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Cariogenicity of a Lactate Dehydrogenase-Deficient Mutant of *Streptococcus mutans* Serotype c in Gnotobiotic Rats

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A lactate dehydrogenase-deficient (Ldh^-) mutant of a human isolate of *Streptococcus mutans* serotype c was tested in a gnotobiotic rat caries model. Compared with the wild-type Ldh-positive (Ldh^+) strains, it was significantly ($\alpha \leq 0.005$) less cariogenic in experiments with two different sublines of Sprague-Dawley rats. The Ldh^- mutant strain 044 colonized the oral cavity of the test animals to the same extent as its parent strain 041, although its initial implantation was slightly but not significantly ($P \geq 0.2$) less. Multiple oral or fecal samples plated on 2,3,5-triphenyltetrazolium indicator medium revealed no evidence of back mutation from Ldh^- to Ldh^+ in vivo. Both Ldh^+ strain 041 and Ldh^- strain 044 demonstrated bacteriocinlike activity in vitro against a number of human strains of mutans streptococci representing serotype a (*S. cricetus*) and serotypes c and e (*S. mutans*). Serotypes b (*S. rattus*) and f (*S. mutans*) and strains of *S. mitior*, *S. sanguis*, and *S. salivarius* were not inhibited. Thus, Ldh mutant strain 044 possesses a number of desirable traits that suggest it should be investigated further as a possible effector strain for replacement therapy of dental caries. These traits include its stability and low cariogenicity in the sensitive gnotobiotic rat caries model, its bacteriocinlike activity against certain other cariogenic *S. mutans* (but not against more inocuous indigenous oral streptococci), and the fact that it is a member of the most prevalent human serotype of cariogenic streptococci.

Lactate dehydrogenase-deficient (Ldh^-) mutants of cariogenic streptococci have been proposed as effector strains for replacement therapy of dental caries (5). The concept has been verified experimentally in rat caries model systems using Ldh^- mutants of *Streptococcus rattus*, formerly *S. mutans* serotype b (8, 9). *S. sobrinus* (formerly *S. mutans* serotypes d and g) has also been mutagenized to Ldh^- (12, 15) and has been found to have low caries-producing activity in hamsters (12). Mutants of *S. mutans* serotype c with low acid-producing activity had reduced virulence in rats; however, their Ldh activity was not determined (14). *S. mutans* serotype c is the most prevalent of the cariogenic mutans streptococci in humans (16) and most frequently associated with the initiation of caries (13), hence there is reason to believe that Ldh^- mutants of this serotype could be employed advantageously as effector strains in replacement therapy of caries. Attempts to obtain such mutants had been unavailing (15) until Abhyankar et al. (1) selected naturally occurring, reduced acid-producing human strains for mutagenesis. Here we report on in vivo caries activity of one such Ldh^- mutant in the gnotobiotic rat test system.

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MATERIALS AND METHODS

Microorganisms. The characteristics of human wild-type Ldh-positive (Ldh^+) *S. mutans* serotype c, strain 041 and its Ldh^- mutant strain 044, obtained by *N*-methyl-*N'*-nitrosoguanidine treatment, have been previously described (1). Briefly, the mutant and wild type utilized glucose to the same extent during growth. Whereas the wild type produced only lactic acid, the mutant produced ethanol, acetic acid, acetoin, and isovaleric acid as end products of glucose metabolism (1). Cells of Ldh^- mutant strain 044 contained less than 1.0% of the Ldh activity of the parent strain 041. In sucrose broth cultures, both strains formed in vitro plaque deposits to the same extent (1). Their genetic relatedness has been confirmed by the identity of their chromosomal DNA patterns on agarose gel after digestion with restriction endonucleases (G. Z. Kulkarni, K. H. Chan, and H. J. Sandham, J. Dent. Res., in press). *S. mutans* PS-14, a type c human oral strain known to produce extensive caries in gnotobiotic rats (6, 7), was included as a positive control in the present study. Attempts to obtain Ldh^- mutants from PS-14 were unsuccessful (15). Stocks of the test organisms were maintained in lyophilized form and coded to conceal their identities in the animal tests.

Inoculum and culture conditions. Cell inocula for the animal studies were grown for 18 h in brain heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.) at 35°C in an atmosphere of 10% carbon dioxide and 90% nitrogen. The optical density at 550 nm was adjusted by dilution with brain heart infusion broth so that each inoculum suspension contained approximately 3.5×10^8 CFU/ml. Media used for evaluation of the degree of microbial colonization of the animals were the following: Mitis-Salivarius medium (MS) (Difco Laboratories, Detroit, Mich.); Trypticase soy agar (BBL) containing 5% defibrinated sheep blood; and 2,3,5-triphenyltetrazolium chloride (TTC)-glucose agar (5) for detection of Ldh^- mutants. The MS and blood plates were incubated at 35°C in 10% CO_2–90% N_2 for 48 h, while the TTC plates were incubated at 35°C in candle jars for up to 7 days. On TTC medium, wild-type strains grew as white colonies and the Ldh^- mutant reduced the TTC to form red colonies.

Gnotobiotic rat model. Germfree outbred Sprague-Dawley rats were obtained from the Charles River in Wilmington, Mass., or from the Gnotobiotics Laboratory of the University of Wisconsin, Madison, Wis. Pregnant females were housed in flexible plastic isolators and fed autoclaved diet L-356 (TEKLAD, Madison, Wis.). The progeny, aged 19

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days, were weaned and transferred to separate isolators; animals were distributed in groups of three of the same sex into stainless steel mesh cages. A separate litter of Charles River rats was used for each test organism in experiment 1. In experiment 2, the Wisconsin rats were all from the same litter. The animals were provided with a vitamin-fortified (4), gamma radiation-sterilized (6 megarads) version of high-sucrose diet 2000 (11) and distilled water ad libitum. Each animal was infected with the appropriate *S. mutans* strain as described below. After 42 days on the test regimen, the animals were sacrificed with chloroform and their jaws were autoclaved and defleshed.

Infection and monitoring procedures. Each test animal had 0.2 ml of the inoculum suspension instilled orally on day one of the experiment. In addition, 1.0 ml of the inoculum was added to 100 ml of the drinking water which was available for the first 2 days. At intervals, oral swabs of the animals were placed in 2 ml of 0.05% yeast extract solution (pH 7.2) and agitated on a Vortex mixer for 10 s before being decimally diluted in yeast extract for plating. Results were reported as log CFU per swab. Freshly voided rat feces were diluted by weight in yeast extract, dispersed in sterile plastic disposable homogenizers (Kontes, Vineland, N.J.), and serially diluted for plating. Results were reported as log CFU per gram of feces. The Student *t* test was used to determine the significance of differences in recoveries between strains at each sampling interval.

Caries scores. The standard rat caries scoring system of Keyes (10) classifies dental lesions by extent and by surface of origin. In this study, many of the teeth of animals infected with wild-type strains PS-14 or 041 had such extensive destruction that the Keyes technique could not be applied. Instead, an abbreviated system was used to rank teeth in order of caries severity (2). Briefly, each first and second mandibular molar was assigned a score from 0 to 4.0 to represent the proportional degree of caries from no detectable decay to complete destruction of the molar crown. The means of the four molar scores per animal were used to calculate the group mean severity score. Composite data were analyzed by the Kruskal-Wallis test, and the significance of intergroup differences was determined by the Mann-Whitney U test for ranked data.

Bacteriocin production. The ability to produce bacteriocin could conceivably provide a competitive advantage for establishment of an effector strain as part of the oral microbial ecosystem. *S. mutans* 041, 044, and spontaneous Ldh^- strain 447-11 were screened for bacteriocin activity against a variety of mutans and other oral streptococci. The producers were spot inoculated on Trypticase soy agar, and the plates were incubated in candle jars at 35°C for 48 h. Then 3.0 ml of Trypticase soy medium containing 0.5% agar and a 10^4 dilution of the indicator organism was overlaid on the surface. The plates were reincubated and examined at 24 h for zones of growth inhibition around the producer strains. Results were recorded as showing zones of no inhibition, partial inhibition, or complete inhibition.

RESULTS

Germfree rats maintained on caries-promoting diet 2000 developed significantly fewer instances of caries ($\alpha \leq 0.005$) when infected with Ldh^- mutant 044 than did animals infected with Ldh^+ strains of *S. mutans*. Caries activity in the mutant-infected animals was minimal and confined to the enamel of the molar fissures, whereas major portions of the molar crowns were destroyed by the wild-type strains. The data are summarized in Table 1 for separate litters infected with each organism and Table 2 for animals which came from a single litter of a different subline of Sprague-Dawley rats that was apparently less susceptible to caries. Weight gains of the animals varied considerably within and between litters (Table 1). The animals infected with strains 041 and 044 in this experiment came from successive litters of the same dam, while those infected with strain PS-14 came from a different dam. This may account for the weight gain discrepancies in the latter group.

TABLE 1. Caries scores and weight gains of gnotobiotic rats[a] infected with *S. mutans* strains

S. mutans strain	Sex[b] (no. tested)	Weight gain (g) ± SD	Caries score ± SD	Total caries score (M + F) ± SD
041 (Ldh^+)	M (3)	158.6 ± 7.1	2.7 ± 0.5	2.7 ± 0.4
	F (3)	126.8 ± 6.5	2.8 ± 0.5	
044 (Ldh^-)	M (6)	148.0 ± 11.5	1.1 ± 0.3	1.2 ± 0.3[c]
	F (3)	115.0 ± 3.0	1.3 ± 0.2	
PS-14 (Ldh^+)	M (3)	138.9 ± 4.2[d]	3.2 ± 0.6	3.1 ± 0.4
	F (3)	112.7 ± 10.7	3.0 ± 0.2	

[a] Charles River Sprague-Dawley subline.
[b] M, Male; F, female.
[c] Significantly different by Mann-Whitney U test ($\alpha \leq 0.005$).
[d] Significantly different from group 041 males ($P \leq 0.002$) by the Student *t* test.

Bacteriologic studies of the animals indicated that the mutant and wild-type strains effectively colonized the animals and that recoveries of each organism were comparable on the three media employed. Pertinent data for experiment 2 are given in Table 3 in the form of log counts per oral swab or per gram of feces. There was no evidence of contaminating organisms on any of the dilution plates. As indicated by the size of the standard deviations, there was a considerable degree of variation in the numbers of microorganisms recovered from animals in the same group. Nevertheless, we could detect no correlation between bacterial counts and caries activity in individual animals or between animals infected with the mutant or the wild-type strain. Initially, at the 7-day sampling interval, recoveries of Ldh^- strain 044 were somewhat lower than recoveries of wild-type strain 041. These differences disappeared at the subsequent sampling intervals; in fact, none of the differences between the two strains was significant at any of the sampling periods (*P*

TABLE 2. Caries scores and weight gains of gnotobiotic rats[a] infected with *S. mutans*

S. mutans strain and sex[b] of rats tested	Weight gain (g) ± SD	Caries score ± SD	Total caries score (M + F) ± SD
041 (Ldh^+)			
M	159.3 ± 13.8	2.1 ± 0.3	2.2 ± 0.23
F	113.7 ± 0.86	2.3 ± 0.2	
044 (Ldh^-)			
M	142.6 ± 6.1	0.43 ± 0.15	0.52 ± 0.18[c]
F	106.5 ± 4.5[d]	0.6 ± 0.2	

[a] Wisconsin Sprague-Dawley subline.
[b] M, Male; F, female. Three animals from each group were tested.
[c] Significantly different by Mann-Whitney U test ($\alpha \leq 0.005$).
[d] Different from group 041 females ($P \leq 0.05$) by the Student *t* test.

TABLE 3. Comparison of recovery of *S. mutans* 041 and Ldh⁻ mutant 044 from gnotobiotic rats

Strain and medium[a]	Log counts per:			
	Oral swab at day:			Feces[b] (g) at day 35
	7	21	35	
S. mutans 041				
MS	5.23 ± 5.02	5.77 ± 5.40	6.11 ± 5.84	10.43 ± 10.15
TSB	5.24 ± 4.92	5.75 ± 5.47	6.13 ± 5.87	10.44 ± 10.04
TTC	5.09 ± 4.76	5.76 ± 5.12	6.12 ± 5.90	10.45 ± 10.12
S. mutans 044				
MS	4.66 ± 4.40	5.76 ± 5.49	5.90 ± 5.67	10.23 ± 9.72
TSB	4.75 ± 4.54	5.73 ± 5.47	5.92 ± 5.82	10.30 ± 9.85
TTC	4.72 ± 4.54	5.79 ± 5.58	5.91 ± 5.80	10.20 ± 9.80

[a] TSB, Trypticase soy blood agar; TTC, triphenyltetrazolium chloride agar.
[b] Recovery determined as log counts per gram of feces ($n = 6$).

≤ 0.2). On the TTC plates, all of the colonies from samples of animals infected with Ldh⁻ strain 044 were typically red, indicating that no revertants to Ldh⁺ occurred. Comparable results occurred in experiment 1 (data not shown). Samples from animals infected with wild-type strains 041 and PS-14 yielded only white colonies on TTC medium, with a single exception. That was the appearance of two red colonies in a 21-day oral sample of a rat infected with Ldh⁺ strain 041. A subculture of one of these colonies, designated 447-11, was a low acid producer and reacted strongly with fluorescein-labeled type c antiserum. Its DNA restriction endonuclease digest pattern was identical to strains 041 and 044 (data not shown). In addition, as described below, it possessed a bacteriocin pattern similar to patterns in those strains. We consider it to be a spontaneous Ldh⁻ mutant of strain 041.

The results of tests for bacteriocinlike activity in strains 041, 044, and the spontaneous Ldh⁻ mutant 447-11 are given in Table 4. Zones of complete or partial inhibition were found for *S. mutans* of serotypes c and e but not for f. Among other mutans streptococci, a human strain of *S. cricetus* (serotype a) was inhibited while a hamster isolate, *S. rattus* (b), and *S. sobrinus* (d and g) were not. The human oral strains of *S. mitior*, *S. sanguis*, and *S. salivarius* tested were unaffected.

TABLE 4. Bacteriocin activity of *S. mutans* 041 Ldh⁺ and its Ldh⁻ mutants against human oral streptococci[a]

Indicator strain (serotype)	Activity[b] found in producer strain:		
	041	044	447-11
S. cricetus (a)			
AHT	+	+	NT[c]
HS-6 (hamster)	−	−	−
S. rattus (b) BHT	−	−	−
S. mutans (c)			
10449	±	+	NT
VA-29	+	+	+
PS-14	±	+	+
DC 34-2	±	±	NT
S. mutans (e)			
B-2	+	+	NT
LM-7	+	+	NT
S. mutans (f) QP 50-1	−	−	−
S. sorinus (d and g) 6715	−	−	−

[a] No activity was found in *S. mitior* CHT, *S. sanguis* M5, and *S. salivarius* HHT.
[b] Results are shown as complete (+), partial (±), or no (−) inhibition.
[c] NT, Not tested.

DISCUSSION

The concept of microbial replacement therapy for dental caries is predicated on the use of an avirulent strain of cariogenic streptococci to preempt or supplant its pathogenic counterpart in vivo. Of the possible metabolic factors that contribute to the virulence of the mutans streptococci, the one most directly linked to caries initiation is the production of lactic acid from simple dietary carbohydrates. The greater cariogenic potential of mutans streptococci over other more numerous acidogenic microorganisms that commonly colonize the dentition has been related to their ability to produce lactic acid at pH levels which inhibit the metabolism of other plaque microorganisms (13). Under conditions of high or frequent sugar intake, this can lead to destruction of the tooth surface. Lactic acid is the strongest of the metabolic acid end products of oral microorganisms (pK_a 3.8), and its production is catalyzed by Ldh. Hillman (5) hypothesized that Ldh⁻ mutans streptococci would have reduced cariogenic potential and that they could be useful effector strains for replacement therapy of caries. The concept was verified experimentally in rats by using Ldh⁻ mutants of a strain of mutans streptococci originally derived from rats and now known as *S. rattus* serotype b (8, 9).

Of the various species and serotypes of mutans streptococci, *S. mutans* serotype c is by far the most prevalent in the human oral cavity and the type most frequently associated with the initiation of caries (13, 16). Although the reasons for the selective colonization of humans by *S. mutans* serotype c are unknown, the fact of its existence suggests that Ldh⁻ mutants of this type may more effectively colonize the human dentition than those types of mutans streptococci that occur less frequently.

In this study, we have examined some of the properties in vivo of Ldh⁻ mutant 044, which was derived from human strain 041 of *S. mutans* serotype c. The gnotobiotic rat caries model employed is a highly sensitive indicator of the caries activity of individual organisms. In addition, because of the virtually unrestricted growth of the infecting organisms, it is a useful system to detect the emergence of mutants or back mutations in vivo (3). Under the test conditions employed, using two different sublines of Sprague-Dawley rats for separate experiments, Ldh⁻ mutant 044 produced significantly fewer instances of caries than did its parent strain 041. Moreover, there was no evidence of back mutation to Ldh⁺ in vivo on TTC plates of oral swabs or fecal samples in either experiment. These findings do not exclude the possibility of back mutation of strain 044 but, taken in conjunction with earlier evidence of its stability for 12 weeks on daily transfer, the indications are that the Ldh⁻ mutant is not likely to back mutate readily in more complex microbial ecosystems (1, 3).

Recoveries of Ldh⁻ strain 044 from oral swabs at the initial (7-day) sampling period were generally lower than in comparable samples from animals infected with the wild-type strains. Although consistent, these differences were not statistically significant. Despite the acknowledged uncertainties in quantitating oral microbial counts in rodents, our findings clearly indicate that the Ldh⁻ mutant implanted readily and persisted as well as its wild-type parent in the gnotobiotic test system. Whether it will be able to establish itself in the mixed microbial flora of conventional animals or humans remains to be determined. One factor that could

facilitate this would be bacteriocin production. Both strain 044 and its parent 041 were shown to possess bacteriocinlike activity in vitro against other human *S. mutans* strains of serotype c and e. *S. mutans* serotype f was not inhibited, nor was a strain of *S. sobrinus* serotypes d and g. Interestingly, a human isolate of *S. cricetus* was inhibited while a hamster-derived strain was not (Table 4). Single strains representing the most numerous nonmutans oral streptococci, *S. mitior*, *S. sanguis*, and *S. salivarius*, were not inhibited. If Ldh^- mutant 044 can be shown to possess a similar spectrum of activity in vivo, its suitability for replacement therapy of caries would be enhanced considerably by the ability to suppress indigenous strains of *S. mutans* serotype c without disturbing the normal balance of the more inocuous oral streptococci.

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