Use of Lysostaphin in the Isolation of Highly
Polymerized Deoxyribonucleic Acid and in the
Taxonomy of Aerobic Micrococcaceae

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By use of the staphyloytic enzyme lysostaphin, a method was devised for isolating and
purifying highly polymerized deoxyribonucleic acid (DNA) from lysostaphin-
susceptible Micrococcaceae. Staphylococcus aureus DNA isolated by this procedure
was an estimated molecular weight of ca. 2 × 10^9 and a residual protein content of
2.3%. The mole percentage of guanine + cytosine (GC) present in the DNA from 21
strains of aerobic Micrococcaceae was determined by buoyant density in cesium chlo-
ride. DNA from 12 biochemically typical members of the genus Staphylococcus gave
a mean GC composition of 35.2 ± 0.5 mole per cent. Four biochemically atypical
Staphylococcus strains and one biochemically typical strain of the genus Micrococcus
(M. candidus) were found to be susceptible to lysostaphin and gave typical Staphy-
lococcus spp. GC base ratios. One biochemically atypical member of the genus Micro-
coccus (M. varians) was not susceptible to lysostaphin and gave a typical Micrococcus
spp. GC base ratio. Lysostaphin susceptibility is an easy test to perform, and the
results of this test appear to correlate with GC base ratio studies of the genera of
Micrococcaceae.

The isolation and purification of highly polym-
erized deoxyribonucleic acid (DNA) from mem-
ers of the family Micrococcaceae is complicated
by the difficulty of liberating the nucleic acids
from the cells of some of these organisms
without subjecting them to shearing stresses.
Blobel (5), by use of phenol extraction, and
Marmur (12), by use of sodium lauryl sulfate,
extracted highly polymerized DNA (with molecu-
lar weights ranging from 8 to 12.8 × 10^9) from
S. aureus. Silvestri and Hill (19) used lysozyme
(on strains of Micrococcaceae susceptible to this
enzyme) and penicillin to induce osmotically
fragile spheroplasts in other strains. Neither these
investigators nor Auletta and Kennedy (1), who
disrupted their micrococal cells with glass beads,
reported the molecular weights of their purified
DNA samples. Both Silvestri and Hill and Auletta
and Kennedy were primarily interested in the
molecular significance of the GC (guanine +
cytosine/total bases) values of the DNA from
strains of Micrococcaceae. They obtained these
GC values by the thermal denaturation (T_m)
method of Marmur and Doty (13).

The discovery and characterization of the
staphyloytic enzyme, lysostaphin, by Schindler
and Schuhardt (17, 18) provided a mild and rapid
method for liberating the nucleic acids and other
cytoplasmic contents from lysostaphin-susceptible
Micrococcaceae. After testing many hundreds of
species and strains of Micrococcaceae and other
families of bacteria, the lytic activity of lysos-
astaphin proved to be specific for members of the
genus Staphylococcus. Our method for isolating the
highly polymerized staphylococcal DNA was
designed to make use of this isolated DNA in
transformation studies on intact cells and on
lysostaphin-induced spheroplasts of selected
strains of Staphylococcus aureus. When trans-
formation efforts failed, we used the isolated
DNA for correlating the guanine + cytosine
(GC) content with the lysostaphin susceptibility
of a group of biochemically typical and atypical
strains of aerobic Micrococcaceae.

MATERIALS AND METHODS

Organisms. The aerobic Micrococcaceae used in
this study (Table 1) were obtained from a variety of
sources. All but two of the cultures (no. 11 and 12,
Table 1) were classified biochemically and for lysosta-
phin susceptibility by E. T. Thomas (Ph.D. Thesis,
The University of Texas at Austin, 1964). The bio-
chemical classification scheme and media used were
those of Baird-Parker (2). This scheme and media,
at the genus level, essentially correlated with the
classification scheme of Breed et al. (Bergey's Manual) and others for these organisms. Six of the strains were judged to be biochemically atypical because they did not produce acid from glucose in anaerobic culture. Stock cultures recovered from the lyophilized state were maintained on 1.5% Trypticase Soy Agar (TSA) slants (BBL), and these cultures were stored at 4 °C. Subcultures of the stocks were made at monthly intervals. Cells for DNA extraction were cultured in Trypticase Soy Broth (TSB) at 37 °C on a reciprocating shaker and were harvested when the late log phase of growth was reached.

**Lysostaphin.** Lyophilized lysostaphin, containing 209 units (18) per mg, was dissolved in 0.05 M tris-(hydroxymethyl)aminomethane (Tri) chloride buffer (6) containing 0.145 M NaCl (Tri buffer) at pH 7.4 to give a stock solution of 200 units/ml. The stock solution was stored in 0.5- or 1.0-ml amounts in screw-capped tubes at −20 °C. For testing, the stock solution was thawed and diluted in cold Tri buffer to yield the desired number of units per ml. The tube containing the diluted lysostaphin was immersed in an ice bath until the lysostaphin was dispensed into the test preparations. Klett standardized suspensions of the test organisms and a control suspension of *S. aureus* FDA 209P were tested for susceptibility to 5 units (1 unit/ml) of lysostaphin by the method described by Schuhardt (see discussion of reference 3).

**DNA and protein determination.** The DNA concentration was estimated by ultraviolet absorption at 260 nm with a DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). The relationship used between optical density (OD) at 260 nm and DNA concentration was 1 OD unit equals 50 μg/ml of DNA. The colorimetric method of Lowry et al. (11) was employed to estimate the protein concentration.

**Method for preparation of DNA.** Logarithmic growth-phase cells were harvested by centrifugation and were washed three times with Tri buffer plus 0.015 M trisodium citrate (TSC), pH 7.4. Lysostaphin-susceptible cells (2 to 4 g, wet weight) were suspended in a total volume of 20 ml of TSC in a large beaker containing a magnetic stirring bar, and the cells were lysed by incubation with lysostaphin at a final concentration of 5 units/ml for 30 to 60 min at 37 °C. Bacteria which were not susceptible to this enzyme nor to lysozyme (*Staphylococcus* K-6-W1 and *Sarcina lutea*) were disrupted by mechanical grinding with alumina at 4 C. *Micrococcus varians* and *Micrococcus lyso- deiticus* were lysed by digesting the washed cells with 10 mg of crystalline lysozyme for 60 to 90 min at 37 C.

The lysates were gently stirred with sodium lauryl sulfate (2%) for 2 hr at 4 C. NaCl was added to a final concentration of 2 m, and, after 15 min, 1.5 volumes of 0.015 M TSC were added. After another 15 min, the residual cellular debris was removed by centrifugation at 4 C, and the viscous supernatant fluid was decanted into a second beaker. The nucleic acids were precipitated by gradually adding 1.5 volumes of 95% ethyl alcohol to the gently swirling fluid. This resulted in a gelled mass of nucleic acids from which the supernatant ethyl alcohol and the soluble extractives were withdrawn by suction, thereby avoiding the necessity of winding the nucleic acids on a glass rod for recovery.

The precipitated nucleic acids were redissolved in a small amount of SSC (0.15 M NaCl plus 0.015 M trisodium citrate, pH 7.0). The ribonucleic acid (RNA) was digested by treatment with 50 μg/ml of ribonuclease for 30 min at 37 °C. Protein was digested by treatment with 100 PUK units/ml of Pronase twice in 24 hr at 37 °C. After RNA and protein digestion, the DNA was precipitated by use of 1.5 volumes of 95% ethyl alcohol in the manner described above. The precipitated DNA was washed with acetone until the wash was clear.

The DNA was redissolved in a small amount of SSC and was reprecipitated by the dropwise addition of 0.54 volumes of isopropanol into the gently swirling solution. The supernatant fluid was discarded, and the DNA was dissolved in 5 to 10 ml of SSC. The DNA solution was centrifuged, if necessary, to remove insoluble material and was stored at 0 °C.

**Molecular weight determination by sedimentation coefficient.** The sedimentation coefficient (S_m,w) was determined at 20 C in a model E analytical centrifuge (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.) at 113,210 × g using a 4°, 12-mm standard cell having a Kel-F centerpiece. Pictures of the DNA boundary during centrifugation were taken at 8-min intervals for 64 min by use of ultraviolet/UV optics. The S_m,w was found by extrapolating to infinite dilution the reciprocal of the S_m,w values obtained at four DNA concentrations. For each centrifugation, the concentration of DNA was measured directly in a spectrophotometer. The molecular weight was calculated from the S_m,w by use of the formula of Doty, McGill, and Rice (7).

**Base composition of DNA from its buoyant density in cesium chloride.** The method and equation of Schildkraut et al. (16) were used to determine the buoyant density in cesium chloride (CsCl) and the GC value of DNA. *Bacillus subtilis* SP8 bacteriophage DNA, with a density of 1.742 g/cm³, was employed as the reference for the determination of the density of the test DNA. The CsCl gradient centrifugation was performed at 25 C in a Spinc model E analytical centrifuge at 145,656 × g for 19 to 21 hr. Tracings of the UV absorption bands of DNA at equilibrium were made with an analytical densitometer (Beckman Instruments, Inc., Fullerton, Calif.).

**RESULTS**

**Yield and relative purity of DNA.** The average yield of DNA obtained from 12 biochemically typical strains of the genus *Staphylococcus* by use of the lysostaphin procedure was 0.82 mg/g (wet weight) of cells. Some strains yielded up to 1.4 mg/g. The DNA preparations gave an average 260 to 280 μA UV absorption ratio of 1.82. Colorimetric analysis (11) indicated an average protein content of 2.3% for these DNA samples.

**Molecular weight determinations.** DNA isolated from *S. aureus* 3189 by the lysostaphin procedure was used to determine the molecular weight. The
results of the sedimentation coefficients at four different DNA concentrations gave a straight line when plotted as the reciprocal of the $S_{20,w}$ values (Fig. 1). When this line was extrapolated to infinite dilutions, an estimated $S_{20,w}$ of 78S was determined. This indicated a molecular weight of about $2 \times 10^6$.

**GC base ratio.** A representative tracing of UV absorption bands of two *S. aureus* BP9 DNA preparations in CsCl is shown in Fig. 2. The *S. aureus* BP9 DNA liberated by mechanical grinding (solid 1.694 line) was compared to *S. aureus* BP9 DNA released by lysostaphin (broken 1.694 line). *B. subtilis* SP8 phage DNA was included as the reference DNA (density, 1.742 g/cm³). Although both *S. aureus* DNA preparations gave the same density (1.694 g/cm³), the broader base of the mechanically liberated DNA indicated some degree of shearing degradation when compared with the base width of the DNA liberated by lysostaphin.

The GC base ratios for the 21 strains of aerobic *Micrococcaceae* are listed in Table 1. The results indicate a remarkable degree of homogeneity in the base composition of the strains of the genus *Staphylococcus* studied. The mean buoyant density for these strains was 1.6943 g/cm³ with a standard deviation of 0.005 and probable error of less than 1%. The mean base composition calculated from the buoyant density results was 35.2 mole percentage of GC with a standard deviation of 0.5.

**Taxonomic significance.** All of the biochemically typical members of the family *Micrococcaceae* listed in Table 1 (cultures no. 1 to 7, 9, 10, 17, 18, 20, and 21) were found to fit the biochemical genera and subgroups of this family as advocated by Baird-Parker (2). On primary testing, six cultures (no. 8, 13 to 16, and 19) were judged to be atypical because they did not produce acid from glucose in the anaerobic depths of the Hugh and Leifson (8) test medium. It was thus impossible biochemically to classify these six cultures as members of the genus *Staphylococcus* or the genus *Micrococcus*. However, all of these atypical strains, with the exception of number 19 (*M. varians*, Table 1), were found to be susceptible to lysostaphin, as was number 17, the biochemically typical member of the genus *Micrococcus* (*M. candidans*).

The six biochemically atypical strains and *M. candidans* were passed through five transplants on the Hugh and Leifson basal medium without glucose, and these strains were then retested for acid production from glucose in the anaerobic base of the complete medium. As a result of this serial passage, cultures 13, 14, 16, and 19 (Table 1) acquired the capacity to produce acid from glucose throughout the medium and could therefore be biochemically classified as members of the genus *Staphylococcus*. Cultures 8 and 15 remained atypical and culture 17 (*M. candidans*) still did not produce acid anaerobically from glucose.

All of the cultures listed in Table 1, with the exception of the last four, were found to be susceptible to lysostaphin. Thus, we found that two biochemically atypical strains (no. 8 and 15) and one biochemically typical member of the genus *Micrococcus* (no. 17) were susceptible to lysostaphin and two biochemically typical members of the genus *Staphylococcus* (no. 18 and 19) were not susceptible to this enzyme. The GC base ratios of the lysostaphin-susceptible strains (no. 8, 15, and
TABLE 1. DNA base ratios (GC) and lysostaphin susceptibility of selected strains of aerobic Micrococcaceae

<table>
<thead>
<tr>
<th>Culture no.</th>
<th>Original designation</th>
<th>Coagulase</th>
<th>Mole % GC</th>
<th>Lysis by lysostaphin</th>
<th>Indicated genus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Staphylococcus aureus FDA 209P</td>
<td>+</td>
<td>35.7</td>
<td>&gt;80</td>
<td>Staphylococcus</td>
</tr>
<tr>
<td>2</td>
<td>S. aureus SCL-1</td>
<td>+</td>
<td>35.7</td>
<td>&gt;80</td>
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<tr>
<td>3</td>
<td>S. aureus 63A1906</td>
<td>+</td>
<td>34.7</td>
<td>&gt;80</td>
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</tr>
<tr>
<td>4</td>
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<td>+</td>
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</tr>
<tr>
<td>5</td>
<td>S. aureus 16851</td>
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<td>&gt;80</td>
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</tr>
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<td>6</td>
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<td>&gt;80</td>
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</tr>
<tr>
<td>7</td>
<td>S. aureus 3189</td>
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<td>35.7</td>
<td>&gt;80</td>
<td>Staphylococcus</td>
</tr>
<tr>
<td>8</td>
<td>S. aureus BP 17</td>
<td>±</td>
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<td>&gt;80</td>
<td>Staphylococcus</td>
</tr>
<tr>
<td>9</td>
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<td>-</td>
<td>34.7</td>
<td>79</td>
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</tr>
<tr>
<td>10</td>
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<td>34.7</td>
<td>69</td>
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<td>11</td>
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<td>-</td>
<td>34.7</td>
<td>&gt;80</td>
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</tr>
<tr>
<td>12</td>
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<td>-</td>
<td>34.7</td>
<td>&gt;80</td>
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</tr>
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<td>-</td>
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<tr>
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<td>34.7</td>
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<td>16</td>
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<td>-</td>
<td>34.7</td>
<td>71</td>
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</tr>
<tr>
<td>17</td>
<td>M. candidus</td>
<td>-</td>
<td>34.7</td>
<td>&gt;80</td>
<td>Staphylococcus</td>
</tr>
<tr>
<td>18</td>
<td>Staphylococcus K-6-W1</td>
<td>-</td>
<td>35.7</td>
<td>0</td>
<td>Micrococcus</td>
</tr>
<tr>
<td>19</td>
<td>M. varians B2158</td>
<td>-</td>
<td>72.4</td>
<td>0</td>
<td>Micrococcus</td>
</tr>
<tr>
<td>20</td>
<td>M. lysodeikticus</td>
<td>-</td>
<td>72.0</td>
<td>0</td>
<td>Micrococcus</td>
</tr>
<tr>
<td>21</td>
<td>Sarcina lutea 18FL</td>
<td>-</td>
<td>71.9</td>
<td>0</td>
<td>Sarcina</td>
</tr>
</tbody>
</table>

* Percentage of reduction in OD at 1 hr.
* The Smith encapsulated strain.
* Isolated from human cases of endocarditis.

17) indicated that these strains were indeed members of the genus *Staphylococcus* and that the strain which was not susceptible to lysostaphin (no. 19), in spite of its ability to produce acid in the anaerobic glucose medium, was an otherwise typical member of the genus *Micrococcus*. The one typical (biochemically and GC base ratio) member of the genus *Staphylococcus* (no. 18, Table 1) which was not susceptible to lysostaphin was the lysostaphin-producing strain K-6-W1.

**DISCUSSION**

Wechman and Catlin (20) found higher deoxyribonuclease production in staphylococci grown in Brain Heart Infusion broth than in staphylococci grown in TSB, and the optimal pH for deoxyribonuclease activity was 8.0. In view of these factors, we chose to grow our cultures in TSB and to adjust our chelating SSC solution to pH 7.0. The inclusion of sodium lauryl sulfate in the purification procedure was recommended by Kay et al. (10) and Jordan (9). We also noted that the common practice of collecting precipitated DNA on a glass rod resulted in a loss of solubility of the DNA in SSC. Consequently, we chose to precipitate the DNA in a beaker, the contents of which were gently swirled during the addition of the precipitant. This resulted in a gelled mass of DNA from which the alcoholic supernatant fluids could be removed by suction and which dissolved rapidly upon addition of SSC.

The residual protein content (2.3%) of our micrococcal DNA was comparable to that reported by Berns and Thomas (4), who used both Pronase and phenol to precipitate their *Haemophilus influenzae* DNA. Massie and Zimm (14, 15) reported that they were able to remove all traces of protein from their *Escherichia coli* and *B. subtilis* DNA by phenol extraction after Pronase digestion.

The molecular weight of the staphylococcal DNA used in our experiments (ca. 2 \( \times 10^9 \)) appeared to be significantly higher than the molecular weight (8 to 12 \( \times 10^9 \)) previously reported for these organisms (5, 12). In addition, in our experiments, the UV absorption curve of DNA liberated from *S. aureus* by the lysostaphin method (Fig. 2) showed a more homogeneous preparation than the DNA liberated by grinding the same cells with alumina. Therefore, we believe that our methods of liberation, precipitation, and recovery of DNA from staphylococci produce less shearing degradation than is obtained by other methods. We realize that highly polymerized DNA is not required for GC base ratio determinations, but, as stated above, our staphylococcal DNA was originally isolated for other purposes.

Both Silvestri and Hill (19) and Auletta and...
Kennedy (1) reported GC base ratios for *Staphylococcus* strains ranging from 30 to 36 mole percentage of GC, with one questionable strain reported as 39% (1). The mean ratios found by these investigators were 32.2 and 33.6%, respectively, and the thermal denaturation method of determining GC values was used. Our results, using the CsCl density gradient method, gave GC base ratios for *Staphylococcus* strains ranging from 34.7 to 35.7% with a mean of 35.2%.

Our results confirm Auletta and Kennedy's finding (1) that there is no single biochemical taxonomic characteristic which will differentiate members of the genera *Staphylococcus* and *Micrococcus*. In fact, we are convinced that there is no multiple biochemical group of characteristics, short of including GC base ratios, which will differentiate these genera. Strain susceptibility to lysostaphin is the closest approximation of a single characteristic which we found to be useful in this differentiation. Had it not been for lysostaphin susceptibility, we would have had no reason to suspect that our *M. candidans* was not a legitimate member of the genus *Micrococcus*. In addition, our strains of *M. violagabriellae* and *M. varians* adapted to the production of acid from glucose in the anaerobic base of the test medium. The former, with a GC value of 34.7, was susceptible to lysostaphin, whereas the latter, with a GC value of 72.4, was not susceptible. Thus, the simple rapid test for lysostaphin susceptibility seems to correlate, in all but one instance, with the much more complicated GC base composition test for differentiating these two genera. The one exception we found, *Staphylococcus* K-6-WI, is the organism which produces lysostaphin. It is conceivable that other lysostaphin-resistant strains of the genus *Staphylococcus* may be found to complicate the taxonomic significance of lysostaphin, but none has been discovered thus far.

**Acknowledgments**

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**Literature Cited**