The Differentiation of *Streptococcus faecalis* and *S. faecium*

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SUMMARY

Enterococci mainly from laboratory collections were separated by established and new or modified tests into two distinct species, *Streptococcus faecalis* and *S. faecium*. Of the established tests only reducing activity and potassium tellurite tolerance completely differentiated the organisms. Of the additional tests the most useful were fermentation of glycerol in a soft agar medium containing fumarate, H_2O_2 formation from polyhydroxy alcohols and the ability to ferment them, H_2O_2 formation on a basal medium containing no added specific energy source, citrate fermentation and dissimilation of malate in the presence of glucose. Catalase activity and the ability to use malate as an energy source were also useful tests but did not completely differentiate the organisms.

INTRODUCTION

The enterococci (from which are excluded the *Streptococcus bovis-equinus* complex) appear to be divisible into two major physiological and biochemical groups, one represented by *S. faecalis* and the other by *S. faecium*. Evidence for such a division can be found in the work of Orla-Jensen (1919, 1943); Olsen (1949); Skadhauge (1950); Sharpe & Shattock (1952); Shattock (1955); Barnes (1956); Lake, Deibel & Niven (1957); Deibel, Lake & Niven (1963). The practical necessity of being able to distinguish the two species has become increasingly evident in relation to the detection of faecal pollution of water supplies and foods. Recent work on the distribution of these species by Kjellander (1960), for instance, revealed that *S. faecalis* was the more numerous species in human faeces and *S. faecium* in animal faeces. Tests for separating *S. faecalis* from *S. faecium* before those devised by Deibel *et al.* (1963) were few and not always satisfactory. The purpose of the present work was to develop new tests which, in conjunction with those described earlier, would assist in the identification of the enterococci. Since the completion and publication of this work in thesis form (Whittenbury, 1961), Deibel *et al.* have reported a comprehensive study of this group of organisms, reviewing the development of its taxonomy and describing new tests which differentiate *S. faecalis* from *S. faecium*. Some of the tests described in the present work are similar to those devised by Deibel *et al.*, but as they were devised independently and differ in certain respects from those of Deibel *et al.* it is considered that they are still worth reporting. Among the organisms studied were strains thought to be typical of *S. faecalis*, *S. faecium* and *S. durans*, and strains which possessed unusual characteristics.

METHODS

Organisms. The strains and their sources are listed in Table 1.

Media. Basal medium agar was: meat extract (Lab-Lemco), 0.5 g.; peptone (Evans), 0.5 g.; yeast extract (Difco), 0.5 g.; Tween 80, 0.05 ml.; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.01 g.; agar (Davis), 1.5 g.; in 100 ml. tapwater, adjusted to pH 6.5 and autoclaved at 121° for 15 min.

Table 1. *Source and identification of cultures used*

Identification	Designation when received	Source
<i>Streptococcus faecalis</i>	<i>S. faecalis</i> 581	NCDO
	„ 370, 775, 2707	NCIB
	„ B 8, H 69 D 5, N 83, s 161	Dr P. M. F. Shattock
	<i>S. liquefaciens</i> 588, 598	NCDO
	„ z 93, Black	Dr T. Gibson
	„ N 97, E IV, GB 122	Dr P. M. F. Shattock
	s 41	Silage
	<i>S. zymogenes</i> 585, 587	NCDO
	<i>S. faecium</i> 942, 943, 944, 945	NCDO
	„ HGH 511, 1 MEC, P ₆ , Hobbs 2678	Dr P. M. F. Shattock
<i>S. faecium</i>	„ 2703 (former NCTC no.)	Dr T. Gibson
	<i>S. faecalis</i> 580, 582	NCDO
	„ 8274	NCIB
	s 1, s 2, s 3, s 4, s 5, s 6, 1, 60, 73, s 32, Motile s 43	Silage
	T 83, SD 1, SD 20, JMA 3	Dr T. Gibson
	<i>S. durans</i> 498	Dr P. M. F. Shattock
	„ Sherman 98 D	Dr T. Gibson

NCDO = National Collection of Dairy Organisms.

NCIB = National Collection of Industrial Bacteria.

NCTC = National Collection of Type Cultures.

Liquid medium was similar to the basal medium agar but without agar.

'Inoculum' medium was similar to basal medium agar but with the agar omitted and glucose 0.5 % (w/v) added.

Soft agar medium, which was used for showing oxygen relationships and fermentative activities, was prepared and inoculated as described previously (Whittenbury, 1963) with the exception that the bromocresol purple content was doubled. Sugars and polyhydroxy alcohols were prepared as Seitz-filtered distilled water solutions and added to give a final concentration in the media of 0.5 % (w/v).

Arginine hydrolysis. The method of Niven, Smiley & Sherman (1942) was routinely used with the exception that Tween 80 was included in the medium. In some studies liquid medium containing L-arginine monohydrochloride 0.3 % (w/v), with or without glucose 0.1 or 1 % (w/v), adjusted to pH 6.5, was used. Ammonia production was determined with Nessler's reagent on a spot plate and glucose disappearance with Benedict's reagent. Cultures were held at 37° in a water bath. Growth was measured nephelometrically at 10–15 min. intervals at the end of the lag phase; pH values were measured electrometrically.

Serine hydrolysis. Liquid medium containing L-serine 0.3 % (w/v) was adjusted to pH 6.5. Ammonia production was determined by Nessler's reagent and acetoin

(indicating dissimilation of pyruvate, the deamination product) by Barritt's (1936) modification of the Voges-Proskauer test.

Ability to initiate growth at pH 9.6. The method of Chesbro & Evans (1959) was used. Additional experiments with the same medium converted to a soft agar were also done.

Tolerance of potassium tellurite. Basal medium agar containing glucose 0.5% (w/v), tellurite 0.04% (w/v) (the concentration recommended by Skadhauge, 1950), adjusted to pH 6.5, was used. The results, erratic at first, became constant when the concentration of the meat extract in the medium was doubled.

Reduction of 2,3,5-triphenyltetrazolium chloride (tetrazolium). This test was adapted from that described by Barnes (1956). Liquid medium containing glucose 0.5% (w/v) was adjusted to pH 6.0. Tetrazolium, 0.01% (w/v) final concentration, was added from a stock solution heated at 100° for 15 min. A heavy inoculum, 3 capillary pipette drops of turbid culture, was added to 4 ml. medium in a test tube. The cultures, incubated at 30°, were examined at 8 hr. Positive cultures were coloured a deep magenta, whilst cultures regarded as negative were either colourless or faintly pink.

Reduction and tolerance of methylene blue in milk and the reduction of litmus in milk. A heavy inoculum, 3 capillary pipette drops, was added to the milk media which were incubated at 30° and read at 8 hr.

Haemolysis. Brown's (1919) method was followed, with nutrient agar containing NaCl 0.5% (w/v) and defibrinated horse blood 3% (w/v). Poured agar plates were incubated for 2 days at 30° and refrigerated overnight. Further tests were made with ox blood in the basal medium agar containing NaCl 1.0% (w/v).

Hydrogen peroxide formation, pseudocatalase and catalase tests. Methods and media (heated blood *o*-dianisidine agar, heated blood agar and glucose agar) described previously (Whittenbury, 1964) were used. In testing for H₂O₂ formation it was essential that the inoculum be taken from aerobically incubated slope cultures.

Malate and citrate dissimilation. Ability to dissimilate malate in the presence and absence of glucose was determined. Liquid medium was used containing DL-malic acid 4.0% (w/v); bromocresol purple, 2.8 ml. of a 1.6% (w/v) ethanolic solution/l. (unless acetoin was being tested for); glucose 1–2.0% (w/v); adjusted to pH 6.0 with KOH and distributed in 4 ml. amounts in 5 × ½ in. test tubes containing Durham tubes. Water agar seals were added to the inoculated tubes; the function of the Durham tubes was to support the seals. Action on malate was judged by increase in pH value, gas (CO₂) production, and, in the absence of glucose, by increase in the amount of growth over that in the basal medium and by acetoin production.

Citrate dissimilation was examined in similar media to those used in the study of action on malate. Citrate (potassium citrate 3.0%, w/v) was added and the medium adjusted to pH 8.0 for routine use. Action on citrate in the absence of glucose was judged by gas production, acetoin formation, increase in pH measured electrometrically, and by increase of growth over that in the basal medium. In the presence of glucose (medium adjusted to pH 6.0) gas (CO₂) production indicated action on citrate.

To examine for gas production from citrate and malate, each tube was sharply tapped to initiate effervescence. In cultures above pH 6.5 bromocresol purple was

frequently bleached except when the culture was in contact with the atmosphere (the top 2 mm. of the agar seal) where it remained oxidized, so allowing an estimate of pH change to be made in these circumstances.

A relatively high concentration of potassium in the medium is essential for active dissimilation of citrate and malate (Keddie, 1959). Consequently pH adjustments of malate media were always made with KOH and potassium citrate was used in the citrate media.

Temperature of incubation. Unless otherwise stated, incubation was at 30°.

RESULTS

The identification of the organisms as enterococci was confirmed by established physiological and biochemical tests which collectively separate the enterococci from other streptococci. These tests are: ability to tolerate 6.5% NaCl, to hydrolyse

Table 2. *Differentiation of the organisms by currently used tests*

	<i>Streptococcus faecalis</i> (no. positive out of 18 strains tested)	<i>Streptococcus faecium</i> (no. positive out of 29 strains tested)
Fermentation of:		
Arabinose	4	20
Melibiose	0	18
Melezitose	12*	0
Sorbitol	18	2†
Glycerol (in the absence of fumarate)	18‡	24 (aerobic)
Tolerance of:		
Tellurite	18	0
Reduction of:		
Methylene blue (1.0 %)	18	0
Tetrazolium (0.01 %)	18	0
Litmus	18	0

* Three strains gave melezitose-fermenting mutants.

† Both strains gave sorbitol-fermenting mutants.

‡ Three strains fermented glycerol aerobically and gave anaerobically fermenting mutants; one strain fermented glycerol aerobically only.

arginine, to withstand heating at 63° for 30 min., to initiate growth at pH 9.6 and to grow at 10° and 45°. Only the ability to grow at pH 9.6 and at 10° and 45° was common to all strains. Where growth at pH 9.6 was tested by the modification of the method of Chesbro & Evans (1959) in which the medium was made into a soft agar, most strains formed a uniform dense growth but *Streptococcus durans* and similar strains gave rise only to a few colonies, suggesting that most of the organisms had not survived. The 'survivors' did not seem to be mutants adapted to alkaline conditions since inocula prepared from them gave only a few colonies, as before. Of the remaining tests arginine hydrolysis was the most useful since only one strain (the motile enterococcus) was negative. This strain was at one time able to hydrolyse arginine but has lost this property within a 4-year period.

The organisms were next subjected to tests commonly used for the differentiation of the enterococci. The results (Table 2) distinguished two main groups which will be discussed as *Streptococcus faecalis* and *S. faecium*. Only the tellurite and reduction tests differentiated the groups completely and the reduction tests (see Methods) had to be modified (Whittenbury, 1956) to achieve a separation. The rate at which tetrazolium, methylene blue and litmus were reduced proved to be a more useful criterion than the ability to reduce these substances; this also seems to have been the view of other workers including Deibel *et al.* (1963). The additional tests that were used are described below and the results are given in Table 3.

Table 3. *Differentiation of the organisms by additional tests*

	<i>Streptococcus faecalis</i>	<i>Streptococcus faecium</i>
Fermentation of glycerol in the presence of fumarate	+	A
H ₂ O ₂ from:		
Basal medium	—	+
Glycerol	—	+*
Sorbitol	—	+*
Mannitol	—	+*
Malate as an energy source	± †	—
Gas from malate + glucose	—	+
Citrate as an energy source at pH 8.0	+	—
Catalase (in presence of heated blood)	± ‡	—

+ = all strains positive, — = all strains negative, ± = some strains positive and some negative, A = negative or aerobic only.

* If substrate used.

† $\frac{2}{15}$ strains negative.

‡ $\frac{1}{18}$ strains positive.

Action on polyhydroxy alcohols

Glycerol. A test separating *Streptococcus faecalis* from *S. faecium* by their action on glycerol, the former fermenting it anaerobically and the latter fermenting it aerobically or not at all, was developed from the work of Gunsalus (1947). He noted that organisms able to ferment glycerol anaerobically did so more rapidly when fumarate was added to the medium as an exogenous hydrogen acceptor. Experiments with a soft agar medium confirmed this finding and revealed that a separation based on glycerol fermentation could only be achieved by adding fumarate (sodium salt 0.5%, w/v) to the medium. In the absence of fumarate one strain of *S. faecalis* (NCIB 370) fermented glycerol aerobically only; three other strains fermented it aerobically and gave anaerobically fermenting mutants. Deibel *et al.* (1963) had found fumarate useful in this test but observed that one strain of *S. faecalis* fermented glycerol aerobically only while one strain of *S. faecium* fermented glycerol anaerobically, so limiting the value of the test. In these instances a further test based on glycerol metabolism might prove useful, namely the ability to form hydrogen peroxide from glycerol. Gunsalus (1947) observed that streptococci which fermented glycerol only aerobically, formed H₂O₂ in the process. Of the strains studied here all those identified as *S. faecium* and able to use glycerol formed H₂O₂ on glycerol heated blood *o*-dianisidine agar. No *S. faecalis* strain,

including the one that showed a strictly aerobic action in the absence of fumarate, formed H_2O_2 on this medium.

Mannitol and sorbitol. Both these hexitols were fermented aerobically and anaerobically by all the *Streptococcus faecalis* strains and by some of the *S. faecium* strains. The hexitol-fermenting *S. faecium* strains, however, formed H_2O_2 on hexitol heated blood *o*-dianisidine agar and the *S. faecalis* strains did not. Therefore inability to ferment these hexitols, or the production of H_2O_2 from them when they were used, proved useful in separating *S. faecium* from *S. faecalis*.

Hydrogen peroxide formation on heated blood o-dianisidine agar

All the organisms identified as *Streptococcus faecium* but none identified as *S. faecalis* formed H_2O_2 on heated blood *o*-dianisidine basal agar. The H_2O_2 reaction by *S. faecium*, although distinct, did not cover such a wide area around the growth as it did when a utilizable polyhydroxy alcohol was present. The addition of glucose to the medium inhibited the production of an H_2O_2 reaction by all but three strains of *S. faecium*. Consistent results for H_2O_2 formation were only obtained when the inocula used had been grown on aerobically incubated slopes. Some strains of *S. faecalis* formed traces of H_2O_2 when the inocula were not fully adapted to aerobic conditions as, for instance, when they were taken from static broth cultures.

Greening on blood agar

Deibel *et al.* (1963) differentiated the non-haemolytic enterococci on sheep blood agar: *Streptococcus faecium* but not *S. faecalis* gave a strong greening reaction. A similar finding was made in the present work with ox blood and horse blood agars. The clearest results were obtained with surface inoculated cultures.

The addition of glucose (1 %, w/v) to the blood media had the effect of lysing red cells under and around the growth of *Streptococcus faecium* but not those under and around the *S. faecalis* growth. In all but three instances the *S. faecium* strains did not cause greening. Since these strains were those which formed H_2O_2 on heated blood *o*-dianisidine glucose agar, the relationship of the greening reaction with H_2O_2 production with unlysed blood agar, with and without added glucose and *o*-dianisidine, was examined. The areas of greening corresponded with the H_2O_2 areas and glucose had a similar effect on both. This circumstantial evidence suggests that greening by the *S. faecium* strains is caused by the H_2O_2 they form.

Catalase activity

Catalase activity on heated blood agar was of some differential value in that 15 of 18 strains of *Streptococcus faecalis* were positive whilst all strains of *S. faecium* were negative. No organism produced pseudocatalase.

Malate and citrate dissimilation

The following tests were developed from methods used to identify strains of *Streptococcus faecalis* and *S. faecium* from silage (Whittenbury, 1956).

Malate. All but two of the strains of *Streptococcus faecalis* used malate as an energy source; the two exceptions had no action on malate. All the strains of *S. faecium* dissimilated malate but did not use it as an energy source. With both types

of organism there was an increase in pH value, indicating decarboxylation; gas (CO₂) production was frequently observed (always when the tubes were heated in boiling water); acetoin was detected, with *S. faecalis* there was a marked increase in amount of growth over that in the basal medium.

A more precise test was obtained when glucose (2.0%, w/v) was included in the medium. In this case *Streptococcus faecalis* acidified the medium and had no effect on the malate. *Streptococcus faecium*, on the other hand, fermented the glucose and simultaneously dissimilated the malate so that initially the pH value decreased but finally rose to pH 7.0 or higher. Gas production was vigorous; a light tapping of the tube at 2–3 days frequently resulted in an effervescence which forced the seals out of the tubes. Acetoin formation from malate was not investigated in this medium since many strains form acetoin from glucose.

Measurements were made of glucose disappearance, pH value and growth in various concentrations of malate and glucose. This showed that the vigorous dissimilation of malate by *Streptococcus faecium* in the presence of glucose reflected a great increase of growth over that developing in the medium containing malate but no added glucose. With *S. faecalis* glucose had a diauxic effect on malate utilization; glucose-grown cocci adapted to malate only when glucose had disappeared from the medium. Consequently in a medium containing excess glucose growth conditions became unfavourable before adaptation was possible. These results indicate that *S. faecalis* and *S. faecium* metabolized malate by different pathways. There is evidence (Whittenbury & Playne, unpublished) which shows that *S. faecium* carries out a non-energy-yielding dissimilation of malate under anaerobic conditions; acetoin, lactate and carbon dioxide are the principal end-products. *Streptococcus faecalis*, on the other hand, carries out an energy-yielding fermentation of malate; acetoin, lactate, formate, acetate, carbon dioxide and ethanol are the major end-products. Acetate and formate are probably the final products of a phosphoroclastic split of pyruvate to formate and acetyl phosphate, the acetyl phosphate giving rise to adenosine triphosphate and acetate.

Citrate utilization. Deibel *et al.* (1963) found the ability to use citrate a valuable test; all their strains of *Streptococcus faecalis* used citrate as an energy source, but only a few strains of *S. faecium* did so. A similar finding (Whittenbury, 1956) led to the development of a medium in which only *S. faecalis* used citrate. It was observed that as the initial pH value of the medium was increased from pH 6.0 to 7.0 fewer strains of *S. faecium* were able to use citrate and in a medium initially at pH 8.0 none did. The changes observed in the absence of glucose were acetoin production, increase in pH value, gas (CO₂) production and increased growth over that in the basal medium. Glucose (2.0%, w/v) had a diauxic effect on citrate fermentation by both types of organism.

Other tests. No other differential tests were devised. An attempt, however, was made to separate the organisms on their metabolism of serine and arginine. Deibel & Niven (1960) reported that *Streptococcus faecalis* and its varieties, but not *S. faecium*, deaminated serine and used it as an energy source. In the present work some strains of both species were found to use serine whilst others did not. Deibel (1960) noted that whereas both species deaminated arginine, only *S. faecalis* used it as an energy source. Strains of both species studied here, however, derived benefit from arginine (0.3%, w/v) in the complex basal medium. Strains of *S. faecium*

obtained least; these gave twice the amount of growth observed in the basal medium, whilst *S. faecalis* gave 4 to 5 times more growth. One strain of *S. faecium* did not hydrolyse arginine; it proved a useful control since the addition of arginine to the basal medium did not affect its growth. Glucose had a diauxic effect on the arginine metabolism of the several strains of *S. faecalis* tested, glucose-grown inocula using arginine only when glucose was no longer detectable. Arginine-grown inocula, however, used both glucose and arginine simultaneously. This diauxic effect was not observed with *S. faecium*.

DISCUSSION

The enterococci were conveniently separated into two species, *Streptococcus faecalis* and *S. faecium*. Among the organisms identified as *S. faecalis* were those recognizable as *S. faecalis* (non-haemolytic and non-proteolytic), *S. zymogenes* (haemolytic and sometimes proteolytic), and *S. liquefaciens* (non-haemolytic and proteolytic); apart from differences in haemolytic, proteolytic and one or two fermentative properties these organisms constitute a fairly homogeneous group. Of the organisms identified as *S. faecium* some were regarded as 'typical'—those fermenting arabinose, melibiose and mannitol and, aerobically only, glycerol—and some were identified as *S. durans*—those fermenting glycerol aerobically only but failing to ferment arabinose, melibiose and mannitol. Haemolysis did not seem to be a reliable characteristic of *S. durans* as one strain (498) lost its haemolytic activity. Deibel *et al.* (1963) also observed the instability of this character among their strains of *S. durans*. Other organisms identified here as *S. faecium* possessed various ranges of properties and occupied intermediate positions between the 'typical' *S. faecium* and *S. durans*. The one motile strain examined and identified as *S. faecium* appears to be similar to the yellow pigmented, motile streptococci described by Graudal (1952), to the motile enterococci described by Langston, Gutierrez & Bouma (1960), possibly to those described in the references listed by Hugh (1959) and to the motile Group D streptococcus mentioned by Sherman (1938). Apart from its ability to ferment inulin and loss of ability to hydrolyse arginine within a 4-year period, it is very similar to the 'typical' *S. faecium*.

Of the established tests used in this study only tellurite tolerance and reducing activity completely differentiated the organisms; the other commonly used tests were, for various reasons, of limited value. Of the new or modified tests used, fermentation of glycerol in a soft agar medium containing fumarate, hydrogen peroxide formation from polyhydroxy alcohols and ability to ferment them, hydrogen peroxide formation on a medium containing no added sugar or polyhydroxy alcohol, citrate fermentation and dissimilation of malate in the presence of glucose seem to be the most useful. Catalase activity and the ability to use malate as an energy source were also useful but did not differentiate the organisms completely. The practical value of these tests in the routine identification of enterococci remains to be assessed from studies of larger collections of enterococci from diverse sources.

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