Taxonomic Studies on Some Group D Streptococci

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Biochemical, menaquinone, fatty acid and DNA analyses were conducted on a number of streptococci of serological group D. The results indicate that S. faecalis, S. faecium, S. casseliflavus and taxa previously designated 'S. avium', 'S. durans' and 'S. faecalis var. malodoratus' are distinct species. Strains previously labelled 'S. faecium var. mobilis' were shown to be identical with S. casseliflavus. The results also indicate that some group D streptococci recently isolated from chickens constitute a new species.

INTRODUCTION

In his classical study, Sherman (1937) classified Streptococcus faecalis and 'S. durans' in his 'enterococcus' division of the streptococci. Streptococcus faecium (Orla-Jensen, 1919) was not designated a separate species because Sherman (1937) considered it to be identical with S. faecalis. The physiologically different taxa, S. bovis and S. equinus were placed in the 'viridans' division (Sherman, 1937). The demonstration of the group D antigen in all of these species (Lancefield, 1933; Shattock, 1949; Skadhauge, 1950; Smith & Shattock, 1962) resulted in disagreement regarding the relationship of S. bovis and S. equinus to the enterococci (see Jones, 1978).

It is not only the relationship of S. bovis and S. equinus to the 'enterococcus' division which is equivocal. The intra- and interspecific relationships of S. faecalis, S. faecium and 'S. durans' are unclear. There is still disagreement about the status of strains of S. faecalis which are haemolytic and/or possess the ability to liquefy gelatin. Deibel & Seeley (1974) divided the species into 'S. faecalis subsp. faecalis', 'S. faecalis subsp. liquefaciens' (non-haemolytic, gelatin liquefied), and 'S. faecalis subsp. zymogenes' (β-haemolytic, gelatin may or not be liquefied). Deibel (1964) and Jones et al. (1972) have cast doubt upon the validity of these subspecies. Streptococcus faecium is now recognized as a well-defined taxon distinct from S. faecalis (see Deibel & Seeley, 1974). It is also generally agreed (see Jones, 1978) that strains previously called 'S. durans' (Sherman & Wing, 1937) should be classified within the species S. faecium. There are, however, a number of reports in the literature of streptococci which cannot be allocated unequivocally to S. faecalis or S. faecium. These include strains isolated by Pette (1955) from Gouda cheese and named by him 'S. faecalis var. malodoratus'. A numerical phenetic study by Jones et al. (1972) which included two strains of 'S. faecalis var. malodoratus' indicated that they were more closely related to S. faecium than to S. faecalis. Other strains possibly related to S. faecium include some motile strains isolated from plant material by Langston et al. (1960) and named by them 'S. faecium var. mobilis'. Mundt & Graham (1968) proposed the name 'S. faecium var. casseliflavus' for a group of similar motile, yellow-pigmented streptococci isolated from vegetation. These strains have since been elevated to species status as S. casseliflavus (Vaughn et al., 1979). The relationship of S. faecalis and S. faecium to 'S. avium' also remains unclear. The species 'S. avium' was proposed by
Nowlan & Deibel (1967) to accommodate streptococci resembling *S. faecalis* and *S. faecium* first described by Guthof (1955), and designated by him streptococci of serological group Q. Although ‘*S. avium*’ was recognized in the 8th edition of *Bergey’s Manual of Determinative Bacteriology* (Deibel & Seeley, 1974), the species is not included in the Approved Lists of Bacterial Names (Skerman et al., 1980). More recently, Barnes et al. (1978) isolated from the intestines of young chickens a number of streptococci of serological group D which were apparently distinct from *S. faecalis, S. faecium* and ‘*S. avium*’.

In the present study mol % G + C, DNA–DNA hybridization, lipid and biochemical studies have been performed on a number of *S. faecalis, S. faecium, S. durans*, ‘*S. casseliflavus*’ and ‘*S. avium*’ strains in an attempt to clarify their taxonomy. In addition, a number of ‘atypical’ (e.g. ‘*S. faecium var. mobilis*, ‘*S. faecalis var. malodoratus*’ etc.) strains have also been examined to establish whether they are separate enough from *S. faecalis* and *S. faecium* to be regarded as separate species.

**METHODS**

*Strains.* The test strains shown in Table 1 were obtained from the National Collection of Dairy Organisms (NCDO), Shinfield, Reading, U.K.

**DNA preparation.** Cultures were grown in 1 l YGPB (Garvie, 1978) at 30 °C from a 0.01 % inoculum using a 7 h culture. On reaching early-stationary phase, the cells were chilled in ice water and harvested by centrifugation. DNA was isolated by the method of Garvie (1976) except that proteinase K (Boehringer) (5 mg ml⁻¹) was substituted for pronase, and only one isopropanol spooling was used. Tritium-labelled DNAs were prepared as above from cells grown in YGPB supplemented with 100 μCi (3·7 MBq) [methyl-⁻³H]thymidine (Amersham) with the exception of *Streptococcus* sp. NCDO 2311, which was grown in YGPB containing 300 μCi thymidine and 250 μCi [⁵⁻³H]uracil.

**DNA base composition determinations.** The DNA base composition was estimated by thermal denaturation in standard saline citrate, as described by Garvie (1978). DNA from *Leuconostoc mesenteroides* NCDO 768 and *Escherichia coli* K12 NCDO 1984 were used as standards.

**DNA–DNA hybridizations.** DNA–DNA hybridizations were performed under optimum conditions using the membrane-filter technique described previously (Garvie et al., 1981). Hybridizations were repeated under stringent conditions to clarify the relationship between strains where approximately 50 % homology had been found under optimum conditions. Labelled DNA was denatured at 100 °C for 10 min each time before use to ensure low binding of the DNA to blank filters.

**Cultivation for lipid studies.** Cells for lipid studies were grown in YGPB (Garvie, 1978) at 30 °C for 3 d. Cultures were checked for purity, harvested by centrifugation, washed with distilled water and freeze-dried.

**Analysis of fatty acids.** Dry cells (100 mg) were degraded by acid methanolysis (Minnikin et al., 1975) and examined by GLC. Analyses were performed on both conventional packed (2 m, OV-1 and 2 m Silar-5CP) and high-resolution capillary (25 m, OV-101) columns. The identity of individual esters was established by comparing their retention times with those of standard mixtures of straight-chain saturated, monounsaturated, anteiso- and iso-methyl branched-chain esters. The presence of unsaturated fatty acid methyl esters was confirmed by hydrogenation. The presence of cyclopropane-ring containing esters was determined by treating hydrogenated samples with bromine (Brian & Gardner, 1968).

**Analysis of isoprenoid quinones.** Isoprenoid quinones were extracted from about 250 mg dried cells (strains NCDO 2311, 2313, 2314) and purified as described previously (Collins et al., 1977). Mass spectra of the quinones were recorded on an AEI MS9 instrument using a direct insertion probe, an ionizing voltage of 70 eV, and a temperature of 200 °C.

**Biochemical tests.** The tests were performed using the API 50CH system (API Products, Basingstoke, U.K.), according to the manufacturers’ instructions. Medium employed was nutrient broth (CM1, Oxoid) containing bromocresol purple (0·002 %, w/v) as indicator. Tests were incubated at 35 °C and readings were made at 3, 24 and 48 h.

**RESULTS**

The mol % G + C contents of the test strains are shown in Table 1. *Streptococcus faecalis* (including subspp. ‘zymogenes’ and ‘liquefaciens’) strains possessed a mol % G + C range of 37·2 to 38·7 (Table 1). A similar range of 37·5 to 38·8 mol % G + C was found in *S. faecium* (including ‘*S. durans*’) strains. *Streptococcus faecium var. mobilis* and *S. casseliflavus* possessed significantly higher values of 41·8 to 43·8 mol % G + C and 42·8 to 43·5 mol % G + C, respectively (Table 1).
Table 1. Strain details and DNA base composition

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<th>Comments*</th>
<th>Mol % G + C</th>
</tr>
</thead>
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</tr>
<tr>
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<td>Isolated from dairy cheese</td>
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* NIRD, National Institute for Research in Dairying, Shinfield, Reading RG2 9AT, U.K.
Table 2. DNA homologies of *S. faecalis* group with other streptococci with optimal hybridization conditions

<table>
<thead>
<tr>
<th>Strain</th>
<th>S. faecalis</th>
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* Not estimated.

* Values >70% in bold type.

Table 3. DNA homologies of *S. faecium*/*S. durans* group and *S. casseliflavus* with other streptococci under optimum and stringent conditions

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* Not estimated.

* Values >70% in bold type.
Taxonomy of streptococci

Table 4. DNA homologies of 'S. avium' and group D streptococci from chicken intestines with other streptococci under optimal and stringent conditions

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<th>Strain</th>
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<th>Streptococcus sp.</th>
<th>'S. faecalis var. malodoratus'</th>
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* Not estimated.
* Values >50% in bold type.

'Streptococcus avium' and the chicken isolates (NCDO 2311, 2313, 2314, 2315) of Barnes et al. (1978) possessed ranges of 39·3 to 39·8 mol % G + C and 39·8 to 40·3 mol % G + C, respectively (Table 1).

The results of DNA-DNA hybridization experiments are shown in Tables 2 to 4. Seven homology groups were recovered. Group 1 consisted of strains highly related to the type strain of S. faecalis (NCDO 581) and contained strains previously designated subspp. 'liquefaciens' and 'zymogenes' (Table 2). In addition, a single strain previously labelled S. faecium (MUTK 1, NCDO 2373, Vaughn et al., 1979) shared a high homology with S. faecalis NCDO 581 (about 81%), and a low homology with the type strain of S. faecium NCDO 942 (about 23%) (Table 3). Homology group 2 consisted of those strains highly related to the type strain of S. faecium (NCDO 942) and included four strains previously designated 'S. durans' (Table 3). Three other strains designated 'S. durans' (including the type strain NCDO 596) formed a third homology group (Table 3) distinct from S. faecium strains under stringent hybridization conditions. Two strains bearing the label S. faecium (NCDO 582, 1258) showed little relatedness to any of the strains examined.

Streptococcus casseliflavus strains, together with two motile, pigmented strains previously designated 'S. faecium var. mobilis' (Langston et al., 1960) formed a fourth homology group (Table 3). Group Q streptococci ('S. avium') formed a fifth homology group (Table 4). Under optimum hybridization conditions, there was approximately 50% homology between these strains and two strains (NCDO 846, 847) isolated from Gouda cheese by Pette (1955), and named by him 'S. faecalis var. malodoratus'. Under stringent hybridization conditions, this apparent relationship between 'S. avium' and 'S. faecalis var. malodoratus' fell to about 15% homology (Table 4), indicating that the two taxa are genetically distinct. The 'group D streptococci' (NCDO 2311, 2312, 2313, 2314, 2315) isolated from the intestines of young chickens by Barnes et al. (1978) also formed a distinct homology group quite unrelated to 'S. avium' or the other streptococci examined (Table 4).

Whole-organism methanolyses of the test strains showed by TLC the presence of single spots corresponding to non-hydroxylated long-chain fatty acid methyl esters. The non-hydroxylated fatty acids were composed of predominantly straight-chain saturated, monounsaturated (cis-vaccenic acid series) and cyclopropane-ring acids (Table 5). Methyl-branched chain...
### Table 5. Percentage fatty acid composition of representative strains

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*Abbreviations for fatty acids are illustrated by the following examples: C₁₄:₀ for the straight-chain saturated tetradecanoic acid; C₁₄:₁ for the monounsaturated tetradecenoic acid; iso-C₁₇:₀ for the iso-acid 15-methylhexadecanoic acid; ΔC₁₉:₀ for cis-11,12-methylenoctadecanoic acid.
Acids were either absent, or present in only trace amounts. Quantitative fatty acid data are given in Table 5.

*Streptococcus faecalis*, *S. faecium* and ‘*S. durans*’ strains produced closely related fatty acid profiles with hexadecanoic (C\(_{16:0}\)) (about 35-40\%) and octadecenoic (C\(_{18:1}\)) (about 30-35\%) acids predominating. In addition, substantial levels of cis-11,12-methyleneoctadecanoic (ΔC\(_{19:0}\)) (about 9-12\%) acid were also present (Table 5). Strains of *S. casseliflavus*, *S. faecium var. mobilis* and the unidentified group D streptococci of Barnes et al. (1978) also possessed hexadecanoic and octadecenoic acids as their major fatty acid types. However, they differed from *S. faecalis*, *S. faecium* and ‘*S. durans*’ in containing significantly lower levels of cyclopropane-ring fatty acids. The chicken isolates of Barnes et al. (1978) possessed only about 1-5\% AC\(_{19:0}\) and strains of *S. casseliflavus* and *S. faecium var. mobilis* either completely lacked ΔC\(_{19:0}\), or possessed only trace amounts (about 0-0.4\%) (Table 5). Strains labelled ‘*S. avium*’ and ‘*S. faecalis var. malodoratus*’ differed from all of the other taxa examined in possessing tetradecanoic (C\(_{14:0}\)) (about 38-44\%) and hexadecanoic (about 31-33\%) acids as their major components. Octadecenoic acid was present in only small amounts (about 10\%) in ‘*S. avium*’ and ‘*S. faecalis var. malodoratus*’ (Table 5).

Components that co-chromatographed with vitamin K were the only respiratory quinones detected in the group D streptococci of Barnes et al. (1978). On mass spectral analysis the quinones produced a base peak at m/e 225, and a second intense peak at m/e 187, in accordance with published data for menaquinones (Collins & Jones, 1981). Peaks corresponding to molecular ions (M\(^+\)) were also observed at m/e 648 and 716 (major component in bold type), attributable to unsaturated menaquinones with 7 and 8 isoprene units (abbreviated MK-7 and MK-8) with MK-8 predominating. The results of mass spectrometry were confirmed by reverse-phase partition TLC.

The biochemical properties of representative strains of *S. faecalis*, *S. faecium*, ‘*S. durans*, *S. casseliflavus*, ‘*S. avium*’, ‘*S. faecalis var. malodoratus*’, and the isolates of Barnes et al. (1978) are presented in Table 6.

**DISCUSSION**

The species *S. faecalis* has become progressively better characterized and now forms a reasonably well-defined taxon (Jones 1978). In the 8th edition of *Bergey’s Manual of Determinative Bacteriology* (Deibel & Seeley, 1974), the species was divided into ‘*S. faecalis* subsp. *faecalis*’, ‘*S. faecalis* subsp. *liquefaciens*’ and ‘*S. faecalis* subsp. *zymogenes*’. Deibel (1964) and Jones et al. (1972) questioned the validity of these subspecies and considered all should be included in one species, *S. faecalis*. The high DNA–DNA homology values (> 70\%) between *S. faecalis* and its former subsp. ‘liquefaciens’ and ‘zymogenes’ lend weight to this view. The homology data also confirm the distinctiveness of the species *S. faecalis* and *S. faecium*. With the exception of *S. faecium MUKTI* (Vaughn et al., 1979) which showed an 81\% homology with the type strain of *S. faecalis* (NCDO 581), the taxa *S. faecalis* and *S. faecium* displayed homology values of about 20–30\%.

*Streptococcus faecium* is now considered to represent a well-defined species and it is generally agreed that strains previously called ‘*S. durans*’ (Sherman & Wing, 1937) should be classified in this species (Jones, 1978). It is worth noting that, although in the numerical phenetic study of Jones et al. (1972) *S. faecium* and ‘*S. durans*’ strains were recovered in the same cluster, they did occupy a large phenetic space. In the present study, the recovery of two DNA homology groups amongst strains designated *S. faecium* and ‘*S. durans*’ (Table 3) indicates that, despite the close phenetic similarity between them (Table 6 and see Deibel et al., 1963; Whittenbury, 1965, Jones et al., 1972), *S. faecium* and ‘*S. durans*’ are worthy of separate species status.

*Streptococcus casseliflavus* strains formed a distinct taxon on the basis of DNA–DNA homologies, thereby confirming the report of Vaughn et al. (1979). It is of interest that the motile strains of Langston et al. (1960) previously designated ‘*S. faecium var. mobilis*’ showed a high homology (about 76–97\%) with the type strain of *S. casseliflavus*, but were only distantly related to *S. faecium* (about 16–20\%) strains. This close relationship between *S. casseliflavus* and ‘*S. faecium var. mobilis*’ was supported by fatty acid analyses (Table 5). *Streptococcus casseliflavus*...
Table 6. Biochemical properties of *S. faecalis*, *S. faecium*, 'S. durans', 'S. avium', 'S. faecalis var. malodoratus', *S. casseliflavus* and the chicken isolates of Barnes et al. (1978)

All strains produced acid from: ribose, galactose, d-glucose, d-fructose, d-mannose, N-acetyl-glucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose and β-gentiobiose. All strains failed to produce acid from erythritol, d-arabinose, α-methyl-xylose, inositol, d-fucose and L-fucose. All strains were catalase-negative.

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<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D-Turanose</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1/2</td>
<td>2/6</td>
<td>+</td>
</tr>
<tr>
<td>D-Lyroxse</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>D-Tagatose</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D-Arabitol</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L-Arabitol</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gluconate</td>
<td>4/5</td>
<td>-</td>
<td>5/6</td>
<td>2/5</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2-Ceto-gluconate</td>
<td>-</td>
<td>2/6</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5-Ceto-gluconate</td>
<td>2/5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
</tbody>
</table>

+, All strains positive; -, all strains negative; 1/2, one of two strains positive etc.
and 'S. faecium var. mobilis' showed very similar fatty acid profiles (Table 5), and could be clearly
distinguished from S. faecium and 'S. durans' strains by the levels of cis-11,12-methyleneoctadeca-
canoic (ΔC19:0) acid present. Streptococcus faecium and 'S. durans' strains possessed significant
amounts of ΔC19:0 (about 10\%), whereas strains of S. casseliflavus and 'S. faecium var. mobilis'
either completely lacked ΔC19:0, or possessed only trace amounts (Table 5). Amstein & Hartman
(1973) also reported the absence, or the presence of extremely low levels of cyclopropane-ring
fatty acids in strains of S. casseliflavus. Collins & Jones (1979a, b), on the basis of menaquinone
analyses, suggested S. casseliflavus and 'S. faecium var. mobilis' should be accommodated in one
taxon. The DNA–DNA homology and fatty acid data accord with this view.

The separate species status given to strains possessing the group Q antigen ('S. avium') by
Nowlan & Deibel (1967) is borne out by the results of the present study. The numerical phenetic
study of Jones et al. (1972) indicated some similarity between 'S. avium' and S. faecium, though
not close enough for 'S. avium' strains to be included in the species S. faecium. In the present
study, 'S. avium' and S. faecium strains shared only about 23–30\% homology. 'Streptococcus
avium' and 'S. faecium' were also quite distinct from each other on the basis of fatty acid profiles
with C14:0 and C16:0 predominating in the former, and C16:0 and C18:1\d preponderating in the
latter (Table 5). The two strains of S. faecalis var. malodoratus' isolated by Pette (1955) from
Gouda cheese showed about 50\% DNA homology with 'S. avium' under optimum hybridization
conditions. The presence of remarkably similar fatty acid profiles within these taxa (Table 5)
supports this relationship. However, in the numerical phenetic study of Jones et al. (1972), 'S.
avium' and 'S. faecalis var. malodoratus' were quite unrelated. This phenetic difference between
'S. avium' and 'S. faecalis var. malodoratus' has been confirmed in the present study (see Table 6).
The phenetic distinctiveness of these two taxa, together with the fact that, under stringent
hybridization conditions DNA–DNA homology values dropped to about 15\%, strongly indicate
that 'S. avium' and 'S. faecalis var. malodoratus' are separate species.

The DNA homology data indicate that the streptococci isolated by Barnes et al. (1978)
represent a distinct species. The presence of MK-8 within these isolates reinforces this
distinctiveness. Strains of S. faecium, 'S. durans', 'S. avium' and 'S. faecalis var. malodoratus' lack
respiratory quinones, whereas S. faecalis and S. casseliflavus contain DMK-9 and MK-7/MK-8,
respectively (Collins & Jones, 1979a, b). The isolates of Barnes et al. (1978) also differ from the
other taxa of serological group D examined on the basis of fatty acid profiles and biochemical
tests.

On physiological and biochemical grounds, S. bovis is quite different from the taxa S. faecalis,
S. faecium, S. casseliflavus, 'S. avium', 'S. durans', S. faecalis var. malodoratus' and the chicken
isolates of Barnes et al. (1978). Preliminary DNA–rRNA homology studies (Weissman et al.,
1966; Garvie & Farrow, 1981) have shown that S. bovis and S. faecalis are only distantly related,
and possibly belong to different genera. DNA–DNA homology values of less than 21\% under
optimum conditions are considered by Johnson (1973) to show species to be unrelated at the
generic level. In the present study, very low homology values (<10\%) were found between S.
bovis and the other group D streptococci examined, thereby confirming the distant relationship
between S. bovis and members of the 'enterococcus' division. In contrast, relatively high
homology values (about 20–50\%) were found between S. faecalis, S. faecium, S. casseliflavus, 'S.
avium', 'S. durans' and the Barnes isolates, indicating these taxa are related at the generic level.

Note added in proof: Since this paper was submitted the chicken isolates of Barnes et al. (1978)
have been named Streptococcus gallinarum (Bridge & Sneath, 1982).

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REFERENCES


