The Classification of Staphylococci and Micrococci from World-wide Sources

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SUMMARY

A study has been made of 607 cultures of Gram-positive and catalase-positive cocci received from workers and collections in different parts of the world. These cultures were examined for a wide range of morphological and physiological characters and representative cultures were further studied to determine the chemical constituents of the organisms. Five hundred and sixty-four of the cultures received were aerobic members of the Micrococcaceae, and of these 96% were classified in the author's groups and subgroups; a further subgroup was, however, introduced to accommodate the not previously studied pink-pigmented micrococci. It appears that the Gram-positive and catalase-positive cocci are best separated into the genus *Staphylococcus* and the genus *Micrococcus* on the ability of members of *Staphylococcus* to grow and produce acid from glucose anaerobically; six subgroups of staphylococci were recognized and eight of micrococci. The relationship of named species, groups and subgroups to the author's classification was examined; several species and groups of micrococci had been incorrectly classified. Thus, *M. denitrificans*, *M. halodenitrificans* and *M. radiodurans* possess characters which suggest that they should be reclassified with the Gram-negative genera and that Abd-El-Malek and Gibson's group IIIB should be classified with the Gram-positive microbacteria.

INTRODUCTION

This paper records the study of 607 cultures of non-halophilic Gram-positive and catalase-positive cocci received from workers and collections in different parts of the world. They were examined by using a wide range of physiological tests; representative organisms were further studied to determine their chemical composition. The organisms studied were named species, groups or subgroups that have been maintained in culture collections or were recent isolates which had been sent to the author as Gram-positive and catalase-positive cocci, or as belonging to the genera *Micrococcus*, *Staphylococcus* or *Sarcina*. Included amongst these organisms were the original strains used in the recent classifications of the Gram-positive and catalase-positive cocci by Abd-el-Malek & Gibson (1948), Shaw, Stitt & Cowan (1951), Pohja (1960), Anderson (1962), Mossel (1962), and Kocur & Martinec (1962). The objects of this work were: to apply the author's proposed classification of the Gram-positive and catalase-positive cocci (Baird-Parker, 1962, 1963) to strains isolated from a wide range of sources; to compare the characters of named species from different collections and habitats; to establish the relationship between the different taxa proposed in recent classifications of these organisms.

METHODS

Table 1 gives the sources of the cultures examined. They had been received on agar slopes or freeze-dried in ampoules. They were checked for purity by plating on the yeast glucose agar described by Baird-Parker (1963) and maintained on slopes of heart-infusion agar stored at 5°; 467 of these cultures were subsequently freeze-dried for further study.

Table 1. *Source of cultures*

Donor	Source	No. of cultures	Description of cultures
Dr J. I. W. Anderson	North sea	8	Marine micrococci
Professor R. Buttiaux	food	4	Staphylococci, micrococci
Miss E. Coster	miscellaneous	7	Named species of aerococci, pediococci
Dr C. M. Cousins	milking equipment	6	Gram-positive catalase-positive cocci
Dr S. T. Cowan	miscellaneous	85	Named species of micrococci, sarcinas, staphylococci, aerococci, pediococci (NCTC collection*)
Dr G. H. Davis	dental	6	Staphylococci
Dr R. H. Deibel	miscellaneous	3	<i>Micrococcus hyicus</i> ; 'denitrifying' staphylococci
Dr E. I. Garvie	miscellaneous	40	Named species of micrococci, sarcinas, staphylococci (NCDO collection†)
Professor N. G. Heatley	sewage	1	Su's (1948) micrococcin-producing micrococcus
Dr A. J. Holding	acid soils	12	Micrococci, sarcinas
Miss M. John	(1) nose	21	Coagulase-negative staphylococci
	(2) air	50	Gram-positive catalase-positive cocci
Dr M. Kocur	miscellaneous	90	Named species of staphylococci, micrococci
Dr J. Liston	sea foods and marine mud	20	Staphylococci, micrococci
Dr E. J. Lowbury	skin burns	50	Staphylococci, micrococci
Dr I. J. McDonald	Cheddar cheese	10	Cocci
Mr J. H. H. Mol	dry sausage	6	Micrococci
Dr D. A. A. Mossel	food	2	<i>Staphylococcus aureus</i> , and a micrococcus
Professor R. G. E. Murray	human	6	'Inhibitory micrococci'
Dr F. P. Ninivaara	dry sausage	1	<i>Micrococcus</i> M53
Mr J. Patterson	pork, Wiltshire bacon and brine	139	Gram-positive catalase-positive cocci
Dr D. M. Pilsbury	human skin	4	<i>Staphylococcus albus</i>
Dr M. S. Pohja	(1) fermented meat products	11	Representatives of Pohja's (1960) subgroups
	(2) dry sausage	4	Micrococci
Dr J. W. Rouatt	miscellaneous	6	Named <i>Micrococcus</i> spp.
Dr H. Williams Smith	chicken, pig, cow, sheep	15	<i>S. aureus</i>
Dr J. M. Shewan	marine	10	Micrococci, cocci (NCMB collection‡)
Dr M. J. Thornley	food	1	<i>M. radiodurans</i>
Dr K. Warnøe	bacon	3	<i>S. aureus</i> , <i>S. epidermidis</i>

* National Collection of Type Cultures; † National Collection of Dairy Organisms; ‡ National Collection of Marine Bacteria.

Examination of morphology and physiological characters

Morphology was examined in hanging-drop preparations and in smears stained by Gram's method. Organisms for these studies were grown at 30° and 37° on yeast glucose agar and nutrient agar, and in nutrient broth. Motility was determined on cultures grown in yeast glucose broth for 6–7 hr at 30°; organisms were subcultured at least once in yeast glucose broth before testing. For studies of physiological characters, organisms were grown at 30° on yeast glucose agar and then subcultured on nutrient agar. Growth from plates of the latter was used to inoculate bottles of nutrient broth. Tests were done by using the media and methods described by Baird-Parker (1963).

Examination of cell and cell-wall components

Forty-five strains of Gram-positive, catalase-positive cocci were examined for sugars, amino sugars, amino acids, teichoic acids, peptides and proteins. Bacteria for these studies were grown by inoculating yeast glucose broth (200 ml.) in 1 l. conical flasks shaken at 120 oscillations/min. for 18–48 hr at 30°. One l. of culture of each organism was grown, giving a yield of 2 to 4 g. wet weight organism. The organisms were washed 3 times with distilled water and then divided into two equal parts. One part was broken to obtain cell-wall and cell-content fractions and the other was used for extracting constituents from whole organisms.

Organisms were broken by shaking with No. 12 ballotini beads at 5° in a Mickle disintegrator; conditions used were those found optimal by Salton & Horne (1951). The ballotini beads were removed by filtration through sintered glass filters (No. 1 porosity) and the cell-wall fragments collected by centrifuging the filtrates at 35,000g for 10 min. The supernatant fluids, containing the cell contents of the disrupted organisms, were concentrated to $\frac{1}{3}$ volume by dialysis in Visking dialysis tubing against polyethylene glycol (mol. wt. 20,000) at 5°; they were stored at –20°.

Amino acids. Cell-wall fragments were cleaned by digestion for 18 hr at 37° in 5 ml. of a 0.5% (w/v) solution of crystalline trypsin (pH 8.6). These were collected by centrifugation at 35,000g for 20 min. and after washing 3 times with distilled water they were hydrolysed with 6 N-HCl and further treated by the procedures described by Davis & Baird-Parker (1959). The amino acids were separated by two-dimensional chromatography on Whatman No. 1 filter paper by using the solvent system of Davis & Freer (1960). The papers were developed by spraying with a solution of ninhydrin (0.25%) in acetone + water + collidine (95 + 5 + 1 by vol.) and heating at 105° for 5–10 min. Isomers of diaminopimelic acid were separated by using the solvent system of Hoare & Work (1957).

Teichoic acids. The procedures of Armstrong *et al.* (1958) and Davison & Baddiley (1963) were used to isolate the teichoic acids. Cell walls (0.25 g. wet wt.) were treated as described by these authors and the purified material containing teichoic acid hydrolysed in 4 N-HCl for 6 hr at 100° (Elliott, 1962). Ribitol and glycerol were separated by chromatography on Whatman No. 1 paper by using the solvent system of Armstrong *et al.* (1958), and were detected by the method of Bean & Porter (1959).

Sugars. Washed organisms (0.75 g. wet wt.) were suspended in 5 ml. of 2 N-sulphuric acid and treated as described by Davis & Baird-Parker (1959). Sugars

were separated by single-dimension chromatography on Whatman No. 1 filter paper by the solvent system of Colombo *et al.* (1960). Sugars were detected by using Roy's (1960) double spray technique.

Amino sugars. Washed organisms (0.75 g. wet wt.) were suspended in 5 ml. of 5 N-HCl and incubated for 2 days at 37°; the HCl was removed under vacuum in a vacuum desiccator containing concentrated H₂SO₄ and NaOH pellets (Salton, 1959). The residues were dissolved in 3 ml. of 10 % (v/v) isopropanol in water and several drops of sp.gr. 0.880 ammonia added to neutralize remaining acid. After filtration through no. 4 porosity sintered glass filters, the filtrates were evaporated to dryness on a steam-bath and the residues dissolved in 0.3 ml. of 10 % (v/v) isopropanol in water. The amino sugars were separated by chromatography on Whatman No. 1 filter paper by using the solvent system of Heyworth, Perkins & Walker (1961) and detected by using Elson & Morgan's (1933) spray.

Peptides and amino acids extractable by acetic acid. Acetic acid extracts of whole organisms were shown by Mattick *et al.* (1956) to be of use in identifying bacteria. The procedure was to suspend 0.5 g. wet weight washed organisms in 5 ml. of 10 % (v/v) glacial acetic acid and to leave at room temperature for 1–2 hr with occasional shaking. The extracts were centrifuged at 10,000g to remove solids and the supernatant fluids evaporated to dryness in a rotary vacuum evaporator; the residues were finally suspended in 0.3 ml. volumes of 10 % (v/v) isopropanol in water. The amino acids and low molecular weight peptides were separated by chromatography on Whatman No. 1 paper by using the solvent system of Davis & Freer (1960) and detected with the ninhydrin + collidine spray used for amino acids.

Electrophoretic separation of cell proteins. Starch gels (Smithies, 1955) were prepared by dissolving 15 % (w/v) Connaught Brand hydrolysed starch in a semi-discontinuous buffer system containing 90 % (v/v) tris + citrate buffer (pH 8.68) and 10 % (v/v) borate buffer (pH 8.2); both buffers used were described by Poulik (1957) but LiOH replaced the NaOH specified in the original borate buffer (Mr A. L. Ogden, personal communication). The concentrated cell-content fractions were applied to strips of Whatman No. 17 filter papers (1.5 cm. × 6 mm.) and inserted 30 mm. from the cathode end of a gel 190 mm. long. A potential of 440 V and a current of 80 mA was applied along the gel, and electrophoresis continued until the moving boundary had migrated 80 mm. past the inserts. Protein bands were developed by staining slices of gel for 1 min. in a saturated solution of naphthalene black in methanol + water + glacial acetic acid (5 + 5 + 1 by vol.) followed by decolorization in several washes of the same solvent.

RESULTS

Preliminary examination of the morphology and catalase production of the organisms received showed that some had been incorrectly classified. Amongst these were three plasma-clotting strains of *Streptococcus faecalis* which had been sent to the author as coagulase-positive staphylococci and two other strains of *S. faecalis*, one of which had been identified as a coagulase-negative staphylococcus while the other had been named *Micrococcus freudenreichii*. Other wrongly classified organisms included several corynebacteria and unidentified Gram-negative rods; a strain of *Serratia marcescens* was received as a culture of *M. roseus*; a yeast was

received labelled as a micrococcus as were two strains of *Aerococcus* (*Pediococcus*) *viridans*. Several Gram-positive and catalase-positive cocci were also found to have been mis-classified as regards their genus or species.

Classification of organisms according to Baird-Parker (1963)

Five hundred and sixty-four of the cultures studied were Gram-positive catalase-positive cocci. With the exception of the pink micrococci, representatives of which had not been previously studied, almost all of the strains could be classified into the Baird-Parker (1963) groups and subgroups. To accommodate these pink pigmented organisms, a further subgroup, *Micrococcus* subgroup 8, was introduced. Characters of the fourteen subgroups are shown in Table 2.

Group 1 (Staphylococcus Rosenbach)

Organisms placed in this group grew and produced acid from glucose when incubated under anaerobic conditions in a mineral salts + yeast-extract medium containing bromocresol purple as pH indicator (Baird-Parker, 1963). With two exceptions, the morphological and physiological characters of the 180 cultures classifiable into this group agreed with previously studied cultures; i.e. they were usually small cocci (0.5–1.5 μ) growing as irregular clusters and producing on agar smooth convex colonies pigmented white, yellow or orange; their physiological characters included growth usually at 45° and sometimes at 10°. They also usually hydrolysed hippurate, proteins, fats and Tweens, and produced acid from galactose, glucose, glycerol, lactose, maltose, mannose and mannitol but not from arabinose, cellobiose, inositol, raffinose or xylose. Other characters included the ability of most strains to reduce nitrate beyond nitrite, and inability to hydrolyse aesculin or to grow on a medium containing ammonium phosphate as sole nitrogen source.

Characters of the subgroups within this group 1 also agreed closely with previous results. Exceptions were two otherwise typical subgroup II organisms which did not form acid from maltose and eight cultures which were acetoin-negative; four of the latter could be classified as acetoin-negative variants of subgroup V or VI. Of the four remaining acetoin-negative organisms only two possessed characters typical of staphylococci. One was coagulase-negative but similar to the coagulase-positive subgroup I organisms in the production of phosphatase and the formation of acid from mannitol in presence of air. However, it differed from sub-group I organisms by not coagulating human or rabbit plasma or forming acid from mannitol anaerobically. The other strain was phosphatase-negative and, of the sugar series tested, produced acid only from galactose, glucose and lactose.

The two apparently atypical staphylococci, both members of Abd-El-Malek and Gibson's group IIIB (NCTC 7567, 7568) appeared to be cocci when first examined. Characters which made me suspect that these were not staphylococci were: good growth on a medium containing ammonium phosphate as sole nitrogen source, hydrolysis of aesculin but not protein, fat, Tweens or hippurate, and formation of acid from a wide range of carbohydrates, including arabinose, cellobiose and xylose. On further examination of the cell morphology of these two organisms it was found that impression preparations of cultures grown for 4 hr on nutrient agar contained mainly short paired rods and that organisms examined in the electron microscope were

Table 2. Complete characters of subgroups

	(Group 1) <i>Staphylococcus</i> [Rosenbach] subgroups						(Group 2) <i>Micrococcus</i> [Cohn] subgroups								(Group 3) <i>Sarcina</i> [Good- sur]	<i>Aerococcus</i> [Williams, Hirsch & Cowan]
	I	II	III	IV	V	VI	I	2	3	4	5	6	7	8		
Colony																
(1) Nodular irregular edge	0	0	0	0	0	0	2	0	0	0	1	0	0	0		
(2) Granular surface, regular edge	0	0	0	0	0	(8)	4 (4)	(22)	2 (10)	0	3 (9)	0	15 (25)	20 (30)		
(3) Smooth convex regular edge	100*	100	100	100	100	93	93	76	88	100	86†	100	62	50		
Pigment																
(1) White	10	97	100	75	75	68	83	93	84	100	75	34	18	0		
(2) Yellow	0	0	0	25	17	28	37	0	8	0	17 (2)	0	83†	0		
(3) Orange	90	0	0	0	8	(4)	0	2 (5)	(6)	0	3 (3)	66	0	0		
(4) Red	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
(5) Violet	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
(6) Translucent	0	3	0	0	0	0	0	0	0	0	0	0	0	0		
Appearance in broth																
(1) Fine deposit	97	71	100	100	100	96	42	41	73	25	19	100	54	70		
(2) Granular deposit	0	(3)	0	0	0	(4)	13	2 (2)	8 (3)	0	3 (5)	0	13 (5)	10 (10)		
(3) Mucoid deposit	(3)¶	(26)	0	0	0	0	21 (26)	12 (41)	6 (8)	(75)	32 (41)	0	10 (18)	(10)		
Size																
< 1 μ	100	77	100	100	34	44	25	5	43	50	6	0	28	10		
1-1.5 μ	0	23	0	0	66	66	75	90	57	50	93	100	72	70		
> 1.5 μ	0	0	0	0	0	0	0	5	0	0	1	0	2	20		
Catalase	100	100	100	100	100	100	100	100	100	100	100	100	100	100		
Glucose																
Acid production under aerobic conditions	100	100	100	100	100	100	100	100	100	100	98 (2)	100	(10)	(60)		
Glucose																
Acid production under anaerobic conditions	100	100	100	100	100	100	0	0	0	0	0	0	0	0		
Coagulase production	100**	0	0	0	0	0	0	0	0	0	0	0	0	0		
Phosphatase production	100	100	0	0	0	0	0	0	(1)	0	(1)	100	(13)	0		
Terminal pH value in glucose	4.4-5.0	4.2-5.1	5.2	4.5-5.6	4.5-5.3	4.4-5.6	4.4-5.6	4.5-5.4	4.5-5.8	4.6-5.7	4.9-5.9	4.7-5.5	6.4-8.2	5.0-5.6		
(av. 4.6)	(av. 4.6)	(av. 4.5)	(av. 4.6)	(av. 4.6)	(av. 4.6)	(av. 4.6)	(av. 4.6)	(av. 4.9)	(av. 4.9)	(av. 4.9)	(av. 5.3)	(av. 4.9)	(av. 6.8)	(av. 5.3)		
Acetoin production	98 (2)	97 (3)	0	100	92	75 (15)	100	98 (2)	98	75 (25)	0	0	(9)	4 (6)		

Table 2 (cont.)

Aerobic acid production from:														
Arabinose	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cellulose	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dextrin	54 (5)	71	100	0	0	0	0	0	0	0	0	0	0	0
Galactose	100	100	100	0	0	0	0	0	0	0	0	0	0	0
Glycerol	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Inositol	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lactose	92 (1)	71	100	0	0	0	0	0	0	0	0	0	0	0
Maltose	99 (1)	97	0	50	100	65	82 (4)	98 (2)	100	95 (1)	75	1 (9)	0	0
Mannose	100	55	0	50	100	17	44	5	22 (2)	50	63 (7)	100	0	0
Mannitol	96 (1)	0	0	0	0	0	0	0	0	0	0	0	0	0
Raffinose	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rhamnose	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Salicin	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Xylose	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hydrolysis of:														
Asculin	8 (10)	3	0	0	0	0	0	0	0	0	0	0	0	0
Hippurate	99 (1)	87 (3)	100	100	100	92 (8)	25 (9)	25 (9)	96	94	89 (11)	0	0	0
Gelatin	63 (3)	68	100	100	100	25 (8)	41 (4)	41 (4)	41 (2)	24 (3)	25	84	90 (2)	0
Clearing of:														
Casein	91 (1)	61 (29)	100	25 (25)	42 (8)	43 (36)	20 (8)	5 (12)	24 (27)	0	8 (11)	33 (44)	31 (4)	0
Egg yolk	92 (2)	68	100	50	17 (17)	61 (11)	21	5	14 (1)	0	7 (4)	16 (14)	0	0
Ammonia from arginine broth	100	81 (6)	100	75	42 (8)	89	50 (18)	64 (7)	90 (6)	100	48 (20)	78 (22)	4 (9)	0
Nitrate														
No reduction	0	16	0	0	0	4	17	32	48	0	16	0	85	100
Reduced to nitrite	6	13	0	0	14	21 (14)	8	30	14	25	41 (1)	56	4 (9)	0
Reduced beyond nitrite	94	71	100	100	86	61	75	38	38	50	22	44	2	0
Lard hydrolysis														
Butter hydrolysis	96	100	0	50	58	64 (7)	42	73 (2)	66 (8)	25	34 (2)	11	97 (7)	0
Tween 20 hydrolysis	96	100	0	50	58	64 (11)	42	76	68 (6)	25	34 (3)	11	23 (14)	0
Tween 40 hydrolysis	93 (5)	100	100	50	58	96	27	35 (5)	46 (1)	0	0	11	54 (2)	0
Tween 60 hydrolysis	91 (7)	100	100	50	50 (8)	96	14	24 (3)	41 (3)	0	0	11	63 (4)	0
Tween 80 hydrolysis	53 (18)	97	100	50	33 (25)	89	14 (4)	24	43 (3)	0	0	11	58 (7)	0
Ammonium phosphate used	0	0	0	0	27 (17)	89	13 (4)	17	26 (1)	0	0	11	19 (7)	0
Growth in the presence of no added NaCl	0	56 (45)	100	100	83 (17)	100	96 (4)	98 (2)	92 (3)	100	99 (1)	89	19 (25)	0
Growth in the presence of 5% NaCl	99 (1)	100	100	100	92 (8)	100	88 (12)	98 (2)	95 (5)	100	100	88 (12)	30 (20)	0
Growth in the presence of 10% NaCl	58 (27)	61 (10)	100	75	25 (33)	64 (14)	21 (4)	51 (20)	70 (11)	75 (25)	100	78 (11)	29 (13)	0
Growth at 10°	98 (14)	4 (11)	0	0	16	61 (8)	72 (8)	69 (11)	71 (7)	100	97	88 (12)	70 (13)	0
Growth at 45°	91 (1)	94	100	100	94	82	41 (4)	37 (5)	40 (6)	0	9 (9)	33	21 (2)	0
Total organisms	80	31	1	4	13	33	25	41	72	4	152	9	38	19

* % of isolates positive.

† Includes 6 cultures with cremated peripheries.

‡ Includes 2 cultures producing yellow and violet colonies (*S. flavocyanus*).§ Includes 1 culture producing yellow and violet colonies (*S. flavocyanus*).

¶ Production of violet pigment on potato and glucose agar but not on nutrient agars.

|| % of isolates weakly positive.

** Two cultures produced only bound coagulase (Duthie, 1954).

definitely rod-like. In their paper Abd-el-Malek & Gibson (1948) mentioned that they were heat-resistant (survived 60° for 30 min.), were unable to grow at 37° and formed colonies with a greenish-yellow pigment. These characters made me suspect that the two organisms were microbacteria. A culture of *Microbacterium lacticum* (NCDO 747) was examined and although it was morphologically distinguishable from Abd-el-Malek and Gibson's two strains it possessed very similar biochemical characters. The results of cell-wall analyses supported the exclusion of these organisms from the genus *Staphylococcus* (see discussion of results of cell analyses).

Group 2 (Micrococcus Cohn)

Group 2 contains cocci which were either unable to produce sufficient acid from glucose to change the indicator bromocresol purple or which grew and produced acid from glucose only in presence of air. They were generally much more variable in size than the staphylococci and formed tetrads as well as irregular clusters of cocci. Their colonies were either smooth and convex or slightly granular and were pigmented white, yellow, orange, red, brown or violet. Their growth in broth was usually either a mucoid or granular precipitate, in contrast to the fine deposit produced by staphylococci.

The eight subgroups differed in their carbohydrate metabolism. Subgroups 1-3 (Baird-Parker, 1963) possessed similar characters to the staphylococci in forming acetoin and an end pH value in glucose broth of generally below 5.0; subgroups 5 and 6 differed from these in attacking a wider range of carbohydrates with a higher end pH value in glucose broth without producing acetoin; subgroup 4 appeared to be intermediate. Subgroups 7 and 8 formed either no acid or only small amounts from glucose.

Some characters of these subgroups differed in some respects from those previously reported (Baird-Parker, 1963). Thus, 20-40% of subgroups 1 and 2 hydrolysed one or more of the Tweens, whereas only weak activity was shown by one organism in the small collection studied previously. Also, a larger number of the members of these subgroups grew at 10° but not at 45°. With the exception of four organisms, the 72 cultures classifiable in *Micrococcus* subgroup 3 could be placed in one or other of the three types previously defined within this subgroup (Baird-Parker, 1963). One of the acetoin-negative organisms was placed in this subgroup rather than with the acetoin-negative subgroups since it hydrolysed Tweens and did not utilize arabinose, xylose or aesculin. The characters of subgroup 5 were mainly the same as those found previously, but the subgroup 6 members, the phosphatase-positive micrococci, were different in that most strains did not hydrolyse Tweens. The pink micrococci placed in subgroup 8 were very similar morphologically and biochemically to subgroup 7 members, but generally differed from the latter in producing more acid from glucose and utilizing a wider range of carbohydrates (see Table 2).

Group 3 (Sarcina Goodsir)

Members of this group grew as regular packets of eight or more cocci. Their physiological characters showed quite wide variations but the majority were similar to *Micrococcus* subgroup 7 or 8.

Relationship of my classification to previous taxonomic groupings

One hundred and ninety-four named cultures were examined and, of these, 178 were established to be Gram-positive catalase-positive cocci. These belonged to

Table 3. *Classification of named species, groups and subgroups according to classification of Baird-Parker (1963)*

Classification according to Baird-Parker (1963)	Original classification
<i>Staphylococcus</i>	
Subgroup I	<i>S. aureus</i> (24)*
Subgroup II	<i>S. albus</i> (2), <i>S. epidermidis</i> , <i>S. saprophyticus</i> (2) ¹ , <i>M. aurantiacus</i> , <i>M. violagabriellae</i> (<i>S. saprophyticus</i> ¹ , <i>S. epidermidis</i> ²)
Subgroup III	<i>M. hyicus</i> (<i>S. lactis</i> ¹)
Subgroup IV	<i>S. epidermidis</i>
Subgroup V	<i>S. albus</i> , <i>S. epidermidis</i> ² , <i>S. saprophyticus</i> (2) ¹ , <i>M. cerolyticus</i> (<i>M. conglomeratus</i> ²)
Subgroup VI	<i>S. albus</i> (<i>S. lactis</i> ¹), <i>S. epidermidis</i> (2) ² , <i>S. saprophyticus</i> (2) ¹
<i>Micrococcus</i>	
Subgroup 1	<i>S. epidermidis</i> , <i>S. saprophyticus</i> (3) ¹
Subgroup 2	<i>S. saprophyticus</i> (<i>S. epidermidis</i> ²)
Subgroup 3	<i>M. candidans</i> (2) (<i>S. saprophyticus</i> ¹ , <i>S. epidermidis</i> ²), <i>M. candidus</i> (2) (<i>S. saprophyticus</i> ¹ , <i>S. epidermidis</i> ²), <i>S. albus</i> (2) (<i>S. saprophyticus</i> ¹ , <i>S. epidermidis</i> ²)
Subgroup 5	<i>M. aurantiacus</i> (<i>S. lactis</i> ¹), <i>M. conglomeratus</i> (2) ² , <i>M. euryhalis</i> (<i>M. varians</i> ²), <i>M. varians</i> (10) ² , <i>S. lactis</i> (8) ¹ , <i>Sarc. aurantiaca</i> (<i>S. lactis</i> ¹), (<i>M. conglomeratus</i> ²), <i>Sarc. lutea</i> (2) (<i>S. lactis</i> ¹ , <i>M. luteus</i> ²). Abd-el-Malek & Gibson's (1948) group IIIA (4) (<i>S. lactis</i> ²); Pohja's subgroups e, i, l, g, d, k, c.
Subgroup 6	<i>M. varians</i> (3) ² , <i>S. lactis</i> ¹ , Pohja's subgroup f
Subgroup 7	<i>M. agilis</i> (<i>S. afermentans</i> ¹), <i>M. flavus</i> (5) (<i>M. luteus</i> (5) ²), <i>M. luteus</i> (8) ² , <i>M. lysodeikticus</i> (4) (<i>S. afermentans</i> (3) ¹ , <i>M. luteus</i> ²), <i>M. sodonensis</i> (<i>M. luteus</i> ²), <i>S. afermentans</i> ¹ , <i>S. flavocyaneus</i> (3) (<i>S. afermentans</i> ¹ , <i>M. luteus</i> (2) ²), <i>Sarc. flava</i> (2) (<i>S. afermentans</i> ¹ , <i>M. luteus</i> ²), <i>Sarc. lutea</i> (<i>M. luteus</i> ²), Abd-el-Malek & Gibson's group IIB (<i>S. afermentans</i> ¹), Abd-el-Malek & Gibson's group IIC (<i>S. afermentans</i> ¹)
Subgroup 8	<i>M. agilis</i> (2) (<i>S. afermentans</i> (2) ¹), <i>M. roseus</i> (2) (<i>S. roseus</i> (2) ¹), Pohja's subgroup h
<i>Sarcina</i>	
	<i>Sarc. aurantiaca</i> (<i>M. luteus</i> ²), <i>Sarc. citrea</i> (<i>M. luteus</i> ²), <i>Sarc. flava</i> (2) (<i>S. afermentans</i> ¹ , <i>M. luteus</i> ²), <i>Sarc. lutea</i> (4) (<i>S. afermentans</i> (2) ¹ , <i>M. luteus</i> (2) ¹), <i>Sarc. marginata</i> (<i>M. luteus</i> ²), <i>Sarc. variabilis</i> (<i>M. luteus</i> ²), <i>M. corralinus</i> (<i>S. roseus</i> ¹), <i>M. citreus</i> (<i>M. luteus</i> ²) <i>M. flavus</i> (4) <i>M. luteus</i> (4) ² , <i>M. flava-roseus</i> (<i>S. roseus</i> ¹), <i>M. luteus</i> (8) ² , <i>M. roseus</i> (4) (<i>S. roseus</i> (4) ¹), <i>M. rubens</i> (<i>S. roseus</i> ¹)

Organisms possessing characters that were very different from their original descriptions: *Staphylococcus* subgroup I: *S. epidermidis* (7)², *S. epidermidis-albus* (1) (*S. aureus*¹, *S. lactis*¹, *M. caseolyticus* (*S. aureus*¹); subgroup II: *S. aureus*; *Micrococcus* subgroup 1: *M. cremoris-viscosi* (*S. lactis*²), *M. citreus*; subgroup 2: *M. caseolyticus*; subgroup 3: *M. luteus* (2) (*S. saprophyticus*¹, *M. conglomeratus*²); subgroup 5: *Sarc. ventriculi* (*M. conglomeratus*²); subgroup 6: *S. aureus*; subgroup 7: *M. candidans* (*S. afermentans*¹), *M. freudenreichii* (*S. afermentans*¹), *M. papyroabsorbens* (*S. lactis*¹); subgroup 8: *M. varians*.

Key:

(24)* = no. of strains studied.

(*S. saprophyticus*¹) Reclassified according to the classification of Shaw *et al.* (1951) by either the National Collection of Type Cultures (Dr S. T. Cowan) or the National Collection of Dairy Organisms (Dr E. I. Garvie).

(*S. epidermidis*²). Reclassified according to the classification of Kocur & Martinec (1962) by Dr M. Kocur of J. E. Purkyne University, Brno, Czechoslovakia.

9 named species of staphylococci, 25 of micrococci, 9 of sarcinas and 12 to groups or subgroups within these genera. Table 3 shows the relationship of these taxa to each other and to the author's groups and subgroups.

The majority of the named species and groups were received through the generosity of Dr S. T. Cowan (Curator of the National Collection of Type Cultures), Dr Ellen I. Garvie (Curator of the National Collection of Dairy Organisms) and Dr M. Kocur (Curator of the Culture Collection of J. E. Purkyne University, Brno, Czechoslovakia). The first two of these sources have reclassified the cultures in their collections according to the scheme of Shaw *et al.* (1951) and the last according to that of Kocur & Martinec (1962). Therefore, to indicate in Table 3 where organisms have been renamed by one or other of these sources, the original name is given followed by the new name in parentheses with a superscript referring to the source of this name. Where the same strain was received from more than one source only one is recorded in Table 3. Also, to avoid confusion, strains whose characters did not in any way correspond to their published descriptions are listed at the end of the Table.

Relationship of classification to habitat

Three hundred and twelve coagulase-negative, Gram-positive catalase-positive cocci were received as isolates from known sources. Table 4 shows the distribution of isolates from habitats from which ten or more organisms were received. For ease of interpretation, the numbers of each subgroup are expressed as a percentage of the total number of isolates from a particular source.

Table 4. *Percentage distribution of coagulase-negative Gram-positive catalase-positive cocci from different sources*

	Sub-group	Human		Air	Soil	Dairy products	Fermented Meats	Marine	Bacon	Brine	Pork
		Nose	Skin								
<i>Staphylococcus</i>	II	62	40	4	0	0	0	0	0	0	0
	IV	0	8	0	0	0	0	10	0	0	0
	V	5	4	8	0	0	0	0	0	0	0
	VI	0	32	6	16	0	0	20	2	0	4
<i>Micrococcus</i>	1	0	4	4	8	12	0	5	8	0	20
	2	18	4	14	0	19	0	5	4	8	32
	3	0	4	46	44	12	0	10	0	4	11
	4	0	0	0	0	0	0	0	4	0	2
	5	5	4	4	16	19	70	20	82	88	27
	6	0	0	2	0	7	10	10	0	0	0
	7	5	0	0	16	0	10	5	0	0	4
	8	0	0	0	0	0	10	5	0	0	0
<i>Sarcina</i>		5	0	12	16	31	0	10	0	0	0
Total organisms		21	25	48	12	16	10	19	46	55	44

Staphylococci predominated among the Gram-positive catalase-positive cocci obtained from human skin and nasal surfaces, whereas micrococci occurred more frequently amongst isolates from air, soil, meat and dairy produce. The predominance of *Staphylococcus* subgroup II and VI organisms on human skin confirms

previous results (Baird-Parker, 1962), as also does the finding that members of *Micrococcus* subgroup 8 are the main micrococci occurring in air samples (Baird-Parker, unpublished). The only coagulase-negative staphylococci received from marine sources were isolates from the skin of freshly-caught cod and herring.

Relationship of classification to chemical components of the cell

Forty-six strains, representative of my groups and subgroups, were examined for chemical components of the cells. Three main cell fractions were examined: whole organisms for sugars, amino sugars and acetic acid-extractable small peptides and amino acids; cell walls for amino acids and teichoic acids; cell contents for proteins.

The cell-wall mucopeptide of all strains contained alanine, glutamic acid and lysine, together with, in most strains varying amounts of aspartic acid, glycine and serine, and in some strains small amounts of leucine, threonine and valine. The results (Table 5) show only small differences in the overall peptide composition of the subgroups. There does, however, appear to be a simplification in the amino acid composition of the peptides as one proceeds from *Staphylococcus* subgroups I and II containing up to 9 amino acid components, through the other staphylococcus and micrococcus subgroups to *Micrococcus* subgroup 8 containing only 4 or 5 amino acids.

Other than ribose, glucose was the only sugar detected in staphylococci and *Micrococcus* subgroups 1-4, whereas glucose, galactose and/or mannose were present in many of the strains belonging to *Micrococcus* subgroups 5-8 and *Sarcina* (Table 5). Thus, with respect to sugars, there was an increase in complexity from *Staphylococcus* subgroup I containing little or no sugar other than ribose to *Micrococcus* subgroup 8 containing ribose and quite large amounts of glucose, galactose and mannose. Glucosamine and muramic acid were present in all the strains examined, with galactosamine in most strains. Variable amounts of teichoic acids were present in the cell walls of most strains but were not detected in walls of either *Micrococcus* subgroup 7 and 8. Glycerol-containing teichoic acids were most commonly found, although these were replaced by ribitol-teichoic acids in *Staphylococcus* subgroup I. Small amounts of ribitol were detected together with glycerol in two members of *Micrococcus* subgroup 2 (Table 5).

Differences in acetic acid-extractable amino acids and peptides were determined by comparing the patterns of ninhydrin-staining spots on two-dimensional chromatograms; no attempt was made to determine the position of individual amino acids by markers or the structure of the peptides by hydrolysis. Two main patterns were distinguishable. One was typical of most staphylococci, members of *Micrococcus* subgroups 1-3 and some members of *Micrococcus* subgroups 5 and 6. The other was shown by the majority of strains of *Micrococcus* subgroup 5 and all strains belonging to *Micrococcus* subgroups 7 and 8 and *Sarcina*.

Electrophoresis of proteins from cell contents yielded a variety of distinct patterns of protein bands. Some of these patterns appeared to be characteristic of certain subgroups, e.g. *Staphylococcus* subgroups II, III, IV and V and *Micrococcus* subgroup 8. Other subgroups, however, gave a variety of patterns within a subgroup, e.g. *Staphylococcus* subgroups I and VI.

Table 5. *Cell and cell-wall components of staphylococci and micrococci*

	No. of cultures											
	2	2	2	2	2	2	2	2	2	1	2	2
Ribitol-teichoic acid	+	+	-	-	-	-	+	+	2	NR	-	-
Glycerol-teichoic acid	- or tr	+	+	+	+	+	+	+	+	+	+	+
Muramic acid	+	+	+	+	+	+	+	+	+	NR	+	+
Glucosamine	+	+	+	+	+	+	+	+	+	NR	+	+
Galactosamine	+	+	+	+	+	+	+	+	+	+	+	+
Valine	+	+	+	+	+	+	+	+	+	+	+	+
Threonine	+	+	+	+	+	+	+	+	+	+	+	+
Serine	+	+	+	+	+	+	+	+	+	+	+	+
Phenylalanine	+	+	+	+	+	+	+	+	+	+	+	+
Lysine	+	+	+	+	+	+	+	+	+	+	+	+
Leucine	+	+	+	+	+	+	+	+	+	+	+	+
Glycine	+	+	+	+	+	+	+	+	+	+	+	+
Glutamic acid	+	+	+	+	+	+	+	+	+	+	+	+
Diaminopimelic acid	+	+	+	+	+	+	+	+	+	+	+	+
Aspartic acid	+	+	+	+	+	+	+	+	+	+	+	+
Alanine	+	+	+	+	+	+	+	+	+	+	+	+
Rhamnose	+	+	+	+	+	+	+	+	+	+	+	+
Ribose	+	+	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+	+	+
Arabinose	+	+	+	+	+	+	+	+	+	+	+	+

++ = present in large amounts; + = present in small amounts; tr = present in trace amounts; NR = no result; +1* = no. of strains positive.

DISCUSSION

A detailed examination was made of the morphology and physiological characters of 607 cocci isolated from different sources and parts of the world. Before this work began, each strain was examined to determine its morphology, stainability by Gram's method and catalase production; this was done to exclude strains which did not correspond to the description: Gram-positive and catalase-positive coccus dividing in more than one plane. One or more of these characters was absent from forty-three of the strains examined. From this examination, there would appear to be two main sources of error in identifying Gram-positive catalase-positive cocci. Thus, cocci can be confused with Gram-positive and Gram-negative rods, with short chains of cocci and with budding yeasts, while citrate-utilizing plasma-clotting strains of faecal streptococci may be identified as coagulase-positive staphylococci. For several reasons it is considered that several of the established *Micrococcus* species are not true members of the Micrococcaceae. Thus, *M. denitrificans*, which is Gram-negative and forms rods in young cultures (Verhoeven, 1957), has a cell-wall mucopeptide which contains a wide range of amino acids characteristic of Gram-negative genera (Baird-Parker, unpublished). In a recent paper Pleva & Kocur (1963), regarded *M. denitrificans* as a valid member of the genus *Micrococcus* although they found that it showed definite rods when examined in the electron microscope. The related species *M. halodenitrificans* is also Gram-negative (Robinson & Gibbons, 1952) and also possesses a characteristic Gram-negative cell wall with respect to both its high lipoprotein content (Smithies, Gibbons & Bayley, 1955) and its wide range of amino acids (Takahashi & Gibbons, 1959). It is also probable that *M. radiodurans* should be reclassified since its cell wall contains lipoprotein and a wide range of amino acids (Work, 1964); the guanine: cytosine ratio of its nucleic acid is also more like to that of the Gram-negative genera (Dr B. Moseley, personal communication). The probable identity of Abd-el-Malek & Gibson's group IIIB with the genus *Microbacterium* on the grounds of morphology and physiological characters has already been mentioned; further evidence for excluding these organisms from the Micrococcaceae is the presence of diaminopimelic acid in their cell walls (Baird-Parker, unpublished).

The non-capsulate cocci which form short chains and grow on salt-containing media, i.e. the faecal streptococci, are frequently confused with coagulase-positive and -negative staphylococci. They are most readily distinguished from the cluster-forming members of the Micrococcaceae by the catalase test (Isaacs & Scouller, 1948; Evans, Buettner & Niven, 1952; Brown & Evans, 1963) and although there are a few reports of catalase-positive faecal streptococci (Langston, Guttierrez & Bouma, 1960) and of catalase-negative staphylococci (Lucas & Seeley, 1955; Jensen, 1963; Solomon & San Clemente, 1963) these reports are rare. Another group of organisms which may be confused with the staphylococci are the aerococci. The latter were originally reported to be catalase-negative but they may decompose hydrogen peroxide under certain conditions (Günther & White, 1961). Also, Clausen (1964) reported the isolation of an *Aerococcus* sp. (*A. catalasicus*) which was nitratase-positive and catalase-positive. It may be necessary, therefore, to use tests other than catalase formation to distinguish these organisms from staphylococci and micrococci. They can be distinguished from micrococci by their fermentative meta-

bolism (Cowan & Steel, 1961) and from the staphylococci by their failure to hydrolyse arginine, proteins, fats or Tweens (Table 2). Aerococci can also be distinguished from staphylococci and from pediococci, which only form catalase on low-glucose media (Felton, Evans & Niven, 1958), by their formation of peroxide. The production of peroxide may be detected by streaking a suspected aerococcus across a heart-infusion agar plate seeded with *Staphylococcus aureus* and incubating at 20° for 8–4 days. The production of peroxide is indicated by inhibition of the staphylococcus in a wide zone surrounding the aerococcus growth; a control plate containing catalase should also be set up, to exclude inhibition due to antibiotic production (Baird-Parker, unpublished).

CLASSIFICATION

The majority of organisms which satisfied the criteria of morphology and catalase production could be classified into the groups and subgroups previously defined (Baird-Parker, 1968). Excluding the pink pigmented micrococci, over 96 % of the remaining organisms could be classified into my thirteen subgroups. A further 3 % of the organisms differed from previously defined subgroups in one main character only and were therefore considered to be variants of these subgroups. Only two organisms, both staphylococci, were sufficiently different from those previously studied to suggest that they should be placed in further subgroups.

The results obtained support the separation of staphylococci from micrococci on ability to grow and produce acid from glucose in the absence of air. This character separates staphylococci as a group of potential pathogens or commensals which are characteristic of animal and human surfaces, from micrococci which are a group of mainly free-living saprophytes which are nutritionally less exacting and morphologically and biochemically more variable than the staphylococci. Contrary to my previous conclusions (Baird-Parker, 1968), to place the packet-forming cocci in the genus *Sarcina* (group 8) could not be justified. This reversal of my conclusions has resulted from a study of the morphology of named *Sarcina* spp. grown under a variety of conditions. When this was done, many of the named *Sarcina* spp. lost their ability to form packets. Of 55 organisms growing as regular packets of cocci on one or more media, only 20 invariably formed packets; under none of the combinations of media and temperature tested would they all form packets. It was further found that the pink-pigmented packet-forming cocci were indistinguishable biochemically and on their chemical constituents from the pink-pigmented micrococci of *Micrococcus* subgroup 8. Therefore, in agreement with Shaw *et al.* (1951) and Kocur & Martinec (1962) it must be concluded that the ability to form cubical packets of cocci is characteristic of certain micrococci and that these, like the tetrad formers, belong mainly to *Micrococcus* subgroups 7 and 8. In the following sections of this paper, the packet-forming cocci will be discussed with the micrococci.

Separation of the Gram-positive and catalase-positive cocci into two groups or genera is supported by the results of Thomas & Schuhardt (1964), who examined the effect of lysostaphin (Schindler & Schuhardt, 1964) on these organisms. They found that staphylococci (defined on ability to grow and produce acid from glucose anaerobically) were lysed by this substance but that the micrococci and sarcinas remained intact.

Group 1 (Staphylococcus Rosenbach)

Subgroup I (Staphylococcus aureus)

The coagulase test is frequently used as sole criterion for recognizing *Staphylococcus aureus*. Ability to clot mammalian blood plasma is not, however, specific for *S. aureus* and by no means all members of this species by clot plasma. Amongst Gram-negative species which may clot plasma, by citrate utilization or coagulase-like activity, are strains of *Pseudomonas aeruginosa* (Frédéricq, 1941), *Serratia marcescens* (Frédéricq, 1946) and *Pasteurella pestis* (Eisler, 1961), and amongst Gram-positive organisms are actinomycetes (Frédéricq, 1946) and strains of *Streptococcus faecalis* (Evans *et al.* 1952). The inability of *S. aureus* to clot plasma may be due to use of unsuitable plasma, the absence of bound and/or free coagulase (Duthie, 1954), or excessive production of fibrinolysin or a coagulase-destroying factor (Lominski, Smith & Morrison, 1953; Munch-Peterson, 1961). The need to control the coagulase test was shown by the finding that seven strains designated as *Staphylococcus epidermidis* on inability to clot human blood plasma were, when tested in this laboratory, found to be typical strains of *S. aureus* which were able to clot rabbit plasma, ferment mannitol and to produce phosphatase; similarly a strain received as *S. epidermidis* had been misclassified because it produced bound coagulase (slide test) but not free coagulase (tube test). The inadvisability of relying solely on the coagulase test for the identification of *S. aureus* was also shown by the receipt of two diphtheroids and five strains of *S. faecalis* which had been classified by their isolators as strains of *S. aureus* because of ability to clot blood plasma. Amongst other strains of *S. aureus* which were examined and found to be of doubtful authenticity (see Table 3) was the propagating strain of phage type 73 (NCTC 8360). In a recent study of the physiological characters of the phage-propagating strains of *S. aureus*, Solomon & San Clemente (1963) reported that phage type 73 showed neither coagulase nor phosphatase activity. Dr I. Lominski (Dr K. J. Steel, personal communication) has shown that this organism will clot sheep plasma but not human or rabbit plasma. When I tested this organism it was coagulase-negative with rabbit plasma but phosphatase-positive, able to utilize glucose and mannitol only in the presence of air, to produce acid from arabinose and grow on a medium containing ammonium phosphate as sole nitrogen source. These characters make the organism unlikely to be a strain of *S. aureus*, and according to my work it should be classified in *Micrococcus* subgroup 6.

Anaerobic utilization of mannitol and the coagulase test were considered by Mossel (1962) to be the most useful criteria for distinguishing *Staphylococcus aureus* from other staphylococci and micrococci. He found that 95 % of coagulase-positive strains from clinical and non-clinical sources fermented mannitol. However, ability to form acid from mannitol anaerobically may depend on the source of organism, since White, Rattray & Davidson (1963) reported that only 73 % of bovine strains of *S. aureus* fermented mannitol. Also in a recent survey Cowan & Steel (1964) found that only 75 % of the coagulase-positive staphylococci they tested were able to ferment mannitol under the conditions defined by Mossel (1962).

Our knowledge of variation in the physiological characters of *Staphylococcus aureus* indicates that a single character such as formation of coagulase cannot be solely relied upon for critical determination of this species. Therefore, before a

coagulase-negative strain is classified with coagulase-negative staphylococci several further tests should be made to exclude the possibility that it is a coagulase-negative variant of *S. aureus*. The best tests for this purpose are the determination of phosphatase production and mannitol fermentation. These tests when used in conjunction with the free and bound coagulase tests (Duthie, 1954) will clearly separate *S. aureus* from related staphylococci; positive recognition of this species is obtained when at least two of these tests are positive. Further confirmation of diagnosis can be obtained by determining phage typability (Parker, 1962), haemolysin pattern (Elek & Levy, 1950) and ability to grow in normal human serum (Fletcher, 1962).

Staphylococcus subgroups II and III

Members of these subgroups produce phosphatase and are distinguished from *Staphylococcus aureus* by not forming acid from mannitol or coagulating plasma; they are separated by acid production from maltose and acetoin from glucose (Table 3). Staphylococci belonging to subgroup III have only been isolated from pig skin while members of *Staphylococcus* subgroup II have been isolated from several sources, although their main habitats would appear to be human and animal surfaces.

It has been suggested that ability to produce phosphatase is associated with pathogenicity and the coagulase test (Barber & Kuper, 1951). However, the majority of coagulase-negative staphylococci from human sources are phosphatase producers (Baird-Parker, 1962; Jacobs, Willis & Goodburn, 1963). Coagulase-negative staphylococci may, however, be associated with, and cause, a number of infective processes (Pereira, 1962). Two coagulase-negative pathogens studied by me were both phosphatase producers; one was a member of *Staphylococcus* subgroup II, the other of *Staphylococcus* subgroup III. The subgroup II organism was a strain of *Micrococcus violagabriellae* Castellani. This organism was isolated by Castellani (Castellani, 1955) from a chronic skin infection occurring in a group of South American marines and was shown by him to give rise to this condition on re-inoculation. Its physiological characters were identical with the apparently non-pathogenic subgroup II staphylococci that were isolated from human skin, but it possessed the additional property of producing a characteristic violet pigment when grown on glucose- or potato-containing media (Castellani, 1955; Steel, 1964). It should be mentioned that the strain studied did not have the same characters as those reported for *M. violagabriellae* by Kocur & Martinec (1963*a*). Reclassification of *M. violagabriellae* in the genus *Staphylococcus* is in agreement with the results of Sneath (1960) and Kocur & Martinec (1963*a*).

The other phosphatase-producing coagulase-negative pathogen studied was a strain of *Micrococcus hyicus* Sompolinsky. This was reported by Sompolinsky (1953) to be the causative organism in an outbreak of contagious impetigo of swine. It possesses similar physiological characters to the subgroup III staphylococci previously isolated from pig skin (Baird-Parker, 1963) but was unable to utilize glycerol (Table 2). *M. hyicus* produces necrotic lesions when subcutaneously injected into mice (Jones, Deibel & Niven, 1963). I confirmed this observation but was unable to produce similar lesions with strains of *Staphylococcus* subgroup III isolated from pig skin. The physiological characters of *M. hyicus* were the same

as those reported by Jones *et al.* (1963), and in support of their conclusions it is suggested that this organism should be transferred to the genus *Staphylococcus*. According to Jacobs *et al.* (1963) potentially pathogenic staphylococci produce deoxyribonuclease as well as phosphatase; this is true of *M. hyicus* (Jones *et al.* 1963). However, deoxyribonuclease activity was shown by several subgroup II staphylococci which I isolated from normal skin and by a number of saprophytic micrococci belonging to subgroups 2, 5 and 7. The correlation between deoxyribonuclease production and potential pathogenicity therefore requires further investigation.

Staphylococcus subgroups IV, V and VI

Organisms received as strains of *Staphylococcus albus*, *S. epidermidis* and *S. saprophyticus* were classified in subgroups IV, V and VI (Table 4). *Staphylococcus* subgroups IV and V possess physiological characters which suggest a relationship with either subgroup II or VI. Thus some strains of *Staphylococcus* subgroups IV were only separated from some members of subgroup VI by their inability to form acid from mannitol and subgroup V members from subgroup II by absence of phosphatase activity. In future considerations of species it is probable that subgroups IV and V will be combined with subgroups VI and II respectively. They are at present retained in separate subgroups as proteins electrophoretically separated from cell contents of members of these subgroups are quite distinct.

Members of *Staphylococcus* subgroup VI are separated from those of subgroup I, II and III by their aerobic utilization of mannitol and the absence of coagulase and phosphatase. They are characteristic of human and animal skins and were found amongst isolates from the skin of freshly-caught fish. However, their presence on fish skin may have occurred through handling of the skin before sampling.

Group 2 (*Micrococcus* Cohn)

Micrococcus subgroups 1, 2, 3 and 4

These subgroups are physiologically closely related and form acetoin as an end product of glucose metabolism. Strains of *Staphylococcus epidermidis* and *S. saprophyticus* were classified in these subgroups as also were cultures originally labelled *S. albus*, *M. candicans* and *M. candidus* but which had been reclassified as either *S. epidermidis* or *S. saprophyticus* (Table 3). As mentioned previously (Baird-Parker, 1962, 1963) these subgroups contain organisms which are distinguished from staphylococci solely on their inability to use glucose under anaerobic conditions. It is with these organisms that the main controversy exists about separation of the Gram-positive catalase-positive cocci into two genera. Evidence can be cited which suggests classification with the staphylococci on the grounds of similar physiological characters and chemical composition, and with the micrococci on grounds of inability to grow anaerobically and to use glucose, and in a number of strains on the ability to produce acid from glucose in a medium containing ammonium phosphate as sole nitrogen source. Unfortunately, tests for anaerobic growth and glucose utilization are fairly critical and a result obtained by one method may not always agree with the result obtained by another method. Recently Cowan & Steel (1964) tested methods which have been used for distin-

guishing staphylococci from micrococci against a comprehensive collection of 87 strains of Gram-positive catalase-positive cocci. They found that 50 of these strains were classifiable as staphylococci by the Hugh & Leifson (1953) test but only 35 of these were classifiable as staphylococci by Baird-Parker's test. Three tests correlated well: these were Baird-Parker's test, the test of Evans, Bradford & Niven (1955), when the medium after incubation was below pH 5.0, and the ability to produce acid from glucose in a peptone medium incubated anaerobically.

A subcommittee set up by the International Association of Microbiological Societies is considering the methods for the separation of staphylococci from micrococci. At the first meeting of the subcommittee it was recommended that a standard test should be introduced to determine the anaerobic utilization of glucose and other carbohydrates by staphylococci. The method proposed was to inoculate growth from an actively growing culture into a tube of a semi-solid agar medium containing peptone + yeast extract + carbohydrate and bromocresol purple as pH indicator and to seal the tube with a layer of liquid paraffin. Tubes are incubated at 37° for 5 days. If, when glucose is present, acid is produced throughout the tube, the organism is a staphylococcus; but if no acid is formed, or acid is only formed at the top of the tube, the organism is a micrococcus.

Micrococcus subgroups 5 and 6

Members of these subgroups oxidize a wider range of carbohydrates than those of subgroups 1-4 and are unable to form acetoin from glucose; the two subgroups are separated by phosphatase production. Subgroup 5 contains a large group of closely related organisms. These have been given a variety of specific names (see Table 3). Shaw *et al.* (1951) and Abd-el-Malek & Gibson (1948) placed these organisms in *Staphylococcus lactis* and group IIIA respectively. Two of the most recent detailed classifications of the micrococci by Pohja (Pohja, 1960; Pohja & Gyllenberg, 1962) and by Kocur & Martinec (1962) have attempted to subdivide these micrococci into a number of subgroups or species. Pohja (1960) used 'numerical taxonomy' (Sneath, 1962) for his studies and subdivided his acetoin-negative micrococci into eleven subgroups; eight of these corresponded to my subgroups 5 and 6; the remainder were either halophilic or pink-pigmented. Primary division of these eight subgroups was based on salt tolerance and further division on optimum salt concentration and the production of acid from arabinose, and urease, gelatinase and nitratase activity. In a further paper Pohja & Gyllenberg (1962) recognized six subgroups of halo-tolerant micrococci and distinguished these on the basis of optimum salt concentration and temperature for growth, the production of acid from arabinose and sorbitol and the hydrolysis of gelatin. Sneath (1962) pointed out that Pohja's subgroups were chosen mainly on arbitrary characters and it appears to me that many of the characters chosen by Pohja from cluster analyses are largely unstable ones. Thus, the salt tolerance of acetoin-negative micrococci can be increased by subculture in media containing increasing concentrations of sodium chloride and may be decreased by subculture in laboratory media of normal salt concentration (Baird-Parker, unpublished). Similarly, optimum temperature for growth is largely dependent on growth conditions. Kocur & Martinec (1962) separated the halo-tolerant organisms belonging to my subgroups 5 and 6 into *Micrococcus varians* Migula, which forms acid from lactose and mannitol, and *M. conglomeratus* Migula

which does not attack either of these carbohydrates. However, in my experience, quite a number of the halotolerant glucose-oxidizing acetoin-negative micrococci oxidize lactose but not mannitol and vice versa. It must be concluded, therefore, that at present there is no satisfactory division of these organisms on physiological characters. It may be possible, however, to separate these organisms on their chemical compositions (Davison & Baddiley, 1963).

Micrococcus subgroups 7 and 8

Members of subgroups 7 and 8 either do not release detectable acid when carbohydrates are utilized or produce only small amounts of acids from these substrates. They also differ from most other micrococci in their inability to grow in the presence of 10% sodium chloride. The two subgroups contain a variety of morphological types, ranging from the irregular clusters of cocci characteristic of the strongly glucose-oxidizing micrococci to the tetrad and the cubical packet formers which were classified in the genus *Sarcina*. The two subgroups are distinguished by the red or pink pigmentation of cocci placed in *Micrococcus* subgroup 8.

The separation of subgroups 7 and 8 solely on the grounds of pigmentation is difficult to justify in the light of observations by several workers that *Micrococcus lysodeikticus*, which is a member of subgroup 7, is able to give stable pink-pigmented mutants (Field & Naylor, 1962; Dr G. W. Gould, personal communication). However, the cell-wall composition of the two subgroups is different (Table 5) as also are the proteins of their cell contents. It has also been shown by Eisenberg & Evans (1963) that two typical members of subgroup 7, *M. sodonensis* and *M. lysodeikticus*, have quite different carbon and nitrogen requirements from *M. roseus*. Therefore on this evidence it would appear justifiable to separate subgroup 7 from subgroup 8 on pigmentation.

The wide variety of morphological and physiological types placed in subgroup 7 is paralleled by the large number of species in which these organisms have been placed (Table 3). *Sarcina* spp. classifiable in *Micrococcus* subgroup 7 include: *S. aurantiaca*, *S. citrea*, *S. flava*, *S. lutea*, *S. marginata*, *S. variabilis*. The endospore-forming organism originally called *S. urea* (Beijerinck) Löhnis possesses similar physiological characters to the packet-forming micrococci but has been shown by MacDonald (1962) to be related serologically and in DNA composition to the Bacillaceae. It has therefore been re-classified as *Planosarcina ureae* Beijerinck by MacDonald (1962) and *Sporosarcina ureae* (Beijerinck) Orla-Jensen by Kocur & Martinec (1963b).

It is currently believed that intensive speciation of the weakly glucose-oxidizing micrococci is not justified and in recent classification they are placed in a single species. For example, Shaw *et al.* (1951) placed them in the species *Staphylococcus a fermentans* ex Castellani and Kocur & Martinec (1962) in the species *Micrococcus luteus* (Schröeter) Cohn. This latter name would appear to be more appropriate as it excludes the implication that they are unable to utilize sugars; results obtained by Rosypal & Kocur (1963) by manometric techniques would indicate that they can all to some extent oxidize a variety of mono- and di-saccharides. Further, in detecting carbohydrate utilization by acid production, the medium used plays a large part in the result obtained (Cowan & Steel, 1964). Thus, Shaw *et al.* (1951) and Rosypal Kocur & Hođák (1963) were unable to show acid production from glucose by *M.*

lysodeikticus growing in a peptone broth, although this organism will produce detectable acid when growing in a mineral-salt yeast-extract medium containing glucose.

Chemical composition of cells in relationship to classification

Several attempts have been made to use chemical components of micro-organisms for the classification of Gram-positive catalase-positive cocci. Cummins & Harris (1956*a, b*) were able to divide nine named species of staphylococci, micrococci, sarcinas and aerococci into three groups according to differences in their cell-wall carbohydrates, amino acids and amino sugars. The first of these groups contained strains of *Staphylococcus aureus*, *S. albus*, *S. citreus*, *Micrococcus luteus* and *Sarcina lutea*; the second a strain of *Micrococcus conglomeratus* and strains of *Aerococcus viridans* and a third two red-pigmented organisms, *M. cinnabarius* and *M. rhodochrous*. However, as pointed out by Cummins & Harris (1956*b*) these groupings cut right across the divisions of these organisms on morphological and physiological grounds. Thus, the second group of Cummins & Harris is unlikely to be of taxonomic significance since it groups a typical micrococcus with strains of the physiologically very different *A. viridans*; their third group must also be disregarded as it contains two rod formers (Shaw *et al.* 1951; Cummins & Harris, 1956*a*). It would appear, therefore, that only the first group of Cummins & Harris is valid since it is the only one to contain only Gram-positive catalase-positive cocci. The characters of this group agree well with my results for a range of different physiological groups of staphylococci and micrococci (see Table 5) and indicate that qualitative differences in cell-wall sugars, amino sugars and amino acids are insufficient to distinguish well-defined groups of these organisms. Determination of these classes of compound is, however, of value in the separation of members of the Micrococcaceae from other coccus-forming genera. For example, I found, in agreement with the results of Salton & Pavlick (1960), that *M. varians* (NCTC 7281) contained diaminopimelic acid (DAP) in its cell wall in place of the lysine characteristic of other micrococci studied. The strains of *M. varians* was also atypical with respect to its morphology and physiological characters and therefore requires reclassification. Also, DAP was found in the cell walls of members of Abd-el-Malek & Gibson's group IIIB and *M. radiodurans* and *M. denitrificans*.

Qualitative differences in the composition of teichoic acids from the cell walls of staphylococci and micrococci were suggested by Davison & Baddiley (1963) to be of use in the classification of these organisms. With the exception of the type culture of *Staphylococcus saprophyticus* (NCTC 7292) ribitol-containing teichoic acids were found only in the cell walls of strains of *S. aureus*; teichoic acids were absent or present only as the glycerol polymer in the other coagulase-negative strains studied. The authors suggested that this ribitol-containing strain of *S. saprophyticus* (a member of *Micrococcus* subgroup 3) is atypical and found that a recently isolated 'pathogenic' strain of this species possessed only glycerol-teichoic acid. However, the occurrence of ribitol-teichoic acid may not be so uncommon amongst coagulase-negative staphylococci since Losnegard & Oeding (1963*a*) reported that two of four strains of *S. epidermidis* they examined contained ribitol-teichoic acids structurally and serologically related to the group A polysaccharide extractable from *S. aureus* (Losnegard & Oeding, 1963*b*). Perhaps it is significant that both these organisms

were isolated from urine. Davison & Baddiley's (1963) results clearly show the presence of at least two distinct glycerol-teichoic acids in organisms classifiable in my *Micrococcus* subgroup 5. Further studies of teichoic acids may therefore aid the classification of these micrococci and form the basis for serological studies of coagulase-negative staphylococci; see Davison *et al.* (1964).

Quantitative differences in the cell-wall amino acids of Gram-positive catalase-positive cocci were examined by Salton & Pavlik (1960), who found distinct differences in the molecular proportions of amino acids in the mucopeptides of staphylococci and micrococci; on a molecular basis the amount of glycine in the cell-wall mucopeptide of staphylococci was 4 times that present in the micrococci studied. The relative molecular proportions of lysine, glutamic acid, glycine and alanine were very similar for members of *Micrococcus* subgroup 7 (*Sarcina lutea*, *S. flava*, *M. tetragenus* and *M. lysodeikticus*); these amino acids were different both in type and proportion in *Micrococcus* subgroup 8 (*M. roseus*). Studies of C-terminal and N-terminal amino acids of the mucopeptide such as those mentioned by Salton (1960) may form an even finer taxonomic tool for classifying these organisms.

Gregory & Mabbit (1957), as part of a study of acetic acid-extractable amino acids and peptides in the classification of micro-organisms, examined representatives of cocci classified according to the scheme of Shaw *et al.* (1951). They found that strains of *Staphylococcus aureus* were distinguishable from *S. saprophyticus* by their pattern of ninhydrin-staining peptides and amino acids, but that a variety of patterns was produced by *S. lactis* strains, some of which were similar to *S. saprophyticus*; *S. roseus* and *S. afermentans* were also not clearly separated. My results are mainly in agreement with those of Gregory & Mabbit, but unlike them clear differentiation of strains corresponding to *S. aureus* and *S. saprophyticus* was not obtained and only two main groups could be distinguished (see Results). Two of the groups that Gregory & Mabbit distinguished chromatographically within *S. lactis* were Abd-el-Malek & Gibson's groups IIIA (*Micrococcus* subgroup 5) and IIIB. They found that the difference between chromatograms of these groups was greater than between the type strains of *S. aureus* and *S. saprophyticus*. This result would support my view that Abd-el-Malek & Gibson's group IIIB organisms are not members of the Micrococcaceae.

Electrophoretic separation of proteins from cell contents by starch-gel electrophoresis and staining of the proteins to demonstrate esterase activity was shown by Norris (1962) to be of use in the classification of *Bacillus* spp. Rosenkast & Clausen (1962) showed that the pattern of proteins separated by starch-gel electrophoresis of autolysed *Staphylococcus aureus* correlated with Cowan's three serological types. The present results indicate that the complex patterns of proteins separated electrophoretically from the cell contents of staphylococci and micrococci may be of value in the grouping of these organisms, but that further work along the lines used by Norris for *Bacillus* spp. may lead to more clear-cut results.

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