ATTEMPT IN CLASSIFICATION OF CATALASE-POSITIVE STAPHYLOCOCCI AND MICROCOCCI[1]

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ABSTRACT

MOSSEL, D. A. A. (Central Institute for Nutrition and Food Research T.N.O., Utrecht, The Nethrlands). Attempt in classification of catalase-positive staphylococci and micrococci. J. Bacteriol. **84**:1140–1147. 1962.—About 390 strains of *Staphylococcus aureus*, isolated from clinical material, and about 190 strains of coagulase-negative staphylococci and micrococci from strictly nonclinical habitats were studied by the following recently recommended biochemical tests: anaerobic dissimilation ("fermentation") of mannitol, gelatin liquefaction, type of growth on tellurite-glycine agar, hydrolysis of urea, and KCN tolerance. The latter three tests appeared either not specific for, or not positive for, most *S. aureus* strains. Virtually all strains of *S. aureus* were gelatin-positive, but 71% of the other types of cocci also liquefied gelatin. Rapid anaerobic breakdown of mannitol, however, was shown by ca. 95% of the strains of *S. aureus*, and late fermentation by an additional 3%. Of 105 obligately aerobic coagulase tive cocci (micrococci), none fermented mannitol; of 40 facultatively anaerobic, coagulase-negative cocci (staphylococci), only 7 (18%) fermented mannitol. Oxidative metabolism of mannitol occurred in only three (<1%) strains of *S. aureus* but was detected in roughly half of the isolates of both groups of coagulase-negative cocci. Pigmentation was confirmed to be of little value, because roughly 50% of both coagulase-positive and -negative strains showed a pale-yellow color on Chapman's mannitol salt agar while 13% of the *S. aureus* strains tested were white. A key to the classification of catalase-positive cocci consistent with that currently used for *Enterobacteriaceae* has been based on these figures.

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In investigations on the ecology and prevention of staphyloenterotoxicosis (staphylococcus food poisoning), a not too complicated identification scheme for organisms isolated in such studies is required. It has been established beyond doubt that coagulase activity may serve as a key criterion for this purpose. The significance of many other biochemical characters seems equivocal, however. Therefore, it was attempted to pursue the ecological approach initiated by Thatcher and Simon (1956), i.e., to study the correlation between source and a few biochemical properties of the catalase-positive cocci. A considerable number of strains recently isolated from various environments by the author and his colleagues abroad, and a few other strains obtained from culture collections, were examined for this purpose.

MATERIALS AND METHODS

Origin of strains. Some 360 strains of *Staphylococcus aureus* were isolated from the human clinical sources listed in Table 1. After preliminary identification in the Clinical Routine Laboratory of the Pasteur Institute, Lille, they were repurified by streaking onto plates of Chapman's (1945) mannitol salt phenol red agar.

The 11 pathogenic strains of bovine origin were obtained from T. M. Higgs, National Institute for Research in Dairying, Shinfield, Berkshire, England. They had been isolated recently from cases of bovine mastitis.

Finally, 11 strains isolated from various meat products were received from A. C. Baird-Parker, Unilever Research Laboratory, Sharnbrook, Bedfordshire, England, and M. S. Pohja, Research Laboratory of the Farmers' Cooperative Packinghouses, Hämeenlinna, Finland.

Sources of the coagulase-negative strains are given in Table 2. The ten strains stemming from the bovine udder were again obtained from Mr. Higgs. Roughly two-thirds of the remainder were recent isolates, obtained from Dr. Baird-Parker,

TABLE 1. *Origin of coagulase-positive strains*

Source of strains	No. of strains
Human milk	125
Dermal and visceral pus	109
Throat swabs	46
Stool	32
Sputum	28
Punctates (pleural and lumbar)	13
Bovine udder	11
Meat products	11
Nasal swabs	6
Urine	5
Vulvo-vaginal swabs	2
Food poisoning	1
Total no. of strains	389

TABLE 2. *Origin of coagulase-negative strains*

Source of strains	No. of strains
Meat and meat products	123
Milk and dairy products	15
Pus	13
Air	11
Bovine udder	10
Ice cream	8
Skin	5
Urine	2
Pastry	1
Total no. of strains	188

Dr. Pohja, and Dr. S. T. Cowan, National Collection of Type Cultures, London, England. The remaining one-third were isolated by the author by spreading drops of 0.1 ml of 10^{-1} dilutions of the commodities studied onto plates (15 cm diam) of mannitol salt phenol red agar, followed by incubation for 48 hr at 37 C and subsequent purification.

Coagulase reaction. Lyophilized rabbit plasma (Difco) was used for all tests, which were carried out following the directions of the manufacturer. In all tests, a coagulase-positive and a coagulase-negative reference strain were used as controls. In over 80 series of tests, not a single erratic result was obtained.

Dissimilation of D*-mannitol.* Evans (1947, 1948) and Evans and Niven (1950) recommended the study of anerobic metabolism (fermentation) of D-mannitol as an important criterion for distinguishing *S. aureus* from other catalase-positive cocci. They also showed that the breakdown of this polyol under aerobic conditions is nonspecific.

Mossel and Martin's (1961) modification of Hugh and Leifson's (1953) test was used for this purpose. It consists of stabbing the strains under investigation into tubes filled to at least 8 cm height with an agar of the following composition: Trypticase (BBL), 10 g; yeast extract (Difco), 1.5 g; NaCl, 5 g; D-mannitol (analytical reagent grade), 10 g; agar, 15 g; bromcresol purple (Difco), 15 mg; water, 1,000 ml; pH = 7.1. All tubes were first heated in a steaming water bath for 10 min, then rapidly cooled in ice-water, and immediately inoculated.

Incubation was continued through 4 days at 36 ± 1 C; readings were carried out after 2 and 4 days. Results were recorded as fermentation of mannitol if the lower and upper parts of the tubes turned distinctly yellow; if only the upper part turned yellow, the result was noted as oxidative attack of mannitol. If, after 4 days of incubation, either no color change was observed or a distinct intensification of the purple color occurred, it was concluded that D-mannitol was not attacked.

Oxygen tolerance. This test was carried out following the procedure recommended by Buttiaux and Gagnon (1959). Tubes (8 mm external diam) containing an agar of the following composition were prepared: Trypticase, 10 g; yeast extract, 2.5 g; glucose, 1 g; cysteine·HCl (analytical reagent grade), 0.5 g; agar, 15 g; water, 1,000 ml; pH = 7.2. All tubes were heated in a boiling-water bath for 10 min, then cooled to 47 ± 2 C and evenly inoculated with a drop (ca. 0.05 ml) of a slightly turbid suspension containing approximately 10^6 viable cells/ml of the strain under investigation.

After incubation for 48 hr at 30 C, the extent of the zone in which colonies developed was observed. A strain was called obligately aerobic if it had formed colonies in the upper few millimeters of the agar only; when uniform development of colonies throughout the tubes occurred, the strain was considered to be facultatively anaerobic.

Gelatin liquefaction. The suspension of the strain used for the determination of its oxygen tolerance was streaked as a 4-cm straight line on freshly dried plates of a slight modification of Frazier's (1926) gelatin agar (Mossel and de

Bruin, 1957). Six strains were tested per plate (9 cm diam).

After 48 hr of incubation at 30 C, plates were developed using Frazier's solution of mercuric chloride. A strain was considered gelatinase-positive if it had digested the gelatin around or under the streak.

Tellurite reduction on tellurite-glycine agar. The suspension of the strain used in the earlier tests was also streaked as 4-cm straight lines on freshly dried plates of tellurite-glycine-lithium chloride agar (Zebovitz, Evans, and Niven, 1955). The dehydrated product marketed by Difco Laboratories, Inc., Detroit, Mich., was used for the preparation of this agar, and the manufacturers' instructions for the preparation of this medium were strictly followed. In all tests, a coagulase-positive and a *Micrococcus*-reference strain were used as controls. Plates were incubated for 48 hr at 37 C.

Hydrolysis of urea. Inclined tubes of Christensen's (1946) urea-agar were used for this purpose. Incubation was for 48 hr at 30 C.

KCN tolerance. Following Gelosa's (1960) suggestion, a representative number of cultures was also examined for growth in the presence of potassium cyanide. The procedure developed by Buttiaux, Moriamez, and Papavassiliou (1956) was used for this purpose. Three loopfuls of a fresh culture of the strain were emulsified in 8-mm tubes containing freshly prepared 0.025% KCN-semi-solid agar and incubated for 24 hr at 30 C. KCN tolerance was indicated by the formation of a zone of intensive growth in the upper few millimeters of the tubes.

Pigmentation. Streak cultures (48 hr at 37 C on mannitol salt phenol red agar) were graded as: golden, weakly golden, or white.

Hydrogen sulfide formation. Following the suggestion of Krynski et al. (1962), a representative selection of strains was tested for the capability of H_2S formation from cysteine or its peptides. The procedure described by Mossel (1961) was used for this purpose. Tubes containing brain heart infusion broth enriched with 0.05% cysteine·HCl were inoculated and then equipped with a folded filter-paper band (ca. 10 cm long and 5 mm diam), impregnated with a saturated solution of lead acetate, and dried subsequently at 55 C. After 48 hr of incubation at 30 C, any blackening of the indicator strips was noted.

TABLE 3. *Correlation between coagulase reaction and anaerobic dissimilation ("fermentation") of* D-*mannitol*

Test	Result*
Coagulase-positive strains	
No. of strains examined	389 (100)
Fermentation within 2 days at 37 C	368 (94.6)
"Late" fermentation (≤4 days)	13 (3.3)
No fermentation	8 (2.1)
Coagulase-negative strains	
No. of strains examined	188 (100)
Fermentation within 2 days at 37 C	9 (4.8)
Oxidation or no attack†	179 (95.2)

* Number of strains; figures in parentheses are percentages of the total number of strains.

† For further details, see Table 4.

TABLE 4. *Mode of metabolism of* D-*mannitol by coagulase-negative strains**

Test	Result†
Obligately aerobic strains (micrococci)	
No. of strains examined	106 (100)
Mannitol oxidized	56 (52.8)
Mannitol not attacked	50 (47.2)
Mannitol fermented	0 (0.0)
Gelatin liquefied	76 (71.7)
Facultatively anaerobic strains (staphylococci)	
No. of strains examined	41 (100)
Mannitol oxidized	16 (39.0)
Mannitol not attacked	18 (43.9)
Mannitol fermented	7 (17.1)
Gelatin liquefied	31 (75.6)

* Examined according to Hugh and Leifson's (1953) principle in the one-tube test of Mossel and Martin (1961).

† Number of strains; figures in parentheses are percentages of the total number of strains.

RESULTS AND DISCUSSION

Anaerobic dissimilation of mannitol. All the strains of *S. aureus* utilized mannitol on Chapman's agar. As can be seen from Table 3, 94.6% of the 389 coagulase-positive strains metabolized mannitol under anaerobic conditions within 2 days, and 97.9% did so when incubation was continued for 4 days. Cowan and Steel (1961), in their latest taxonomic key for medical bacteri-

ology, defined a biochemical criterion as "valid" for determinative purposes when 80% or more of strains show the characteristic reaction. Anaerobic breakdown of mannitol can therefore definitely be considered a highly significant property of coagulase-positive staphylococci, hence of *S. aureus*. This was entirely confirmed by the results of the examination of the 188 coagulase-negative strains also shown in Table 3. Of these, only 4.8% fermented mannitol. It is essential to take into account that all these cultures, according to Table 4, were facultatively anaerobic strains, hence staphylococci of the *S. saprophyticus* (*S. epidermidis*) type (Fairbrother, 1940). None of the 106 micrococci examined metabolized mannitol under anaerobic conditions, although 52.8% oxidized this polyol in the upper layer of the medium, probably via the phosphogluconate pathway (Raj et al., 1960; Duryee et al., 1961).

My results, therefore, fully confirm the earlier observations of Evans (1947, 1948) and Evans and Niven (1950). Hence, it is all the more surprising that neither Shaw, Stitt, and Cowan (1951) nor Thatcher and Simon (1957) seem to have been able to accept Evans' results as a base for ending the long-standing confusion about the taxonomy of the catalase-positive cocci. In other recent studies on staphylococci, such as those of Schaub and Merritt (1960), Grün and Pulverer (1960), Angyal (1961), Clark, Moore, and Nelson (1961), Fink (1961), Krynski et al. (1962), Kimler (1962), and Vogelsang, Wormnes, and Östervold (1962), no attention was paid to anaerobic dissimilation of mannitol. It may well be that failure to choose the right type of carbon source, or lack of familiarity with a reliable routine test for anaerobic metabolism of carbohydrates, has been the cause of this lack of acceptance of Evans' results.

With regard to the former aspect, we have been able to confirm the results of Colwell (1939), Evans and Niven (1950), and Garvie, Higgs, and Neave (1961), showing that anaerobic dissimilation of, e.g., glucose has not nearly the same degree of specificity for *S. aureus* as has the anaerobic breakdown of mannitol. It must be stated again that unless the study of mannitol metabolism is carried out under adequate anaerobiosis it has very little differential value. Hussemann and Tanner (1949) found that ca. 75% of coagulase-negative staphylococci formed acid from mannitol in the conventional aerobic broth test, while Dickscheit's (1961*a*, *b*) recent investigations showed that about 80% of the micrococci which he examined formed acid from mannitol in conventional open-tube sugar "fermentation" tests. We have completely confirmed these findings with our micrococci and coagulase-negative staphylococci.

Roughly 2% of our strains of *S. aureus* did not ferment mannitol. This figure competes favorably with the data of Clark et al. (1961), wherein 6% of a rather low number of *S. aureus* strains appeared mannitol (aerobic)-negative. Kimler (1962), summarizing many years of experience in a clinical laboratory, stated that ca. 1% of *S. aureus* strains are mannitol-negative; hence, it may be safely assumed that false negatives of this type will be of such a low order of magnitude as to be considered insignificant.

Liquefaction of gelatin. Of the total of 577 strains, 99.2% of *S. aureus* cultures but only 71.3% of coagulase-negative staphylococci and micrococci were gelatinase-positive. These figures are, at least qualitatively, in agreement with the results of Levy (1952), who found that about 98% of *S. aureus* but only 60% of other catalase-positive cocci liquefied gelatin. The lower figures for *S. aureus* found by Angyal (1961), Clark et al. (1961), and Krynski et al. (1962) may well have been caused by the use of a less sensitive or less reliable technique than that of Frazier. However this may, our results clearly demonstrate that gelatin liquefaction is not too useful a criterion for determinative purposes within the catalase-positive cocci.

Oxygen tolerance. In Table 4, the data obtained have been further broken down along the line suggested by Abd-El-Malek and Gibson (1948), Evans, Bradford, and Niven (1955), Breed (1956), and Hill (1959). All obligately aerobic strains were considered micrococci, whereas all facultatively anaerobic strains were grouped as staphylococci. Although none of the strains of *S. aureus* oxidized mannitol, 52.8% of micrococci and only 39.0% of the coagulase-negative staphylococci did so. None of the micrococci fermented mannitol in the true sense, but 17.1% of the coagulase-negative staphylococci did.

It thus seems that the coagulase-negative staphylococci form the phylogenetic link between the type species, *S. aureus*, and the typical aerobic micrococci. While they all seem capable of thriving under anaerobic conditions, their metabolism

of mannitol tends to be oxidative rather than fermentative, although true fermenters are not at all rare.

Tellurite reduction. All 155 strains of *S. aureus* examined grew as black colonies on tellurite-glycine agar, confirming the figures of Hoeprich, Croft, and West (1960), who also found that all of their strains of *S. aureus* formed black colonies on this agar. However, 67.9% of 134 of our coagulase-negative cocci also showed this type of growth. This is in agreement with the results obtained by Jay (1961), who demonstrated that of 140 tellurite-glycine-positive cocci, isolated from meats, only 10% were coagulase-positive. Thus, "characteristic" growth of cocci on tellurite-glycine agar may not be reliable for counting or isolating *S. aureus*.

This lack of specificity of tellurite-glycine agar, in combination with its repeatedly shown inhibitory properties for primary isolates (Chapman, 1949; Burdin, Schmitt, and de Lavergne, 1959; Sevel and Plommet, 1960; Innes, 1960; Menolasino, 1960; Moore and Nelson, 1960, 1962; Baird-Parker, 1962), had already been noted some years ago (Mossel, 1956). Therefore, we have not since used this medium for the isolation of catalase-positive cocci from foods (Mossel and Krugers Dagneaux, 1959) or other sources.

Hydrolysis of urea. Hydrolysis of urea did not appear to be a useful criterion for the classification of catalase-positive cocci. In fact, 80.0% of the 30 coagulase-positive strains, and 61.3% of the 31 coagulase-negative organisms, had a urease. These results contrast sharply with those of Roemer and Schmitz (1951), Kielwein (1960), and Angyal (1961), who found that saprophytic cocci were almost always urea-positive but *S. aureus* strains usually were not. These results also disagree with those of Fusillo and Jaffurs (1955), who found that only 20% of their coagulase-positive organisms were urea-positive, although only 87% of their coagulase-negative cocci showed this character.

However, our results are in agreement with those of Cowan, Shaw, and Williams (1954), who found the type species of *S. aureus* urea-positive, and with the figures of Krynski et al. (1962) and of Seneca, Peer, and Nally (1962), establishing that ca. 86 and 69%, respectively, of a large number of coagulase-positive strains were urea-positive.

KCN tolerance. Growth in the presence of KCN did not appear to be a constant character in *S. aureus*, with 37.9% of 29 strains being positive. Growth was moderately frequent (14.8% positive) in the 27 coagulase-negative cocci studied. This character, therefore, also appears to be an inefficient criterion for the classification of the catalase-positive cocci.

Pigmentation. The typical, golden-yellow zeaxanthin pigment formed by *S. aureus* on suitable media (Steuer, 1956) has long been used as a means of identification of this organism. However, Hussemann and Tanner (1949), Cowan (1956), Clark et al. (1961), and Jay (1962) noted that many genuine *S. aureus* strains might not form this pigment, or form only traces.

Our results confirm this trend. In perfect agreement with the figures obtained by Jay (1962), we found that 13.4% of the 97 coagulase-positive strains examined formed white colonies on Chapman's (1946) medium even though this is considered to favor chromogenesis (Chapman, 1945). By discarding white colonies found on plates inoculated with foods, one may therefore quite frequently miss coagulase-positive strains. It is recommended, rather, to test also a representative number of such isolates for anaerobic breakdown of mannitol or coagulase formation or both.

Also, although 39.2% of 97 strains of *S. aureus*, compared with only 7.9% of 63 strains of coagulase-negative cocci, formed typically golden colonies, almost equal percentages (47.4 vs. 52.4) of both types of cocci formed weakly golden pigmented colonies on Chapman's agar. This further substantiates the limited importance of pigmentation as a diagnostic criterion.

Formation of H_2S from cysteine. In agreement with Krynski et al. (1962), I found that 92.9% of the 28 coagulase-positive staphylococci examined formed H_2S from a suitable cystine-containing broth. However, because approximately the same percentage (88.9) of 18 coagulase-negative strains examined desulfhydrized cysteine, such a test is not helpful in rapid distinction of *S. aureus* from similar organisms.

The results presented in this paper confirm the efficacy of the key given in *Bergey's Manual* (Evans, 1957). The anaerobic dissimilation of mannitol is, in combination with the coagulase test, the most useful criterion to distinguish *S.*

TABLE 5. *Suggestion for the classification of staphylococci and micrococci*

Genus and species	Oxygen relation	Metabolism of mannitol	Coagulase test
Staphylococcus aureus	Facultatively anaerobic	Fermentation	Positive
S. saprophyticus	Facultatively anaerobic	D*	Negative
Micrococcus†	Obligately aerobic	Oxidation or none	Negative

* Different dissimilation mechanisms observed.

† Species: *M. denitrificans, M. flavus, M. freudenreichii, M. luteus, M. roseus,* and *M. ureae* (as in *Bergey's Manual*).

aureus from other catalase-positive cocci. Also, coagulase-negative staphylococci can be readily distinguished from micrococci by the oxygen-tolerance test. These broad dividing lines are summarized in Table 5.

As for classification to the species level, I support Fairbrother's (1940) suggestion of designating all coagulase-negative and most mannitol-nonfermenting staphylococci as *S. saprophyticus*, because I have been unable to find any reliable criterion for further subdivision.

With regard to the micrococci, I have been able to identify most of my isolates as the species indicated in Table 5, although one or two characters might occasionally be in disagreement with the description of the species in *Bergey's Manual.*

The suggestion in Table 5, therefore, attempts no more than to present a key to the determination of catalase-positive cocci isolated from various habitats, of the same order of consistency as Ewing and Edwards' (1960) recent new approach to the classification of the *Enterobacteriaceae.*

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