APPENDIX 5

Classification of Nonhaemolytic Streptococci

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There is much to be learned about the systematic relationships of the streptococci, and new techniques, for instance the study of cell-wall composition, need to be applied to increase our knowledge of these relationships. Moreover, the knowledge that we already have can perhaps be made more effective by the use of electronic computers. This paper reports some of our attempts to apply these techniques to the study of streptococci.

In brief, we have a collection of strains that has been studied by a wide range of tests. The collection is not representative of any natural population, but the majority of the strains are of human origin; some were freshly isolated, others were from culture collections. Three hundred forty-two of the strains can, in our opinion, be put in established or previously proposed taxa, and only 4 strains cannot be given some sort of label. This suggests that previous workers have made a comprehensive catalogue, but because many of the divisions are based on a single diagnostic feature, the value of the process remains doubtful. The most time-consuming test that we use is the study of cell-wall composition, and it has been carried out on 213 strains. This paper is concerned mainly with those strains that could conceivably be called Streptococcus viridans and for which we have complete data.

The Lancefield Group Reaction

In the classification of streptococci, there has been a tendency to equate "serological group" with "species," and this has led to the removal of any strain that gives a group precipitation from the species named S. viridans (or mitis). The Lancefield group reaction is without doubt the most reliable single method of identification we have. Some group antigens appear to define a homogeneous species, such as group B and S. agalactiae; the same is practically always true of S. pyogenes (group A). But there are strains in which a group reaction conflicts with other evidence. Some of these conflicts can be attributed to the choice of immunizing strain. Thus, some sera labeled "group O," including the one that we have used, contain antibodies not only to the

"O" antigen but also to an antigen common to group O haemolytic streptococci, some greening streptococci and S. pneumoniae. It is well known that there are two "groups" labeled "H." Strain "Challis" belongs to one and strain "Perryer" to the other. And more strains will be called "K" if strain "Levy" is used for immunizing rabbits rather than strain "Perrot." The sera we have used are prepared against the strains "Challis" (group H) and "Perrot" (group K). The difficulties mentioned above can be overcome by general agreement on vaccine strains and methods of testing. A more profound difficulty is raised by, for example, the Streptococcus of group A that has more in common with indifferent strains of group F than with S. pyogenes. Furthermore, group D reactions can be obtained with strains that are not even streptococci. For the purposes of classification, as opposed to identification, we would therefore regard serological grouping as just one of many relevant tests.

Results of Computer Analysis

The results from 216 strains (10 of which were pediococci) were subjected to "cluster analysis" in a computer programme devised by Jeffrey Harrison of I. C. I. who with his staff carried out all computer procedures with this programme. The computer was instructed to determine the similarity coefficient of each strain in relation to every other strain and then print out, at a number of different significance levels, the clusters for which this relationship is hardly due to chance; as the significance level falls the clusters contain more strains and new but less homogeneous clusters are formed. A number of limitations are imposed on the clusters: a strain in one cluster cannot be the centre of a new cluster, that is the clusters can be related thus

Each cluster had to contain at least five strains not present in any other cluster; this limitation meant that a cluster of S. pyogenes could not be formed as we had not included sufficient strains in the collection.

At the 99 significance level the computer allotted 88 of the 216 strains to 7 clusters. The attributes of the clustered strains are listed in Table 1; two clusters, one of enterococci and one of pediococci, are omitted.

Table I. Computer-formed Clusters at 99% Significance Level
(clusters of enterococci and pediococci are omitted)

No. of strains	Cluster No.				
	1	2	3	4	5
	19	8	18	8	8
Serological reactions	A 1 C 2 P 9 O 1	H 7	K 1 KM 1 O 10	B 7 G 1	K 6
Only Ottens antigens 2					
Chains	15	5	18	8	5
Pairs	4	3	0	0	3
Haemolysis	0	6	3	7	0
Greening	0	2	15	1	0
Indifferent	19	0	0	0	8
Bile soluble	0	0	0	0	0
Optochin sensitive	0	0	0	0	0
Bacitracin sensitive	0	0	6	4	5
Nitrofurazone sensitive	0	5	16	3	0
5% sucrose agar, type of colony formed:					
normal	19	3	18	8	1
adherent	0	0	0	0	0
fleshy	0	0	0	0	7
Growth on:					
40% bile	15	4	0	8	5
10% bile	18	8	0	8	8
MacConkey agar	2	0	0	7	0
Survival at 60° 30 mins.	0	0	0	0	0
Growth at 45°	1	0	0	0	1
Growth in:					
6.5% NaCl broth	0	0	0	0	0
4.0% NaCl broth	1	0	0	7	0
NH ₃ from arginine	19	8	0	8	0
Hippurate hydrolysis	0	0	1	7	0
CAMP test	0	0	0	7	0
Starch hydrolysis	0	0	2	0	0
Sugar reactions at 2 days:					
maltose	19	8	17	8	8
glycerol-					
aerobic	0	0	0	6	0
anaerobic	0	0	0	0	0
mannitol	0	0	0	1	0
sorbitol	0	0	0	0	0
arabinose	0	0	0	0	0
lactose	16	7	16	4	6
sucrose	19	8	17	7	8
trehalose	18	8	2	8	8
raffinose	0	1	8	0	4
salicin	19	8	0	8	8
inulin	0	7	1	0	1
aesculin	16	7	0	0	8
Hyaluronidase	2	0	2	7	0
Glucuronidase	0	0	0	5	0
V.P. test	19	0	1	1	3
Tellurite broth:					
1/1000	2	1	0	0	1
1/4000	14	6	2	5	7
1/10000	19	8	10	7	8
Cell wall residues:					
glycerol	0	0	0	0	0
anhydrosorbitol	0	0	18	0	0
rhamnose	19	8	0	8	8
rhamnose - trace	0	0	4	0	0
glucose	19	7	17	7	8
galactose	15	7	18	8	8
galactosamine	16	3	16	1	3
fucosamine	0	1	0	0	0

Two of the clusters consist of well recognized "species" — S. agalactiae (cluster 4) and S. salivarius (cluster 5). Cluster 4 contains the 7 strains of S. agalactiae that were in the data fed to the computer and also one haemolytic strain of Lancefield group G of the large-colony variety. At the 80% significance level, an additional group G strain is found in the cluster, but both these strains are also members of an additional cluster formed of group G strains.

Cluster 5 is made up of 7 of the 10 strains of S. salivarius and an additional group K strain that appears to differ in that it fails to produce mucoid colonies on sucrose agar. These clusters seem satisfactory.

Cluster 1 contains 13 indifferent streptococci of Lancefield's groups A, C, F or G, 2 strains lacking a group antigen but containing an Ottens antigen, and 4 strains that are serologically unreactive. Included in the last are some strains of S. milleri sent to us by Professor Guthof. His description of this species in 1956 notes the absence of group reactions. However, the indifferent streptococci with the serological reactions listed above are so like the strains that are serologically unreactive in physiological behaviour and in cell-wall composition that we classify them all together. The strains in cluster 1 are thus homogeneous in a number of physiological tests such as the splitting of arginine, the Voges-Proskauer test and resistance to bacitracin and nitrofurazone.

Cluster 2 in Table I contains many destran-forming strains and some additional strains of group H that fail to produce dextran. The close relationship between group H and S. sanguis is well known, and we find Dr. Niven classifying them together in the 7th edition of Bergey's Manual.

Cluster 3 is made up of strains with a combination of distinctive properties. The cell wall contains a polymer which yields anhydrosorbitol on acid hydrolysis. Most streptococci contain readily detectable amounts of rhamnose in the cell wall, but strains in this cluster show only trace amounts of rhamnose or none at all. Most streptococci split arginine, those in cluster 3 do not, and acid is produced from a notably small range of sugars. The streptococci in this cluster also fail to grow on 10% bile agar. The greening strains show wide zones of colouration, and when grown in broth the upper portion of the broth is often clear.

Aggregation of Additional Strains to the Computer Clusters

One view of the reactions of bacteria is that relatively homogeneous centres of population are linked by intermediate forms. If this view is correct, and it has much to commend it, then we should be concerned not only with what might be termed typical specimens but also the premissible range of species. We have, therefore, examined the full collection of strains to determine which additional strains might be aggregated to the computer-formed clusters, and the results of this analysis are presented in Table II for 3 possible species, based on clusters 1, 2 and 3.

The strains in cluster 1 conform quite well to Guthof's S. milleri and are so named in Table II; a total of 36 strains seem reasonably included.

The strains added to cluster 2 to form the S. sanguis aggregate in Table II are largely additional strains of group H and dextranformers. Some strains included here as serologically unreactive would be called group H if "Perryer" (F90A) were used instead of "Challis" as the immunizing strain for the production of group H antiserum. Others of this alternate group H (for example K208) have been placed in S. viridans aggregate in Table II on the basis of cell-wall composition, failure to split arginine, sensitivity to bile and sugar reactions; it seems that the more homogeneous grouping is achieved by basing the group H on the strain "Challis" rather than on the strain "Perryer." Four strains of group K were aggregated to this species as they seemed closely related to these strains and unlike the strains of group K in the S. viridans aggregate. A total of 32 strains seems reasonably included here.

A total of 35 strains is included in the S. viridans aggregate which is formed about cluster 3 of Table I. With many strains there was little difficulty in deciding with which cluster they should be aggregated. Occasional strains did seem to be truly intermediate in their reactions. Several ways of adding further strains were tried, but division on the basis of cell-wall composition seemed to give the highest number of correlated features. Difficulty was encountered with a few strains of S. sanguis (for example NCTC 7864, S. sanguis II) which differ from this aggregated species mainly in their ability to produce dextran from sucrose. They were, therefore, separated from the other dextranformers and included in the S. viridans aggregate.

Table II.

Aggregated Species

	<u>S.milleri agg.</u>	<u>S.sanguis agg.</u>	<u>S.viridans agg.</u>
No. of strains	26	22	25
Serological reactions	A 2 C 2 F 13 G 3 Only Ottens antigens 7	H 14 K 4	K 4 O 13
Chains	25	25	34
Pairs	11	7	1
Haemolysis	0	9	9
Greening	3	20	26
Indifferent	23	3	0
Bile soluble	0	0	0
Optochin sensitive	0	0	0
Bacitracin sensitive	3	9	9
Nitrofurazone sensitive	2	23	25
5% sucrose, type of colonies			
formed: normal	26	19	31
adherent	0	13	4
Growth on: 40% bile agar	19	14	1
10% bile agar	26	26	5
MacConkey agar	4	3	1
Survival at 60°: 30 min.	0	0	0
Growth at 45°	1	0	0
Growth in: 6.5% NaCl broth	0	0	0
4.0% NaCl broth	2	1	1
NH ₃ from arginine	25	27	3
Hippurate hydrolysis	0	0	1
CAMP test	0	0	0
Starch hydrolysis	1	8	5
Sugar reactions at 2 days:			
maltose	26	32	34
glycerol: aerobic	0	0	0
anaerobic	0	0	0
mannitol	1	2	0
sorbitol	1	0	0
arabinose	0	0	0
lactose	30	27	31
sucrose	25	32	35
trehalose	22	29	9
raffinose	3	9	19
salicin	25	29	2
inulin	3	13	2
aesculin	25	15	2
Hyaluronidase	7	0	5
Glucuronidase	0	0	0
V.P. test	22	2	8
Tellurite broth: 1/1000	2	1	0
1/4000	23	15	8
1/10000	25	26	25
Cell wall residues:			
glycerol	0	1	0
anhydrosorbitol	0	1	32
rhamnose	26	22	0
rhamnose - trace	0	0	16
glucose	25	30	31
galactose	28	26	35
galactosamine	25	7	28
fucosamine	0	5	1

CONCLUSIONS

We suggest that the strains in our collection that might colloquially be termed S. viridans can be allotted to three species. One aggregate species is clearly related to the S. milleri proposed by Professor Guthof. We suggest that the act of classifying together the indifferent streptococci of Lancefield's groups A, C, F and G with strains in which group reactions are as yet unknown brings together like strains and separates them from unlike strains. The cluster of these strains in Table I is both homogeneous and distinctive, and we think it would serve as a basis for the amendment of Professor Guthof's definition. One other named organism found in this cluster is Streptococcus MG, but this is not a Latin binomial.

In the other two aggregates, little account has been taken of group reactions, but for a different reason. Here, the group reactions H, K and O are, at present, so imprecise that it would be hazardous to use them as determinant characters.

The "S. sanguis aggregate," formed largely of strains that produce dextran or react with a group H serum, is the least satisfactory of the 3 aggregated species. The classification together of strains such as these is not new, but we have been unable to find in our data anything that sharply separates this aggregate from the S. milleri aggregate. However, the modal points as determined in the computer clearly differ, and adequate definition of the limits of the aggregate may be possible when further tests have been carried out on these strains.

Cluster 3 in Table I would, in our opinion, serve as a basis for the definition of S. viridans (or S. mitis). The attributes of these strains come within the description given to S. mitis by Dr. Niven in the 7th edition of Bergey's Manual. Further work needs to be done, particularly on the serological reactions of the strains, but we suggest that the cluster is distinctive and serves as the centre of a useful aggregate.