Neutral Lipids in the Study of Relationships of Members of the Family Micrococcaceae

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Received for publication 15 July 1971

The organisms studied were those of the family Micrococcaceae which cannot participate in genetic exchange with Micrococcus luteus and those whose biochemical and physiological characteristics appear to bridge the genera Staphyloccocus and Micrococcus. The hydrocarbon compositions of M. luteus ATCC 4698 and Micrococcus sp. ATCC 398 were shown to be similar to those previously reported for many M. luteus strains, consisting of isomers of branched monoolefins in the range C25 to C31. However, Micrococcus sp. ATCC 398 differed somewhat by having almost all C29 isomers (approximately 88% of the hydrocarbon composition), Micrococcus spp. ATCC 401 and ATCC 146 and M. roseus strains ATCC 412, ATCC 416, and ATCC 516 contained the same type of hydrocarbon patterns, but the predominant hydrocarbons were within a lower distribution range (C23 to C27), similar to Micrococcus sp. ATCC 533 previously reported. The chromatographic profile and carbon range of the hydrocarbons of an atypical strain designated M. candidans ATCC 8456 differed significantly from the hydrocarbon pattern presented above. The hydrocarbons were identified as branched and normal olefins in the range C16 to C22. Studies of several different strains of staphylococci revealed that these organisms do not contain readily detectable amounts of aliphatic hydrocarbons. The members of the family Micrococcaceae have been divided into two major groups based on the presence or absence of hydrocarbons. With the exception of M. candidans ATCC 8456, this division corresponded to the separation of these organisms according to their deoxyribonucleic acid compositions.

The aliphatic hydrocarbon contents of various Micrococcus luteus strains (formerly designated M. lysodeikiticus, Sarcina flava, S. lutea, and S. subflava), of their interstrain hybrids, and of Micrococcus sp. ATCC 533 (formerly S. lutea) have been reported (30). The hydrocarbons, occurring in groups of four isomers, were identified as acyclic monoolefins containing methyl branches in the iso and anteiso configurations (1, 27, 29, 30). The presence of hydrocarbons in micrococi contrasted sharply to the absence of hydrocarbons in staphylococci (30). From the limited available evidence, a possible correlation among the results from the studies of guanine-cytosine (GC) molar content of deoxyribonucleic acid (DNA) (2, 6, 21, 23, 24), transformation studies (13, 16, 17), and the analysis of the hydrocarbon compositions are discussed.

This paper presents evidence on the aliphatic hydrocarbon and fatty acid compositions of members of the family Micrococcaceae, with special attention to the organisms that are genetically incompatible with M. luteus (16) as well as to those whose biochemical and physiological characteristics appear to bridge the genera Staphylococcus and Micrococcus (3, 4, 7).

MATERIALS AND METHODS

Organisms. The organisms studied were Micrococcus spp. ATCC 401 and ATCC 146 (formerly M. conglomeratus and S. aurantiaca, respectively); M. roseus strains ATCC 412, ATCC 416, and ATCC 516; Micrococcus sp. ATCC 398 (formerly M. luteus); M. luteus ATCC 4698 (formerly M. lysodeikiticus); Staphylococcus sp. NCTC 1557 (formerly Baird-Parker Micrococcus subgroup 1), B-P5 (formerly Baird-Parker Micrococcus subgroup 2), B-P47 (formerly Baird-Parker Micrococcus subgroup 5), and NCTC 1463 (formerly Baird-Parker Micrococcus subgroup 6); and M. candidans ATCC 8456. The staphylococci above, which have been previously recorded as micrococi (4), are being considered as members of the species S. saprophyticus.
(Baird-Parker, personal communication). The strains ATCC 401, ATCC 146, and ATCC 398, as well as another strain discussed in this report, ATCC 533 (formerly S. lutea), are designated as Micrococcus species because of the uncertainty regarding their taxonomic status. The organisms designated B-P originally were from the collection of A. C. Baird-Parker, University Research Laboratory, Bedford, England; those designated NCTC were from the National sources of Type Cultures, London, England. Original sources and descriptions of the other organisms are given by Kloos (16).

Culturing conditions. M. candidans ATCC 8456 was grown in Brain Heart Infusion Broth (Difco) or Trypticase Soy Broth (BBL), both supplemented with 0.5% yeast extract, on a rotary shaker at 37°C. All other organisms were grown to early stationary phase in Trypticase Soy Broth on a rotary shaker at 25°C, except for strains of Staphylococcus species which were grown at 37°C. In addition, M. candidans ATCC 8456 and strains of Staphylococcus species were cultivated in 100 ml of Brain Heart Infusion Broth plus 0.5% yeast extract and in 100 ml of Trypticase Soy Broth, respectively, with both containing 50 μCi of sodium acetate-1-14C, as described previously (31); M. roseus ATCC 412 was cultivated in the presence of sodium acetate-1-14C in both media. All cells were harvested by centrifugation and washed twice with 0.9% NaCl solution.

It was shown previously (1, 26, 30) that variations in the nutrients and in the age of the cells can result in changes in the relative proportions of aliphatic hydrocarbon components. These changes, however, do not affect the overall hydrocarbon compositions of the cells.

Extraction and column fractionation. Cells were extracted by a modification (15) of the method of Bligh and Dyer (5). The chloroform-soluble materials were fractionated on silicic acid columns as described previously (28). Hydrocarbons were eluted with n-hexane (nano grade). All solvents used in this study were purchased from Mallinckrodt Chemical Works, St. Louis, Mo., and were redistilled before use. Procedures for handling the samples and for the preparation for analyses have been presented (22, 27, 28, 30).

Analytical methods. The dry weights of the extracted cellular materials were obtained by drying the samples to a constant weight in vacuo. The total hydrocarbon content, on a weight basis, was determined by calibration of the gas chromatographic peak areas with known amounts of hydrocarbon standards.

A sample of the n-hexane eluate fractionated from the extract of M. candidans ATCC 8456 on a silica gel column was hydrogenated catalytically (Pt), in an atmosphere of H2, to reduce the unsaturated hydrocarbons present.

The fatty acids were liberated from the lipid components and methylated by refluxing the lipid samples in a mixture of methanol and HCl as described by Kates (14).

The aliphatic hydrocarbons and fatty acid methyl esters were analyzed on an F & M model 5750 Gas Chromatograph equipped with dual-flame ionization detectors. Chromatograms were obtained by using stainless steel columns (62 m by 0.05 cm and 93 m by 0.075 cm) coated with Apiezon L (a high-temperature grease) and Igepal CO 990 (nonyl phenoxyl polyoxy-ethylene ethyl alcohol), respectively. The identities of all components separated by gas chromatography were determined by comparing their retention times with those of established standards (27, 29, 30). 14C-labeled samples were plated on aluminum planchets and counted with a thin end-window Geiger-Müller counter.

Thin-layer chromatography and autoradiography. Glass plates were spread with Silica Gel G (Stahl) and heat-activated at 120°C for at least 2 hr. The plates were developed in lined tanks by the ascending method with a two-step system employing the solvents benzene-diethyl ether-ethanol-acetic acid (50:40:2:0.2, v/v); and hexane-diethyl ether (96:4, v/v), as described by Freeman and West (9). Components were made visible by exposure to iodine vapor or by spraying with dichromate-saturated sulfuric acid and charring. 14C-labeled spots were detected by radioautography on Kodak non-screen X-ray film.

Determination of GC molar content of DNA. DNA was isolated from the organisms with the procedures outlined by Kloos (16). The thermal denaturation temperature (Tm) and the molar per cent GC contents were determined by the methods of Marmur and Doty (20). The solvent used for the DNA was standard saline-citrate (0.15 m NaCl plus 0.15 m trisodium citrate, pH 7.0).

RESULTS

Hydrocarbon composition of genus Micrococcus. It was previously shown by gas chromatographic analyses that all micrococci studied have qualitatively similar aliphatic hydrocarbon compositions (30). The hydrocarbons in the range C22 to C31 consisted of families of monoolefinic isomers containing methyl branches in the iso, or anteiso, or iso-anteiso configurations, symmetrically and asymmetrically disposed on the ends of the chains (29, 30). The positions of the double bond in each isomer are in the approximate center of the chains (29). The hydrocarbon compositions for many of the organisms reported here are the same as those previously reported for other members of the family Micrococcaceae (30), including the variations in carbon chain length previously observed in the gas-liquid chromatography-derived hydrocarbon patterns. The nature of the hydrocarbon distribution patterns for this group of organisms has been documented (27, 29, 30); only the differences among patterns are presented to aid in classifying this group of microorganisms (Table 1).

Differences among hydrocarbon patterns were seen in the predominant carbon fractions of the different organisms. The major fractions of M. roseus strains ATCC 412, ATCC 416, and ATCC 516 were the C24 and C25 hydrocarbons. The C25 fraction was predominant in Micrococcus sp. ATCC 146, with appreciable quantities of the C23, C24, C26, and C27 hydrocarbons also present. The predominant fractions in Mi-
### Table 1. Characteristics of members of family Micrococaceae

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Moles % GC in DNA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ability to transform &lt;i&gt;M. luteus&lt;/i&gt;</th>
<th>Aliphatic hydrocarbons (fractions greater than 5 moles %)</th>
<th>Fatty acids (fractions greater than 3 moles %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;i&gt;Micrococcus luteus&lt;/i&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>FD 533</td>
<td>68.3</td>
<td>+</td>
<td>C27, C28, C29</td>
<td>C14, C15, C16</td>
</tr>
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<td>&lt;i&gt;M. luteus&lt;/i&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ATCC 272</td>
<td>73.7</td>
<td>+</td>
<td>C25, C27, C28, C29</td>
<td>C15</td>
</tr>
<tr>
<td>&lt;i&gt;M. luteus&lt;/i&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ATCC 381</td>
<td>72.8</td>
<td>+</td>
<td>C25, C26, C27, C28, C29</td>
<td>C14, C15, C16</td>
</tr>
<tr>
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<td>72.0</td>
<td>+</td>
<td>C27, C25, C28, C29</td>
<td>C13, C14, C15</td>
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<tr>
<td>&lt;i&gt;M. luteus&lt;/i&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ATCC 540</td>
<td>72.9</td>
<td>+</td>
<td>C27, C28, C29, C30</td>
<td>C14, C15, C16, C17</td>
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<tr>
<td>&lt;i&gt;M. luteus&lt;/i&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ATCC 7468</td>
<td>73.4</td>
<td>+</td>
<td>C25, C26, C27, C28, C29</td>
<td>C14, C15, C16, C17</td>
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<td>&lt;i&gt;M. luteus&lt;/i&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ATCC 4698</td>
<td>69.2</td>
<td>+</td>
<td>C27, C28, C29</td>
<td>C13, C14, C15, C16, C18</td>
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<td>&lt;i&gt;M. luteus&lt;/i&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ISU</td>
<td>69.8</td>
<td>+</td>
<td>C27, C28, C29</td>
<td>C14, C15, C17</td>
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<tr>
<td>&lt;i&gt;M. roseus&lt;/i&gt;</td>
<td>ATCC 412</td>
<td>72.8</td>
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<tr>
<td>&lt;i&gt;M. roseus&lt;/i&gt;</td>
<td>ATCC 416</td>
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<td>&lt;i&gt;M. roseus&lt;/i&gt;</td>
<td>ATCC 516</td>
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<td>ATCC 146</td>
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<td>&lt;i&gt;Micrococcus sp.&lt;/i&gt;</td>
<td>ATCC 398</td>
<td>66.3</td>
<td>-</td>
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<td>C15, C17</td>
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<tr>
<td>&lt;i&gt;Staphylococcus aureus&lt;/i&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>655</td>
<td>34.1</td>
<td>-</td>
<td>None</td>
<td>C15, C16, C17, C18, C19, C20</td>
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<tr>
<td>&lt;i&gt;Staphylococcus sp.&lt;/i&gt;</td>
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<td>None</td>
<td>C15, C16, C18, C20</td>
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<td>&lt;i&gt;Staphylococcus sp.&lt;/i&gt;</td>
<td>NCTC 1557</td>
<td>32.7</td>
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<td>None</td>
<td>C14, C15, C16, C18, C20</td>
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<td>&lt;i&gt;Staphylococcus sp.&lt;/i&gt;</td>
<td>B-P 5</td>
<td>33.2</td>
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<td>None</td>
<td>C13, C14, C15, C16, C17, C18, C20</td>
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<td>&lt;i&gt;Staphylococcus sp.&lt;/i&gt;</td>
<td>B-P 47</td>
<td>33.9</td>
<td></td>
<td>None</td>
<td>C13, C14, C15, C16, C17, C18, C20</td>
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<tr>
<td>&lt;i&gt;Staphylococcus sp.&lt;/i&gt;</td>
<td>NCTC 1463</td>
<td>33.4</td>
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<td>C14, C15, C16, C18, C20</td>
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<tr>
<td>&lt;i&gt;M. candidans&lt;/i&gt;</td>
<td>ATCC 8456</td>
<td>37.1</td>
<td></td>
<td>C18, C19, C20</td>
<td>C14, C15, C16, C17, C18</td>
</tr>
</tbody>
</table>

<sup>a</sup> Hydrocarbon and fatty acid analyses previously reported (30).

<sup>b</sup> Superscript numbers indicate reference for guanine plus cytosine (GC) determinations made by other investigators.

<sup>c</sup> +, DNA from organism was capable of transforming <i>Micrococcus luteus</i>; -, no transformation of <i>M. luteus</i>. ISU was achieved with DNA from organism (13, 16, 17, 30). No designation indicates no available information.

**croccoccus** sp. ATCC 401 were the C25, C26, and C27 hydrocarbons. <i>M. luteus</i> ATCC 4698 contained a predominance of the C29 hydrocarbon fraction. Approximately 88% of the hydrocarbons of <i>Micrococcus</i> sp. ATCC 398 was present as C29 isomers. The hydrocarbons of the organisms presented were within the range C22 to C29, inclusive, with the exception of <i>Micrococcus</i> sp. ATCC 398, whose hydrocarbons ranged from C25 to C31. The hydrocarbon compositions in these micrococci comprise 18 to 22% of the total lipids.

**Analysis of n-hexane fraction of <i>M. candidans</i> ATCC 8456.** The yield of aliphatic hydrocarbons from cells of <i>M. candidans</i> ATCC 8456 was 20 to 22% of the total lipids. The hydrocarbons of <i>M. candidans</i> ATCC 8456 (Table 2) ranged from C16 to C22, significantly lower than the range observed for other micrococci. On the other hand, the chemical nature of the <i>M. candidans</i> hydrocarbons was predominantly branched olefins, which are somewhat characteristic of microccci hydrocarbons. The branching characteristics, determined through the retention-time values of the unsaturated and saturated hydrocarbons, were tentatively identified as methyl branches occurring in the iso and anteiso configurations. A thorough examination of these hydrocarbons is required to determine their exact structures.

**Analysis of n-hexane fractions of selected strains of Staphylococcus species.** The gas chromatographic analysis of the n-hexane fractions of strains of <i>Staphylococcus</i> species revealed no acyclic branched monoolefins common to microccci. Radioactivity measurements of the total lipids extracted from the staphylococci grown in the presence of <sup>14</sup>C-acetate showed that components in the lipid fractions had readily incorporated <sup>14</sup>C atoms; however, the n-hexane fractions isolated by column chromatography revealed no measurable radioactivity. Autoradiograms of the total n-hexane fractions showed no radioactivity after 4 weeks of exposure. On the other hand,
Hydrocarbons with Igepal parentheses Nitrogen flow were: steel stainless br-A-C22 br-A-C21 n-A-C19 br-A-C19 516; Micrococcus ATCC 8456 grown in Trypticase Soy Broth 

<table>
<thead>
<tr>
<th>Hydrocarbons</th>
<th>Retention time (min)</th>
<th>Relative moles %</th>
</tr>
</thead>
<tbody>
<tr>
<td>br-Δ-C16</td>
<td>18.5 (17.25)</td>
<td>0.3</td>
</tr>
<tr>
<td>br-Δ-C16</td>
<td>19.0 (17.75)</td>
<td>0.2</td>
</tr>
<tr>
<td>br-Δ-C17</td>
<td>23.25 (21.25)</td>
<td>0.2</td>
</tr>
<tr>
<td>n-Δ-C17</td>
<td>25.5 (23.6)</td>
<td>0.2</td>
</tr>
<tr>
<td>br-Δ-C18</td>
<td>28.25 (25.75)</td>
<td>10.2</td>
</tr>
<tr>
<td>br-Δ-C18</td>
<td>29.0 (26.5)</td>
<td>1.3</td>
</tr>
<tr>
<td>br-Δ-C19</td>
<td>33.25 (31.25)</td>
<td>0.6</td>
</tr>
<tr>
<td>br-Δ-C19</td>
<td>34.0 (32.0)</td>
<td>0.1</td>
</tr>
<tr>
<td>n-Δ-C19</td>
<td>35.75 (33.75)</td>
<td>9.8</td>
</tr>
<tr>
<td>br-Δ-C20</td>
<td>39.0 (36.5)</td>
<td>49.7</td>
</tr>
<tr>
<td>br-Δ-C20</td>
<td>39.75 (37.25)</td>
<td>26.1</td>
</tr>
<tr>
<td>br-Δ-C21</td>
<td>46.0 (41.75)</td>
<td>0.2</td>
</tr>
<tr>
<td>br-Δ-C21</td>
<td>47.5 (43.0)</td>
<td>0.1</td>
</tr>
<tr>
<td>n-Δ-C21</td>
<td>56.5 (44.25)</td>
<td>0.5</td>
</tr>
<tr>
<td>br-Δ-C22</td>
<td>56.0 (47.5)</td>
<td>0.2</td>
</tr>
<tr>
<td>br-Δ-C22</td>
<td>57.5 (48.75)</td>
<td>0.1</td>
</tr>
</tbody>
</table>

* Branching configurations (br) tentatively identified as methyl branches in the iso and anteiso configurations. Unsaturation (Δ); normal structure (n).

Gas chromatographic separation was obtained on a stainless steel capillary column (93 m by 0.075 cm) coated with Igepal CO-990. Temperature programmed at approximately 2 °C/min from 110 to 172 °C and held isothermally. Nitrogen flow rate ca. 15 ml/min. Values in parentheses are those obtained for the hydrogenated hydrocarbons. Retention time values for authentic standards were: C16, 19.8; C17, 24.0; C18, 22.8; C19, 33.7; and C20, 40.2.

diluted 14C-hydrocarbon samples of M. roseus ATCC 412 (0.0004%) and M. candicans ATCC 8456 (0.0008 to 0.0025%) were readily detected with a thin-end-window Geiger-Müller counter and by autoradiographic analysis after 2 weeks of exposure.

Fatty acid compositions. The fatty acid compositions of M. roseus ATCC 412, ATCC 416, and ATCC 516; Micrococcus spp. ATCC 146, ATCC 401, and ATCC 398; and M. luteus ATCC 4698 were qualitatively similar to each other and to many other members of the family Micrococcaceae previously established (27, 30, 31). The saturated fatty acids consisted of iso and anteiso methyl-branched structures and occasional normal structures for the odd-numbered carbon chains and iso and normal structures for even-numbered carbon chains in the range C13 to C18, inclusive. The predominant components were the iso and anteiso C15:0 fatty acids. The fatty acids of M. candicans ATCC 8456 differed from the typical fatty acid pattern only by containing significantly greater quantities of both C18:0 and C18:1 fatty acids.

The fatty acid methyl ester patterns of Staphylococcus sp. NCTC 1557, B-P5, B-P47, and NCTC 1463 were qualitatively similar to the fatty acid compositions previously presented for S. aureus (18, 19, 30) and Staphylococcus sp. ATCC 10875 (formerly Gaffkyana tetragena) (30). The fatty acid patterns differed from those of the Micrococcus species by having significant quantities of the normal C16:0, C18:0, and C20:0 fatty acids.

DNA base composition. The GC molar content of DNA was determined in certain members of the family Micrococcaceae not previously studied (Table 1). All the organisms can be divided into two groups according to GC content (high, 66.3 to 73.7 moles %; or low, 32.7 to 37.1 moles %).

DISCUSSION

The results of this study are in accord with a previous report (29) that the hydrocarbon compositions consisting of isomers of branched olefins are characteristic of certain organisms in the family Micrococcaceae. The proposal for differentiating between Micrococcus species and Staphylococcus species on the basis of the hydrocarbon compositions (30) is also supported in this paper. Micrococcus sp. ATCC 401 and ATCC 146; M. roseus ATCC 412, ATCC 416, and ATCC 516; Micrococcus sp. ATCC 398; and M. luteus ATCC 4698 all contain hydrocarbon profiles generally similar to the hydrocarbon profiles of many micrococci (30). These organisms also have similar fatty acid contents and GC contents in DNA of 66.3 to 73.7 moles % (Table 1). Strains of Staphylococcus species with GC molar contents in DNA of 32.7 to 33.9 moles % (10) contained no detectable hydrocarbons; the fatty acid contents were similar to those in S. aureus (18, 19, 30). The nature of the fatty acid compositions and the DNA base ratios of those organisms without hydrocarbons are characteristic different from those of the hydrocarbon-containing members (Table 1).

The division of members of the family Micrococcaceae into two major groups on the basis of the presence or absence of hydrocarbons corresponds generally to the separation of these organisms on the basis of DNA base composition. M. candicans ATCC 8456, however, was an exception. This strain has a GC molar content in DNA of 37.1 moles %, close to but slightly higher than the GC molar content in most Staphylococcus strains. However, the normal menaquinone pattern reported in this organism (12) is generally characteristic of staphylococci. Unlike staphylococci with GC molar contents in the 30% range, this organism has aliphatic hydrocarbons and a fatty acid pattern more similar to the fatty acid patterns of micrococci with GC
molar contents in the 65 to 75% range. M. candi-
cans ATCC 8456, an interesting exception to the
proposed scheme for dividing the organisms into
two groups, may represent a genus that should be
separated from Micrococcus and Staphylo-
coccus.

The hydrocarbon compositions of many micro-
cocci differ characteristically with respect to
carbon chain lengths. It was previously reported
that the hydrocarbon pattern of Micrococcus sp.
ATCC 533 (formerly S. lutea; major hydrocar-
bons C25, C26, C27) differed somewhat from the
patterns of M. luteus strains studied (major
hydrocarbons C27, C28, C29) (30). Micrococcus
sp. ATCC 533, distinguished from M. luteus
FD533 (formerly S. lutea), also differs by failing
to transform M. luteus (16). [S. lutea FD533
serving as a donor in transformation was inad-
vertently presented as S. lutea ATCC 533 in pre-
vious reports (16, 17).] In this paper it was shown
that the hydrocarbon patterns of Micro-
coccus spp. ATCC 401 and ATCC 146 and M.
roese ATCC 412, ATCC 416, and ATCC 516
resemble the pattern of Micrococcus sp. ATCC
533. Those organisms were also unable to trans-
form M. luteus (16). Micrococcus sp. ATCC
398, reported to be an atypical M. luteus (11,
23) unable to transform M. luteus (16), has a
hydrocarbon pattern somewhat different from
those of the typical M. luteus strains previously
reported (30). The Micrococcus sp. ATCC 398
pattern consists almost entirely of C29 isomers,
a structure commonly observed in the hydrocar-
bons of M. luteus hybrids cultivated under the
same environmental conditions (30). The dif-
ference between the hydrocarbon distribution
patterns of M. luteus and those of the Micrococcus
species and M. roseus correlated with differences
in cell wall mureins (25). M. luteus strains have
a murein composition with an interpeptide bridge
consisting of a complete peptide subunit, L-
alanyl-L-glutamyl-L-lysyl-L-alanine, whereas
M. roseus and Micrococcus spp. ATCC 533,
ATCC 401, and ATCC 146 have the L-lysyl-L-
alanyl type. The murein of Micrococcus sp.
ATCC 398 is unique, having cross-linkage with
the peptides γ-L-glutamyl-glycine.

ACKNOWLEDGMENTS

We are indebted to Margaret Muscelwhite and Katherine
Kelly for their technical assistance in isolating DNA and deter-
mining the GC contents of strains used in this study.

This investigation was supported by research grant GB-
8719 from the National Science Foundation and by Public
Health Service grant AI-08255 from the National Institute of
Allergy and Infectious Diseases.

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