Classification of Micrococci on the Basis of Deoxyribonucleic Acid Homology

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SUMMARY

The DNA homology relationships of 25 micrococci (15 strains of *Micrococcus*, eight strains of *Sarcina* and two strains of *Staphylococcus*) were studied by the deoxyribonucleic acid hybridization method using nuclease S_1 , an endonuclease specific for single-stranded DNA molecules. Nineteen of the strains were classified into three groups. Group I contained *Micrococcus lysodeikticus* IAM1056, *M. luteus* IAM11010, *M. flavus* IAM12005 and IAM12006, *Sarcina flava* IAM12007 and IAM12008, *S. subflava* IAM12009, *S. lutea* ATCC381 and ATCC382, and *M. luteus* IAM11006. Group II contained *M. roseus* IAM1315, ATCC412, ATCC185 and IAM1295. Group III contained *S. lutea* IAM1099, IFO3232 and ATCC383, *M. varians* ATCC399 and *Staphylococcus lactis* ATCC15306. *Micrococcus luteus* IAM1097, *M. varians* ATCC19099 and ATCC19100, *M. conglomeratus* IAM1459 and IAM1470, and *St. aureus* IAM1011 could not be assigned to any of the three groups.

The grouping corresponds to that derived from the results of differential lysis by lysozyme, 'lytic enzyme 2' from *Cytophaga* sp., or *Streptomyces albus* G enzyme; and to types of peptidoglycan in the cell walls and genetic transformation. The usefulness of classification based on sensitivity to various lytic enzymes was demonstrated. Group I probably coincides with *M. luteus* of *Bergey's Manual of Determinative Bacteriology* (1974), and groups II and III with *M. roseus* and *M. varians* respectively.

INTRODUCTION

Since 1961, many investigators have applied various DNA hybridization methods to the elucidation of the genetic relationships between a variety of bacteria (Schildkraut, Marmur & Doty, 1961; McCarthy & Bolton, 1963; Brenner *et al.*, 1969, 1972; Okanishi, Akazawa & Umezawa, 1972; Boling, 1972; De Ley *et al.*, 1973). The DNA hybridization method using nitrocellulose membranes is common today, but this method has the disadvantage that DNA fixed on nitrocellulose membranes is released during incubation at high temperatures (Okanishi & Gregory, 1970). Recently, nuclease S_1 isolated from *Aspergillus oryzae*, which is specific for single-stranded nucleic acid (Ando, 1966), was successfully used in improving the nitrocellulose-membrane DNA hybridization method. This endonuclease digests only the single-stranded DNA portion that has not hybridized. Crosa, Brenner & Falkow (1973) studied the nucleotide sequence relatedness between homologous and heterologous chromosomal and plasmid DNA by the DNA hybridization method with nuclease S_1 and showed that this method permitted an accurate, reproducible and rapid determination of the extent of polynucleotide sequence homology. In this study, we examined the DNA homology relationships, by DNA hybridization followed by nuclease S_1 digestion, among species of the genus *Micrococcus*, including aerobic *Sarcina* strains which had been proved to belong to the genus *Micrococcus* by Kocur & Martinec (1965) and Hubalek (1969).

Table 1. *Strains used, and their lytic enzymes*

Species	Source*	Other names or numbers*	Lytic enzyme†
<i>Micrococcus lysodeikticus</i>	IAM1056	ATCC4698, <i>M. luteus</i> CCM169	1
<i>M. luteus</i>	IAM11006	CCM840, <i>M. ureae</i> ATCC408	1
<i>M. luteus</i>	IAM11010	CCM410, <i>S. lutea</i> ATCC272	1
<i>M. luteus</i>	IAM1097	ATCC398, CCM810	1
<i>M. flavus</i>	IAM12005	ATCC400, CCM210	1
<i>M. flavus</i>	IAM12006	ATCC14452	1
<i>M. varians</i>	ATCC399		3
<i>M. varians</i>	ATCC19099		1
<i>M. varians</i>	ATCC19100		1
<i>M. conglomeratus</i>	IAM1459	ATCC19101, CCM2134	1
<i>M. conglomeratus</i>	IAM1470	ATCC19102, CCM2135	1
<i>M. roseus</i>	ATCC185		2
<i>M. roseus</i>	ATCC412		2
<i>M. roseus</i>	IAM1315	CCM679, <i>M. rubens</i> ATCC186	2
<i>M. roseus</i>	IAM1295	ATCC9815	2
<i>Sarcina flava</i>	IAM12007	ATCC147	1
<i>S. flava</i>	IAM12008	ATCC540	1
<i>S. subflava</i>	IAM12009	ATCC7468, <i>M. luteus</i> CCM1048	1
<i>S. lutea</i>	IAM1099	ATCC9341	3
<i>S. lutea</i>	IFO3232		3
<i>S. lutea</i>	ATCC381		1
<i>S. lutea</i>	ATCC382		1
<i>S. lutea</i>	ATCC383		3
<i>Staphylococcus aureus</i>	IAM1011	ATCC6538p	2
<i>St. lactis</i>	ATCC15306	NCTC7564	3

* IAM, Institute of Applied Microbiology, Tokyo, Japan. ATCC, American Type Culture Collection, Maryland, U.S.A. CCM, Czechoslovak Collection of Micro-organisms, Brno, Czechoslovakia. NCTC, National Collection of Type Cultures, London, England. IFO, Institute for Fermentation, Osaka, Japan.

† 1, Lysozyme; 2, 'lytic enzyme 2'; 3, *Streptomyces albus* G lytic enzyme.

METHODS

Bacteria. The strains employed, together with the lytic enzymes used to prepare their DNA, are listed in Table 1.

DNA preparation. Bacteria were collected by centrifuging at the beginning of the stationary phase. DNA was isolated by a modified version of the method of Saito & Miura (1963). Bacteria were incubated at 37 °C in one of three ways: 1, with lysozyme (Sankyo, Tokyo, Japan) in SSC buffer (0.15 M-sodium chloride-0.015 M-sodium citrate); 2, with 'lytic enzyme 2' (Kyowa Hakko Kogyo, Tokyo, Japan; a product from *Cytophaga* sp. including *N*-acetylmuramyl-L-alanine amidase and peptidases) in 50 mM-Tris-HCl, pH 8.5; or 3, with *Streptomyces albus* G lytic enzyme (Petit, Munoz & Ghuysen, 1966; kindly provided by Professor S. Kotani, Department of Dentistry, University of Osaka, Japan) in 5 mM-citrate buffer pH 4.0, as indicated in Table 1. Lysis was completed by addition of sodium lauryl sulphate (SLS) to give a final concentration of 0.4 % (w/v). The viscous suspension obtained was incubated with 1 mg of pronase E/ml (Kaken Kagaku, Tokyo, Japan) for 4 h at 37 °C. An equal amount of phenol saturated with 0.1 M-trizma base was added and mixed for 20 min at 4 °C. The aqueous phase containing DNA was separated by centrifuging at 3000 rev./min for 10 min, and 6000 rev./min for 15 min. DNA was precipitated by adding twice the volume of cold ethanol and spooled on to a glass rod. The spooled DNA was dissolved in 0.1 × SSC and the concentration of the solution was adjusted to SSC by adding 10 × SSC. The DNA solution was incubated with 50 µg pancreatic ribonuclease/ml (Sigma)

for 60 min at 37 °C, then 50 µg of pronase E/ml was added and the solution was incubated for a further 30 min at 37 °C. The phenol extraction and ethanol precipitation procedures were repeated. Ribonuclease and pronase E treatments were also repeated in most cases. DNA concentrations were estimated spectrophotometrically, assuming an extinction of 1.0 at 260 nm to be equal to 50 µg ml⁻¹.

Labelled DNA was prepared by the same method from bacteria grown in the basal medium containing added H₃³²PO₄ (carrier free, New England Nuclear, 0.8 mCi/50 ml medium). Basal medium contained: 1 M-Tris-HCl buffer solution (pH 7.4), 10 ml; glucose, 0.4 g; neopeptone (Difco), 0.5 g; Na₂SO₄, 0.05 g; salt solution, 10 ml (stock composed of 5.4 g NaCl, 3.0 g KCl, 1.1 g NH₄Cl, 0.1 g MgCl₂, 0.01 g CaCl₂, 0.2 mg FeCl₃ and 100 ml water) and distilled water to 100 ml. Over 90 % of the isotope was incorporated into the bacteria and the radioactivity of prepared DNA was 1 × 10⁵ to 4 × 10⁵ c.p.m./µg.

DNA was sheared to between 5 × 10⁵ and 1 × 10⁵ daltons by adding NaOH to 0.5 M and incubating at 100 °C for 7 min. The molecular weight of the DNA was estimated by agarose gel electrophoresis with reference to λ-phage DNA treated with an endonuclease EcoRI (Aaij & Borst, 1972; Allet *et al.*, 1973). The DNA was then heated at 100 °C in 0.1 × SSC for 15 min and cooled rapidly in ice-water. The complete denaturation was checked by a nuclease S₁ digestion test.

DNA digestion by nuclease S₁. The reaction mixture (7.5 ml) contained: 0.1 M-sodium acetate (pH 4.5); 0.2 mM-ZnSO₄; 0.03 M-NaCl; 1600 units of nuclease S₁/ml (Seikagaku-kōgyō, Tokyo, Japan) and 20 µg of DNA/ml. After incubation at 35 °C for 40 min, 2.5 ml of 20 % (w/v) trichloroacetic acid (TCA) was added. The TCA-insoluble DNA was collected on a nitrocellulose membrane filter (Sartorius, Göttingen, Germany; catalogue no. 11306, pore size 0.45 µm), washed with cold 5 % (w/v) TCA (30 ml), and the radioactivity on the dried filter was determined with a toluene-based scintillator in a liquid scintillation spectrophotometer. One unit of nuclease S₁ is defined as the activity that produces 1 µg acid-soluble deoxypentose-P in 30 min, as determined by the diphenylamine method.

DNA hybridization. Radioactive DNA (0.1 µg, 1 × 10⁴ c.p.m.) from the reference strain, and unlabelled DNA (100 µg; denatured and sheared at 100 °C for 7 min in 0.5 M-NaOH) from various strains, were neutralized and mixed in 0.1 × SSC (0.6 ml). Sodium chloride (0.15 ml of 2.0 M) was added to give a final concentration of 0.4 M and the mixture was incubated with shaking at 75 or 85 °C for 12 to 18 h. The mixture was then treated with nuclease S₁ and the radioactivity of the hybridized DNA was determined as described previously. The values were corrected for the radioactivity resistant to nuclease S₁ digestion which appeared by self-annealing of labelled DNA. The homology value is expressed as the percentage of radioactivity in heterologous hybridized DNA relative to that in homologous hybridized DNA.

Thermal stability of the hybridized DNA. The hybridized DNA solution was diluted with distilled water to four times its volume, heated at various temperatures for 15 min, and cooled rapidly. Dissociated DNA was digested by nuclease S₁. The thermal stability of the hybridized DNA was expressed by plotting the percentage of DNA dissociated (DNA digested by nuclease S₁) against temperature.

Fig. 1

Fig. 2

Fig. 1. Kinetics of DNA digestion by nuclease S_1 . Thermally denatured or native DNA ($0.5 \mu\text{g}$ of ^{32}P -labelled *S. flava* 1AM12007 DNA supplemented with $100 \mu\text{g}$ of unlabelled calf thymus DNA) was incubated with $1600 \text{ units ml}^{-1}$ of nuclease S_1 at 35°C for various times. The DNA concentration of the reaction mixture was $20 \mu\text{g ml}^{-1}$. DNA hydrolysis was expressed as the percentage count recovered in the TCA-soluble fraction. ●, Native DNA; ○, denatured DNA.

Fig. 2. Effect of nuclease S_1 concentration on the digestion of thermally denatured and native DNA. Thermally denatured or native DNA was incubated with various concentrations of nuclease S_1 at 35°C for 15 min. Other conditions were as for Fig. 1. ●, Native DNA; ○, denatured DNA.

RESULTS

DNA digestion by nuclease S_1

Figure 1 shows the kinetics of DNA digestion by 1600 units/ml of nuclease S_1 . Hydrolysis of thermally-denatured DNA reached a maximum at 96 % after 20 min, but native DNA was not hydrolysed during the same period of time. (Hydrolysis of native DNA never exceeded 5 % in the standard experiments.) In the early stages of the reaction (1.5 min), some digestion of native DNA was observed: this phenomenon was often encountered when the NaCl concentration was below 0.1 M , but the reason for it is not known. The digestion of native DNA was also observed when nuclease S_1 was used in high concentrations (Fig. 2). At concentrations above 2000 units/ml , nuclease S_1 digested 15 % of native DNA in 15 min. Nuclease S_1 action was found to depend upon ionic strength as reported by Sutton (1971). In our case, the rate of DNA hydrolysis in the presence of 0.03 M-NaCl was 94 % of that in the absence of NaCl, and the inhibitory effect of NaCl on DNA hydrolysis increased with the NaCl concentration. The action of nuclease S_1 on denatured and native DNA was independent of DNA concentration between 0.5 and $20.1 \mu\text{g ml}^{-1}$.

DNA hybridization

The renaturation reaction of fully-denatured DNA is strongly influenced by the salt concentration and the temperature. Optimal renaturation is known to occur at about 25°C below the denaturation temperature, T_m , and with a salt concentration above 0.4 M (Marmur & Doty, 1961; Wetmur & Davidson, 1968). The *Micrococcus* and aerobic *Sarcina* DNAs that we studied had a high GC content (66 to 77 %) (Kocur, Bergan & Mortensen, 1971) and their T_m values in SSC were around 98°C . For example, the T_m of *M. lysodeikticus* was 99.5°C (Marmur & Doty, 1962). As the T_m was found to be very nearly a linear function of the logarithm of the ionic strength (Owen, Hill & Lapage, 1969), the T_m of the *M. lysodeikticus* in 0.4 M-NaCl (the concentration used in this study) was expected to be above 100°C , and it was difficult to determine it accurately. Therefore, we chose the hybridization temperatures of 75 and 85°C and compared the results.

Table 2. DNA hybridization at 75 and 85 °C

³²P-labelled DNA of *M. lysodeikticus* (0.1 µg) was incubated with unlabelled DNA of various strains (100 µg). Hybridization is expressed as a percentage relative to the homologous system at each temperature.

	Percentage of labelled DNA hybridized	
	75 °C	85 °C
<i>M. lysodeikticus</i> IAM1056	100	100
<i>M. flavus</i> IAM12006	82	84
<i>S. flava</i> IAM12007	77	81
<i>M. luteus</i> IAM11006	45	29
<i>M. luteus</i> IAM1097	10	6
<i>S. lutea</i> IFO3232	11	1
<i>S. lutea</i> IAM1099	6	2
<i>M. roseus</i> IAM1315	8	2

Table 2 compares the extent of hybridization of ³²P-labelled *M. lysodeikticus* DNA with that of various other strains at 75 and 85 °C. Hybridization was expressed as a percentage relative to the homologous system at each temperature. For strains showing high homology (> 80 %), the values were practically independent of temperature: for other strains (homology > 50 %), the values at 85 °C were lower than at 75 °C. The higher temperature tended to discriminate the DNA sequence homology more clearly but essentially the same DNA homology was observed at 75 and 85 °C. We chose 75 °C as the hybridization temperature in the following experiments. The kinetics of DNA hybridization at 75 °C is shown in Fig. 3. In this experiment, we subtracted from the count the radioactivity due to self-annealing of ³²P-labelled DNA which increased with time and reached 5 % after 21 h. When ³²P-labelled *M. lysodeikticus* DNA was hybridized with the homologous DNA, the percentage of hybridized DNA increased with time and reached a plateau at 83 % after 9 h. In subsequent experiments, the extent of hybridization of the homologous system varied from 74 to 96 %. On the other hand, when the labelled *M. lysodeikticus* DNA was hybridized with heterologous *M. luteus* IAM1097 DNA, the hybridization reached a plateau at 7 % after 4 h. In each DNA hybridization experiment, two or three samples were assayed after between 12 and 18 h of incubation to ensure that the plateau had been reached.

DNA homology and differential lysis by lytic enzymes

Table 3 shows DNA homology values of test strains with the reference strains of *M. lysodeikticus* IAM1056, *S. lutea* IAM1099 and *M. roseus* IAM1315 by DNA hybridization at 75 °C, together with the mole percentage of guanine plus cytosine (% GC) of DNAs (Kocur *et al.*, 1971), the peptidoglycan types of cell walls (Schleifer & Kandler, 1972) and the results of classification by cell-wall lytic enzymes. Repeats of most of the hybridization experiments gave essentially the same results. Nineteen strains fell into three groups on the basis of homology values. Group I contained nine strains with high homology (76 to 93 %) to *M. lysodeikticus* IAM1056, and *M. luteus* IAM11006 which had 48 % homology to this reference strain. Group II consisted of four strains with high DNA homology (80 %) to *M. roseus* IAM1315. Group III consisted of five strains which showed homologies of between 27 and 91 % to *S. lutea* IAM1099 but did not show significant homology to *M. lysodeikticus* IAM1056. *Micrococcus luteus* IAM1097, *M. varians* ATCC19099 and ATCC19100, and *M. conglomeratus* IAM1459 and IAM1470 showed low homology to all three reference strains, and were not considered to belong to any of the three groups. *Staphylococcus aureus* IAM1011 which is a

Table 3. DNA homology of the various strains to the reference strains of *M. lysodeikticus* IAM1056, *S. lutea* IAM1099 and *M. roseus* IAM1315 by DNA hybridization at 75 °C

Group	Strain	Percentage DNA hybridized to reference strains					GC (%)*	Peptidoglycan type†	Lytic enzyme
		<i>M. lysodeikticus</i> IAM1056		<i>M. roseus</i> IAM1315	<i>S. lutea</i> IAM1099				
I	<i>M. lysodeikticus</i> IAM1056	100	100	3	8	7	73.3, 72.8	A2	Lysozyme
	<i>M. luteus</i> IAM11010	79					73.7, 70.0	A2	
	<i>M. flavus</i> IAM12005	91			8		72.8	A2	
	<i>M. flavus</i> IAM12006	84			9				
	<i>S. flava</i> IAM12007	87			10				
	<i>S. flava</i> IAM12008	85			6		70.0, 68.6	A2	
	<i>S. subflava</i> IAM12009	93			7		73.4, 70.0	A2	
	<i>S. lutea</i> ATCC381		76		10		73.4, 72.8	A2	
	<i>S. lutea</i> ATCC382		79		8		72.0	A2	
	<i>M. luteus</i> IAM11006	48			7		71.0, 72.0	A2	
II	<i>M. roseus</i> IAM1315	10		100	14		69.8, 68.0	A3 α	Kyowa's 'lytic enzyme 2'
	<i>M. roseus</i> ATCC412			83	17	6	72.8, 69.0	A3 α	
	<i>M. roseus</i> ATCC185			83	13	10	71.0, 68.0	A3 α	
	<i>M. roseus</i> IAM1295			82	12		73.3, 70.0	A3 α	
III	<i>S. lutea</i> IAM1099	8		6	100	100	71.0	A3 α	<i>Streptomyces</i> <i>albus</i> G enzyme
	<i>S. lutea</i> IF03232	9			99	91			
	<i>S. lutea</i> ATCC383		9		40	36		A3 α	
	<i>M. varians</i> ATCC399		7			35		A3 α	
	<i>St. lactis</i> ATCC15306		6			27	69.3, 69.0	A3 α	
	<i>M. luteus</i> IAM1097	11		2	6		66.0, 66.3	A4 α	Lysozyme
	<i>M. varians</i> ATCC19099		13			7		A3 α	
	<i>M. varians</i> ATCC19100		9	5		6		A3 α	
	<i>M. conglomeratus</i> IAM1459	6		1	8	3	71.4, 68.0	A4 γ	
	<i>M. conglomeratus</i> IAM1470	8		2	7	2	72.0, 69.0	A4 γ	
	<i>St. aureus</i> IAM1011	4			5		37.7, 35.7	Ao	'Lytic enzyme 2'

* Results from Kocur *et al.* (1971).† Results from Schleifer & Kandler (1972). Peptidoglycan types were: A2, L-Lys-D-Ala-L-Lys-D-Glu (Gly)-L-Ala; A3 α , L-Lys-L-Ala₃₋₄; A4 α , L-Lys-Gly-L-Glu; A4 γ , *m*-Dpm-D-Glu₂; Ao, L-Lys-Gly₅. (*meso*-Diaminopimelic acid: *m*-Dpm).

typical member of Staphylococcaceae did not show significant homology to *M. lysodeikticus* IAM1056 or *S. lutea* IAM1099.

The reproducibility of the homology values obtained by this method was tested by duplicate runs of experiments using *S. lutea* IAM1099 as reference strain (Table 3). Another check was carried out by changing the reference strain reciprocally, i.e. *S. flava* IAM12007 DNA was labelled and renatured with unlabelled *M. lysodeikticus* and *S. lutea* IF03232 DNAs. The homology values obtained were 87 and 12 % respectively, showing good agreement with the 87 and 9 % obtained when *M. lysodeikticus* was used as the reference strain (Table 3).

Thermal dissociation of hybridized DNA

Laird, McConaughy & McCarthy (1969) showed that the T_m of heteroduplex DNA decreased with the increase in mispaired bases. To examine the extent of mispairing in hybridized DNA, we studied the thermal dissociation profiles of hybridized DNA. Figure 4 shows thermal dissociation profiles in 0.1 M-NaCl of a homoduplex of *M. lysodeikticus*

Fig. 3

Fig. 4

Fig. 3. Kinetics of the hybridization of homologous and heterologous DNA at 75 °C. ^{32}P -labelled DNA of *M. lysodeikticus* IAM1056 (0.1 μg) was incubated with 100 μg of *M. lysodeikticus* IAM1056 (homologous) or *M. luteus* IAM1097 (heterologous) at 75 °C for various times. After hybridization, the unhybridized DNA was digested by nuclease S_1 . Hybridized DNA is expressed as the percentage radioactivity recovered in the TCA-insoluble fraction. ●, Heterologous DNA; ○, homologous DNA.

Fig. 4. Thermal dissociation profile of hybridized DNA. After ^{32}P -labelled DNA of *M. lysodeikticus* IAM1056 (0.1 μg) was incubated with 100 μg of unlabelled *M. lysodeikticus* IAM1056 DNA (homologous) or *S. flava* IAM12007 DNA (heterologous) at 75 °C for 15 h, the hybrid DNA solution was diluted with three volumes of distilled water (0.1 M with respect to NaCl), heated at various temperatures for 15 min and cooled rapidly followed by the addition of nuclease S_1 . Dissociated DNA is expressed as the percentage radioactivity recovered in the TCA-soluble fraction. ●, Heterologous DNA; ○, homologous DNA.

IAM1056 DNA and a heteroduplex of *M. lysodeikticus* IAM1056 DNA with *S. flava* IAM12007 DNA after DNA hybridization at 75 °C. The T_m of *M. lysodeikticus* IAM1056 homoduplex was 97 °C. This agreed with the T_m value of 95 °C reported for DNA from the same strain of *M. lysodeikticus* under similar conditions (Owen *et al.*, 1969), which suggested that pairing was complete in the hybridized homoduplex. On the other hand, the T_m of the heteroduplex with *S. flava* IAM12007 was 93.5 °C, which was 3.5 °C lower than that of the homoduplex. Anderson & Ordal (1972) reported that ΔT_m , the decrease in T_m of a heteroduplex, increased linearly with the decrease in percentage of DNA homology. *Sarcina flava* IAM12007 showed 87 % homology to *M. lysodeikticus* IAM1056. Our results agreed with the relationship given by Anderson & Ordal (1972), i.e. 4.25 % decrease in homology per degree C of ΔT_m .

DISCUSSION

We have classified 25 strains of Micrococcus, Sarcina and Staphylococcus: 19 fell into three groups (groups I, II, III) but six strains did not belong to any of these groups (Table 3). Our classification agrees with the grouping by types of peptidoglycan in bacterial cell walls (Schleifer & Kandler, 1972). All bacterial strains of group I have A2 type peptidoglycan and were lysed with lysozyme. Group II and group III strains have the A3 α type peptidoglycan. Group II strains were lysed with 'lytic enzyme 2' but not with lysozyme, while group III strains were lysed with *Streptomyces albus* G lytic enzyme but not with lysozyme or 'lytic enzyme 2'. The differential action of 'lytic enzyme 2' on group II and group III strains suggests the existence of some difference in cell-wall constitution between the two groups. From these results, it seems that bacterial strains of genetically related groups have similar cell-wall structures. It should be noted, however, that five strains which did not belong to

any of the three groups were lysed with lysozyme, and *M. varians* ATCC19099 and ATCC19100 having A₃ type peptidoglycan did not belong to group II or group III.

Genetic homology among various bacterial strains can be estimated by genetic transformation, which is known to parallel the results obtained with DNA hybridization. Kloos (1969) found that *M. lysodeikticus* ATCC4698 (IAM1056), *M. flavus* ATCC400 (IAM12005), *S. flava* ATCC540 (IAM12008), *S. lutea* ATCC272 (*M. luteus* IAM11010), *S. lutea* ATCC381, *S. lutea* ATCC382 and *S. subflava* ATCC7468 (IAM12009) among many others were able to transform adenine, histidine and tryptophan auxotrophs of *M. lysodeikticus* strain ISU to prototrophy at relatively high frequency, while *M. roseus* ATCC412 and ATCC9815 (IAM1295), *M. rubens* ATCC186 (*M. roseus* IAM1315), *M. varians* ATCC399, *M. luteus* ATCC398 (IAM1097) and others failed to transform them. Taking Kloos' and our results together, it is clear that all strains belonging to our group I had high transforming ability toward *M. lysodeikticus*, while the strains not belonging to group I invariably failed to transform. Thus the results of transformation support our grouping by DNA homology.

In *Bergey's Manual of Determinative Bacteriology* (1974) the genus *Micrococcus* is divided into three species—*M. luteus*, *M. roseus* and *M. varians*—by physiological and biochemical characteristics and the GC content of DNA. This classification essentially agrees with our grouping on the basis of DNA homology, i.e. our group I corresponds to *M. luteus*, group II to *M. roseus* and group III to *M. varians*. The reference strains of group I and II are the neotype strains of *M. luteus* and *M. roseus*. As Table 3 shows, the homology among group II strains is very high (> 80 %), while group III consists of two types of strains, one showing over 90 % DNA homology and the other showing between 27 and 36 % homology to *S. lutea* IAM1099. Furthermore, *M. luteus* IAM11006 in our group I shows an exceptionally low DNA homology value compared with other strains of group I. Further investigation might justify division of groups I and III.

Five strains of *Micrococcus* could not be assigned to any of the three groups on account of their low DNA homology to the reference strains used. *Micrococcus luteus* IAM1097 was not considered to belong to group I because of its low GC content (66 %); different peptidoglycan type (A₄) and menaquinone pattern (Jeffries *et al.*, 1965); and other characteristics (Kloos, 1969), which is in agreement with our results. *Micrococcus varians* ATCC19099 and ATCC19100, and *M. conglomeratus* IAM1459 and IAM1470 were isolated from petroleum brines in Japan by Iizuka & Komagata (1965), and the latter two strains have a peptidoglycan type (A₄) which is not found among the strains of the three groups. Possibly these five strains belong either to some groups other than the three groups of *Micrococcus* or to some genus or genera other than *Micrococcus*. Any conclusion, however, must await further investigation.

This study also confirms the usefulness of the hybridization method using nuclease S₁ in estimating DNA homology among various bacterial strains, as claimed earlier by Crosa *et al.* (1973). The method is convenient for treating many samples and gives fairly reproducible results even if the labelled reference DNA and unlabelled DNA are exchanged. This is probably due to the endonucleolytic nature of nuclease S₁, which removes the unpaired portion of DNA very precisely, even distinguishing nicks on T5 bacteriophage DNA (Shishido & Ando, 1975).

Another feature of this study is the evaluation of cell-wall lytic enzymes as an accurate, reproducible and rapid method for classification. The three groups determined by the DNA hybridization procedure coincide fairly well with the results of a simple cell lysis test by three types of cell-wall lytic enzymes. The test by lytic enzymes sometimes gives closer classification than that by the peptidoglycan types studied by Schleifer & Kandler (1972).

More detailed taxonomic studies will be made possible by introducing other lytic enzymes.

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