Evidence that L-(-)-Lactate Dehydrogenase Deficiency Is Lethal in Streptococcus mutans

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In order to construct an effector strain for the replacement therapy of dental caries, we wished to combine the properties of low-level acid production and high-level colonization potential in a strain of Streptococcus mutans. To this end, we made a deletion in the lactate dehydrogenase (LDH) gene cloned from the bacteriocin-producing S. mutans strain JH1000. However, we were unable to substitute the mutant for the wild-type allele by transformation with linear DNA fragments. The mutated gene, carried on a suicide vector, was shown by Southern analysis to integrate into the JH1000 chromosome to yield transformants carrying both the wild-type gene and mutated LDH gene. Three spontaneous self-recombinants of one heterodiploid strain were isolated by screening 1,500 colonies for a loss of the tetracycline resistance encoded by the gene used to mark the LDH deletion. In all three cases, Southern analysis showed that a loss of tetracycline resistance was accompanied by a loss of the mutated LDH gene, resulting in restoration of the wild-type genotype. However, screening the same number of colonies for self-recombinants that did not make lactic acid during anaerobic growth in Todd-Hewitt broth failed to identify clones in which the wild-type allele was lost. A second, simpler screening of more than 80,000 colonies grown aerobically on glucose tetrazolium medium to identify low-level-acid-producing colonies was also unsuccessful. These results are interpreted as indicating that LDH deficiency is lethal in S. mutans under the cultivation conditions used in these experiments. The physiological bases for this hypothesis are described.

In the past, lactic acid production by fructose-1,6-diphosphate (FDP)-dependent L-(-)-lactate dehydrogenase (LDH) was shown to be an important virulence factor for Streptococcus rattus (11). LDH-deficient mutants were substantially less cariogenic than the wild-type organism in both laboratory and rodent models. As a result, LDH deficiency has been proposed as one aspect of a strategy to construct an effector strain for the replacement therapy of dental caries.

Following chemical mutagenesis and aerobic incubation on glucose tetrazolium medium, LDH-deficient mutants of S. rattus BHT-2 (5) and Streptococcus cricetus AHT (unpublished data) were recovered as red colonies. However, we have been unable to isolate LDH-deficient mutants from any of a large number of laboratory and fresh S. mutans strains, including the bacteriocinogenic strain JH1000 (9), which has colonization properties that make it particularly suitable for use in replacement therapy (8).

Abhyankar et al. (1) did succeed in isolating an LDH-deficient mutant from a fresh isolate of S. mutans. This isolate was distinctive in the pattern of fermentation end products it generated during growth in limiting glucose under anaerobic conditions. In particular, it produced relatively high levels of ethanol and acetate and a low level of lactic acid. The authors speculated that a preexisting mutation in an alternate pyruvate-dissimilating pathway was necessary to support viability in the presence of an LDH deficiency.

The LDH-deficient S. mutans strain isolated by Abhyankar et al. was obtained following mutagenesis with nitrosoguanidine, which raises concerns about cryptic mutations and reversion of the mutation in the LDH gene. Furthermore, this strain has no known properties, such as bacteriocin production, that promote its ability to colonize the human oral cavity. To overcome these problems, and to ensure stable and specific mutation of the LDH gene in effector strain construction, we cloned the LDH gene from strain JH1000 into Escherichia coli (7). In vitro disruption of the gene was achieved by deletion of the promoter and a major portion of the protein coding sequence (3). A tetracycline resistance gene from S. mutans, which is also expressed in E. coli, was inserted at the deletion site as a marker for selection. Southern analysis demonstrated that strain JH1000 carries a single copy of the LDH gene in its chromosome. Consequently, exchange of the mutated gene for the wild-type gene should give rise to an LDH-deficient mutant in a single step.

In this report we describe experiments that show that single-step exchange of the mutated LDH gene for the wild-type LDH gene does not occur via natural transformation. However, when carried on a suicide vector, the mutated LDH gene can be introduced into strain JH1000 at a low frequency by natural transformation. The mutated LDH gene became integrated into the chromosome by homologous recombination to produce clones that were heterodiploid for LDH. Spontaneous recombination events which excised the mutated LDH gene were observed to occur at a relatively high frequency, but none which eliminated the wild-type gene were found, suggesting that LDH deficiency in S. mutans is lethal under the culture conditions employed.

MATERIALS AND METHODS

Chemicals, media, bacterial strains, and plasmids. Chemicals and antibiotics were from Sigma Chemical Co., St. Louis, Mo. Except where indicated, S. mutans strains were grown aerobically overnight in brain heart infusion broth or
Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) or in candle jars on plates of the same media containing 1.5% agar. E. coli strains were grown overnight with aeration in Luria-Bertani broth or on plates of the same medium containing 1.5% agar. Glucose tetrazolium plates were prepared by the method of Miller (14) and contained 1% (wt/vol) glucose. Ampicillin and tetracycline were added to plates and broth at 30 and 10 μg/ml, respectively, as required. S. mutans JH1000 (9), JH145 (6), and NG8 (12) and E. coli MC1061 (2) have been described previously. Plasmids used in this study are described in Results.

**Genetic methods.** Restriction enzymes and T4 ligase were obtained from New England BioLabs, Inc. (Beverly, Mass.), and were used with the buffer supplied, according to the conditions specified by the manufacturer.

Chromosomal DNA was isolated from S. mutans as previously described (7). Plasmid DNA was purified by dye-buoyant density centrifugation (13). DNA fragments from restriction enzyme digestions were excised from agarose gels (0.6 to 1.0%) and purified by electroelution as described by Maniatis et al. (13).

Southern hybridizations were performed with the Enhanced Chemiluminescence Gene Detection System from Amersham International PLC (Amersham, England). The supplier’s protocols were used without modification for hybridization and for DNA probe preparation. Nitrocellulose filters were exposed to Hyperfilm-ECL (Amersham).

Transformation of S. mutans JH1000 and NG8 was performed by the methods of Lee et al. (12).

**Screening for lactic acid production.** Isolated colonies of S. mutans clone JDM4 were picked with sterile toothpicks and inoculated into microtiter wells containing 100 μl of Todd-Hewitt broth plus tetracycline. The plates were incubated anaerobically (85% nitrogen, 10% carbon dioxide, and 5% hydrogen) for 3 days at 37°C. Lactic acid production was determined semiquantitatively by placing a 5-μl sample from each well into a fresh microtiter well and adding 5 μl of a cocktail containing 0.25 M potassium phosphate buffer (pH 7.4), 20 mg of NAD per ml, 2 mg of nitroblue tetrazolium per ml, 2 mg of phenazine methosulfate per ml, and 50 U of L(+)-lactate dehydrogenase from rabbit muscle (Sigma Chemical Co.) per ml. The microtiter plates were incubated for 20 min at 37°C. The color of each well was compared with those of standards consisting of Todd-Hewitt broth plus 0, 5, 10, 20, or 40 mM L(+)-lactic acid (Sigma Chemical Co.) which were treated as described above. Microtiter well cultures of strain JH145 (ldh) and the wild-type strain, JH1000, were tested as negative and positive controls, respectively.

**LDH assay.** Cell extracts of S. mutans strains were prepared from 100-ml overnight cultures grown in Todd-Hewitt broth supplemented with 0.5% (wt/vol) glucose. Cells were washed and resuspended in 1 ml of 0.1 M potassium phosphate buffer (pH 6.2) and ruptured with a French press at 14,000 lb/in². The extract was clarified by centrifugation at 14,000 × g for 30 min at 4°C. LDH activity was assayed as the pyruvate-dependent oxidation of NADH with and without FDP as previously described (7). The values presented are the level of FDP-dependent NADH oxidation corrected by subtracting the level of FDP-independent NADH oxidation. In all cases, the latter represented <5% of the total activity. The protein concentrations of cell extracts were determined with the BCA protein assay kit (Pierce Chemical Co., Rockford, Ill.).

**RESULTS**

In a previous study, a pBR322-based plasmid, p10-5, which carries the wild-type LDH gene from S. mutans JH1000 (7) was isolated. The LDH gene was inactivated (3) by deletion of the promoter and approximately two-thirds of the protein coding sequence, followed by insertion of the tetracycline resistance gene from pVA981. In E. coli strains transformed with the resulting plasmid, p3-5, the LDH gene was completely disabled, as evidenced by their growth phenotype on glucose tetrazolium medium and the absence of enzyme activity in cell extracts. Plasmid p3-16, which was used in the studies described here, is identical to p3-5 except that the tetracycline resistance gene is inserted in the opposite orientation.

Plasmid p3-16 was digested with SpeI, and a 5.8-kb fragment containing the mutated LDH gene plus 1.0 kb of 5' flanking DNA and 0.7 kb of 3' flanking DNA was recovered by DNAase gel electrophoresis. When 0.5 μg of the fragment was used to transform JH1000 to tetracycline resistance, no transformants were obtained. The readily transformable S. mutans strain NG8 also failed to yield tetracycline-resistant clones when treated in the same fashion.

Because of our inability to create the LDH-deficient clone directly by allelic exchange, we attempted a two-step process involving the creation of a heterodiploid intermediate. First, since S. mutans is not naturally resistant to ampicillin, p3-16 was partially digested with DraI to remove a 0.7-kb fragment containing the ampicillin resistance gene. The resulting 10.8-kb fragment was gel purified, religated, and used to transform E. coli MC1061 with selection for tetracycline resistance. Colonies which arose following overnight incubation were purified on the selective medium and tested for ampicillin sensitivity by replica patching. Plasmid DNA from four ampicillin-sensitive, tetracycline-resistant clones was mapped with restriction enzymes to confirm the predicted deletion of the ampicillin gene.

One confirmed plasmid, pDM4, was used to transform JH1000 (Fig. 1). By using 2 μg of cesium chloride-purified DNA, three clones (JDM1, -2, and -3) were obtained following anaerobic incubation on selective medium containing tetracycline. One additional clone (JDM4) was obtained from aerobically incubated plates. More than 600 colonies were obtained when the naturally transformable S. mutans strain NG8 was used as a recipient.

Southern blot analysis of SpeI-digested chromosomal DNA from JDM2, -3, and -4 was performed. When an SpeI-Hpal fragment of p10-5 containing a portion of the LDH gene which was not deleted in p3-16 was used as a probe, two bands were observed (Fig. 2B). The size of the lower band (3.3 kb) agreed with the size predicted from the previously reported (7) restriction map for the wild-type gene. Only the lower band was observed in DNA from JH1000, further indicating that this band contained the wild-type gene. Furthermore, in SpeI digests of chromosomal DNA of JDM2, -3, and -4, only this band was reactive with an Hpal fragment of p10-5 that spanned the portion of the LDH gene that was deleted in p3-16 (Fig. 2C). The upper band contained a fragment with the mutated LDH gene, as evidenced by its reaction when a HincII fragment of pVA981 containing the tetracycline resistance gene was used as a probe (Fig. 2A). The size of this band (5.0 kb) also agrees with the prediction based on the size of the wild-type fragment (3.3 kb) minus the size of the deletion (1.7 kb) plus the size of the pVA981 insert (3.5 kb). These results are
consistent with the hypothesis that the transformants arose as a result of Campbell-type recombination and are heterodiploid for the LDH gene. The wild-type and mutant genes are located on the chromosome, separated by vector DNA.

Recombination between the mutated and wild-type LDH genes in heterodiploid transformants should lead to wild-type revertants and LDH-deficient mutants at approximately equal frequencies. To demonstrate that these revertants and mutants occur and to determine the frequencies at which they occur, we first sought self-recombinants in which the mutated LDH gene was eliminated. Three overnight cultures of JDM4 were grown in Todd-Hewitt broth without tetracycline, decimally diluted, and spread on brain heart infusion agar. After 2 days of anaerobic incubation, 500 colonies from each culture were replica patched onto brain heart infusion agar with or without tetracycline. After overnight incubation, three tetracycline-sensitive clones were identified, one from each culture. An in vitro assay of crude cell extracts showed wild-type levels of LDH activity for all three clones, as they oxidized 19.13, 18.66, and 18.74 μmol of NADH per min per mg of protein, compared with 19.26 and 18.39 μmol/min/mg for controls JH1000 and JDM4, respectively. Southern blot analysis confirmed that each of these clones had a single LDH gene that migrated coincidently with the wild-type gene in JH1000 (Fig. 3A) and that none of them contained DNA homologous to that of the tetracycline resistance gene of pVA981 (Fig. 3B). These results are consistent with the hypothesis that homologous recombination between the mutated and wild-type LDH genes in JDM4 had occurred and eliminated the mutated gene in each of the tetracycline-sensitive clones.

![Diagram](image)

**FIG. 1.** *ldh* heterodiploid construction by transformation of JH1000 with pDM4. Symbols: , *S. mutans* DNA; , *ldh* gene; , Tet' gene of pVA981; , pBR322 DNA. Abbreviations: S, SpeI; H, HpaI. Probes 1, 2, and 3 were used in the blots shown in Fig. 2 and 3.

![Southern blots](image)

**FIG. 2.** Southern analysis of chromosomal DNA from JH1000 transformed with pDM4 and controls. Lane 1, lambda DNA digested with HindIII; lane 2, Tet' gene from pVA981; lanes 3, 4, and 5, chromosomal DNA of JDM2, JDM3, and JDM4, respectively, digested with SpeI; lane 6, chromosomal DNA of JH1000 digested with SpeI; lane 7, plasmid p10-5 containing the wild-type *ldh* gene digested with HpaI. The blot was probed with the Tet' gene of pVA981 (Fig. 1, probe 1) (A), an SpeI-HpaI fragment of p10-5 containing a portion of the LDH gene which was not deleted in pDM4 (Fig. 1, probe 2) (B), and an HpaI fragment of p10-5 that was deleted in pDM4 (Fig. 1, probe 3) (C).
strain NG8 was also untransformable with this fragment provided preliminary evidence that an LDH deficiency is lethal in \textit{S. mutans} under the cultivation conditions employed for selection of transformants.

We were able to introduce the mutated LDH gene as part of a circular DNA molecule which required a single-cross-over event to create heterodiploid transformants. The results of Southern blot analysis are consistent with the hypothesis that the mutated and wild-type genes are situated on the chromosome, separated by vector DNA.

One of the heterodiploids was found to undergo spontaneous homologous recombination which eliminated the mutated LDH gene. This event occurred at a frequency of 1/500. The alternative outcome of this recombination event, elimination of the wild-type LDH gene, was not observed in 1,500 colonies of the heterodiploid screened for decreased lactic acid production. Although anaerobic cultivation conditions are more likely to be compatible with LDH deficiency (see below), we also performed screening which used tetrazolium medium (which works only under aerobic conditions) to identify LDH-deficient self-recombinants. Screening of more than 80,000 colonies failed to identify a single colony for which red coloration gave an indication of decreased acid production. These results provide additional support for the hypothesis that LDH deficiency is lethal in \textit{S. mutans} under the cultivation conditions used in the screening.

The reliance of \textit{S. rattus} LDH mutants on acetoin production as a major route of pyruvate metabolism, especially under conditions of aerobic growth (6), can be interpreted to indicate that LDH deficiency may impose constraints on the maintenance of cell homeostasis. One possible constraint is indicated by the fact that the biosynthesis of acetoin has no obvious advantage over that of pyruvate itself as an end product of metabolism in terms of energetics or balance of oxidation and reduction. Therefore, acetoin may be formed because pyruvate excretion is rate limiting and pyruvate may be toxic at high intracellular concentrations. Formation of acetoin from two molecules of pyruvate represents an efficient means for the mutant to reduce intracellular levels of pyruvate.

A second possible explanation for the lethal effect of LDH deficiency may be found in the facts that the conversion of pyruvate to lactate serves as a major sink for electrons generated by glyceraldehyde-3-phosphate dehydrogenase. Anaerobically growing LDH mutants of \textit{S. rattus} are able to compensate for their metabolic deficiency by increasing their production of ethanol (6). Under aerobic conditions, this pathway is less utilized, so other sinks for electron disposal must be employed. Higuchi (4) demonstrated that the ability of \textit{S. rattus} to grow on mannitol in the presence of oxygen correlated extremely well with high levels of NADH oxidase and superoxide dismutase.

Most strains of \textit{S. mutans} appear to have less flexibility for dealing with excess reducing equivalents generated by glycolysis, particularly under aerobic conditions. It was shown (4) that the growth of two of three \textit{S. mutans} strains on mannitol was severely inhibited by oxygen, and this correlated with a relative lack of NADH oxidase and superoxide dismutase activities. If the addition of a single excess reducing equivalent per molecule of substrate utilized, as in the case of mannitol consumption, has toxic effects on wild-type \textit{S. mutans}, an excess of two electrons generated by glucose metabolism in an LDH-deficient mutant would likely have lethal consequences.

Like other streptococci, \textit{S. rattus} and \textit{S. mutans} appear to
share the same pathways for pyruvate metabolism, as indicated by the similarity in their end products under various culture conditions. It is likely, therefore, that \textit{S. rattus} is able to accommodate a deficiency in LDH while \textit{S. mutans} is not because the species differ somehow in their regulation of the enzyme activities that catalyze these pathways. Given the flexibility afforded by multiple pathways for pyruvate metabolism (10), we predict that culture conditions which will be permissive for LDH deficiency in \textit{S. mutans} will be able to be found. Experiments are under way to test this prediction.

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REFERENCES