STUDIES ON STREPTOCOCCI RESEMBLING
STREPTOCOCCUS MILLERI AND ON AN
ASSOCIATED SURFACE-PROTEIN ANTIGEN

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PLATES XXXVII-XXXIX

GUTHOF (1956) described a study on 92 strains of streptococci isolated from
88 dental abscesses. Samples for culture were obtained from the neck or face
by puncture or incision of the lesions from the outside. Guthof allotted each
strain to one of several groups on the basis of biological tests, and at the same
time tried to classify the strains according to established taxa, distinguishing
clearly between those that did and those that did not carry one of the Lancefield
group antigens. Among the non-haemolytic and greening strains, he defined
a new species, Streptococcus milleri.

Colman and Williams (1965, 1967) and Colman (1968, 1969) used cell-wall
analysis, numerical classification, and DNA transformation to classify a large
series of laboratory strains of streptococci. These studies suggested a close
taxonomic relationship between Guthof's S. milleri and non-haemolytic
streptococci of the serological groups A, C, F, and G. Also, non-haemolytic
strains possessing the type antigens described by Ottens and Winkler (1962),
but no Lancefield group antigen, were clustered with S. milleri, as was Streptococcus MG (Mirick et al., 1944; Willers, Ottens and Michel, 1964). In the
computer classification studies of Colman (1968) and Mejäre (1975a)
β-haemolytic strains with minute colonies also joined the S. milleri cluster.

The aim of our investigation was to study streptococci isolated from deep
pyogenic infections in the face and neck. Most of the strains used in this
investigation were obtained from sources similar to those in Guthof's study.

MATERIALS AND METHODS

Cultures examined

Ninety-nine strains of streptococci were isolated in the years 1972–75, largely from
specimens sent to our bacteriological laboratories in Cologne from patients with the clinical
diagnosis of actinomycosis or suspected actinomycosis. The sources of the strains are
summarised in table I. Although most of the streptococci originated from infections in the
face or neck, a few non-haemolytic strains with a similar colonial appearance from other
sites were included. Strains from the face and neck had been obtained either by puncture or

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**TABLE I**

Sources of the streptococcal strains studied

<table>
<thead>
<tr>
<th>Streptococci isolated</th>
<th>Number of strains isolated from cervico-facial sites</th>
<th>other sites*</th>
</tr>
</thead>
<tbody>
<tr>
<td>with <em>Actinomyces israelii</em></td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>with other organisms</td>
<td>62</td>
<td>5</td>
</tr>
<tr>
<td>in pure culture</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Total†</td>
<td>90</td>
<td>9</td>
</tr>
</tbody>
</table>

* The infections took the form of: abscesses (5), empyema (3), and wound infection (1).
† In addition, 37 strains from culture collections were examined.

by incision through the skin. Two patients yielded two distinguishable strains; thus the 99 isolates came from 97 different patients. Most of these streptococci occurred in mixed culture with other aerobes or anaerobes, including *Actinomyces israelii*. Most of the strains were isolated in primary culture on various solid media in the reduced oxygen tension provided by the method of Fortner (1928). Thirty-seven strains of streptococci from culture collections were also examined for the purpose of numerical analysis.

**Bacteriological methods**

The tests described below were performed on all 136 strains. The methods were derived from procedures commonly employed in the identification of streptococci and were based either on descriptions in the literature or on personal communications, but because there were some major modifications the tests are outlined here.

Unless stated otherwise, all cultures were incubated at 35°C. In general an overnight culture in Heart Infusion Broth (Difco), supplemented with Yeast Extract (Difco) 0.3% (w/v), and incubated anaerobically in a GasPak jar (Baltimore Biological Laboratories), served as inoculum for fluid media and subcultures on plates.

*Catalase.* Production of the enzyme was tested by adding a few drops of a H₂O₂ 3% (w/v) solution to a 48-h culture on Brain Heart Infusion Agar (Difco) supplemented with horse serum 1% (v/v). The cultures were examined for bubbles of gas.

*Pigment formation.* This was sought on the culture subsequently used for the catalase test.

*Optochin sensitivity.* Taxo P disks (BBL) were applied to the surface of Brain Heart Infusion Agar plates containing defibrinated sheep blood 5% (v/v). The plates had previously been inoculated with the test organism by streaking with a cotton swab. After incubation for 2 days, zones of inhibition were recorded as a measure of sensitivity, and lack of inhibition was considered to indicate resistance.

*Bacitracin sensitivity.* Taxo A disks (BBL) were used on the same plate as that employed for testing sensitivity to optochin.

*Salt tolerance.* Heart Infusion Broth was supplemented with NaCl to bring the concentration to 2, 4 or 6.5% (w/v). After inoculation, the cultures were covered with sterile liquid paraffin and incubated for 4 days; turbidity was taken to indicate salt tolerance.

*Atmospheric requirements.* These were determined by inoculating three plates of Brain Heart Infusion Agar containing blood 5% (v/v). One was incubated in air, the second in
air with CO₂ 5% (v/v), and the third anaerobically in a GasPak jar. The presence or absence of growth was recorded after 2 days’ incubation.

Haemolysis was tested on Tryptose Blood Agar (Difco) containing sheep blood 5% (v/v) after anaerobic incubation for 2 days. Complete clearing around the colony was defined as β-haemolysis and greening as α-haemolysis.

Potassium tellurite tolerance was tested on Brain Heart Infusion Agar containing horse serum 1% (v/v) and potassium tellurite 0.01% or 0.04% (w/v). Filter-sterilised potassium tellurite solution was added after cooling of the medium to 48°C. The plates were incubated for 2 days, and the formation of readily visible black colonies was regarded as indicating tolerance. A tellurite-free control plate was always used.

Growth on bile. The ability to grow on bile agar was tested on Tryptose Blood Agar Base containing (1) horse serum 1%, v/v, and (2) dehydrated Oxgall (Difco) at concentrations that provided the equivalent of 40% and 10% final concentrations of bile. Bile-free control plates were also inoculated. Plates were incubated for 3 days.

Growth at 45°C was tested by inoculating tubes containing Brain Heart Infusion (Difco) supplemented with Yeast Extract 0·3% (w/v). The broth was covered with liquid paraffin and incubated in a waterbath for 5 days. Turbidity indicated ability to grow at 45°C.

Hydrolysis of aesculin was tested in Heart Infusion Broth containing aesculin 0·1% (w/v) and incubated for 4 days in a GasPak jar. Then 0·6 ml of a solution of FeCl₃, 7% (w/v) was added to 6 ml of the culture, and blackening was recorded as a positive reaction.

Hydrolysis of sodium hippurate was determined by growing the strains—anaerobically in a GasPak jar—for 4 days in tubes containing Brain Heart Infusion supplemented with sodium hippurate 1% (w/v). In the test, 0.25 ml of H₂SO₄ solution 50% (w/v) was added to 1 ml of culture supernate. Formation of a precipitate of benzoic acid crystals was accepted as a positive result.

Production of extracellular polysaccharide from sucrose was tested by fractional precipitation with ethanol as described by Hehre and Neill (1946). The cultures were grown for 5 days in a GasPak jar in 10 ml of medium, pH 7·5, containing the following: Trypticase (BBL) 14 g, Yeast Extract (Difco) 5 g, sucrose or glucose 80 g, sodium acetate 2 g, potassium carbonate 0·55 g, distilled water 1 litre.

Voges-Proskauer (VP) test. For the VP test, strains were grown for 2 days in the following medium, pH 7·3, in a GasPak jar: Trypticase 10 g, Yeast Extract 5 g, glucose 5 g, K₂HPO₄ 5 g, distilled water 1 litre. The test was performed as described by Holdeman and Moore (1973).

Liquefaction of gelatin was tested in the following medium pH 7·1: Bacto-Gelatin (Difco) 150 g, Trypticase 10 g, Yeast Extract 5 g, glucose 2 g, distilled water 1 litre. The tubes were inoculated by stabbing with a straight wire, sealed with liquid paraffin and incubated for 8 days. Before reading the test, the tubes were refrigerated for 2 h.

Proteinase attacking casein. Screening for proteinase other than gelatinase was done by applying a single streak of inoculum broth to a medium, pH 7·1, that consisted of Brain Heart Infusion Agar and Casein, Purified (Difco) 2% (w/v). The plates were read after 2 days. A zone of turbidity around the streak of growth indicated proteinase activity. Plates were also flooded with HCl 10% (w/v), and examined for zones of clearing around the streak of growth.

Fermentation of carbohydrates was tested in tubes containing Phenol Red Broth Base (Difco) and 1% (w/v) of the following filter-sterilised carbohydrates: glucose, lactose, melibiose, sucrose, trehalose, melezitose, raffinose, inulin, salicin, mannitol, sorbitol, L-arabinose, xylose, galactose, arbutin, maltose, ribose, cellobiose, glycerol. Where necessary, the final pH was adjusted to 7·4. After inoculation the medium was covered with liquid paraffin and incubated for 7 days.

Arginine dihydrolase. The ability to produce ammonia from arginine was tested by growing the strains in Decarboxylase Base Moeller (Difco) containing L-arginine monohydrochloride 1% (w/v) and Yeast Extract 0·1% (w/v). Liquid paraffin was layered on the surface of the medium. An alkaline reaction after 5 days' incubation indicated a positive test result.
Nicotinamide adenine dinucleotide glycohydrolase (NADase). Production of this enzyme was tested by the method of Lüticken et al. (1976).

Survival at 60°C for 30 min. For this test the tubes containing the inoculum broth were immersed in a waterbath at 60°C for 30 min. The tubes were then rapidly cooled to room temperature in a waterbath and centrifuged; the bacterial sediment was streaked out on Brain Heart Infusion Agar plates containing sheep blood 5% (v/v). These plates were incubated for 3 days.

Reduction of 2,3,5-triphenyl tetrazolium chloride (TTC) was tested as follows: molten Brain Heart Infusion Agar was cooled to 48°C and 1·5 ml of a filter-sterilised TTC solution (1%) were added to each litre of the medium. Plates were inoculated by streaking in the usual manner and were incubated for 2 days. Growth of red colonies indicated the ability to reduce TTC.

Gas-liquid chromatography for determining the end products of glucose metabolism was performed by the method of Holdeman and Moore (1973).

Serological procedures

Antisera for groups A, B, C, D, E, F, G, H, K, L, N, O, P, and U were made in our own laboratories with streptococcal vaccine strains kindly supplied by Dr R. C. Lancefield, Rockefeller University, New York, and by Dr W. R. Maxted, Central Public Health Laboratory, Colindale, England. No satisfactory antisera for groups M and Q were available at the time of this investigation. Group reactions for groups R, S, and T were not performed because the reference strains strongly reacted with group-D serum.

Typing sera for the types II, III, and IV of group F (Ottens and Winkler, 1962) were produced in the usual manner by inoculating rabbits with pepsin-treated, heat-killed cells of the appropriate streptococcal reference strains obtained from Dr J. M. N. Willers, University of Utrecht, Netherlands. No vaccine strain for type I was available at the time of this investigation; type V of Ottens and Winkler (1962) has proved not to be a distinct type upon further investigation (Willers, personal communication, 1976). The "z3" and "z5" (HS 8920-de Moor) strains described by Willers and Alderkamp (1967) and Willers, Michel and Benner (1973a) were also used for the production of precipitating rabbit antibodies. Vaccines prepared from these two strains were not pepsin-treated. Cells of strain NCTC10708 (S. milleri) that had been disrupted by ultrasonic treatment were used as immunogen in one rabbit.

Extracts for serological grouping were made from all freshly isolated strains by (1) the hydrochloric acid method (Swift, Wilson and Lancefield, 1943), (2) the formamide method (Fuller, 1938), and (3) the nitrous acid method (El Kholy, Wannamaker and Krause, 1974). Lancefield extracts were prepared from the reference strains to confirm their group reaction. From certain selected reference strains (see Results) formamide extracts were also prepared. Hydrochloric acid extracts of all strains were made from the centrifugates of cultures in 80-ml volumes of Todd Hewitt broth; each centrifugate was treated with 0·5 ml of 0·2M HCl in saline. The extracts were used for precipitin tests with the type sera and the antisera prepared against strains z3 and z5. All precipitin tests were performed as agar double-diffusion tests on microscope slides, covered with agarose 1% in distilled water. An antisera prepared against strain z3 was also used for capillary precipitin tests as described by Swift et al. (1943) for streptococcal M typing. Hydrochloric acid extracts that gave a precipitin reaction with this antisera in the capillary tube test, but not in the agar double-diffusion test, were concentrated 5-fold in an Amicon Minicon A25 concentrator and retested by the agar method.

Hydrochloric acid extracts from the z3 vaccine strain, and any other similar extract, from clinical isolates or reference strains of streptococci, reacting with z3 antiserum, were treated with pepsin as follows. The extracts were adjusted to pH 2 with 0·2M HCl and Pepsin (Boehringer, Mannheim) was added to a final concentration of 0·1 mg per ml; the mixtures were incubated for 2 h at 37°C, cooled in an ice bath, neutralised with 0·2M NaOH and, after concentration in an Amicon Minicon B15 or A25 concentrator to half the original
STREPTOCOCCUS MILLERI

volume, tested in agar precipitin tests. Hydrochloric acid extracts treated in the same way, but without the addition of pepsin, served as controls.

Trypsin treatment of strains NCTC8037, NCTC10708 and z3 was also performed. For this purpose an equal volume of a Trypsin (Boehringer, Mannheim) solution 0.1% in 0.066M phosphate buffer, pH 8.0, was added to an extract. After incubation for 2 h at 37°C, the mixture was cooled in an ice bath, and a further volume of a 0.2% solution of Trypsin Inhibitor from Soy Bean (Boehringer, Mannheim) in phosphate buffer, pH 7.0, was added. Concentration and testing was carried out as for the pepsin-treated extracts.

In a further experiment, whole cells of strains NCTC8037, NCTC10708 and z3 were each grown overnight in 200 ml of Todd Hewitt Broth (Difco), washed twice in saline, and then divided into two equal portions. One portion was incubated for 2 h at 37°C in 2 ml of an 0.2% solution of trypsin in phosphate buffer pH 8.0. The other portion, serving as a control, was treated in the same way except that trypsin was not added. The centrifugates of both portions were then washed twice in cold physiological saline, and Lancefield extracts were prepared from the cells by means of 0.2M HCl at the usual rate of 0.5 ml per centrifugate. Cultures of these strains were also treated with pepsin. For this purpose the deposits were suspended in 0.5 ml of 0.2M HCl in saline (pH 2.0), and 0.05 ml of pepsin (1 mg per ml, in distilled water) or 0.05 ml distilled water (control) was added. After incubation for 2 h at 37°C the samples were neutralised with 0.2M NaOH. After two washings in saline, the cells were extracted with HCl as described above for the trypsin-treated cells.

The rabbit antiserum produced against the z3 strain was used for cross-absorption experiments. For this purpose heat-killed cells of strains NCTC8037, NCTC10708 or z3 were added to an equal volume of the antiserum. These suspensions were incubated for 2 h at 37°C and refrigerated overnight. If the absorption was not complete, the treatment of the serum was repeated.

Numerical taxonomic analysis

In the numerical taxonomic analysis, 55 characteristics were used, but those properties common to all strains were excluded. The programme used ("cluster 3") had been developed at the Department of Biometry at the University of Minnesota Hospitals. This clustering method is called a "minimum spanning tree" (Hartigan, 1975). It can be summarised as follows: each of n subjects provides a vector of k observations. The elements of the measurement vector are subjected to a multiplicative transformation so that the standard deviation is one when computed over all subjects on the transformed scale. In the transformed space, all clustering is based upon the ordinary Euclidean distance between subjects. The first cluster is formed by joining together the two subjects closest to one another. Later, two clusters join if their edges are closer together than are those of any other pair of clusters.

RESULTS

Bacteriological examination

Fluid cultures of the patients’ strains were examined microscopically; all were gram-positive cocci in chains. The other characteristics that were common to all strains, and were therefore not used in the numerical analysis, were failure to produce catalase, resistance to optochin, and ability to ferment glucose.

In the computer analysis the majority of the streptococci isolated from our patients joined a single large cluster. Copies of the dendrogram are available from one of us (R. L.) on request. We considered the clustered organisms to be S. milleri-like for the following reasons. Two S. milleri reference strains (NCTC10708 and NCTC10709) originally isolated by Guthof, Streptococcus MG, and all group-F reference strains were found in the cluster. Six of these
reference strains were also grouped as *S. milleri* in the numerical classification studies of earlier workers. Seventy-nine of the 99 freshly isolated strains were thus tentatively classified as *S. milleri*. The majority of these strains (80 of 99) were able to grow aerobically with minute colonies, but 5% CO₂ almost always

### TABLE II

**Biochemical characteristics* of the streptococcal strains**

<table>
<thead>
<tr>
<th>Test</th>
<th>Number of positive results† shown by</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>streptococci clustered as <em>S. milleri</em></td>
</tr>
<tr>
<td></td>
<td>Strains isolated from patients (n = 79)</td>
</tr>
<tr>
<td>Fermentation of melibiose</td>
<td>3</td>
</tr>
<tr>
<td>sucrose</td>
<td>77</td>
</tr>
<tr>
<td>trehalose</td>
<td>72</td>
</tr>
<tr>
<td>melezitose</td>
<td>0</td>
</tr>
<tr>
<td>raffinose</td>
<td>4</td>
</tr>
<tr>
<td>inulin</td>
<td>3</td>
</tr>
<tr>
<td>salicin</td>
<td>75</td>
</tr>
<tr>
<td>mannitol</td>
<td>4</td>
</tr>
<tr>
<td>sorbitol</td>
<td>0</td>
</tr>
<tr>
<td>arabinose</td>
<td>9</td>
</tr>
<tr>
<td>xylose</td>
<td>0</td>
</tr>
<tr>
<td>galactose</td>
<td>78</td>
</tr>
<tr>
<td>arbutin</td>
<td>76</td>
</tr>
<tr>
<td>maltose</td>
<td>79</td>
</tr>
<tr>
<td>ribose</td>
<td>1</td>
</tr>
<tr>
<td>cellulobiose</td>
<td>70</td>
</tr>
<tr>
<td>glycerol</td>
<td>0</td>
</tr>
<tr>
<td>Growth in the presence of 2-0% NaCl</td>
<td>78</td>
</tr>
<tr>
<td>6-5% NaCl</td>
<td>0</td>
</tr>
<tr>
<td>0-01% K tellurite</td>
<td>74</td>
</tr>
<tr>
<td>0-04% K tellurite</td>
<td>1</td>
</tr>
<tr>
<td>10% bile</td>
<td>73</td>
</tr>
<tr>
<td>Growth at 45°C</td>
<td>4</td>
</tr>
<tr>
<td>Splitting of aesculin</td>
<td>67</td>
</tr>
<tr>
<td>hippurate</td>
<td>0</td>
</tr>
<tr>
<td>Formation of dextran</td>
<td>1</td>
</tr>
<tr>
<td>levan</td>
<td>0</td>
</tr>
<tr>
<td>pigment</td>
<td>0</td>
</tr>
<tr>
<td>gelatinase</td>
<td>0</td>
</tr>
<tr>
<td>proteinase (casein)</td>
<td>0</td>
</tr>
<tr>
<td>arginine dihydrolase</td>
<td>78</td>
</tr>
<tr>
<td>NADase</td>
<td>0</td>
</tr>
<tr>
<td>Reduction of triphenyl tetrazolium chloride</td>
<td>2</td>
</tr>
<tr>
<td>Resistance to bacitracin (0-04 units)</td>
<td>79</td>
</tr>
<tr>
<td>60°C for 30 min.</td>
<td>0</td>
</tr>
</tbody>
</table>

* Only characteristics present in at least 80% or less than 20% of the streptococci clustered as *S. milleri* are listed here.

† Percentages are given in parenthesis.
enhanced growth and colony size. This was true for the *S. milleri*-like strains as well as for the streptococci not clustered together with *S. milleri*. Two freshly isolated strains were unable to grow in air either with or without CO₂, but they were thought to be streptococci because lactic acid alone was detected as an end product of glucose metabolism (see Holdeman and Moore, 1973); they did not belong to the *S. milleri* cluster. Seventeen other isolates grew only in air with 5% CO₂, or anaerobically. In the *S. milleri* cluster, 12 strains (14%) showed a similar requirement for CO₂. The 37 streptococcal reference strains—including those classified as *S. milleri*—were able to grow aerobically without CO₂.

Only a few freshly isolated strains exhibited α- or β-haemolysis on blood agar plates incubated anaerobically. The *S. milleri* group (including the reference strains) contained 74 (86.0%) non-haemolytic (indifferent) strains, two (2.3%) α-haemolytic strains and 10 (11.6%) β-haemolytic strains. Nine of the β-haemolytic *S. milleri*-like strains produced minute colonies under aerobic conditions, and the remaining strain required CO₂ for aerobic growth. These β-haemolytic streptococci joined the *S. milleri* cluster at lower levels of similarity than most of the non-haemolytic strains.

Table II lists other characteristics present in at least 80% or less than 20% of the strains brought together as *S. milleri*. A single difference only was found between the biochemical reactions of the non-haemolytic and the β-haemolytic *S. milleri*-like strains: lactose was fermented by 63 (82.9%) of the non-haemolytic strains, but by only one of the 10 β-haemolytic isolates.

### Serological examination

The serological reactions of the *S. milleri*-like strains are summarised in table III. In our hands identical grouping results were achieved with each of the three extraction procedures. The group-F antigen was detected in 30 strains and the group-C antigen in four. The four group-C strains were completely non-haemolytic, but none of the group-F strains showed β-haemolysis. Among

<table>
<thead>
<tr>
<th>Group antigen</th>
<th>Number of strains</th>
<th>Number of strains with type-II antigen</th>
<th>type-III antigen</th>
<th>“sm” protein antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>4 (4)</td>
<td>0</td>
<td>2 (2)</td>
<td>3 (3)</td>
</tr>
<tr>
<td>F</td>
<td>30 (26)</td>
<td>5 (4)</td>
<td>5 (4)</td>
<td>20 (18)</td>
</tr>
<tr>
<td>None</td>
<td>52 (49)</td>
<td>0</td>
<td>1 (1)</td>
<td>33 (31)</td>
</tr>
<tr>
<td>Total strains</td>
<td>86 (79)</td>
<td>5 (4)</td>
<td>8 (7)</td>
<td>56 (52)</td>
</tr>
</tbody>
</table>

Numbers in parenthesis give numbers for *S. milleri*-like streptococci isolated from patients.
the latter, five possessed also the type-II antigen, but the type-III antigen was found only in non-haemolytic *S. milleri* strains. No type-IV strain was detected in our series. No freshly isolated strain possessing a type-II or type-III antigen was found outside the *S. milleri* cluster.

Hydrochloric acid extracts from 56 (65.1%) of the 86 *S. milleri*-like streptococci reacted in the capillary precipitin test with an antiserum prepared in our laboratory against the z3 strain. The z3 vaccine strain is a mutant of a type-III minute-streptococcal strain, obtained by serial culture in the presence of type-III antiserum and is said to lack a carbohydrate type-antigen but to possess a carbohydrate antigen that resembles a group antigen and has been designated "z3" (Willers and Alderkamp, 1967). When acid extracts of certain group-F strains were examined in the slide double-diffusion system with antiserum prepared in our laboratory against the z3 strain, a line of precipitate appeared that did not coalesce with the group- or type-antigen lines. The reactions obtained with an acid extract of the group-F type-III strain NCTC8037 and three antisera (group F, type III, and z3) are shown in fig. 1(a). The reaction between the acid extract and the z3 antiserum is shown by a sharp line. Fig. 1(b) shows that a corresponding line did not develop when a formamide extract was used. This led us to believe that the reactions with the z3 antiserum were based on the presence of one or more protein antigens, subsequently referred to as "sm" antigens. To examine this possibility, crude HCl extracts reacting with z3 antiserum were treated with highly purified proteolytic enzymes as described above and tested in double-diffusion and capillary-tube tests, before and after the treatment.

The sm-protein precipitin reactions demonstrated with z3 antiserum were completely abolished by treating the streptococcal extracts with pepsin. Trypsin destroyed the antigen in HCl extracts of strains NCTC8037, NCTC10708 and z3. The protein antigen reacting with the z3 antiserum could also be removed from whole cells of the three strains by pepsin or trypsin treatment; HCl extracts of the treated cells did not form a precipitate with z3 antiserum. Pepsin- or trypsin-treated acid extracts or acid extracts from enzyme-treated cells of the group-F type-III strain, NCTC8037, still reacted in the agar precipitin test with the group-F antiserum and the type-III antiserum, but not with z3 antiserum; the precipitin lines were similar to those obtained with a formamide extract of strain NCTC8037 and illustrated in fig. 1(b).

In capillary precipitin tests the reaction with z3 antiserum occurred in 56 (65.1%) of the 86 strains clustered as *S. milleri*. This reaction was found in only five freshly isolated strains not included in the *S. milleri* cluster, but was found in 52 of the 79 strains of *S. milleri* isolated from patients.

All acid extracts that yielded precipitin lines with the z3 antiserum prepared in our laboratory were compared with extracts of the strains NCTC8037, NCTC10708 and z3. Most of the former extracts gave lines fusing with the lines of strains NCTC8037 and z3, but with some strains the precipitin line completely coalesced with that of NCTC10708, and spur formation occurred with the lines of strains NCTC8037 or z3, suggesting either partial identity or the involvement of two protein antigens. The reactions of HCl extracts of
FIGS. 1a and 1b.—Comparison of agar-gel precipitation reactions of hydrochloric acid and formamide extracts from a group-F type-III streptococcus (strain NCTC8037). The wells marked 1, 2, 3 and 4 contained group-F antiserum, 23 antiserum, type-III antiserum and group-F antiserum respectively. In fig. 1a the central well (marked L) contained a hydrochloric acid extract prepared by the method of Lancefield. In fig. 1b the central well (marked F) contained a formamide extract prepared by the method of Fuller.
**Streptococcus milleri**

Fig. 2.—Reactions of hydrochloric acid extracts from strains NCTC8037 (well 1) and NCTC10708 (well 2) with Z3 antiserum (central well), showing spur formation by the precipitin lines of the two extracts.
Fig. 3.—Double-diffusion reactions between hydrochloric acid extracts of strains NCTC 10708 (well 2) and NCTC 8037 (well 3) and antisera to whole cells of strain 23 (central well) and to sonically disintegrated cells of strain NCTC 10708 (wells 1 and 4).
NCTC8037 and NCTC10708 with the z3 antiserum prepared in our laboratory are demonstrated in fig. 2, in which spur formation can be seen. Seventeen of the 56 S. milleri-like strains that had reacted with the z3 antiserum in the capillary precipitin test gave a line of complete identity with an extract of NCTC10708, but showed spur formation with the extracts of strains NCTC8037 and z3. One such strain was found outside the S. milleri cluster. From cross-absorption experiments with the z3 antiserum and cells of strains NCTC8037, NCTC10708 and z3 it may be concluded that two protein antigens (or two antigenic determinants on a single molecule) were involved: cells of strains NCTC8037 and z3 absorbed from the antiserum its capacity to react with extract of strain NCTC10708, while cells of strain NCTC10708 absorbed the antibodies to the homologous protein antigen but not the ability to react with extracts of strains NCTC8037 and z3. Pepsin-treated cells of strain NCTC8037 did not absorb from the z3 antiserum its ability to react with the protein antigens in acid extracts of strains NCTC8037, NCTC10708 and z3.

The involvement of two antigens may be deduced from the double-diffusion experiment shown in fig. 3. A rabbit antiserum against sonically disintegrated cells of strain NCTC10708 (wells 1 and 4) produced a fusing precipitin line with acid extracts of strains NCTC10708 and NCTC8037, whereas z3 antiserum gave a separate precipitin line with the extract of strain NCTC8037 only.

DISCUSSION

This study supports the thesis (Colman and Williams, 1965, 1967; Colman, 1968, 1969) that S. milleri constitutes a distinct group within the oral streptococci. Although we used only 55 characteristics for numerical classification, the results are in good agreement with those of Colman and Williams (1967), Colman (1968), Edwardsson (1974) and Mejär (1975a).

Most of the organisms in the S. milleri cluster fermented sucrose, trehalose, salicin, arbutin, maltose and cellobiose, and the non-haemolytic strains generally fermented lactose in addition. Many did not ferment raffinose, inulin, mannitol or sorbitol, but most grew in the presence of 10% bile and of 0-01% but not 0-04% potassium tellurite. Aesculin was hydrolysed and ammonia was produced from arginine by nearly all the strains. Only two dextran-producing strains, one of which was Streptococcus sanguis strain NCTC7863, were found in the S. milleri cluster, but no levan-producing strain occurred. All the S. milleri-like organisms were resistant to bacitracin. The β-haemolytic strains, which joined the cluster at a below-average level of similarity, differed from the non-haemolytic strains by their inability to ferment lactose. All group-F strains except one were found within the S. milleri cluster; type-II and type-III antigens (Ottens and Winkler, 1962) were exclusively present in S. milleri strains, with the exception of NCTC10446 (group T). The characteristics of the S. milleri-like strains outlined here correspond well with other descriptions (Ottens and Winkler, 1962; Colman and Williams, 1967; Edwardsson, 1974; Mejär, 1975; Mejär and Edwardsson, 1975). The findings of this study are consistent with the view that streptococci resembling S. milleri in their physiological properties should be so named, even if they possess a group A, C, F or
G antigen, and whether or not they are β-haemolytic (Colman and Williams, 1967, 1972; Parker and Ball, 1976; Poole and Wilson, 1976). The finding of one or two protein antigens ("sm" antigens) in many of the S. milleri-like strains, including β-haemolytic strains, gives for the first time serological evidence of the close relationship of organisms within this group. The experiments with proteolytic enzymes ruled out the possibility that one of the precipitin lines occurring with the z3 antiserum (obtained from four rabbits in our laboratory) was due to the group antigen-like carbohydrate of strain z3. A single sample of antiserum, from a single rabbit, showed in addition to the protein-antigen precipitin line a faint precipitin line with HCl and formamide extracts of the strain z3, probably due to a reaction with the group antigen-like z3 carbohydrate. Further studies are needed on the nature and the specificity of these proteins. The protein antigens may well have been responsible for the difficulties described by Bliss (1937) with vaccine strains modified by culturing them in homologous immune serum. In reports of studies (Willers and Alderkamp, 1967; Willers et al., 1973b) on the z3 strain, no mention was made of protein antigens; however, formamide extracts were employed in much of this earlier work, and the purification procedures used in preparing other extracts may have reduced or eliminated such antigens.

Our study supports the view of Colman and Williams (1972) that the "minute" (Long and Bliss, 1934; Bliss, 1937; Deibel and Niven, 1955) β-haemolytic streptococci should be accepted as varieties of S. milleri. We would not classify the β-haemolytic S. milleri-like strains in a separate species, called Streptococcus anginosus, as proposed by Cowan (1974). Although the name S. anginosus has been used for non- or β-haemolytic streptococci resembling S. milleri (Deibel and Seeley, 1974), priority should be given to the designation S. milleri because type strains of this organism are available, and S. milleri has been described more precisely (Guthof, 1956) than S. anginosus (Andrewes and Horder, 1906).

S. milleri has been shown to be indigenous to the human oral cavity (Bowden, Hardie and Slack, 1975; Mejäre and Edwardsson, 1975). The frequency with which it occurs in infected dental-root canals (Ottens and Winkler, 1962; Mejäre, 1975a and b) and in cervico-facial infections of dental origin, either in pure or in mixed culture suggests some pathogenic potential. This can also be concluded from the report of Parker and Ball (1976) in which, in a large series of strains, S. milleri was the species most frequently isolated (29.3% of cases) from purulent lesions in internal organs. It was associated particularly with brain abscesses, meningitis, pleural empyema, and intra-abdominal abscesses. Other reports, such as those by Reid and Davidson (1976) and Facklam (1977), also describe S. milleri-like streptococci isolated from pyogenic liver abscesses, brain abscesses or other types of infections. Bacteraemia has also been reported (Bannatyne and Robson, 1974; Parker and Ball, 1976), and these organisms have been isolated in association with acute appendicitis (Wort, 1975; Bannatyne and Randall, 1977). No extracellular products have been described other than hyaluronidase (Colman and Williams, 1967, 1972) and deoxyribonuclease (Lütticken and Wannamaker,
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1975); these may contribute to the pathogenicity of S. milleri. The sm protein antigens detected in this study may possibly contribute to the virulence of S. milleri, as does M protein in S. pyogenes. The experiments with proteolytic enzymes suggest that these sm proteins are located at or near the surface of the streptococcal cell, giving support to the hypothesis that they may play a role in virulence. We have examined only strains from pyogenic infections—mainly dental abscesses—and it would therefore be useful to examine the protein antigens of S. milleri-like streptococci from infections in other sites and from healthy persons.

SUMMARY

Ninety-nine strains of streptococci were isolated from 97 cases of pyogenic infections, most of which involved the teeth. Physiological and serological tests were performed on these streptococci and on 37 strains of streptococci from culture collections. The results were used for a numerical classification. Seventy-nine of the strains isolated from patients formed a cluster with Streptococcus milleri and group-F reference strains, and were therefore considered as streptococci resembling S. milleri. By the use of an antiserum prepared against strain 23, protein antigens were demonstrated in acid extracts of 65% of the strains of S. milleri. These antigens were found in only five strains not included in the S. milleri cluster.

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