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DNA Base Composition and Taxonomy of Some Micrococci

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SUMMARY

The present report gives the results of studies on the % guanine+cytosine (GC) content of the DNA of 29 strains designated as *Micrococcus*, by using the methods of Marmur & Doty (1962) and Frédéricq, Oth & Fontaine (1961). In 17 strains agreement was found between their taxonomic position and their % GC content. Five micrococci formed a very tight cluster around a mean of 50% GC. It is recommended to classify them in the genus *Planococcus* Migula. From the DNA base compositions these cocci form a group hitherto not described, intermediate between the genera *Staphylococcus* and *Micrococcus*. *Micrococcus cryophilus* McLean, Sulzbacher & Mudd, and *Micrococcus cerolyticus* Friedman & Kern differ in their % GC values from those of the genus *Micrococcus* and should be discarded from the genus. It is confirmed that % GC content in the DNA in the genus *Micrococcus* lies within the range of 65–75%.

INTRODUCTION

The usefulness of the study of DNA base compositions for the taxonomy of bacteria has been emphasized by several authors. This approach has given important results in the problem of the classification of aerobic Gram-positive cocci. It was pointed out by Silvestri & Hill (1965) that aerobic Gram-positive cocci can be divided into two groups on the % guanine+cytosine (GC) content of their DNAs. The first group, with a low % GC in DNA, corresponds to the genus *Staphylococcus* and the second group, with a high % GC, corresponds to the genus *Micrococcus*. These conclusions were confirmed by the present authors in a previous paper (Boháček, Kocur & Martinec, 1965).

Equally significant is the application of this method in solving problems of intrageneric classification. As regards the species within the genus *Micrococcus* and their DNA base compositions, data on only some of the species can be found in the literature (Belozersky & Spirin, 1960; Marmur, Falkow & Mandel, 1963). With the exception of reports by Silvestri & Hill (1965), Auletta & Kennedy (1966) and Rosypalová, Boháček & Rosypal (1966), the % GC contents of DNA have not directly been used in the classification of the genus *Micrococcus*. The object of the present paper was to analyse the DNA base composition in species of the genus *Micrococcus* and to find whether the % GC content was in agreement with their classification by other characters.

METHODS

In the present study 29 strains of micrococci were investigated; they are listed in Table 1.

Most of the strains were grown on yeast-extract glucose agar (g./l.): yeast-extract, 5; peptone, 5; glucose, 10; agar, 20; pH 7·2. *Micrococcus halodenitrificans* was grown on the following medium: (g./l.): beef-extract, 5; peptone, 5; NaCl, 80; agar, 20; pH 7·2. *M. litoralis* was grown on the following medium (g./l.): yeast extract, 5; peptone, 5; glucose, 10; KCl, 2; $MgSO_4.7H_2O$, 25; NaCl, 150; agar, 25; pH 7·8. The marine micrococci were cultivated in the following medium (g./l.): beef extract, 10; peptone, 10; agar, 20; in sea water, 750 ml., tap water, 250 ml.

Table 1. *List of organisms used*

Name	CCM no.*	Other names or numbers
Micrococcus luteus	132	
	149	
	169	*M. lysodeikticus* ATCC 4698; NCTC 2665
	337	*Sarcina lutea* ATCC 382
	409	
	410	*Sarcina lutea* ATCC 272
	840	*M. ureae* ATCC 408
	1674	
Micrococcus roseus	679	*M. rubens* ATCC 186
	837	ATCC 416
	385	ATCC 185
	560	ATCC 179
	633	ATCC 412
	168	
	1405	*Staphylococcus roseus*
Micrococcus litoralis	2226	*Sarcina morrhuae*
Micrococcus sp.	740	*M. conglomeratus*
	825	*M. conglomeratus*
	836	
	2087	ATCC 401
Micrococcus cerolyticus	901	ATCC 12559
Micrococcus denitrificans	982	NCIB 8944; ATCC 13543
	1396	
Micrococcus halodenitrificans	286	ATCC 13511
Micrococcus radiodurans	1700	ATCC 13939
	1701	UI
Micrococcus cryophilus	900	ATCC 12226
Micrococcus aquivivus	316	ATCC 14404
Micrococcus eucinetus	2388	XQ 58
	2389	XQ 40

* CCM = Czechoslovak Collection of Microorganisms, University J. E. Purkyně, Brno.

The organisms were incubated at 30° for 24 hr with the exception of *Micrococcus litoralis*, which was incubated for 6 days, and *M. cryophilus* which was incubated at 10° for 2 days.

For the isolation of DNA a method combining chloroform deproteinization with phenol deproteinization was used, since when following the procedure of Marmur

(1961) it was found difficult with some DNA samples to decrease the protein content below 1% even after performing chloroform deproteinizations 8 times. Strains sensitive to lysozyme were incubated with 0·5 mg. lysozyme/ml. bacterial suspension in a NaCl + EDTA solution at 37° for 10–60 min. A 25% (w/v) solution of sodium dodecyl sulphate was then added to make the final concentration 2%; lysis was then complete at 60° in 10 min. After the mixture had been cooled to room temperature, sodium perchlorate was added to M concentration and one volume of a mixture of chloroform + isoamyl alcohol (24 + 1, v/v). After 30 min. shaking and 20 min. centrifugation at 5000 rev./min. the upper layer was removed and precipitated with 1·5 volume ethanol. The resulting fibrous DNA precipitate was dissolved in 10-fold dilution of standard saline citrate buffer pH 7 (0·15 M-NaCl + 0·015 M-sodium citrate, pH 7; hereafter SSC) and after its solution the saline citrate buffer concentration was adjusted to SSC with 10 × SSC and ribonuclease (5 × cryst. Reanal, Hungary) added 50 μg./ml. After incubation for 30 min. at 37°, NaCl was added to the solution to M concentration and a further equal volume of re-distilled phenol saturated with M-NaCl + 0·1 M-tris (pH 9). After shaking for 20 min. at 4°, the mixture was centrifuged at 75,000*g* for 30 min. and the clear supernatant layer precipitated with one volume of ethanol.

Deproteinization with a mixture of chloroform + isoamyl alcohol (24 + 1) was done twice and as a rule, after the second deproteinization, no interlayer of protein was formed after centrifugation. The DNA sample was precipitated with one volume of ethanol again. By this procedure, the amount of phenol in the sample decreased below 0·5%; this was detected spectrophotometrically by measuring the ratio $E_{260}:E_{235}$ in a medium of 0·1 M-NaOH (Boháček, 1966). The ethanol-precipitated DNA was dissolved in a 10-fold diluted SSC and 1/10 volume of 3 M-sodium acetate in 10^{-3} M-EDTA added. The DNA was precipitated by adding 0·55 to 0·7 volume of re-distilled 2-propanol with constant stirring. After twice washing in 75% (w/v) ethanol in water the DNA was dissolved in phosphate + EDTA (0·01 M-sodium phosphate + 0·001 M-EDTA, pH 7) buffer (PE).

For the measurement of the DNA melting curves a spectrophotometer, type Beckman DU, was used. The cell holder compartment was thermostated at both sides by two thermospacers for circulating hot water from a U-10 ultra thermostat. The temperature was measured with a rod thermometer directly in one of the cuvettes. The melting curves were not corrected for thermal expansion of the solution. The solvent used was 10^{-2} M-sodium phosphate buffer (pH 7) + 10^{-3} M-EDTA which contained the following substances per liter of solution: 0·608 g. $NaH_2PO_4.2H_2O$, 2·18 g. $Na_2HPO_4.12H_2O$, 0·372 g. disodium salt of EDTA, 1 ml. N-NaOH. An example of a melting curve and its evaluation is given in Fig. 1.

The % GC content was calculated according to the equation $T_m = 51 + 0{\cdot}45\,(GC)$ derived from the dependence of T_m on the GC content determined in DNA by paper chromatography (unpublished results) and according to the results from the method of Frédéricq *et al.* (1961). The width of the transition interval (ΔT) was established as the difference between temperatures from 17 to 83% of the over-all increase in E_{260} and the value $2\sigma = (\Delta T - 3).2{\cdot}5$ was calculated from it. The values 2σ and 3σ served then to express graphically the heterogeneity of the sample according to De Ley & van Muylem (1963).

As a second control method for determining the % GC content in isolated samples

of DNA, the method of Frédéricq *et al.* (1961) was used. This method is based on the fact that the extinction ratio of 260:280 mμ (E_{260}:E_{280}) measured in a medium of 0·1 M-acetic acid (pH 3) is dependent on the % GC content in DNA. The samples of DNA were dissolved in a PE medium in a concentration of approximately 2 mg./ml. Before measurement, 0·1 M-acetic acid was added to the final concentration of DNA, 20–30 μg/ml. The measurements of O.D. at 260 and 280 mμ respectively were made with a type Zeiss VSU-1 spectrophotometer. For pH measurements a pH-meter type PYE-Master was used.

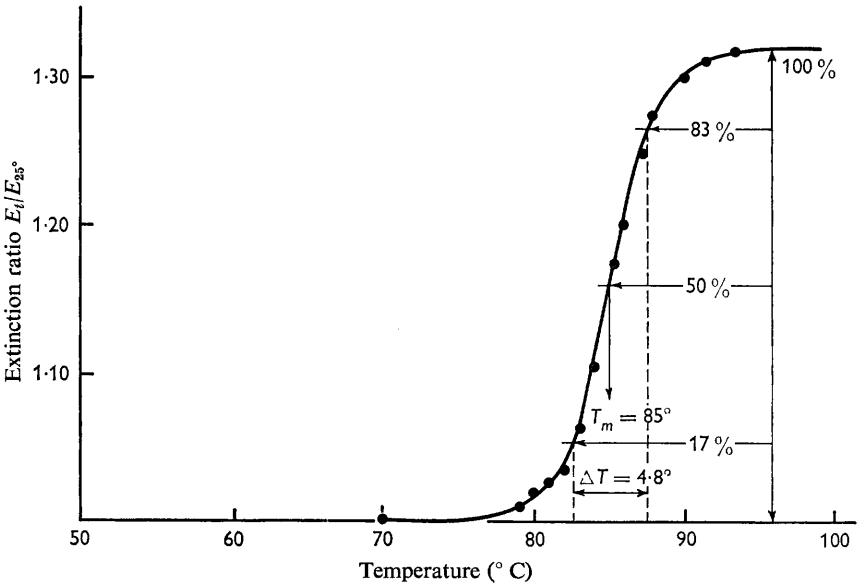

Fig. 1. Example of the evaluation of the melting curve of DNA of *Micrococcus luteus*, no. 409. Ordinate extinction ratio E_{260} at the given temperature (t) to E_{260} at the basal temperature 25°. The over-all increase in extinction ratio is designated as 100 %, T_m corresponds to 50 % of the over-all increase in E_{260}. From the course of the melting curve within the temperature range of 17–83 % E_{260}, the interval ΔT ($\Delta\frac{2}{3}$) is subtracted, from which 2σ = ($\Delta T - 3$) 2·5 is calculated.

RESULTS AND DISCUSSION

For determining the % GC content in the DNA of the micrococci examined, two different methods were used; besides the method of determining the % GC content from the T_m value (Marmur & Doty, 1962) we also used the method of determining % GC according to the ratio E_{260}:E_{280} at pH 3 (Frédéricq *et al.* 1961). The difference between the values obtained by the two methods were within the range of 1–3 % GC (see Table 2). Greater differences between the results given by the two methods would indicate the presence of impurities in the DNA preparations. In our opinion, the method recommended by Frédéricq *et al.* (1961) is useful for a rapid and orientating determination of % GC. However, since this method does not furnish data on the heterogeneity of DNA molecules it is advisable to use in parallel the method of T_m determination to obtain more detailed information.

The results (Table 2) show that the strains examined can be divided into three groups. The first, most numerous, group (Fig. 2) includes 17 strains (8 *Micrococcus luteus*,

5 *M. roseus*, 3 *Micrococcus* spp., 1 *M. litoralis*) whose % GC in DNA was in agreement with their classification as given by Kocur & Martinec (1962). Our results also confirm the opinion of these authors that aerobic Gram-positive yellow-pigmented cocci which do not attack glucose should be classified as *M. luteus*. The % GC values found in strains of *M. luteus* and *M. roseus* are in agreement with the data given by Belozersky & Spirin (1960) and by Silvestri & Hill (1965). In contrast, Rosypalová *et al.* (1966) found only 66·3% GC in the DNA of the type culture of *M. luteus* ATCC 398. Only one strain from our series (*M. luteus* CCM 169, NCTC 2665) was also studied by Silvestri & Hill (1965). Their result 72·8% GC agrees with the value of 73·3% GC found by us.

Table 2. *DNA base composition of some micrococci*

Name	CCM no.	T_m (°C)	% GC	2σ	$E_{260/280}$ pH = 3	% GC
Micrococcus luteus	132	83·0	71·0	2·5	1·130	67·0
	840	83·0	71·0	2·5	1·050	72·0
	1674	83·2	71·5	0	1·090	71·0
	337	83·6	72·3	4·5	1·100	70·0
	169	84·0	73·3	2·0	—	—
	410	84·2	73·7	2·5	1·100	70·0
	149	84·6	74·6	3·0	1·070	73·0
	409	85·0	75·5	3·30	1·080	72·0
Micrococcus roseus	679	82·0	69·0	3·0	1·120	68·0
	837	82·4	69·7	3·0	1·100	70·0
	385	83·0	71·0	4·5	1·120	68·0
	560	83·0	71·0	2·5	1·100	70·0
	633	83·8	72·8	0·75	1·100	69·0
	168	73·2	49·3	0·5	1·400	45·0
	1405	73·2	49·3	4·5	1·330	50·5
Micrococcus litoralis	2226	80·5	65·0	4·0	1·170	63·5
Micrococcus spp.	740	77·4	58·6	8·5	1·250	53·5
	836	81·7	68·3	1·5	1·165	64·0
	2087	81·7	68·3	0	1·165	64·0
	825	82·2	69·5	0	—	—
Micrococcus cerolyticus	901	67·0	35·6	5·0	14·90	39·0
Micrococcus denitrificans	982	79·8	64·0	4·5	1·160	64·0
	1396	80·6	66·3	1·5	1·130	67·0
Micrococcus halodenitrificans	286	80·2	65·0	0·5	1·140	66·0
Micrococcus radiodurans	1700	82·0	69·0	3·25	1·140	66·0
	1701	80·4	65·3	4·3	1·145	65·5
Micrococcus cryophilus	900	69·9	41·3	3·5	1·410	44·5
Micrococcus aquivivus	316	74·0	51·2	3·5	1·375	47·5
Micrococcus eucinetus	2388	72·6	48·0	3·25	1·370	47·5
	2389	73·6	50·3	4·0	1·350	49·0

The second heterogeneous group of strains (Fig. 2) consisted of those which would appear to need reclassification since they substantially differed in their % GC content from the value for the genus *Micrococcus*. This group contains *Micrococcus* sp. 740, *M. cerolyticus* 901, and *M. cryophilus* 900. With the exception of strain 740, these strains also differed in their cultural and biochemical properties from the species within the genus *Micrococcus*. *M. cerolyticus* had already been found by us to be identical on biochemical properties with *Staphylococcus epidermidis*. The finding of a low % GC

value (35·6–39·0) is compatible with the suggestion that this is a strain of *S. epidermidis*. As Marmur *et al.* (1963) reported, we also found a low % GC (41·3%) in the DNA of *M. cryophilus*. These results support the opinion of Mazanec, Kocur & Martinec (1966) that *M. cryophilus* should be discarded from the genus *Micrococcus*. The % GC values of *M. denitrificans*, *M. halodenitrificans* and *M. radiodurans* are lower than in most strains within the genus *Micrococcus*; these results agree with those of Marmur *et al.* (1963) and Moseley & Schein (1964). The above species were proposed by Baird-Parker (1965) for transfer to Gram-negative genera.

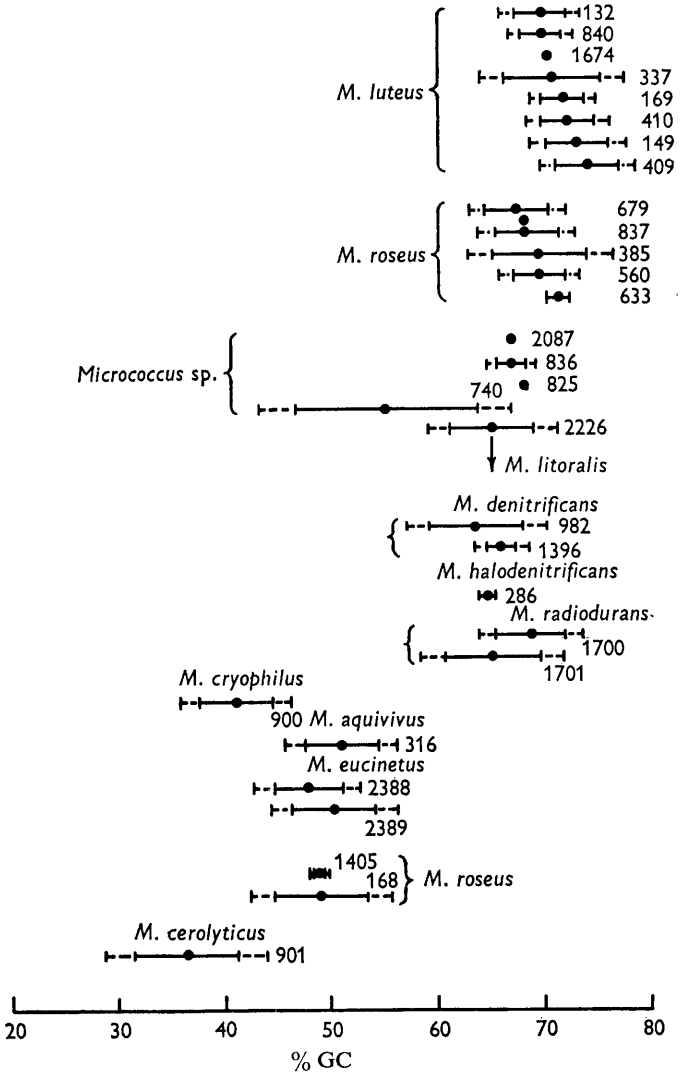

Fig. 2. Survey of the mean base composition and the exponential distribution of DNA molecules of various micrococci. The strains are designated by numbers (see Table 1). The horizontal lines are the projections of the Gaussian curves on the plane of the paper, their maxima being average % GC. The full horizontal lines represent % GC$\pm 2\sigma$, and encompass about 95% of the DNA molecules. The total length of each full line+dashed line represents % GC$\pm 3\sigma$ and encompasses about 99·7% of the DNA molecules.

The third group, comprising five strains, is of special interest from the standpoint of the % GC content in DNAs and its variability. The group includes two strains of *Micrococcus eucinetus*, two of *M. roseus* (168 and 1405), and one of *M. aquivivus*. These strains are interesting not only because they occupy an intermediate position in their % GC content (48–51 %) between the values for the genera *Staphylococcus* and *Micrococcus*, but also because they form a tight cluster around a mean value of 50% GC. Also interesting is the fact that three of these strains are marine strains, and in the case of the other two strains (*M. roseus* CCM 168 and 1405) the possibility of an original marine habitat cannot be excluded. Our results support to some extent Belser's (1964) conception of an evolutionary affinity among marine bacteria. Of course, in contrast to Belser's (1964) finding of a very tight cluster (around 41 % GC in DNA) among Gram-negative marine bacteria, our Gram-positive marine micrococci form a very tight cluster around a mean of 50% GC. On the other hand, we found (unpublished data) that some marine micrococci. e.g. *M. maripuniceus* ATCC 14399 had 73% GC similar to that of species of *Micrococcus*, but *M. euryhalis* ATCC 14389 had 33% GC in DNA, similar to the species of the genus *Staphylococcus*. Consequently, before general conclusions about the phylogeny of marine bacteria can be drawn from the above results, many more strains of these bacteria must be investigated. Since the strains of marine cocci studied by us show marked differences in their % GC content from species of *Micrococcus* it is recommended that these cocci should be discarded from the genus *Micrococcus* and a more suitable taxonomic position found for them. Komagata (personal communication) recommends that *M. eucinetus* should be placed in the genus *Planococcus* Migula in view of its motility and % GC content in DNA. In our opinion, for the same reason the strains designated as *M. aquivivus* CCM 316 and *M. roseus* CCM 168 and 1405 should also be placed in the genus *Planococcus*.

The results obtained in the present work support the correctness of the methodological principles suggested by Silvestri & Hill (1965), and also confirm the correctness of Evans's (1965) proposal to recognize only two species within the genus *Micrococcus*: *M. luteus* and *M. roseus*.

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