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Use of Antibody to Membrane Adenosine Triphosphatase in the Study of Bacterial Relationships[1]

THERESA L. WHITESIDE, AUGUST J. DE SIERVO,[2] AND MILTON R. J. SALTON
Department of Microbiology, New York University, School of Medicine, New York, New York 10016

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An antiserum to Ca^{2+}-activated adenosine triphosphatase from membranes of *Micrococcus lysodeikticus* cross-reacted in agar gels with membrane adenosine triphosphatases from other pigmented micrococci and related species. Species of *Micrococcus* and *Sarcina* showed different levels of inhibition of adenosine triphosphatase activities in heterologous reactions with antiserum. Inter- and intraspecific relationships based on the inhibition reaction were compared with an independent parameter, namely the quantitative and qualitative composition of the bacterial membrane phospholipids and fatty acids. The guanine plus cytosine contents in the deoxyribonucleic acid of the species studied correlated well with the serological cross-reactivity of adenosine triphosphatases from their membranes. The types of cross-bridges found in the peptidoglycans of these cocci were also compared with the other properties. The results suggest that an antiserum specific for a major membrane protein may be a reliable and most useful adjunct in studying bacterial serotaxonomy.

The value of serological cross-reactivity in studies of bacterial relationships has been well recognized. Its usefulness seems to have been largely limited, however, to intraspecific differentiations, such as the typing of gram-positive pathogenic cocci. Comparative bacterial serology relied almost exclusively on antigenic characteristics of cell surface components, such as capsules, walls, and flagella. Relatively few bacterial proteins, especially those characterized as enzymes, were studied from the comparative immunological view point (21, 25, 31). The elegant work of Stanier et al. (25) on the immunological properties of two inducible enzymes in the β-ketoadipate pathway showed clearly that enzymes can be of great value as antigens for serotaxonomic purposes.

Proteins of the bacterial membrane are, in general, not readily accessible because of their insolubility as lipid-protein complexes. They are more difficult to isolate and purify, and thus do not lend themselves easily to comparative serological study. Nevertheless, once resolved and characterized they could be potential aids in the serological classification of microorganisms. Indeed, the study of the antigenic properties of the membrane-bound proteins of *Mycoplasma* proved to be of diagnostic value for this group (2). We recently reported details of the preparation and properties of an antiserum specific for Ca^{2+}-dependent adenosine triphosphatase from membranes of *Micrococcus lysodeikticus* (30). This antiserum enabled us to compare the immunological properties of membrane adenosine triphosphatases from different micrococci and sarcinae. The validity of this serological relationship was further explored by comparing adenosine triphosphatase cross-reactivity with the base compositions and other chemical characteristics of the species studied. Several microorganisms of uncertain taxonomic status have also been included in this study.

MATERIALS AND METHODS

Cultures and growth conditions. Table 1 lists the cultures used and the sources from which they were obtained. All of the cultures were grown in the PWYE broth (5% peptone, 0.1% yeast extract, 0.5% NaCl) under aerobic conditions at 30 C, unless otherwise specified. *M. roseus* R27 was grown in Neopeptone broth (Difco). PWYE broth was supplemented with 0.5% glycerol to grow *Bacillus subtilis* and with 1% glucose to grow the *Micrococcus* sp. The anaerobic organisms,

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[2] Present address: Department of Microbiology, University of Maine, Orono, Me. 04473.

TABLE 1. *List of the microorganisms used, sources, and strain designations*

Name of organism	Strain no. and source
Micrococcus lysodeikticus ...	NCTC 2665
M. tetragenus	170 (from V. Skerman)
Sarcina flava	NCTC 7495
S. lutea	Laboratory strain
M. roseus	NCTC 7520
M. roseus	R 27
M. varians	NCTC 7281
Micrococcus sp.....	From O. Kandler
M. conglomeratus	NCTC 2677
M. caseolyticus	116 (from V. Skerman)
M. rhodochrous	NCTC 7510
Corynebacterium coelicolor ..	From H. L. Jensen
Sporosarcina ureae	CCM 981
Sarcina maxima	11 (from E. Canale-Parola)
S. vertriculi	AL 2 (from E. Canale-Parola)
Bacillus subtilis	42 (from L. Mindich)

Sarcina ventriculi and *S. maxima*, were grown and maintained as described by Holt and Canale-Parola (13).

Preparation of membrane adenosine triphosphatase. The selective release method described by Muñoz et al. (20) was used for isolation of adenosine triphosphatases from membranes of those bacteria which were sensitive to lysozyme. Bacteria not lysed by lysozyme were broken by sonic disintegration with glass beads (0.22 mm; Minnesota Mining and Manufacturing Co., Minneapolis, Minn.) or, as in the case of the anaerobic *Sarcina* species, in a Hughes press. Membrane adenosine triphosphatase was released into the cytoplasm during sonic oscillation and was then separated from cell debris and walls by differential centrifugation (30,000 × *g*, 30 min). The cytoplasmic fraction was precipitated with ammonium sulfate (enzymatic grade, Mann Research Corp.), and the adenosine triphosphatase-rich fraction was obtained at 30 to 50% saturation. Some cross-reacting adenosine triphosphatases were purified further by gel filtration on Sephadex G200 (20). Crude or purified adenosine triphosphatases were tested for cross-reactivity with the antiserum to *M. lysodeikticus* adenosine triphosphatase by the double-diffusion method in agar (8, 30).

Preparation of antiserum. Antiserum to isolated, purified Ca^{2+}-dependent adenosine triphosphatase was prepared in rabbits as described earlier (30). The gammaglobulin fraction was obtained by means of several successive ammonium sulfate precipitations (8).

Immunodiffusion and immunoelectrophoresis. Double diffusion in agar gels and electrophoresis of the enzymes were performed as described earlier (30).

Inhibition experiments. Purified or crude adenosine triphosphatases from different membranes were used in the inhibition experiments. The levels of inhibition were not dependent on the purity of adenosine triphosphatases used, provided the antibody was in excess. In all cases, preliminary titration curves were run to determine the smallest amount of antibody needed for maximal inhibition. A constant volume (50 μliters) of undiluted, *M. lysodeikticus* anti-adenosine triphosphatase (gammaglobulin fraction) was added to a constant volume (100 μliters) of appropriately diluted enzyme preparation. The mixture was incubated for 30 min at 25 C and immediately after addition of the substrate (ATP) and $CaCl_2$, the adenosine triphosphatase activity was assayed exactly as described by Muñoz et al. (20). To determine control levels of enzymatic activity (i.e., no antibody present), samples of each enzyme solution were incubated with a diluent [0.03 M tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride buffer, *p*H 7.5] instead of the antibody. As a further control measure, samples of each enzyme solution were incubated with normal rabbit gamma globulin at concentrations equivalent to those of the antibody (30).

Phospholipid determination. Cells were grown in sidearm flasks in PWYE medium containing ^{32}P (2.5×10^5 counts per min per ml). The turbidity of the cultures was followed, and cells were harvested in the stationary phase, washed twice with 0.05 M Tris-hydrochloride buffer (*p*H 7.5) and resuspended in a constant volume of the same buffer. Samples were then taken for dry-weight determinations. The remainder of the suspension was centrifuged to obtain a well packed pellet of cells. Lipids were extracted quantitatively with chloroform-methanol (1:2, v/v) by the method of Bligh and Dyer (5). The lipid extracts were dissolved in chloroform and stored at −70 C. Polar lipids were separated on silica gel-loaded paper (Whatman SG81) by the procedures described by Marinetti (18) with chloroform-methanol-diisobutyl ketone-acetic acid-water (45:15:30:20:4, v/v) as the solvent system (32). Major phospholipids of *M. lysodeikticus* were deacylated as described by White and Frerman (29) and identified by comparative chromatography with authentic standards. Tentative identifications of other lipids were made from R_F data, staining with Rhodamine 6G, and tests for free amino groups and vicinal hydroxyl groups as described by Kates (14).

Autoradiograms were made from Rhodamine-stained chromatograms with Dupont Cronex 4 X-ray film. To quantitate the ^{32}P-labeled phospholipids, the spots were cut out and counted in a gas-flow counter.

Total phosphorus was determined by the method described by Ames and Dubin (1). Glycerol phosphate (Sigma Chemical Co., St. Louis, Mo.) was used as a standard. The weight of phospholipids was estimated by assuming an average molecular weight of 700 g/mole of P.

Fatty acid analysis. Lipids extracted from the stationary-phase cells were deacylated in methanolic 0.2 M KOH (29). The chloroform phase containing the fatty acids was removed and evaporated under a stream of N_2. In the case of *M. rhodochrous* and *Corynebacterium coelicolor*, whole cells were saponified as described by Lanéelle et al. (16). Fatty acids were methylated with diazomethane. The crude fatty acid methyl esters were purified by preparative thin-layer chromatography on silica gel plates in petroleum ether-ether (8:2, v/v). The purified methyl esters, located with Rhodamine B, were eluted from silica gel with ether. Fatty acid methyl esters were identified by gas chromatography in an F & M model 400 gas chromatograph fitted with a hydrogen-flame ionizing detector. The nonpolar column [copper, 0.25 inch (~0.64 cm) diameter, 6 ft (~1.83 m) length] was packed with 10% silicone GE SE 30 on 60 to 80 mesh Chromosorb W (Applied Science Laboratories, Inc., State College, Pa.). Temperature was pro-

grammed at approximately 7.5 C per min from 180 to 250 C and held isothermally. Methyl esters from bacterial lipids were identified by co-chromatography with standards and by comparing their retention values with those of the standards (Supelco Inc., Bellefonte, Pa.). Fatty acids were quantitated from the peak areas obtained by multiplying the peak heights by the widths at half peak heights.

RESULTS

Serological reactions of ATPases. Figure 1 shows cross-reactions of several bacterial adenosine triphosphatases with the antiserum to *M. lysodeikticus* adenosine triphosphatase. *M. tetragenus*, *S. flava*, and *S. lutea* all gave lines of complete identity with no spur developing even after prolonged incubation of the slides. Adenosine triphosphatases from two strains of *M. roseus*, *M. conglomeratus*, and *C. coelicolor* (*not shown*) exhibited only partial cross-reaction, as indicated by the presence of distinct spurs. In the case of *M. varians*, the precipitin lines were weak, but again spurs were evident. However, adenosine triphosphatases from membranes of *M. caseolyticus*, *M. rhodochrous*, *S. ureae*, and *B. subtilis* showed no reaction at all with the *M. lysodeikticus* anti-adenosine triphosphatase.

When the enzymes from membranes of cross-reacting species were purified, electrophoresed in agar gels, and reacted with the antiserum to *M. lysodeikticus* adenosine triphosphatase, they all gave a characteristic single, anodal band (Fig. 2). Electrophoretic mobilities of *S. flava* and *S. lutea* adenosine triphosphatases were identical to that of *M. lysodeikticus* adenosine triphosphatase, whereas the weakly and partially cross-reacting adenosine triphosphatase from *M. varians* exhibited a slightly different electrophoretic mobility.

One simple and precise method for immunological comparison of enzymes is based on measurements of inhibition of catalytic activity by the specific antibody (31). In our case, cross-reactivity of different membrane adenosine triphosphatases with the antiserum to *M. lysodeikticus* adenosine triphosphatase could be measured quantitatively because the antiserum inhibited their enzymatic activities to different extents. Table 2 gives values of the maximal levels of inhibition of different membrane adenosine triphosphatases by excess antiserum. It is apparent that membrane adenosine triphosphatases of pigmented, strongly cross-reacting cocci, closely related to *M. lysodeikticus*, are inhibited almost as effectively as *M. lysodeikticus* adenosine triphosphatase. The antibody is less effective on the weakly cross-reacting adenosine triphosphatases, such as those of *M. varians* and *M. conglomeratus*. Adenosine triphosphatases of the "taxonomically controversial" *M. rhodochrous* and *S. ureae*, and adenosine triphosphatase of the unrelated *B. subtilis* showed no inhibition by the antibody. Such a result is in agreement with their greater taxonomic distance from *M. lysodeikticus*. It appeared that the closer the relationship between the different cocci, the greater was the structural similarity of their membrane adenosine triphosphatases as determined serologically. No adenosine triphosphatase activity could be detected in the cytoplasm or in the membrane wall fractions of the anaerobic sarcinae. The assay was performed over a wide range of *p*H and in the presence of different cations. It is evident that further investigations would be needed with these species.

Phospholipid and fatty acid composition. Attempts were then made to determine whether the relationships based on the inhibition reaction would correspond to those based on an entirely different characteristic of the bacterial membrane, namely quantitative and qualitative composition of its phospholipids. Phospholipids, carotenoids, and menaquinones were shown to reside exclusively in the membranes of gram-positive bacteria (27, 23). Lipids from the stationary-phase cells grown in the presence of ^{32}P were extracted with chloroform-methanol and then subjected to autoradiography and quantitative analysis of the phospholipids. Tables 3 and 4 summarize quantitative differences among the major phospholipids from membranes of the organisms studied. All of the four yellow-pigmented cocci, listed first in Table 4, are quite similar with respect to the quantitative composition of their lipids. All four gave precipitin lines of identity when their membrane adenosine triphosphatases were reacted against antibody in the agar diffusion test. *S. lutea* differed from the others in that it had a low cardiolipin content. Its adenosine triphosphatase was inhibited the least of the four by the antibody. Although the quantitative phospholipid composition of other cocci varied from species to species, these quantitative differences were not as striking, in our view, as the qualitative differences revealed in the patterns of membrane phospholipid composition.

The four most strongly cross-reacting micrococci (*M. lysodeikticus*, *S. flava*, *S. lutea*, and *M. tetragenus*) gave almost identical chromatographic patterns of phospholipids (Fig. 3). Three major phospholipids —cardiolipin, phosphatidyl glycerol, and phosphatidyl inositol—and one glycolipid were detected in all. *M. roseus*, which gave strong partial cross-reaction lacked phosphatidyl inositol. (The two strains used here had identical chromatographic patterns.) On the other hand, in the weakly cross-reacting *M. varians*, in addition to the absence of phosphatidyl inositol,

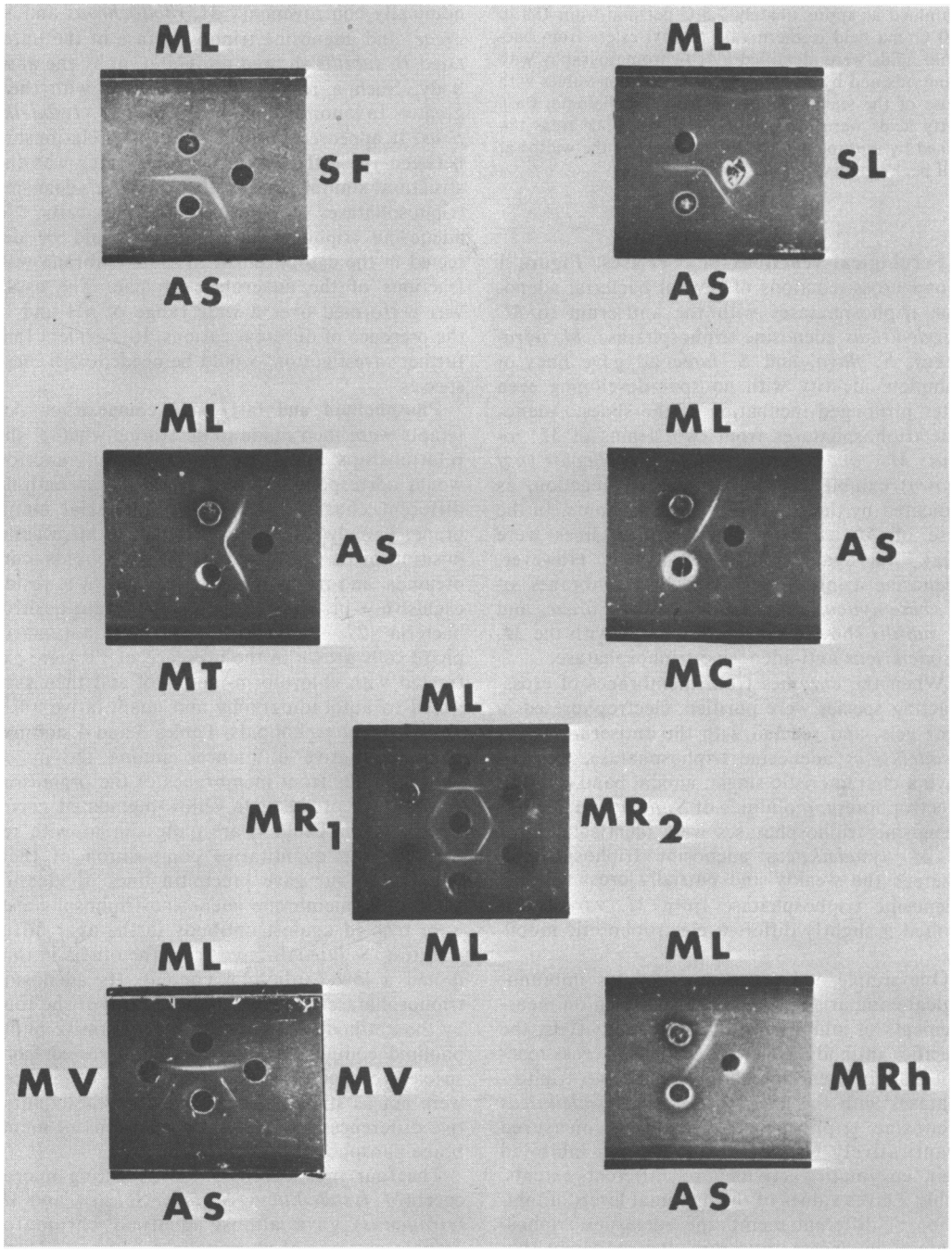

FIG. 1. *Reaction of adenosine triphosphatases from membranes of various pigmented micrococci with the antiserum to M. lysodeikticus adenosine triphosphatase. The anti-adenosine triphosphatase serum (AS) was reacted in double-diffusion agar plates with partially purified adenosine triphosphatase from membranes of S. flava (SF), S. lutea (SL), M. tetragenus (MT), M. conglomeratus (MC), two strains of M. roseus (MR_1 and MR_2), M. varians (MV), M. rhodochrous (MRh), and also with membrane adenosine triphosphatase from M. lysodeikticus (ML) as the homologous control.*

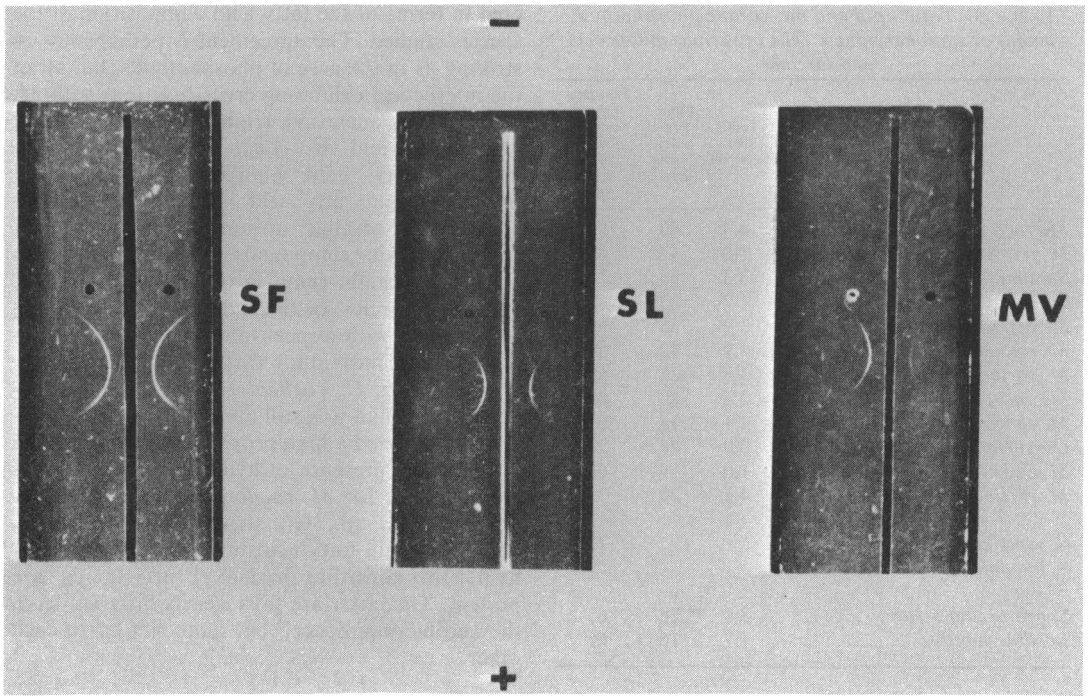

FIG. 2. *Immunoelectrophoresis of purified adenosine triphosphatases from membranes of S. flava (SF), S. lutea (SL), and M. varians (MV). Adenosine triphosphatase from membranes of M. lysodeikticus was placed in each left well and electrophoresed to provide a standard for the homologous system. The antiserum to M. lysodeikticus adenosine triphosphatase was placed in the troughs.*

TABLE 2. *Inhibition of adenosine triphosphatases from membranes of different gram-positive cocci by the antiserum to Micrococcus lysodeikticus adenosine triphosphatase*

Microorganism	Inhibition by excess antibody (%)
M. lysodeikticus	100
M. tetragenus	89
Sarcina flava	87
M. roseus	84
M. roseus R 27	79
S. lutea	75
M. conglomeratus	36
M. varians	35
Corynebacterium coelicolor	30
M. rhodochrous	0
M. caseolyticus	0
Sporosarcina ureae	0
Bacillus subtilis	0

we detected a phospholipid (X_1) tentatively identified as phosphatidic acid, and observed a low cardiolipin content and an unidentified polar lipid (X_2). The weakly cross-reacting *M. conglomeratus* also lacked phosphatidyl inositol and had two other characteristic, minor phospholipid components, one of which seemed to be aminoacyl phosphatidyl glycerol. *C. coelicolor* was remarkably similar to *M. lysodeikticus* in its qualitative phospholipid composition, but differed in that it showed a great abundance of glycolipids. Finally, of the cocci which did not cross-react, *M. caseolyticus* was quite conspicuous as the one species which possessed large quantities of aminoacyl phosphatidyl glycerol. *M. rhodochrous* and the two anaerobic sarcinae contained two unidentified phospholipids (X_3 and X_4), which gave positive reactions for vicinal hydroxyl groups. The anaerobic sarcinae also contained large amounts of phosphorus-free, unidentified polar lipids. *S. ureae* and *B. subtilis* stand apart from the others because of the presence of the ninhydrin-positive phosphatidyl ethanolamine.

In general, there appeared to be good agreement between the serological cross-reactivity among the cocci and the nature of the major phospholipids of their membranes. Thus, on the basis of the results presented here, we can suggest that there are likely to be greater differences in the qualitative phospholipid composition of the membranes as intra- or interspecific relationships become more distant.

Table 5 shows that a similar general correlation exists if interspecific relationships are consid-

TABLE 3. *Total lipid and membrane phospholipid content of stationary-phase cells of various micrococci and sarcinae*

Organism	Total lipid, % (w/w)	Lipid P/g of dry cells (μmoles)	Phospholipid content (as per cent of total lipid)
Micrococcus lysodeikticus	4.3	50	82
M. tetragenus	1.2	12	67
Sarcina flava	3.3	36	77
S. lutea	2.7	23	60
M. roseus	3.7	35	66
M. roseus R 27	4.9	42	59
M. varians	3.3	23	59
Micrococcus sp.	2.3	20	62
M. conglomeratus	2.1	18	61
Corynebacterium coelicolor	10.0	28	20
M. caseolyticus	1.0	9	64
M. rhodochrous	4.1	20	35
S. ventriculi			22
S. maxima			9
Sporosarcina ureae	3.1	28	63
Bacillus subtilis	1.7	16	66

ered in terms of the fatty acid composition of the species studied. The agreement is perhaps not as striking as in the case of phospholipids, but all of the micrococci exhibiting cross-reactions with *M. lysodeikticus* adenosine triphosphatase, including *M. varians* and *M. conglomeratus*, have very similar fatty acid compositions. The C_{15} branched-chain fatty acid is the major component, with smaller amounts of C_{16} or C_{17} branched-chain compounds, or both, and with some C_{16} straight-chain fatty acids also present. In other species distinct differences were observed, both with respect to the kind and the proportions of individual fatty acids. In weakly cross-reacting *C. coelicolor*, the C_{15} branched-chain compound was still predominant. *M. caseoliticus* contained a high proportion of unsaturated C_{18}, and the presence of hydroxy fatty acids was characteristic for *M. rhodochrous*. In the anaerobic *Sarcina* sp., C_{16} straight-chain and C_{19} branched-chain fatty acids are the major components; in addition, hydroxy fatty acids are present. The latter are thus clearly different from the aerobic micrococci, but quite similar to each other.

TABLE 4. *Composition of lipids from stationary-phase cells of various micrococci and sarcinae*

Organism	Relative mole per cent composition of phospholipids[a]									Non-P lipids		
	CL	X_1	PG	X_3[b]	PI	AAPG	X_4[b]	X_5	PE[c]	GL_1	GL_2	X_2
R_F ranges	.73–.87	.63–.72	.43–.63	.20–.34	.22	.19	.13–.26	.08	.70	.78	.66	.33
Micrococcus lysodeikticus	38.8	2.4	46.1		12.7					±	+	
M. tetragenus	31.0	1.3	58.8		8.9						+	
Sarcina flava	36.5		55.6		7.9						+	
S. lutea	1.1		89.6		9.3					+	+	
M. roseus	25.5		74.5								+	
M. roseus R 27	34.8		64.6								+	
M. varians	9.6	2.3	88.1									+
Micrococcus sp.	31.0	5.0	62.7									+
M. conglomeratus	27.4	1.2	69.5			1.3					+	
Corynebacterium coelicolor	59.8		24.3		15.6					++	++	
M. caseolyticus	10.0	1.7	52.5			35.8						
M. rhodochrous	2.0		61.7	24.8			11.5					
S. ventriculi	14.5	7.9	31.4	16.5			29.7				±	
S. maxima	3.0	6.5	32.2	40.7			17.6				±	
Sporosarcina ureae	27.9		72.1						+			
Bacillus subtilis	3.6	1.8	77.8			16.8			+			

[a] To calculate relative mole per cent compositions, we assumed 2 moles of P/mole for cardiolipin and 1 mole of P/mole for other phospholipids. Abbreviations used are: CL, cardiolipin (diphosphatidyl glycerol); PG, phosphatidyl glycerol; PI, phosphatidyl inositol; AAPG, aminoacyl phosphatidyl glycerol; GL, glycolipid; PE, phosphatidyl ethanolamine; X, unidentified compounds. Symbols used: +, present; ++, present in large amounts; ±, appear to be present in small amounts.

[b] These unidentified phospholipids were positive for vicinal hydroxyl groups. Components within the given R_F range may not necessarily be identical.

[c] Detected by ninhydrin and autoradiography. Since the small amount of phosphatidyl ethanolamine co-chromatographed in this system with phosphatidyl glycerol, it was not quantitated separately.

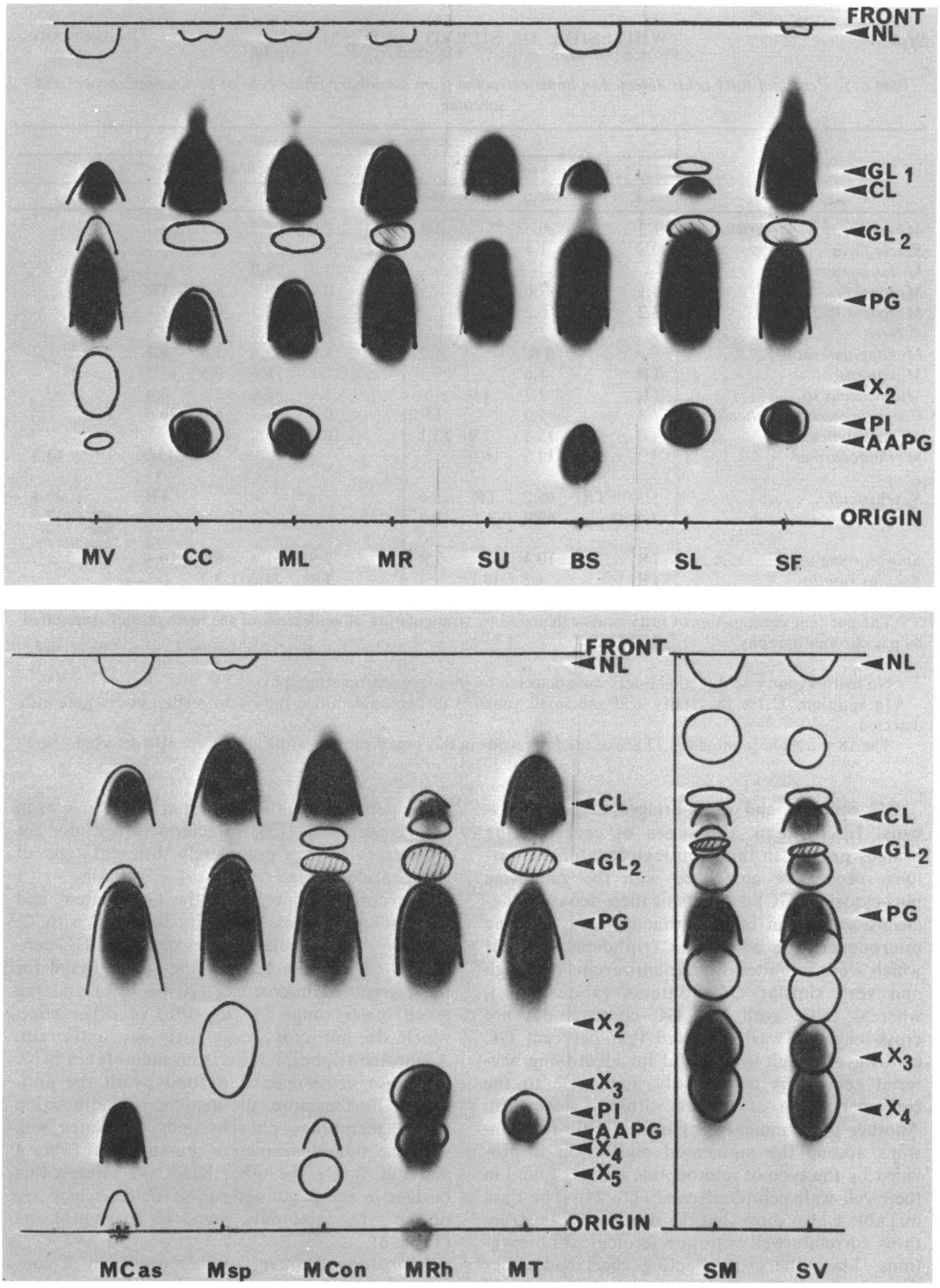

FIG. 3. *Autoradiograms of* ^{32}*P-labeled phospholipids extracted from various Micrococcus and Sarcina species. Chromatograms were developed with Rhodamine 6G to identify unlabeled lipids (marked as open or hatched spots). Lipid extracts of the following microorganisms were chromatographed: M. varians (MV), C. coelicolor (CC), M. lysodeikticus (ML), M. roseus (MR), S. ureae (SU), B. subtilis (BS), S. lutea (SL), S. flava (SF), M. caseolyticus (MCas), Micrococcus sp. (Msp), M. conglomeratus (Mcon), M. rhodochrous (MRh), M. tetragenus (MT), S. maxima (SM), and S. ventriculi (SV). R_F values and other abbreviations are given in Table 4. A minor, unknown component (referred to as X_1 in Table 4) occurring just above phosphatidyl glycerol (MCas, Msp, and MV) was tentatively identified as phosphatidic acid.*

TABLE 5. *Principal fatty acids detected in lipids extracted from stationary-phase cells of various micrococci and sarcinae*

Organism	Per cent composition of fatty acids[a]											
	Saturated straight chain					Branched chain						
	14:0	15:0	16:0	17:0	18:0	13:0	14:0	15:0	16:0	17:0	18:0	19:0
Micrococcus lysodeikticus ...	1.3		0.5		TR[b]	2.4	2.0	91.3	0.7	1.7	TR	
Sarcic flava	0.8		1.4			3.2	6.8	89.0				
M. tetragenus						3.3	3.6	93.0				
M. roseus	3.1		7.0				0.9	80.8	8.2	TR		
M. roseus R 27	3.2		6.2				3.0	79.5	6.2	1.8		
S. lutea							1.9	88.0	5.1	2.8		
M. conglomeratus			TR		1.2		1.6	83.4	5.6	8.2		1.2
M. varians	TR		1.6				1.1	76.6	9.5	11.2		
Micrococcus sp	TR		2.7	4.0			1.7	80.6	4.6	6.4		
Corynebacterium coelicolor[c] .	1.8		7.0		TR		0.6	57.7	5.9	26.6	TR	
M. caseolyticus[d]	4.0	2.8	15.5	2.9	13.1		10.7	3.2	13.3			
M. rhodochrous[e]		12.5	19.5	16.0						15.6		13.5
S. ventriculi	5.3	TR	65.2	TR	5.6					TR		20.1
S. maxima	3.4		69.0		10.0						TR	17.5
Sporosarcina ureae	TR		10.4		TR		2.9	61.4	8.7	16.4	TR	
Bacillus subtilis	TR		4.2	18.2			TR	74.3	3.2			

[a] The per cent composition of fatty acids calculated by triangulation of peak areas of the methyl esters separated by gas chromatography.

[b] Trace.

[c] No hydroxy fatty acid methyl esters were detected by thin-layer chromatography.

[d] In addition, 27.7% 18:1 fatty acid and small amounts of two unidentified fatty acid methyl esters were also detected.

[e] The 18:1 acid accounted for 22.8% of the fatty acids in this organism. Nocardic acids were also detected.

GC contents and cross-bridges in peptidoglycans. In Table 6, the types of cross-linking bridges present in the peptidoglycans of the various species are compared with the guanosine plus cytosine (GC) contents of their deoxyribonucleic acid. It can be seen that all cross-reacting micrococci, the adenosine triphosphatases of which were inhibited by the antiserum, had high and very similar GC contents (about 70%), whereas those with low GC contents did not cross-react. It was suggested that per cent GC content, although very useful for identifying bacterial genera, is not equally applicable to the characterization of species within a genus (3). Another good indicator of interspecific relationships among the pigmented micrococci is provided by the type of interpeptide bridges found in their cell-wall peptidoglycans (11, 24). The data in Table 6 also show that the peptidoglycan structures correlate well with our serological observations. Thus, the cross-reacting cocci had either the *M. lysodeikticus*, "head to tail" type of bridges or the L-Lys-L-Ala_3 bridges in their peptidoglycans (11). Weakly cross-reacting *M. varians* has a different cross-bridge, which contains *m*-diaminopimelic acid. However, its peptidoglycan has the α-carboxyl groups of the D-glutamic acid in the peptide subunit substituted by glycine, as in *M. lysodeikticus* (24). Bacteria which do not cross-react have a completely different type of interpeptide linkage.

The correlation between the GC content and the serological cross-reactivity observed with *C. coelicolor* is particularly noteworthy. Its GC content of 65.3% falls between the values found for the pigmented micrococci (70 to 73%) and the much lower range (30 to 40%) of other cocci which do not cross-react with our antiserum. Adenosine triphosphatase from membranes of *C. coelicolor* cross-reacted partially with the antiserum. Furthermore, the qualitative composition of the membrane phospholipids correlates well with the other parameters presented in Table 4 and Fig. 3. On the other hand, the interpeptide bridges in the peptidoglycan of *C. coelicolor* are of the type commonly found in corynebacteria (Table 6).

Sporosarcina ureae, another organism of controversial taxonomic status, showed no serological relationships between its adenosine triphosphatase and those of the pigmented micrococci. Moreover, the serological results were supported by the fact that its membrane phospholipids and fatty acids, its GC (42.9%) content and the type

of peptidoglycan cross-bridge were somewhat similar to those found in *B. subtilis* (Tables 4 and 6, Fig. 3). Of all the micrococci with high GC contents, *M. rhodochrous* was the only species whose membrane adenosine triphosphatase did not cross-react with our antiserum. However, despite its high GC content (70.4%), analysis of its phospholipids and fatty acids showed that it was very different from the other pigmented micrococci. Indeed, the presence of nocardic acids (Table 5) in this microorganism indicates that it would be more appropriately named as *Nocardia rhodochrous*, a suggestion which has already been made for other strains of *M. rhodochrous* (12).

DISCUSSION

The results of the investigations presented here support the now well recognized view that the comparative immunology of bacterial enzymes constitutes one of the most precise and valuable methods of analysis available to taxonomists (10, 21, 25). As pointed out by Wilson and Kaplan (31), an immunological comparison of enzymes has many advantages. It can be performed more rapidly than protein sequencing or fingerprinting; only the enzyme used as immunogen needs, to be pure; and cross-reactions with homologous enzymes from other species can be measured quantitatively in crude extracts. Of the several quantitative methods of comparison available, we chose one based on the inhibition of an enzyme (adenosine triphosphatase) by its antiserum. Although the immunodiffusion experiments provided evidence for immunological relationships between adenosine triphosphatases of various pigmented micrococci, the quantitative inhibition of membrane adenosine triphosphatases by the antiserum enabled us to estimate the degree of immunological homogeneity among these adenosine triphosphatases. We are aware that for a more accurate serological study of a larger number of organisms, the microcomplement fixation method (28) might have been a more suitable choice. However, in dealing with a membrane-associated enzyme, we were more interested in relating our immunological observations to similarities or differences of other membrane components, rather than in measuring homology of an enzyme as an expression of taxonomic distance.

Although we have used a relatively small "sample" of species in this study, in general our quantitative data on enzyme inhibition agree quite well with the existing taxonomic schemes for *Micrococcaceae* (6, 22), with our own observations on membrane phospholipid and fatty acid compositions of micrococci, and with the qualitative data on hydrocarbon composition of various micrococci reported by Tornabene et al. (26). All aerobic, gram-positive, pigmented cocci possessing membrane adenosine triphosphatases cross-reacting with our antiserum and GC contents above 70% had strikingly similar phospholipids and fatty acids (Tables 4 and 5, Fig. 3), and their peptidoglycans contained interpeptide linkages of either *M. lysodeikticus*-type or L-Lys-L-Ala_3-type (Table 6). Within this group, adenosine

TABLE 6. *GC contents of DNA and types of interpeptide linkage in peptidoglycans of various micrococci*

Organism	GC content (moles %)[a]	Interpeptide linkage in peptidoglycan[b]
Micrococcus lysodeikticus	73.3	*M. lysodeikticus*-type (11)
Sarcina flava	72.4	*M. lysodeikticus*-type (11)
M. tetragenus	73.0	Not determined
S. lutea	73.0	*M. lysodeikticus*-type (11)
M. roseus	73.5	L-Lys-L-Ala_3-type (11)
M. roseus R 27		L-Lys-Thr-L-Ala_3-type (11)
M. conglomeratus	69.4	L-Lys-L-Ala_3-type (24)
M. varians	72.4	*m*-diaminopimelic acid-D-Glu_2-type (24)
Micrococcus sp		*m*-diaminopimelic acid-D-Glu_2-type (24)
Corynebacterium coelicolor	65.3	L-Lys-(Ser, Ala, Thr)[c]
M. caseolyticus	44.4	L-Lys-(Gly_4, Ser_1)[c]
M. rhodochrous	70.4	Not determined
S. ventriculi	30.6[d]	Not determined
S. maxima	28.6[d]	Not determined
Sporosarcina ureae	42.9	L-Lys-Gly-D-Glu (24)
Bacillus subtilis	43.0	Direct (11)

[a] Determinations performed by Manley Mandel.
[b] References in parentheses.
[c] Determinations performed in O. Kandler's laboratory.
[d] Data from Canale-Parola et al. (9).

triphosphatases of the yellow pigmented cocci were inhibited to the greatest extent and gave precipitin lines of complete identity with adenosine triphosphatase of *M. lysodeikticus*. These cocci have been classified on the basis of their GC content as *M. luteus* by Boháaek et al. (6). The organisms so grouped contain *M. lysodeikticus*-type bridges in their peptidoglycans (11), their deoxyribonucleic acid species are able to transform *M. lysodeikticus* genetically (15), and they were recently found to contain similar aliphatic hydrocarbons (26). Adenosine triphosphatases of other pigmented cocci (*M. roseus*, *M. conglomeratus*) gave lower levels of inhibition and partial cross-reaction with the test antiserum. This subgroup also includes strains unable to transform *M. lysodeikticus* and was collectively named *M. roseus* by Boháček et al. (6). They all have peptidoglycans with L-Lys-L-Ala_3-type bridges (11, 24). We were able to distinguish between these two subgroups of pigmented micrococci immunologically.

M. varians NCTC 7281, unlike all other micrococci, was reported to contain *m*-diaminopimelic acid in cross-bridges of the peptidoglycan (24). Since it was also believed to be morphologically and physiologically different, a suggestion has been made to separate it from other micrococci (4). Our experiments have shown that its adenosine triphosphatase cross-reacts with the antiserum to *M. lysodeikticus* adenosine triphosphatase. Moreover, the major phospholipid and fatty acid components of its membranes do not differ basically from those of the other micrococci studied.

Sporosarcina ureae, a packet-forming coccus, is morphologically similar to species of the *Micrococcaceae*. Yet, in its ability to produce endospores (19), its motility under certain conditions (17), its GC content and some of its biochemical properties (7), it is reminiscent of the genus *Bacillus*. Membrane phospholipids of *S. ureae* CCM 981 are qualitatively different from those of micrococci (Fig. 3) and somewhat similar to those of *B. subtilis* (e.g., presence of phosphatidyl ethanolamine). Differences in fatty acid composition are less conspicuous. Its adenosine triphosphatase does not cross-react with the antiserum to micrococcal adenosine triphoshatases. Thus, our results are in agreement with the proposal of Boháček et al. (7) to separate. *S. ureae* from other aerobic and anaerobic *Sarcina* species and to reclassify it in a separate genus.

It is evident that whenever a microorganism seems to have affinities with different taxonomic groups, as in the case of *S. ureae*, or where its taxonomic status is unclear, as with *M. varians* NCTC 7281, an immunological comparison of a major membrane protein and quantitative and qualitative comparisons of other membrane components can provide additional valuable criteria for classification. In addition, we demonstrated that the antiserum to a membrane-associated enzyme can be used successfully in comparative immunological studies of enzymes from different species, and that such studies are of importance in bacterial taxonomy.

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