Aldolase of Lactic Acid Bacteria: Immunological Relationships Among Aldolases of Streptococci and Gram-Positive Nonsporeforming Anaerobes

JACK LONDON, NINA M. CHACE, AND KIMBERLY KLINE

National Institute of Dental Research, Bethesda, Maryland 20014

Antiserum prepared against a *Streptococcus faecalis* fructose diphosphate aldolase (EC 4.1.2.13) was used to measure the extent of immunological homology between the reference enzyme and aldolases of various streptococci and gram-positive nonsporeforming anaerobic bacteria. The majority of streptococci surveyed were isolated from the oral cavity or blood samples. Strains within the species *Streptococcus mutans* Clarke and *Streptococcus mitis* (mitior) possessed aldolases that exhibited marked antigenic heterogeneity and thereby contrasted sharply with previously studied species of *Streptococcus* (London and Kline, 1973). Varying degrees of cross-reactivity were observed between the aldolases of certain species of *Eubacterium*, *Butyribacterium*, and *Propionibacterium* and the anti-streptococcal aldolase serum. Evidence is also presented confirming an earlier report by Neimark (1974) that aldolases of *Acholeplasma* species react with the anti-*S. faecalis* aldolase serum.

In a previous study, the fructose diphosphate (FDP) aldolase (EC 4.1.2.13) of *Streptococcus faecalis* ATCC 27792 (formerly strain MR) was used as a reference point to establish evolutionary relationships among various homofermentative lactic acid bacteria by determining the degree of immunological reactivity between their aldolases and a specific anti-*S. faecalis* aldolase serum (18). Despite significant differences in the quaternary arrangement of the subunits of this enzyme (17), their primary structure was sufficiently conserved to permit a comparison of their antigenic determinants. A preliminary phylogenetic map of streptococci, lactobacilli, and pediococci was constructed from both qualitative and quantitative immunological data (18).

In the present study, the streptococcal branch of the phylogenetic map is confirmed and extended with new immunological data for aldolases of previously untested strains. Also, immunological heterogeneity has been detected among the aldolases from strains of streptococci within the species *Streptococcus mutans* and *Streptococcus mitis* (mitior). The data indicate that neither species is homogeneous. A strong reaction between the aldolase of *Butyribacterium rettgeri* and anti-*S. faecalis* aldolase serum prompted the survey reported here for other immunologically related aldolases among gram-positive nonsporeforming anaerobic bacteria. The survey includes organisms belonging to the genera *Eubacterium*, *Propionibacterium*, and *Arachnia*.

MATERIALS AND METHODS

Organisms. The strain designations of the organisms are listed in Table 1. With the exception of strains of *S. mutans* and *S. mitis* (mitior), all cultures of streptococci used in this study were a gift of Richard Facklam (Center for Disease Control); strains of *S. mutans* and *S. mitis* (mitior) were obtained from the National Institute of Dental Research Collection and the American Type Culture Collection (ATCC), respectively. Strains of *Eubacterium cylindroides* were kindly supplied by Lillian Holdeman (Virginia Polytechnic Institute); *B. rettgeri* was provided by Charles Wittenberger (National Institute of Dental Research). All other gram-positive nonsporeforming bacteria and *Acholeplasma* strains were obtained from the ATCC.

Maintenance and cultivation of the microorganisms. The streptococci were all maintained on N.I.H. thioglycolate broth (Difco Laboratories, Detroit, Mich.). For enzymological and immunological studies, 2 liters of complex streptococcal medium (16) was inoculated with 10 ml of a 12-h-old culture of the appropriate organism and incubated at 37 C for 18 h. Cells were harvested by centrifugation and washed twice with cold 0.05 M potassium phosphate buffer, pH 7.0. Washed cell pellets were stored at -40 C until needed.

Strains of *Eubacterium*, *Butyribacterium*, and *Arachnia* were maintained and cultivated in the prereduced peptone-yeast extract medium of Holdeman and Moore (12) supplemented with 1% (wt/vol) glucose. Two-liter batch cultures were grown from a 5% inoculum in volumetric flasks fitted with pyrogallol-K₂CO₃ seals to maintain anaerobiosis at 30 C for 24 to 48 h and processed as described above.

The propionibacteria were cultured at 30 C in a medium described by van Niel (thesis, Der Tech-

TABLE 1. Strain designations of organisms

<i>Streptococcus</i>	<i>Propionibacterium</i>
<i>S. avium</i> (group Q) ^a SS-559	<i>P. acnes</i> ATCC 6919
<i>S. bovis</i> DS-25-72	<i>P. freudenreichii</i> ATCC 6207
<i>S. equinus</i> DS-1908-71	<i>P. arabinosum</i> ATCC 4965
<i>S. faecalis</i> subsp. <i>liquefaciens</i> PS-853-69	<i>P. intermedium</i> ATCC 14072
<i>S. faecalis</i> subsp. <i>liquefaciens</i> PB-1733-71	<i>P. jensenii</i> ATCC 4867, 4868
<i>S. mitis</i> ATCC 9895	<i>P. pentosaceum</i> ATCC 4875
<i>S. mitis</i> ATCC 15909	<i>P. peterssonii</i> ATCC 4870
<i>S. mitis</i> ATCC 15910	<i>P. rubrum</i> ATCC 4871
<i>S. mitis</i> ATCC 15911	<i>P. thoenii</i> ATCC 4874
<i>S. mitis</i> ATCC 15912	<i>P. zeae</i> ATCC 4964
<i>S. mitis</i> ATCC 15914	
<i>S. mutans</i> SL-1 (d) ^b	<i>Eubacterium</i>
<i>S. mutans</i> Bergergren (d) ^b	<i>E. limosum</i> ATCC 8486
<i>S. mutans</i> 6715 (d) ^b	<i>E. cylindroides</i> 3594, 3696
<i>S. mutans</i> 01H1 (d) ^b	<i>E. aerofaciens</i> ATCC 25986
<i>S. mutans</i> 10449 (c) ^b	
<i>S. mutans</i> JC-2 (c) ^b	<i>Acholeplasma</i>
<i>S. mutans</i> BHT (b) ^b	<i>A. granulareum</i> ATCC 19168
<i>S. mutans</i> FA1 (b) ^b	<i>A. laidlawii</i> ATCC 14089, 25937
<i>S. mutans</i> E49 (a) ^b	<i>A. xanthum</i> ATCC 25176
<i>S. sanguis</i> "Wicky" SS-979	
<i>S. sanguis</i> "Channon" SS-982	<i>Listeria</i>
<i>S. sanguis</i> "Blackburn" SS-983	<i>L. grayi</i> ATCC 25400
<i>S. sanguis</i> (F90A) SS-546	<i>L. monocytogenes</i> ATCC 15313, 19111
<i>S. sanguis</i> (K208) SS-547	<i>L. murrayi</i> ATCC 25401
<i>Streptococcus</i> sp. (group K) ^a SS-665	
<i>Streptococcus</i> sp. (group P) ^a SS-558	<i>Others</i>
<i>Streptococcus</i> sp. (group S) ^a SS-981	<i>Arachnia propionica</i> 14157
<i>Streptococcus</i> sp. (group T) ^a SS-840	<i>Butyribacterium rettgeri</i> ATCC 10825
<i>Streptococcus</i> sp. (group U) ^a SS-740	<i>Staphylococcus epidermidis</i> ATCC
<i>S. zooepidemicus</i> SS-259	155, 14852, 12228

^a Lancefield group antigen.^b Bratthall serotype; the serotype of *S. mutans* strain Bergergren was serotyped by George Hageage (personal communication).

nische Hooge School, Delft, The Netherlands, 1928) modified to contain yeast extract, 1% (wt/vol), tryptone, 1% (wt/vol), and glucose added as a sterile solution to a final concentration of 1%; the pH was adjusted to 7.2 before sterilization. Strictly anaerobic conditions were not necessary for the cultivation of this group of bacteria. Inoculated screw-cap test tubes filled with medium almost to the brim produced copious growth. Batch cultures were grown in 1-liter screw-cap bottles filled to the neck with medium after inoculation with 10 ml of the appropriate organism. After 24 h of incubation, the cultures were harvested by centrifugation and treated as above.

Strains of *Listeria*, *Staphylococcus*, and *Acholeplasma* were cultured in media 44, 3, and 243, respectively, as described (1).

Preparation of cell-free extracts. Approximately 4 g (wet weight) of cell paste was suspended in 10 ml of 2,2-bis(hydroxymethyl)-2',2',2'-nitrilotriethanol buffer, pH 6.5, containing 10 mM β -mercaptoethanol (BTME) and subjected to ultrasonic disruption in a Branson model 185 sonifier. Operating at 80% of maximum power, cell suspensions of streptococci were treated for 15 min, and other gram-positive non-sporeforming bacteria and *acholeplasma* were treated

for 8 min. All operations were carried out at 8 C. The crude extracts were centrifuged at 30,000 $\times g$ for 30 min and the clarified supernatant fluids were decanted. Glycerol was added to all cell-free preparations to a final concentration of 20% (vol/vol), and the crude FDP aldolase preparations were stored at -40 C until used.

Biochemical determinations. The molecular weights of the various aldolases were estimated from their gel filtration profiles on a Sephadex G-200 column (2.5 by 95 cm) that had been calibrated with ribonuclease, chymotrypsinogen A, ovalbumin, bovine serum albumin, rabbit muscle aldolase, and human 7S gamma globulin according to the procedure of Andrews (2). After the application of 10 to 20 mg of extract protein, the aldolase activity was eluted from the column with BTME buffer containing 0.1 M KCl by using the upward-flow technique. The procedures required to obtain accurate and reproducible protein values for crude extracts are described elsewhere (17).

Electrophoretic mobility coefficients of the aldolases were determined on 7.5% polyacrylamide gel columns. The methods used to prepare the gel columns and the detection of aldolase activity on the columns have been described elsewhere (18).

Aldolase activity in cell-free extracts was determined spectrophotometrically according to the procedure of Groves et al. (10). A unit of enzymatic activity is reported as micromoles of FDP cleaved per minute per milligram of protein.

Immunological procedures. The purification of the reference antigen, *S. faecalis* ATCC 27792 FDP aldolase, and the preparation of the anti-aldolase serum have been previously described (18).

Immunodiffusion experiments were performed by using the Stollar and Levine modification (26) of the Ouchterlony double-diffusion technique. Sample wells contained between 0.2 and 0.8 units of aldolase activity, and the center well was charged with sufficient antiserum to produce a sharp precipitin line. The results of immunodiffusion experiments are summarized by using the convention of Gasser and Gasser (9). Discussions dealing with the interpretation of these results have been published elsewhere (9, 18) and will not be repeated here.

Micro-complement fixation experiments were carried out according to the procedure of Wasserman and Levine (27); minor modifications of this technique appear elsewhere (19). Guinea pig complement was standardized by the method of Hook and Muschel (13). Anti-aldolase sera were used at a dilution of 1:75,000 or 1:90,000 with the homologous antigen. Homologous antigen in crude extracts of *S. faecalis* ATCC 27792 was used in the range of 0.8 to 8 μ g of protein, whereas heterologous antigens were used in concentration ranges as great as 4 to 40 μ g of protein. Since the comparative amounts of complement fixed by both homologous and heterologous antigens were linear and parallel functions of the logarithm of the antibody concentration (23), data from these experiments were directly comparable. From such data, the index of dissimilarity (ID) was calculated. In those instances where the percentage of complement fixed by the heterologous (het) system was essentially equal to the percentage of complement fixed by the homologous (hom) system ($\pm 10\%$), the ID was calculated by the following equation:

$$\frac{\text{AB dilution}_{\text{hom}}}{\text{AB dilution}_{\text{het}}} \times \frac{\% \text{ complement fixed}_{\text{hom}}}{\% \text{ complement fixed}_{\text{het}}}$$

The ID values were also calculated according to the equation of Champion et al. (4); for the aldolase system, the term $m = 178$. The ID values obtained by the two equations were comparable to one another only when the percentage of complement fixed by the homologous system was nearly equal to that of the heterologous system. In those instances where it was not possible to raise the antibody concentration sufficiently to make the two values nearly equal, the equation of Champion et al. (4) was used to calculate the ID value. To facilitate a comparison of data from the aldolase study with immunological surveys of other microbial proteins, results of the micro-complement fixation experiments are also expressed as immunological distance units (21) where the immunological distance = $\log \text{ID} \times 100$.

Results of micro-complement fixation and immunodiffusion studies were corroborated with antibody inhibition experiments. Since the anti-aldolase serum

contained low but detectable levels of rabbit aldolase, the serum was heated at 56 C for 30 min to destroy all residual enzyme activity. One to 40 μ l of heated antiserum was added to the previously described enzyme assay mixture (10) and, after a 5-min incubation at ambient temperature, the reaction was started with substrate. Any interaction between aldolase and anti-aldolase serum generally produced an inhibition of enzymatic activity. Such inhibition is indicative of shared structural homology between the heterologous and homologous antigen (enzyme). Serum obtained from the rabbits before immunization served as a control to insure that the enzyme inhibition was specifically induced by the antibodies against the aldolase.

RESULTS

Biochemical characteristics of aldolase from the gram-positive nonsporeforming anaerobes. In a previous study of the immunologically related aldolases from the lactic acid bacteria, the five groups of aldolase of differing molecular weight were shown to be a manifestation of the five subunit aggregation patterns, which range from dimer to hexamer (17). Although there is only one form of the enzyme within a species, two or three forms have been observed within a genus. Three of the five forms of aldolase have been found among the anaerobes examined in the present study (Table 2). The three forms of the aldolase are found as follows: (i) the relatively large enzyme found in all *Propionibacterium* species and in *Arachnia propionica*, whose estimated molecular weight

TABLE 2. Physical characteristics of aldolases from gram-positive nonsporeforming anaerobes

Organism	Estimated mol wt of aldolase	R_f of aldolase
<i>Eubacterium aerofaciens</i>	56,000	0.78
<i>E. limosum</i>	61,000	0.75
<i>E. cylindroides</i> 3696	60,500	0.69
<i>E. cylindroides</i> 3594	61,000	0.69
<i>Butyribacterium rettgeri</i>	116,000	0.56
<i>Propionibacterium jensenii</i>		
ATCC 4867	170,000	0.36
<i>P. freudenreichii</i>	164,000	0.36
<i>P. intermedium</i>	180,000	0.34
<i>P. petterssonii</i>	200,000	0.33
<i>P. zeae</i>	164,000	0.33
<i>P. rubrum</i>	176,000	0.33
<i>P. acnes</i>	200,000	0.32
<i>P. thoenii</i>	176,000	0.32
<i>P. arabinosum</i>	200,000	0.32
<i>P. pentosaceum</i>	180,000	0.32
<i>Arachnia propionica</i>	164,000	0.34

ranges from 164,000 to 200,000 and which resembles the hexameric form found in most species of pediococci (17); (ii) the 60,000-molecular-weight enzyme found in *Eubacterium* species, which is similar to the aldolase of streptococci; and (iii) the *B. rettgeri* enzyme with a molecular weight of 116,000, whose size is comparable to that of the aldolase found in most lactobacilli.

A classification of these enzymes according to their electrophoretic mobility rates in polyacrylamide gels also produced three distinct divisions (Table 2). This is perhaps not too surprising since the gels act, in part, as molecular sieves and separate these proteins according to their size as well as their net charge. However, this procedure provides a convenient means of distinguishing between three of the four genera studied. Aldolases from species of *Eubacterium* migrated most rapidly, followed by the *B. rettgeri* aldolase. The aldolases of propionibacteria were the least mobile in anionic gels.

Immunological differentiation of the aldolases from gram-positive nonsporeforming anaerobes. The aldolases of the anaerobes were arranged in the order of their relatedness to the *S. faecalis* reference enzyme by double-diffusion experiments. Results of a number of multiple cross-matches are summarized in Fig. 1. The *B. rettgeri* aldolase exhibits the greatest degree of immunological similarity to the reference enzyme and therefore leads the following order of decreasing antigenic similarity: *B. rettgeri* >

S. faecalis

FIG. 1. Hierarchy of immunological relatedness among aldolases of gram-positive nonsporeforming anaerobic bacteria. The arrowhead indicates the dominant antigen in the cross-match on immunodiffusion plates; a single spur is produced against the well of the less related antigen (a pattern of partial identity). The equal sign indicates that the pairwise match produced a confluent precipitate (a pattern of identity or apparent identity).

Eubacterium cylindroides > *E. limosum* = *E. aerofaciens* > *Propionibacterium* species. Although the aldolase of the 10 species of *Propionibacterium* reacted only weakly with the anti-streptococcal aldolase serum, the precipitin lines in the Ouchterlony plates were confluent with one another, indicating apparent identity. Initially the precipitin lines were concave to the sample well; however, concentrating the antigen twofold so that 2 units of enzyme activity could be conveniently added to the sample well produced arcs convex to the sample well. Extracts containing the *A. propionica* aldolase reacted very weakly with the anti-aldolase serum to produce a faint hazy precipitate. The weak cross-reaction precluded any meaningful cross-matches with aldolases from the other anaerobic bacteria.

Cross-matches between the *B. rettgeri* aldolase and the aldolases of *Streptococcus lactis*, *Pediococcus acidilactici*, *Lactobacillus xylosus*, and *Microbacterium thermosphaetum* produced double or crossed spurs (Fig. 2). The pattern of nonidentity produced by the cross-matches is indicative of antigenic divergence (9, 18) with regard to the reference protein and necessitates the creation of a new group or evolutionary line to accommodate the aldolases of the *Eubacterium*, *Butyrivacterium*, and *Propionibacterium* species.

Quantitative serology of aldolases from gram-positive nonsporeforming anaerobes. With only one exception, the results of the micro-complement fixation experiments and immunodiffusion results allow a corresponding ordering of the species. A clear conflict exists between the results of the two procedures as regards the *E. limosum* aldolase. Immunodiffusion experiments indicated that *E. limosum* aldolase is antigenically similar to the *E.*

B. rettgeri

FIG. 2. Reactions of immunological nonidentity produced by five intergeneric cross-matches on Ouchterlony plates. The X denotes the formation of double spurs when comparing the aldolases of the five bacteria with anti-*S. faecalis* serum.

TABLE 3. Results of micro-complement fixation experiments with aldolases of gram-positive, nonsporeforming anaerobes

Organism	Index of dissimilarity	Immunological distance ^a
<i>Butyribacterium rettgeri</i>	7.75 ± 1	89
<i>Eubacterium limosum</i>	8.2 ± 1	91
<i>E. cylindroides</i> (3594)	33 ± 6	152
<i>E. cylindroides</i> (3696)	34 ± 4	153
<i>E. aerofaciens</i>	108 ± 9	208
<i>Arachnia propionica</i>	>220	
<i>Propionibacterium jensenii</i> (4867)	145 ± 20	216
<i>P. jensenii</i> (4868)	148 ± 18	217
<i>P. zeae</i>	185 ± 21	226
<i>P. intermedium</i>	190 ± 12	228
<i>P. acnes</i>	191 ± 17	228
<i>P. peterssonii</i>	212 ± 14	232
<i>P. pentosaceum</i>	215 ± 12	233

^a Log ID × 100.

aerofaciens enzyme (Fig. 1), whereas micro-complement fixation data indicated that the aldolases of *E. limosum* and *B. rettgeri* are serological equivalents of one another (Table 3). The problems inherent in these observations will be discussed later.

Antibody-mediated inhibition studies. To provide additional evidence that the antibodies

prepared against the *S. faecalis* aldolase were, in fact, interacting with the immunologically distant aldolases of *A. propionica* and the propionibacteria, aldolase activity in cell-free extracts of these organisms was assayed in the presence and absence of the anti-aldolase serum. Only 2 to 4 µl of antiserum was needed to completely inhibit 0.05 units of *S. faecalis* aldolase activity, whereas 30 to 40 µl of the same antiserum was required to produce a 50% inhibition of 0.04 to 0.05 units of the aldolase activity present in extracts of *Propionibacterium pentosaceum*, *P. thoenii*, *P. freudenreichii*, *P. rubrum*, *P. arabinosum*, and *A. propionica*. The addition of 50 µl of preimmunized rabbit serum neither enhanced nor inhibited the aldolase activity in the respective extracts.

Immunological characterization of streptococcal aldolases. In the original study, 23 species of *Streptococcus* were surveyed and assigned to one of five aldolase antigen groups on the basis of their cross-reactivity in immunodiffusion experiments (18). It seemed desirable to extend the present study to include streptococci of interest to medical and dental microbiologists and to examine isolates possessing the less common Lancefield antigens. Figure 3 depicts cross-matches of 13 strains of streptococci and includes as reference points the aldolases from *S. faecalis* (antigen group I), *S. lactis* (antigen group II), *Streptococcus bovis* (antigen group III), and *Streptococcus salivarius* (anti-

FIG. 3. Results of intra- and interspecific comparisons of 13 streptococcal aldolases on immunodiffusion plates. The symbols are explained in Fig. 1.

gen group IV) categorized in the earlier work. The aldolase from a Lancefield Q-type *Streptococcus* appears to be the antigenic equivalent of the *S. faecalis* enzyme. Microorganisms possessing this antigen have already been classified as atypical enterococci, since all Q-type isolates also bear the Lancefield group D antigen (11, 25); however, the specific epithet *Streptococcus avium* has been suggested for this group of organisms because they are readily differentiated from the other group D streptococci (20). The aldolase of *Streptococcus zoepidemicus* cannot be assigned to one of the five original antigenic groupings (18) since it clearly falls between the group N streptococci (antigen group II) and *S. bovis* (antigen group III). A comparison of this aldolase and aldolases from other Lancefield group C (animal) streptococci (18) points out yet another difference; all of the other group C aldolases fall into aldolase-antigen group V. The five strains of group H streptococci produce lines of identity with one another and with the *S. bovis* aldolase, which places them in antigen group III. A Lancefield group E strain fell into aldolase antigen group III, whereas group P and S aldolases were relegated to aldolase antigen group V (Fig. 3).

Aldolases from nine strains of *S. mutans* Clarke were compared with one another and with the aldolases of *S. bovis* ATCC 9809 and *Streptococcus pyogenes* ATCC 14289. Immunodiffusion experiments segregated the aldolases into two clear and distinct antigenic groups (Fig. 4). The first group, which contains all of the Bratthall (3) serotype *d* strains, produced

confluent lines of precipitation with *S. bovis* ATCC 9809 (antigen group III). In sharp contrast, the aldolases from Bratthall serotype *a*, *b*, and *c* strains produced a pattern of apparent identity with one another and the *S. pyogenes* aldolase (antigen group V). The failure of these strains to cluster within a single antigen group as the enterococci, *S. bovis*, *S. salivarius*, and *Streptococcus sanguis* did, suggests that the species *S. mutans* is composed of a heterogeneous collection of streptococci.

The same situation appears to exist within the species *S. mitis* (*mitior*). Immunodiffusion experiments with aldolases from six of the Williamson *S. mitis* serotypes uncovered three distinct antigenic groups (Fig. 5) that corresponded roughly to aldolase-antigen groups III, IV, and V. These appear in the order: ATCC 15909 = ATCC 15911 = ATCC 15912 > ATCC 15914 = ATCC 9895 > ATCC 15910.

Quantitative serology of the streptococcal aldolases. Table 4 lists the indexes of dissimilarity and immunological distances for the streptococcal aldolases as calculated from the results of micro-complement fixation experiments. The ordering of the aldolases was in complete agreement with the immunodiffusion results. However, the increased sensitivity of the former method exposes subtle divergences in the antigenic structure of the respective enzymes that are not apparent from double-diffusion experiments. For example, the aldolase of *S. faecalis* subsp. *liquefaciens*, which was essentially homologous with the reference *S. faecalis* enzyme, differed significantly from the aldolase of *S. faecalis* subsp. *liquefaciens* strain

S. bovis

FIG. 4. Results of cross-matches of nine strains of *S. mutans* in immunodiffusion experiments. The symbols are defined in Fig. 1.

S. faecalis MR

FIG. 5. Results of cross-matches of six strains of *S. mitis* (*mitior*) in immunodiffusion experiments. The symbols are explained in Fig. 1.

TABLE 4. *Indexes of dissimilarity of streptococcal aldolases*

Organism	Index of dissimilarity	Immunological distance ^a
<i>Streptococcus faecalis</i> subsp. <i>liquefaciens</i> (835-69)	1.06 ± 0.2	2.5
<i>S. faecalis</i> subsp. <i>liquefaciens</i> (1773-71)	1.51 ± 3	18
<i>S. avium</i> group Q (SS-559)	2.6 ± 0.4	42
<i>Streptococcus</i> sp. group K (SS-665)	22 ± 3	134
<i>S. zooepidemicus</i> group C (SS-259)	27 ± 3	143
<i>S. bovis</i> variant (27-72)	30 ± 5	148
<i>Streptococcus</i> sp. group U (SS-740)	49 ± 5	169
<i>Streptococcus</i> sp. group P (SS-558)	70 ± 13	184
<i>Streptococcus</i> sp. group T (SS-840)	88 ± 9	194
<i>Streptococcus</i> sp. group S (SS-981)	98 ± 7	199
<i>S. sanguis</i> strains (group H)		
"Wicky" strain (SS-975)	30 ± 4	147
Strain F90A (SS-546)	32 ± 5	150
"Channon" strain (SS-982)	35 ± 5	154
"Blackburn" strain (SS-983)	35 ± 6	154
Strain K208 (SS-547)	40 ± 4	160
<i>S. mutans</i> strains		
SL-1; (type d) ^a	35 ± 5	154
Bergergren (type d)	41 ± 2	161
6715 (type d)	42 ± 1	162
+ O1H1 (type d)	45 ± 5	165
10449 (type c)	85 ± 5	193
JC-2 (type c)	85 ± 6	193
BHT (type b)	89 ± 7	195
FA-1 (type b)	109 ± 4	204
E-49 (type a)	131 ± 7	211
<i>S. mitis</i> (<i>mitior</i>) strains		
ATCC 15909 (type I) ^b	18 ± 1	125
ATCC 15912 (type IV)	23 ± 4	136
ATCC 15911 (type III)	33 ± 2	152
ATCC 15914 (type VII)	70 ± 2	184
ATCC 9895 (type V)	80 ± 6	190
ATCC 16910 (type II)	88 ± 8	194

^a Bratthall serotypes.^b Williamson serotypes.

1773-71. *S. avium*, the group Q streptococcus, was clearly differentiable from either of the above organisms. The *S. sanguis* group may also be undergoing diversification; the aldolases of strains "Wicky," "Channon," "Blackburn," and SS-546 exhibit a slightly greater degree of similarity to the reference enzyme than the aldolases of strains SS-547 and ATCC 10556 (18). As expected, the *S. mitis* (*mitior*) aldol-

ases were very heterogeneous, ranging from an ID value of 18 to 88. Similarly, the distribution of ID values among *S. mutans* aldolases was also very large. It is especially interesting to note that the Bratthall *d* serotypes clustered as a distinct entity, whereas the *a*, *b*, and *c* serotypes were dispersed between ID values of 81 and 131. The significance of this observation will be discussed later.

In addition to the above, Table 4 also lists a number of unspicated streptococci with distinctive Lancefield group antigens; these data provide the map with loci for the group P, T, S, and U antigens. The ID value for the aldolase of a variant of *S. bovis*, strain 27-72, corresponds rather well with those of ATCC strains (18). As expected, the *S. zooepidemicus* aldolase was immunologically distinct from the other group C streptococci (18). The two strains bearing the Lancefield K antigen, SS-665 and *S. mitis* ATCC 15910, contrasted with each other in two respects. First, the aldolase of SS-665 had a significantly lower index of dissimilarity than *S. mitis* ATCC 15910, whose ID value was more consonant with those of the *S. salivarius* strains (Lancefield group K also). Second, an estimation of the size of these two aldolases disclosed that the enzyme from strain SS-665 was typical of the streptococci with an estimated molecular weight of 60,000. However, *S. mitis* ATCC 15910 possessed a 130,000-molecular-weight aldolase that appeared to be unique to strains of *S. salivarius*; it is conceivable that this organism may be a phenotypic variant of this latter species.

Cross-reactions with anti-streptococcal aldolase and aldolases from other bacteria. The aldolases from a number of species belonging to three hitherto untested genera of bacteria were surveyed for cross-reactivity with anti-streptococcal aldolase. Neimark (Abstr. Annu. Meet. Am. Soc. Microbiol., M250, p. 108, 1974) has already published a preliminary report describing the cross-reactions between the anti-*S. faecalis* aldolase and three species of *Acholeplasma*, which are in the order *Acholeplasma* *laidlawii* > *A. xanthum* > *A. granularum*. Our results confirm his observations. It was the similarity in the FDP activation mechanism of the lactate dehydrogenases found in both the streptococci (H. C. Neimark, Abstr. Int. Assoc. Microbiol., p. 13, 1973) and acholeplasmas that led to an immunological comparison of their aldolases. Therefore, when a report by Schleifer and Kocur (24) appeared recently describing an FDP-activated lactate dehydrogenase from *Staphylococcus epidermidis*, the aldolases of three strains of this organism were tested for

cross-reactivity with anti-streptococcal aldolase serum. No cross-reaction between the anti-serum and staphylococcal extracts was observed; however, the relatively low levels of aldolase activity may account for the failure to detect an antibody-antigen interaction. The anti-aldolase serum also failed to react with extracts of *Listeria murrayi*, *L. monocytogenes*, and *L. grayi*.

DISCUSSION

The cross-reactions between the anti-*S. faecalis* aldolase serum and enzyme preparations of certain gram-positive nonsporeforming anaerobes suggest that the aldolases of these organisms share a substantial degree of structural homology with aldolases of the lactic acid bacteria (18). In general, such a finding suggests that both groups of bacteria share a common ancestor. Initially, these observations appear to conflict with the general practice of classifying bacteria according to their phenotypic characteristics, which has, in the past, mandated a distinct separation of *Eubacterium*, *Butyrivacterium*, and *Propionibacterium* from genera belonging to the family *Lactobacillaceae* (12). However, the phenotypic differentiation of these two groups of organisms can be reconciled with the common progenitor hypothesis inferred from the present study if it is assumed that the rate of evolution of different species of proteins is not constant (8, 14). Hence, a highly conserved aldolase may evolve far less rapidly than many of the proteins that impart a particular biochemical phenotype to a specific group of microorganisms. This latter event is probably potentiated by environmental pressures that eventually bestow a selective advantage on that group of organisms through the acquisition of new traits.

Deoxyribonucleic acid (DNA) hybridization studies by Johnson (14) established that the nine species of propionibacteria still form a relatively uniform genetic group. The biochemical and serological data reported here support his findings. However, the immunological distance between the reference enzyme and the aldolases of the propionibacteria is so great that minor antigenic differences which might serve to differentiate the respective species cannot be detected. Like the pediococci, the propionibacteria all possess the large form of the aldolase, which presumably is an aggregate of six or more structurally conserved subunits having a molecular weight of 28,000 or 29,500 (17). In contrast to most of the other genera studied, evolutionary alterations affecting the size of the aldolase aggregate have not yet occurred.

The conflicting data that resulted from the immunological characterization of the *E. limosum* aldolase cannot be readily explained, since it is a general practice in our laboratory to use micro-complement fixation assays to confirm and amplify the results of immunodiffusion experiments. Although the micro-complement fixation assay does not directly measure the interaction of antigen and antibody, the complications that usually manifest themselves as "anti-complementary activity" were not apparent in the antigen and antibody controls. This problem is particularly annoying because an unambiguous result would have been useful in resolving the taxonomic status of *B. rettgeri* and *E. limosum*. Holdeman and Moore (12) have suggested that *B. rettgeri* and *E. limosum* are the same organism. Although the micro-complement fixation data support this position, the immunodiffusion data do not. Until more definitive serological evidence is available, the differences in the molecular weights and electrophoretic mobility coefficients of the two aldolases can be used to build a persuasive case for the separation of the two organisms as distinct taxonomic entities. Current studies with another protein marker should resolve this question.

E. cylindroides and *E. aerofaciens*, like *E. limosum*, also possess a 60,000-molecular-weight aldolase. Although the size of these enzymes closely approximates that found in the majority of the streptococci, it remains to be seen whether the aldolases of these three anaerobes are dimeric aggregates of the smaller 28,000-molecular-weight streptococcal subunit or the larger 29,500-molecular-weight subunit found in lactobacilli and pediococci (17).

The findings discussed above make it necessary to enlarge the phylogenetic map published earlier (18) to accommodate the gram-positive nonsporeforming anaerobic bacteria. A modified map that integrates the new data on the gram-positive nonsporeforming anaerobic bacteria and presents a more precise alignment of the other related microorganisms based on a comparative study with anti-*Pediococcus cerevisiae* aldolase sera will be published in the near future.

In the original streptococcal aldolase study, strains of the various species tested were assigned to one of five distinct antigen groups by immunodiffusion cross-match (18). At that time, it was thought that the aldolase of *Streptococcus equisimilis* ATCC 9598 might serve as the nidus for the sixth and most immunologically distant group. The creation of yet another antigenic group or subgroup may be warranted

for the placement of the *S. zooepidemicus* aldolase, which appears to be intermediate to group II, the group N streptococci, and group III, the *S. bovis* cluster. The correlation between the aldolase antigen grouping and the Lancefield serotypes is good for some species and poor for others. For example, the Lancefield group D, H, and N streptococci all cluster rather nicely (Fig. 6); exceptions have been cited for the Lancefield group C, K, and E streptococci.

The putative species *S. mutans* Clarke has already been shown to be composed of at least five distinct serotypes (3) and four genotypes (5). Data presented here confirm the heterogeneity of this species. The Bratthall *d* serotypes appear as an evolutionarily distinct group as compared to the *a*, *b*, and *c* serotypes (Fig. 6). Whereas the former cluster among the *S. bovis* and *S. sanguis* strains, the latter are located among and beyond the pyogenic streptococci. It is clear from the cell wall serology, DNA homology, and protein relatedness studies that *S. mutans* is presently populated by a number of divergent groups of organisms that share a significant number of unique phenotypic characteristics (6, 7). This paradox may, in part, result from selective pressures acting at two different levels of complexity. Genotypic differences are probably the result of normal mutational events that tend to produce divergence within taxons. However, the highly specialized environment in which these microorganisms must live and compete may impose very stringent conditions upon them that give rise to isologous sets of enzymes and, in turn, produce a homogeneous or convergent phenotype. Gasser and Gasser described a similar situation among the lactobacilli (9). It is apparent, there-

fore, that in some instances the taxonomic differences arising from an attempt to use both phenotypic and genotypic characters to define a species such as *S. mutans* are irreconcilable. *S. mitis* (*mitior*) also appears to be a heterogeneous collection of streptococci. Although DNA pairing data are not yet available, cell wall serology (28) and aldolase similarity studies indicate extreme heterogeneity. The members of this species are spread over much of the aldolase map (Fig. 6).

In addition to accommodating the gram-positive nonsporeforming anaerobes, the relatedness map will also include members of *Mycoplasmatales*. Several species of *Acholeplasma* appear to be closely related to the streptococci (Neimark, Abstr. Int. Assoc. Microbiol., p. 13, 1973; Abstr. Annu. Meet. Am. Soc. Microbiol., M250, p. 108, 1974). The failure to detect cross-reactions between anti-*S. faecalis* aldolase and extracts of *Staphylococcus epidermidis* and three species of *Listeria* does not exclude the possibility that the respective aldolases share some primary sequence homology, since the serological techniques used in these studies can only detect relatively high levels of similarity (22). Finally, it is worth reemphasizing a point made earlier (18). The positions of the various species of bacteria in the aldolase-based phenetic maps are a function of the enzymes' rate of evolutionary change. If another enzyme with the same rate of primary sequence substitution as aldolase were selected as a reference protein, the map positions of the various species of lactic acid would probably remain the same. However, the use of an enzyme whose primary structure is less conserved than that of the aldolase as a reference

FIG. 6. *Streptococcus* branch of the proposed phylogenetic map based on antigenic similarities of the FDP aldolase. The lower arm simply shows the position of other organisms in that section of the branch.

protein would be expected to produce a greater degree of evolutionary divergence and differentiation in a map comprised of the same group of bacteria. In contrast, a more conserved protein would compress and blur the antigenic distinctions observed in the aldolase study and thereby establish closer evolutionary ties within this group of bacteria. A complete appreciation of the phylogenetic relationships between the various species of lactic acid bacteria will not be realized until a number of proteins representing a spectrum of amino acid substitution rates are compared.

REPRINT REQUESTS

Address reprint requests to: Dr. Jack London, Laboratory of Microbiology and Immunology, National Institute of Dental Research, National Institutes of Health, Bethesda, Md. 20014.

LITERATURE CITED

1. American Type Culture Collection. 1974. Catalogue of strains, 11th ed. American Type Culture Collection, Rockville, Md.
2. Andrews, P. 1964. Estimation of the molecular weight of proteins by Sephadex gel filtration. *Biochem. J.* **91**:222-233.
3. Bratthall, D. 1970. Demonstration of five serological groups of streptococcal strains resembling *Streptococcus mutans*. *Odontol. Revy* **21**:143-152.
4. Champion, A. B., E. M. Prager, D. Wachter, and A. C. Wilson. 1974. Microcomplement fixation, p. 397-416. In C. A. Wright (ed.), *Biochemical and immunological taxonomy of animals and plants*. Academic Press Inc., London.
5. Coykendall, A. L. 1971. Genetic heterogeneity in *Streptococcus mutans*. *J. Bacteriol.* **106**:192-196.
6. Edwardsson, S. 1968. Characteristics of caries inducing human streptococci resembling *Streptococcus mutans*. *Arch. Oral Biol.* **13**:637-646.
7. Fitzgerald, R. J. 1968. Plaque and caries. *Alabama J. Med. Sci.* **5**:239-246.
8. Florkin, M. 1966. A molecular approach to phylogeny. Elsevier Publishing Co., Amsterdam.
9. Gasser, F., and C. Gasser. 1971. Immunological relationship among lactic dehydrogenases in the genera *Lactobacillus* and *Leuconostoc*. *J. Bacteriol.* **106**:113-125.
10. Groves, W. E., J. Calder, and W. J. Rutter. Fructose diphosphate aldolase. II. *Clostridium perfringens*, p. 486-491. In S. P. Colowick and N. O. Kaplan (ed.), *Methods in enzymology*, vol. 9. Academic Press Inc., New York.
11. Guthof, O. 1955. Über eine Neue Serologische Gruppe Q. *Zentralbl. Bakteriol. Parasitenk. Infektionskr. Hyg. Abt. 1 Orig.* **164**:60-69.
12. Holdeman, L. V., and W. E. C. Moore. *Anaerobe laboratory manual*. VPI Anaerobe Laboratory, Blacksburg, Va.
13. Hook, W. A., and L. H. Muschel. 1964. Anticomplementary effect and complement activity of human sera. *Proc. Soc. Exp. Biol. Med.* **117**:292-297.
14. Johnson, J. L. 1973. Use of nucleic acid homologies in the taxonomy of anaerobic bacteria. *Int. J. Syst. Bacteriol.* **23**:308-315.
15. King, J. L., and T. H. Jukes. 1969. Non-Darwinian evolution. *Science* **164**:788-798.
16. London, J. 1968. Regulation and function of lactate oxidation in *Streptococcus faecium*. *J. Bacteriol.* **95**:1380-1387.
17. London, J. 1974. Variations in the quaternary structure of three lactic bacteria aldolases. Evidence for the existence of a class I and class II aldolase in *Lactobacillus casei*. *J. Biol. Chem.* **249**:7977-7983.
18. London, J., and K. Kline. 1973. Aldolase of lactic acid bacteria: a case history in the use of an enzyme as an evolutionary marker. *Bacteriol. Rev.* **37**:453-478.
19. London, J., E. Y. Meyer, and S. R. Kulczyk. 1971. Detection of relationships between *Streptococcus faecalis* and *Lactobacillus casei* by immunological studies with two forms of malic enzyme. *J. Bacteriol.* **108**:196-201.
20. Nowlan, S. S., and R. H. Deibel. 1967. Group Q streptococci. I. Ecology, serology, physiology, and relationship to established enterococci. *J. Bacteriol.* **94**:291-296.
21. Prager, E. M., and A. C. Wilson. 1971. The dependence of immunological cross-reactivity upon sequence resemblance among lysozymes. I. Microcomplement fixation studies. *J. Biol. Chem.* **246**:5798-5989.
22. Prager, E. M., and A. C. Wilson. 1971. The dependence of immunological cross-reactivity upon sequence resemblance among lysozymes. II. Comparison of precipitin and micro-complement fixation results. *J. Biol. Chem.* **246**:7010-7017.
23. Sarich, V. M., and A. C. Wilson. 1966. Quantitative immunochemistry and evolution of primate albumins: microcomplement fixation. *Science* **154**:1563-1566.
24. Schleifer, K. H., and M. Kocur. 1973. Classification of staphylococci based on chemical and biochemical properties. *Arch. Microbiol.* **93**:65-85.
25. Smith, D. G., and P. M. F. Shattock. 1964. The cellular location of antigens of Group D, N and Q. *J. Gen. Microbiol.* **34**:165-175.
26. Stollar, D., and L. Levine. 1963. Two-dimensional immunodiffusion, p. 848-854. In S. P. Colowick and N. O. Kaplan (ed.), *Methods in enzymology*, vol. 6. Academic Press Inc., New York.
27. Wasserman, E., and L. Levine. 1961. Quantitative microcomplement fixation and its use in the study of antigenic structure by specific antigen-antibody inhibition. *J. Immunol.* **87**:290-295.
28. Williamson, C. K. 1964. Serological classification of viridans streptococci from the respiratory tract of man. In C. A. Leone (ed.), *Taxonomic biochemistry and serology*, p. 607-622. Ronald Press Co., New York.