Menaquinone Determination in the Taxonomy of Micrococcaceae

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(Accepted for publication 29 July 1968)

SUMMARY

Staphylococci, micrococci and strains intermediate in guanine + cytosine (GC) content between these genera were all found to contain menaquinones. Two different types of menaquinone, described as 'normal' and 'hydrogenated' were revealed. Distinct and stable menaquinone patterns, formed by the percentages of individual isoprenologues, were found to characterize certain previously proposed species, groups or subgroups within the family. Studies on the distribution of menaquinones have also demonstrated heterogeneity within certain subgroups, notably Baird-Parker's *Micrococcus* subgroup 7, and the divisions within the yellow-pigmented micrococci revealed by Rosypal, Rosypalová & Hořejš have been substantiated. Normal menaquinones were found in strains extending over a wide range of reported GC ratios (31.0 to 61.4 %). These strains included staphylococci and non-pigmented micrococci, classified according to Baird-Parker, and also marine strains and others of uncertain taxonomic position. Hydrogenated menaquinones, however, were restricted to pigmented micrococci within the range 66.3 to 73.3 % GC. There is thus evidence of a correlation between pigmentation, high GC ratio and menaquinone type. Menaquinone assay may contribute to the classification of Micrococcaceae and thus complement existing techniques in chemotaxonomy.

INTRODUCTION

Menaquinones, vitamins of the K₂ group, are 2-methyl-1,4-naphthoquinones with an unsaturated polyisoprenoid side chain (MK); partially hydrogenated variants also occur in which at least one isoprene unit is saturated (MK(H)). Isoprenologues are designated by the number of isoprene units (*n*) in the side chain (Fig. 1). Mena-

Fig. 1. Menaquinones, vitamins of the K₂ group, are 2-methyl-1,4-naphthoquinones with unsaturated polyisoprenoid side chains; *n* = number of isoprene units.

quinones have been extracted from both Gram-positive and Gram-negative aerobic bacteria (review by Pennock, 1966) and also from *Bacteroides* and *Veillonella* species (Gibbons & Engle, 1964); one isoprenologue, MK-4, has been isolated from animal

tissues (Martius & Esser, 1959) and also from a strain of *Staphylococcus aureus* (Cawthorne *et al.* 1967). Earlier investigations (Jeffries *et al.* 1967*a*) revealed the presence of menaquinones in all strains of staphylococci and micrococci examined. Several different menaquinone (MK) patterns, formed by different percentages of individual isoprenologues, were found and it was suggested that distinct MK patterns characterize *S. aureus* and certain accepted *Micrococcus* species. Subsequent studies of further strains of micrococci confirmed the taxonomic significance of their MK patterns (Jeffries *et al.* 1967*b*). The present paper describes our extended examination of aerobic Micrococcaceae and includes representatives of the genera *Staphylococcus* and *Micrococcus*, together with a number of mostly marine strains of intermediate and, at present, uncertain classification. Reported figures for guanine + cytosine content (GC ratio) in the DNA of the strains studied ranged from 31.0 to 74.0 %.

Baird-Parker (1965) divided the aerobic catalase-positive, Gram-positive cocci into two groups. Strains fermenting glucose were placed in group 1 (*Staphylococcus*) and those attacking glucose oxidatively, or not at all, in group 2 (*Micrococcus*). On physiological characters, Baird-Parker further divided the staphylococci into six subgroups, subgroup I representing *S. aureus*; the micrococci fell into eight subgroups, two of which corresponded to the accepted species *Micrococcus luteus* and *M. roseus*. More recently, Rosypal, Rosypalová & Hořejš (1966), in studies mainly on pigmented micrococci, proposed a classification into groups based on the GC content of their DNAs; the strains were further divided into subgroups on similarity of biochemical and physiological characters. They proposed that, pending further data on the GC content of species within the Micrococcaceae, strains of GC content within the range 30.7 to 36.4 % should be classified as *Staphylococcus* species and that only strains of GC content within the range 66.3 to 73.3 % should, at present, be accepted in the genus *Micrococcus*; attention was also drawn to a strain intermediate in GC content between these two genera. On DNA base composition Boháček, Kocur & Martinec (1967*a*) divided a number of strains previously classified as micrococci into three groups. The first group comprised strains with GC ratio of 63.5–73.0 % and included *M. luteus* and *M. roseus*, the second group consisted mainly of strains requiring reclassification, since they differed in GC content and also in other characters from the suggested ranges for the genera *Micrococcus* and *Staphylococcus*. Their third group consisted of motile strains, probably of marine origin, which were intermediate in GC content between the ranges for the genera *Staphylococcus* and *Micrococcus*, and it was suggested that these strains may be transferred to the genus *Planococcus*. Kocur & Martinec (1965) proposed that the genus *Sarcina* should include only the anaerobic species with fermentative metabolism and that the species *Sarcina lutea* should be considered identical with *M. luteus*. Canale-Parola, Mandel & Kupfer (1967) showed that the strict anaerobes, *Sarcina ventriculi* and *Sarcina maxima*, are distinct from aerobic micrococci in DNA base composition.

METHODS

Strains and cultivation. The strains studied included certain of those examined by Baird-Parker (1965), by Rosypal *et al.* (1966) and additional strains received from the Czechoslovak Collection of Micro-organisms (Brno), the *Staphylococcus* Reference Laboratory (Central Public Health Laboratory, London), and other sources (Table 1).

On receipt, all cultures were examined for purity and pigment production on nutrient agar at 26°, 30° and 37° after incubation for 1 week; agar-slope subcultures were then stored at room temperature or at 4°. With the exception of *Micrococcus morrhuae* (see below), Lemco agar (blood agar base, Oxoid Ltd.) was found to be suitable for the cultivation of all strains, including those of marine origin. Nutrient agar plates inoculated with peptone-water suspensions of growth from freshly prepared agar-slope cultures were incubated for 48 hr and the surface growth harvested for MK assay. Staphylococci were grown at 37° and micrococci at 30°; in strains growing well at 30° and 37° the MK patterns were found to be similar at both temperatures. With most strains between 1 and 3 g. wet weight of organism were extracted, requiring the inoculation of between 10 and 30 Petri dishes; with strains yielding only small amounts of MK quantities up to 9 g. wet wt organism were prepared. The extreme halophile, *M. morrhuae*, did not grow on the nutrient agar and a medium of high salt concentration (Payne, Sehgal & Gibbons, 1960) was used for maintenance of cultures and preparation of suspensions for extraction. Thirty plates inoculated from each strain of *M. morrhuae* were incubated at 37° for 3 weeks in sealed tins containing flasks of water to maintain high humidity.

Menaquinone assay

Extraction of lipids. Since menaquinones are light-sensitive all operations were done in subdued light. The bacterial growth scraped from the surface of the medium was dispersed in ethanol (15 ml.) to remove water. The dispersion was centrifuged (1250 g; 5 min.) and the supernatant liquor decanted; ethanol (15 ml.), containing 50 mg. pyrogallol as antioxidant, was added to the residue and the mixture then boiled under reflux for 30 min.; after cooling the supernatant fluid was removed. Water (25 ml.) was added to the combined ethanolic liquors, which were then extracted with light petroleum (b.p. 40 to 60°, 3 × 25 ml.). This extract (extract A) was then evaporated and dried by azeotropic distillation under reduced pressure with benzene + ethanol (1 + 1, v/v). To test the efficiency of ethanolic extraction the residue remaining from this process was further subjected to alkaline digestion. Since this procedure destroys some MK (Jeffries *et al.* 1967*a*) quantitative results must be treated with caution; however, we have no evidence that this results in changes in the isoprenologue pattern. The residue remaining from each 1 g. wet wt of extracted organisms was dispersed in 4 ml. ethanolic pyrogallol (pyrogallol 5%, w/v, in ethanol, prepared freshly each day); 2 ml. aqueous potassium hydroxide solution (KOH, 88 g.; water 54 ml.) was then added and the mixture boiled under reflux for 30 min. After cooling and the addition of water (10 ml.) the lipid was extracted with ether (3 × 25 ml.). The ether extracts were pooled, washed free from alkali with water and evaporated; the lipid extract (extract B) was then dried.

Thin layer chromatography. Extracts A and B were treated identically, but separately. Each extract was dissolved in a small volume of light petroleum and applied as a streak to a 20 × 10 cm. plate coated with a 300 μ layer of silica gel (Merck, A.G., Darmstadt) containing sodium fluorescein (50 p.p.m.). A spot of a pure MK solution was placed beside the streak to act as a marker and the chromatoplate was developed with benzene + light petroleum (1 + 1, v/v) to a distance of 8.5 cm. Menaquinones quench the green fluorescence when the plates are viewed under ultraviolet light, and thus appear purple against a green background; a marker of pure MK (R_F 0.4 in this

Table 1. *Classification and sources of strains*

Classification		Strains
STAPHYLOCOCCUS		
<i>Staphylococcus aureus</i> (Baird-Parker, 1965, group 1, subgroup I)		
	II	OXFORD 'H', WO133, B-P1650, WOOD 46, CI309, CI341, C2936, C2956,
	III	C2976, C2981, C3512, C4532, C4601, C5422, C5437, C5490, C6828, C6830,
	IV	C8973, NCTC4163, NCTC8319, NCTC8325, NCTC8327, NCTC8329,
	V	NCTC8331, NCTC8354, NCTC8356, NCTC8357, NCTC8358, NCTC8363,
	VI	NCTC8507, NCTC8509, NCTC8511, NCTC9315, NCTC9717, NCTC9754,
		NCTC9789, NCTC10033, NCTC10039, NCTC10455, NCTC10457
		A197, A4, A5, A194
<i>S. aureus</i> (Brown, Sandvik, Scherer & Rose, 1967). Bovine strains, of proteinase groups G & H and 1 strain of undetermined group, not classifiable in Baird-Parker's system.		
<i>S. epidermidis</i>		
	II	B-P12, B-P14, B-P317
	III	B-P34, B-P1654
	IV	B-P19, B-P35, NCTC4276, NCTC7291, M/C2
	V	M/C122, M/C193, M/C199, M/C223, M/BR11218
	VI	WO143, B-P21, B-P32, NCTC5955, NCTC7944
Micrococcus (non-pigmented)		
	1	B-P1652, M/GP2, M/QWA8
	2	B-P5, B-16
	3	B-P3, B-P10, NCTC7292
	5	B-P45, B-P46, B-P47, B-P114, NCTC189
	6	B-P1653, NCTC8360, B-P8, B-P24
	7	B-P25, B-P26, B-P29, B-P89 (ATCC8456)
Baird-Parker, 1965, group 2, subgroup		
Micrococcus (pigmented)		
	1a (Baird-Parker, 1965, Micrococcus subgroup 7), yellow pigmented	CCM144, CCM210, CCM247, CCM248, CCM265, CCM266(i), CCM309, CCM331,
	1a (Baird-Parker, 1965, Micrococcus subgroup 7), non-pigmented variant	CCM559, CCM851, CCM852, CCM853, CCM1335
	1b (Baird-Parker, 1965, Micrococcus subgroup 8), pink-pigmented	CCM266(ii)
	2a yellow-pigmented	CCM146, CCM633, CCM706, CCM385
	3a grey-violet-pigmented, <i>M. cyaneus</i>	CCM208, CCM547, CCM884, CCM1044
	3b yellow-pigmented, <i>M. luteus</i> , neotype strain	CCM856
		CCM810 (ATCC398)
Rosypal, Rosypalová & Hořejš, 1966, subgroup		

system) also aided identification. The MK band was scraped from the plate and extracted from the silica gel with ether vapour.

Reversed phase paper chromatography. Zinc-ammine solution was prepared by dissolving zinc oxide (16 g.) and ammonium carbonate (25 g.) in a mixture of ammonium hydroxide (150 ml.) and water (600 ml.); 20 ml. of 0.1 % (w/v) aqueous fluorescein solution was then added. Sheets of Whatman no. 4 grade 'chromatography paper', 23 × 57 cm., were immersed in the zinc ammine solution, hung to dry at room temperature and then baked in a fan-draught oven at 95 to 100° for 1 hr. A portion of the sample was applied to a paper and partially overlapped with marker MKs. Since interfering substances were present the overlapping technique was necessary to ensure accurate identification of the MK isoprenologues. The remainder of the sample was applied to another paper, which was used for quantitative estimation of MKs. The phase of the papers was reversed by immersion in liquid paraffin solution (3 %, w/v, solution liquid paraffin B.P. in light petroleum, b.p. 60–80°). After evaporation of the light petroleum solvent the papers were developed in a filter-paper lined, vapour-saturated tank, by descending chromatography with 95 % (v/v) ethanol in water for 5 hr. at 27°. The papers were then dried with warm air and examined under ultraviolet light. Separation and migration of MKs in reversed-phase chromatography are related to isoprenoid side-chain length, thus MK-6 migrates farther than MK-7. After elution from the quantitative paper, the separate MK isoprenologues were measured.

Measurement and identification of isolated menaquinones. When examined by ultraviolet spectrometry menaquinones showed maxima at 239, 249, 260 and 270 nm. The absorption at 285 nm. was very small and the difference in absorption value (ΔE), observed at 285 and at 270 nm., was used as the basis of the assay. It was assumed that irrelevant absorption over this short range would have been constant. $\Delta E_{1\text{cm}}^{1\%}$ values determined experimentally in our laboratories on synthetic MKs were as follows: MK-9, 222; MK-8, 238; MK-7, 244; MK-6, 272. To complete the identification of MKs a complete u.v. scan from 230 to 350 nm. was made to ascertain that all four absorption maxima were present. Hydrogenated menaquinones, when present, ran more slowly than normal menaquinones on paper chromatography and thus occupied a position on the paper chromatogram between the normal isoprenologues.

RESULTS

The results of the menaquinone assays are shown in Tables 2*a* and *b*. Table 3 indicates the correlation between representative values for GC content in DNA and the type of menaquinone produced.

Menaquinones identified as MK-6, MK-7, MK-8 and MK-9 (referred to as 'normal' MKs, designated MK-n, with R_F values 0.318, 0.213, 0.143, 0.100, respectively) were found in staphylococci, micrococci and strains of intermediate uncertain classification. However, certain strains of micrococci with high GC values yielded menaquinone fractions which, when resolved in our paper chromatographic system, gave substances with R_F values 0.275, 0.182, 0.124 and 0.086. These substances gave a u.v. spectrum typical of menaquinones. Calculation of their R_F values suggested that they were partially hydrogenated MKs (referred to as 'hydrogenated' MKs and designated MK-n (H)). This conclusion was substantiated by their behaviour on thin layers of silver nitrate-impregnated silica gel.

Classification	No. of strains	Percentage of individual menaquinone isoprenologues*			
		MK-9	MK-8	MK-7	MK-6
STAPHYLOCOCCUS					
<i>Staphylococcus aureus</i> (Baird-Parker, 1965, group I, subgroup D)†	41	12 ± 5	73 ± 4	15 ± 5	0
<i>S. aureus</i> (Brown, Sandvik, Scherer & Rose, 1967, bovine strains)	4	0	1 ± 1	5 ± 2	94 ± 2
<i>S. epidermidis</i>					
II	3	0	53 ± 12	47 ± 12	0
III, B-P34	1	0	5	95	0
III, B-P1654	1	0	83	17	0
IV	4	0	17 ± 6	83 ± 6	0
IV, M/C2	1	0	0	100	0
V	3	0	7 ± 6	93 ± 6	0
V, M/C199	1	0	58	42	0
V, M/C223	1	0	Trace	Trace	0
VI	4	0	16 ± 8	84 ± 8	0
VI, NCTC7944	1	4	4	92	0
MICROCOCOCCUS (non-pigmented)					
I, B-P1652	1	0	0	0	100
I, M/GP2	1	0	Trace	100	Trace
I, M/QWA8	1	0	0	Trace	0
2	2	0	0	100	0
3	3	0	5 ± 5	95 ± 5	0
5	5	0	0	95 ± 9	5 ± 9
6	3	0	15 ± 13	85 ± 13	0
6, B-P24	1	0	0	0	100
7	3	0	0	0	100
7, B-P89 (ATCC8456)	1	0	0	0	100
<i>Micrococcus morrhuae</i>					
CCM889	2	0	Trace	0	0
CCM2226	1	0	Trace	Trace	0
1	1	0	Trace	Trace	Trace
STRAINS OF UNCERTAIN CLASSIFICATION					
Rosypal, <i>et al.</i> 1966, subgroup 4a, CCM250	1	0	70	30	0
<i>M. corallinus</i> , CCM678 (ATCC966)	1	85	9	6	0
<i>M. eucinetus</i> , CCM2387	1	0	50	25	25
<i>M. eucinetus</i> , CCM2389	1	0	50	50	0
<i>Micrococcus</i> spp. (marine), CCM1849	1	0	0	Trace	0
<i>Micrococcus</i> spp. (marine), CCM2069	1	0	Trace	0	0
<i>Micrococcus</i> spp. (marine), CCM2104	1	0	Trace	Trace	0

* Mean values with standard deviations (individual results are obtainable from the authors).

† Ten additional strains of *S. aureus* examined after alkali digestion gave MK patterns similar to those shown in the table (Baird-Parker group I, subgroup D).

Strain number abbreviations as in Table I.

Table 2b. *Distribution of hydrogenated menaquinones in pigmented micrococci*

Micrococcus (pigmented)	Classification	No. of strains	% of individual menaquinone isoprenologues*					
			MK-9 (H)	MK-8 (H)	MK-7 (H)	MK-6 (H)	MK-5 (H)	
Rosypal, <i>et al.</i> 1966, subgroup 2a	1a (Baird-Parker, 1965, Micrococcus subgroup 7)	13	0	84±6	16±5	0	0	
	1a (non-pigmented variant, CCM266(ii))	1	0	64	36	0	0	
	1b (Baird-Parker, 1965, Micrococcus subgroup 8)	4	21±2	74±4	5±3	0	0	
	3a CCM856 (<i>M. cyaneus</i>)	4	1±1	15±10	82±8	2±2	0	
	3b CCM810 (ATCC 398)	1	94	6	0	0	0	
Boháček, <i>et al.</i> 1967a	(<i>M. luteus</i> neotype strain)	1	80	20	0	0	0	
	CCM132 (<i>M. luteus</i>)	1	0	12	83	5	0	
	CCM169 (<i>M. luteus</i>)†	1	0	21	0	0	0	
CCM classification	(<i>M. roseus</i>)	3	14±4	79±6	7±1	0	0	
	CCM134 (<i>M. luteus</i>)	1	11	77	2	1	9	
	CCM840 (<i>M. luteus</i>)	1	4	79	17	0	0	
Baird-Parker, 1965, Micrococcus subgroup 8, B-P97 (ATCC966)	CCM840(ii) non-pigmented variant (<i>M. luteus</i>)	1	7	81	3	0	9	
	CCM166 (<i>M. luteus</i>)‡	1	0	26	11	0	10	
		1	95	5	0	0	0	

‡ The following normal menaquinone isoprenologues were also present:
MK-9, 12 %; MK-8, 30 %; MK-7, 11 %.

Strain number abbreviations as in Table 1.

* Mean values with standard deviations (individual results are obtainable from the authors).

† The following normal menaquinone isoprenologues were also present:
MK-9, 13 %; MK-8, 28 %; MK-7, 21 %; MK-6, 17 %.

Table 3. Correlation between guanine + cytosine content (% GC) in DNA of various cocci and type of menaquinone produced

Name or designation of strain	Strain no.	Guanine + cytosine content in DNA		Menaquinones
		% GC	Reference	
<i>Staphylococcus aureus</i> (Baird-Parker, 1965, group 1, subgroup I)*	NCTC4163	31.0	1	Normal (MK-n)
<i>Micrococcus canadensis</i> (Baird-Parker, 1965, group 2, subgroup 7)*	ATCC8456, B-P89	36.4	2	
<i>Staphylococcus epidermidis</i> (Baird-Parker, 1965, group 1, subgroup VI)*	NCTC7944	36.5	1	
<i>Micrococcus</i> sp. (marine)	CCM1849	39.6	3	
<i>M. eucinetus</i>	CCM2387	40.0	3	
<i>Micrococcus</i> sp. (marine)	CCM2069	42.2	3	
<i>Micrococcus</i> sp. (marine)	CCM2104	42.2	3	
<i>M. eucinetus</i>	CCM2389	50.3	4	
Rosypal, <i>et al.</i> 1966	CCM250	54.2	5	
<i>M. morrhuae</i>	CCM859	57.1	3	
<i>M. morrhuae</i>	CCM537	57.8	3	
<i>M. corallinus</i> (non-pigmented)	CCM678, ATCC966	59.6	3	
<i>M. morrhuae</i>	CCM889	61.0	3	
<i>M. morrhuae</i>	CCM2226	61.4	3	
{ Rosypal <i>et al.</i> 1966	{ Micrococcus subgroup 3b†	66.3	5	
	{ Micrococcus subgroup 2a†	68.0	5	
	{ Micrococcus subgroup 1b†	70.8	5	
	{ Micrococcus subgroup 1a†	73.3	5	

* GC content in DNA within suggested range for *Staphylococcus* spp. (Rosypal *et al.* 1966)
† GC content in DNA within suggested range for *Micrococcus* spp. (Rosypal *et al.* 1966).
References: 1, Silvestri, L. G. & Hill, L. R. (1965); 2, Boháček, J., Kocur, M. & Martinec, T. (1967b); 3, M. Kocur, personal communication; 4, Boháček, *et al.* (1967a); 5, Rosypal, *et al.* (1966).
Strain number abbreviations as in Table 1.

Although, for a given strain, the yield of MK was found to vary somewhat between cultures grown on different occasions, the isoprenologue ratios (MK patterns) were found to be constant. The reasons for this variation in MK yield are not known and our limited experiments yielded negative results. However, it has been shown, with a vitamin-K dependent strain of *Fusiformis melaninogenicus*, that MKs diffuse into agar from growing bacteria and this may represent a variable loss, since we only extracted the bacterial mass and not the culture fluid. The amounts of MK extracted ranged from a trace (less than 1 $\mu\text{g.}$) to more than 400 $\mu\text{g./g.}$ wet wt organism. In general, the highest yields were from the *Staphylococcus aureus* strains and the lowest were from the strains originally isolated from marine sources, including the extreme halophile, *Micrococcus morrhuae* (Table 2a). The MK patterns attributed to strains producing very small amounts of MK should be regarded as approximate, since the errors involved in such calculations may be considerable; but the identification of the type of MK produced by these strains was, in all cases, unequivocal.

Menaquinone patterns

As shown in Table 2a, MK-7 and MK-8 were common to both staphylococci and certain micrococci; with the exception of one strain of uncertain classification (CCM 678, ATCC966, discussed below), MK-9 was found only in staphylococci. Menaquinone-6, although present as the major isoprenologue in strains of a physiologically distinct group of coagulase-producing staphylococci, was not found in any other strains within this genus. Menaquinone-6 was, however, present in certain non-pigmented micrococci, and in some strains it was the only isoprenologue detected. Hydrogenated MKs were restricted to yellow, pink and violet-pigmented strains (*Micrococcus luteus*, *M. roseus*, *M. cyaneus*) and the non-pigmented variant colonies derived from two *M. luteus* strains (Table 2b). As discussed below, normal MKs were present in strains ranging widely in GC content, whereas hydrogenated MKs were extracted only from strains within the highest GC range.

The MK patterns of all strains have remained stable after repeated subculture and also after freeze-drying.

DISCUSSION

Staphylococcus

Staphylococcus aureus. With the exception of a single culture, described below, all the coagulase-producing strains comprising the accepted species, *S. aureus*, classifiable in group 1, subgroup I of Baird-Parker (1965) showed a single and characteristic MK pattern, with MK-8 as the major isoprenologue. MK-9 and MK-7 were present as minor isoprenologues in all strains, but their relative proportions differed between strains. The strains studied varied widely in sensitivity to antibiotics and in bacteriophage type; they included the twenty-one propagating strains of the International Phage Typing series.

Four cultures of *Staphylococcus aureus* NCTC7511 were received from different laboratories; this strain has been widely distributed as propagating strain 53 of the International Phage Typing series. Three of the cultures gave the MK pattern characteristic of *S. aureus* Baird-Parker group 1 subgroup I as shown in Table 2a. One culture, however, which had been employed in genetical studies by Korman (1963), revealed an anomalous MK pattern (MK-8, 17%; MK-7, 48%; MK-6, 11%;

MK-5, 15 %; MK-4, 9 %) including two isoprenologues, MK-4 and MK-5, not previously encountered in a bacterium. This culture was indistinguishable, in bacteriophage studies, from the other three cultures of NCTC8511. Detailed studies on the menaquinones of these cultures of NCTC8511, and on mutants allegedly derived from one of them, were described by Jeffries, Harris & Price (1967*c*). Identical GC ratios (32.4 %) were found in the culture of NCTC8511 giving an anomalous MK pattern and in one of the cultures of this strain from another source (L. R. Hill, personal communication).

Four bovine strains, isolated by Brown, Sandvik, Scherer & Rose (1967), were coagulase and phosphatase positive but did not produce acetoin from glucose or ferment maltose or mannitol. These strains, with features common to both *Staphylococcus aureus* (Baird-Parker group I subgroup I) and *S. epidermidis* (Baird-Parker group I subgroup III), were therefore not classifiable in Baird-Parker's system; they were, moreover, clearly separable on MK pattern from the strains of *S. aureus* studied by Baird-Parker (1965), all of which fell into his subgroup I.

Staphylococcus epidermidis. Of the twenty strains classified in Baird-Parker's subgroups II to VI, all except one of the subgroup IV strains, which contained MK-7 only, produced both MK-7 and MK-8. Two strains (NCTC7291, 7944), one in subgroup IV and one in subgroup VI, also produced small amounts of MK-9; MK-6 was not detected in any of the *S. epidermidis* strains. The strains could be broadly divided into two groups, according to the relative proportions of MK-7 and MK-8. The ratio of MK-7:MK-8: varied from about 0.5 to 10, or even higher; the highest ratios tending to predominate within subgroups IV, V and VI. A strain of the violet-pigmented *Micrococcus violagabriellae* (strain B-P317 in Table 1), now reclassified as a subgroup II *Staphylococcus* (Baird-Parker, 1965), showed an MK pattern similar to that of the two other strains of subgroup II staphylococci examined.

Micrococcus

Non-pigmented strains. The nineteen strains examined belonged to Baird-Parker's *Micrococcus* group 2, subgroups 1, 2, 3, 5, 6 and 7; they produced normal MKs only. MK-7 was the major isoprenologue in most strains and in ten of them either MK-7 or MK-6 was the sole isoprenologue detected. Menaquinone-8 was found, in relatively small amounts, in a few strains. Certain MK patterns were common to strains in different Baird-Parker subgroups. Strain NCTC8360, formerly classified as *Staphylococcus aureus* and alleged to clot sheep plasma, but not human or rabbit plasma (K. J. Steel, cited by Baird-Parker, 1965) was found by Baird-Parker to belong to *Micrococcus* subgroup 6. The MK pattern of this strain supports its separation from *S. aureus*.

Pigmented strains. In contrast to the non-pigmented strains, considered above, the yellow and pink-pigmented strains classified in Baird-Parker's subgroups 7 and 8 respectively, together with other pigmented strains classified by Rosypal *et al.* (1966), produced hydrogenated MKs (Table 2*b*). All except one of the pigmented strains B-P97, ATCC966) were from the Czechoslovak Collection of Micro-organisms and included representatives of the groups and subgroups proposed by Rosypal *et al.* and a few of the strains studied by Boháček *et al.* (1967*a*).

Two species (*Micrococcus luteus*, *M. roseus*) are recognized in the genus *Micrococcus* (Evans, 1965) but, in the tests described by Baird-Parker (1966), pigment is the only character which reliably separates these species from each other, their separation from

micrococci in the other subgroups being mainly on negative evidence. Differences in cell-wall composition and in the protein contents of the cocci are regarded as criteria justifying the separation of the species, although stable pink-pigmented mutants are alleged to have been produced from a yellow-pigmented strain classifiable in Baird-Parker subgroup 7 (G. W. Gould, cited by Baird-Parker, 1965).

Baird-Parker (1965) classified the yellow-pigmented *Micrococcus luteus* strains, together with non-pigmented strains, in *Micrococcus* subgroup 7, but our MK studies show that this subgroup is not homogeneous: of the strains examined by us all the yellow-pigmented ones contained hydrogenated MKs, and the non-pigmented strains normal MKs. We suggest a division of these strains into two subgroups, based on type of menaquinone produced. This separation is further supported by the higher sensitivity of the yellow-pigmented strains to lysozyme (unpublished observations). Non-pigmented variants (CCM266 (ii); CCM840 (ii)) isolated in our laboratory from two yellow strains have, like the parent strains, contained hydrogenated MKs.

Rosypal *et al.* (1966) divided the yellow-pigmented *Micrococcus* strains into three groups on differences in GC content within the range 66.3 to 73.3 %; they found that strain ATCC398 proposed as the type strain of *M. luteus* by Breed (1952), was in a different group (3) from the other strains classified as *M. luteus* which they studied and placed in group 1. These authors considered that, since the name of the type species should not be changed, the application of the specific name *luteus* to strains within their subgroup 1a, corresponding to *Micrococcus* subgroup 7 of Baird-Parker, should be questioned. Although the significance of minor differences in GC content, such as those found by Rosypal *et al.*, remains to be substantiated, a correlation has been found between the proposed subgroups of Rosypal *et al.* and MK patterns. Although the yellow-pigmented subgroup 2a strains of Rosypal *et al.* were alleged to correspond to *Micrococcus* subgroups 5 and 6 of Baird-Parker, we were unable to confirm this finding; we found that, in the tests recommended by Baird-Parker, these strains more closely resembled those in subgroup 7.

*Correlation between MK patterns and the classification of micrococci
proposed by Rosypal et al. (1966)*

Subgroup 1a. Thirteen strains were tested; all were yellow and some produced violet pigment also. They gave a distinct MK pattern, with MK-8(H) as the main isoprenologue and MK-7(H) as minor; small amounts of MK-6(H) were found in some strains and one strain also produced a trace of MK-9(H). Strain CCM266 yielded unstable variants lacking pigment (226 (ii)); in MK pattern these were identical with the pigmented parent strain (266 (i)).

Subgroup 1b. The four strains examined produced pink pigment and revealed a single MK pattern, with MK-8(H) as major and MK-9(H) and MK-7(H) as minor isoprenologues. The presence of MK-9(H) and the lower relative percentage of MK-7(H) differentiates this subgroup from the strains in subgroup 1a; MK-8(H) as the main isoprenologue is common to both subgroups, and perhaps supports their suggested close relationship.

Subgroup 2a. Four strains (two previously classified as *Micrococcus varians* and two as *M. conglomeratus*) showed a single MK pattern, in which MK-7(H) predominated, MK-8(H) was present in all strains and two of them also contained small amounts of MK-6(H) or MK-9(H).

Subgroups 3a and 3b. The single strains comprising these two subgroups gave a similar and distinct MK pattern in which MK-9(H) was the main isoprenologue and MK-8(H) was the minor. *Micrococcus cyaneus* (CCM856) represents subgroup 3a and produces grey/violet rough colonies. It was regarded by Kocur & Martinec (1963) as an atypical strain of *M. luteus*; Rosypalová *et al.* (1966), however, regarded this species as distinct. The type strain of *M. luteus* (ATCC398) represents subgroup 3b.

Subgroup 4a. The single strain in this subgroup (CCM250), differing in GC ratio (56.4 %) from staphylococci and micrococci, contained normal MKs only; the pattern (MK-8, 70 %; MK-7, 30 %) resembles that of certain coagulase-negative staphylococci.

The yellow-pigmented *Micrococcus* strains studied by Rosypal *et al.* (1966) fell into three ranges in GC content; each has now been shown to have a distinct MK pattern. Furthermore, the subgroup 1a strains have been shown to be sensitive to lysozyme by a plate incorporation method, whereas the subgroup 2a strains were relatively resistant (unpublished observations).

The menaquinone patterns of other yellow and pink-pigmented Micrococcus strains studied. Three pink-pigmented strains classified as *M. roseus* by Boháček *et al.* (1967a) gave an MK pattern identical with the strains of this species classified by Rosypal *et al.* (1966) in their subgroup 1b, thus all seven *M. roseus* strains examined were homogeneous in MK pattern.

Two allegedly non-pigmented strains of *Micrococcus luteus*, unlike the thirteen strains of this species classified in subgroup 1a of Rosypal *et al.* (1966), produced an MK pattern similar to that of the *M. roseus* strains. On nutrient agar at 30° one of these strains (CCM840; GC 71 %) produced numerous colonies lacking pigment, together with a single yellow colony; on subculture these colonies remained stable with respect to pigmentation. Although the MK patterns of the two colony types were similar, the non-pigmented variant produced an additional isoprenologue (tentatively identified as MK-5(H)), which was not encountered in any of the other strains in our series. The presence of MKs of the hydrogenated type suggests that the non-pigmented variant colonies (CCM840, (ii)) unlike the non-pigmented strains in Baird-Parker subgroup 7 which produced normal MKs, arose as variants from an originally yellow-pigmented strain. The other strain (CCM134), received as nonpigmented, produced a pale yellow pigment at 30° which intensified at room temperature; the MK pattern of this strain was also like that of the *M. roseus* strains. A pink-pigmented strain (B-P97, ATCC966) of *M. corallinus* (Baird-Parker *Micrococcus* subgroup 8) showed an MK pattern similar to that of the type strain of *M. luteus*, classified in subgroup 3 of Rosypal *et al.* Menaquinone patterns may thus substantiate the suggested close relationship between the yellow and pink-pigmented strains at present accorded specific status.

Two yellow-pigmented *Micrococcus luteus* strains (CCM169, GC 73.3 %; CCM166, GC 73.4 %) each contained normal and hydrogenated menaquinones; we have not encountered a mixture of normal and hydrogenated menaquinones in any of the other strains examined.* However, it should be stressed that hydrogenated menaquinones have been found to be characteristic of pigmented micrococci and have not been detected in any other strains.

Strains of uncertain classification

Strains originally described as *Micrococcus* species, and subsequently found to be of intermediate position in GC content (48 to 51 %) between the suggested ranges for the genera *Staphylococcus* (30.7 to 36.5 %) and *Micrococcus* (66.3 to 73.3 %) were described by Boháček *et al.* (1967*a*). These authors suggested that certain motile strains, including the light-brown pigmented *M. eucinetus* should, in view of their % GC, motility and marine habitat, be transferred to the genus *Planococcus*. The two strains of *M. eucinetus* examined (GC 40 and 50.3 %) each produced less than 20 µg. MK of the normal type/g. wet wt organism. Three other strains of salmon-pink pigmented marine micrococci (CCM1849, 2069 and 2104) were received from Dr M. Kocur, together with estimates of their GC content (39.6 to 42.2 %); these strains also yielded normal MKs, but in insufficient amounts for quantitative estimation of individual isoprenologues. Normal MKs (mainly MK-8) were found in very small quantities, necessitating the extraction of between 7 and 9 g. wet wt organism, in four strains of the red-pigmented *M. morrhuae*, grown for 3 weeks on a special medium of high salt content formulated for halophilic cocci; a fifth strain (CCM2224) grew relatively poorly, yielding only 2.4 g. wet wt organism, from which we were unable to extract sufficient MK for characterization. The GC content of the strains of *M. morrhuae* ranged from 57.1 to 61.4 % (M. Kocur, personal communication).

A strain submitted as a non-pigmented variant of *Micrococcus corallinus* (CCM678, ATCC966) was classified by us on negative characters and placed in Baird-Parker's *Micrococcus* subgroup 7. This strain, with a reported GC ratio of 59.6 % (M. Kocur, personal communication), has a value somewhat lower than those within the range for pigmented *Micrococcus* strains. This strain produced small quantities of menaquinones of the normal type but, as shown in Table 2*a*, the MK pattern, with MK-9 as major isoprenologue, was not encountered in any other strain that we examined. The GC ratio of the pink-pigmented strain, ATCC966, B-P97, has not been determined, but the MK pattern, with hydrogenated isoprenologues (Table 2*b*) suggests that the alleged non-pigmented variant strain is not related to it.

The strain with 56.4 % GC in DNA, representing subgroup 4*a* of Rosypal *et al.* (1966) discussed above, like the other strains intermediate in position between micrococci and staphylococci, also yielded normal MKs.

Strains NCTC7292 and NCTC189 were received as *Staphylococcus saprophyticus* and *S. lactis*, respectively. Both strains were submitted to Dr A. C. Baird-Parker, who classified them as micrococci (NCTC7292 in *Micrococcus* subgroup 3; NCTC89 in *Micrococcus* subgroup 5). In the standard medium for the determination of anaerobic utilisation of glucose with a paraffin oil seal, proposed by the International Subcommittee on Staphylococci and Micrococci (1965), we found that NCTC7292 produced acid only at the top of the tube, and NCTC189 did not produce acid. Mortensen & Kocur (1967) confirmed that, although neither strain visibly fermented glucose in the proposed standard medium containing the indicator bromocresol purple, both strains formed acid weakly and slowly, as revealed by potentiometric pH determination of anaerobic cultures, in a fluid medium containing glucose. These two strains, together with others of different GC ratios, were thus found by Mortensen & Kocur to be intermediate between strains rapidly fermenting glucose, all of which were of low GC ratio (staphylococci), and strains forming little if any acid from glucose by oxidation

and all of high GC ratio (micrococci). The GC ratios of strains 7292 and 189 (Table 1) suggest their classification with the genus *Staphylococcus*, but this is not supported by their relative resistance to novobiocin, determined according to Mitchell & Baird-Parker (1967). The presence of MK-6 in both strains suggests that they may be closer to strains at present classified as non-pigmented micrococci.

Micrococcus candicans, ATCC8456, B-P89, was classified on physiological characters in Baird-Parkers' *Micrococcus* subgroup 7; however, the GC ratio of this strain, reported as 36.4% by Boháček, Kocur & Martinec (1967*b*), suggests that it should be classified in the genus *Staphylococcus*. As shown in Table 2*a*, all four non-pigmented subgroup 7 *Micrococcus* strains produced normal menaquinones; but strain ATCC8456 showed a different pattern from the other three strains, on which GC values have not been established.

It would appear, from the literature, that GC ratios have been determined on a disproportionately large number of strains of pigmented micrococci and on relatively few staphylococci and non-pigmented micrococci. A few non-pigmented strains, at present classified as micrococci on biochemical and physiological characters, have GC ratios which suggest that, in fact, these strains should not be classified in the genus *Micrococcus* (A. C. Baird-Parker, personal communication).

Association between menaquinone type and GC ratio

In addition to the values shown in Table 3, GC ratios, which fell within the range 66 to 74 %, were provided for all the pigmented micrococci received from the Czechoslovak Collection of Micro-organisms. Thus 45 of the 129 strains of *Micrococcaceae* on which we have determined menaquinones were of known GC content.

According to the type of menaquinone produced, aerobic *Micrococcaceae* may be divided into two groups. The first, broader, group would include strains with GC ratios lower than 61.4 %. The second group would comprise all strains producing hydrogenated menaquinones and would include those at present classified as *Micrococcus luteus* and *M. roseus* (% GC 66 to 74). Within these groups, subgroups may be defined on MK patterns. It will be interesting to see whether further data on GC ratios supports the differences suggested by the menaquinone distribution.

We are grateful to Dr M. Kocur, Curator, Czechoslovak Collection of Micro-organisms, who supplied the strains of marine cocci and most of the pigmented micrococci, together with GC values where they were not available from the literature. To Dr A. C. Baird-Parker we are indebted for providing strains representative of his proposed classification and also for many helpful discussions during the course of our work. For supplying other strains, together with details of isolation or classification, we are pleased to record our thanks to Mrs E. Asheshov, Dr M. T. Parker, Dr S. P. Lapage and Dr R. G. Mitchell. Dr O. Isler (Hoffman la Roche, Basle, Switzerland) kindly provided samples of synthetic menaquinones. For constructive criticism during the course of this work and in its presentation we thank Mr S. A. Price and Dr J. Green.

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