool to generate defined Lac⁻ deletion mutants of *S. sanguis* and to study the integration process. The *S. cremoris* sequences which promote integration are also being more precisely defined and can serve to direct integration into the *S. sanguis* chromosome. Knowledge of the *S. cremoris*-*S. sanguis* system may be a prerequisite for studying similar Lac⁺ regions in view of the homology detected between *S. cremoris* Lac⁺ DNA and cellular DNA from strains *S. lactis* ATCC 11454, *S. mutans* 6715-10, and *S. faecalis* JH1.

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