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

PHILLIP H. KLESIUS AND V. T. SCHUHARDT

Department of Microbiology, The University of Texas, Austin, Texas 78712

By use of the staphylolytic 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 gave an estimated molecular weight of ca. 2×10^8 and a residual protein content of 2.3%. The mole percentage of guanine + cytosine (GC) present in the DNA from 21 strains of aerobic *Micrococceae* was determined by buoyant density in cesium chloride. 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. candicans*) were found to be susceptible to lysostaphin and gave typical *Staphylococcus* spp. GC base ratios. One biochemically atypical member of the genus *Micrococcus* (*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 polymerized deoxyribonucleic acid (DNA) from members 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 molecular weights ranging from 8 to 12.8×10^6) 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 micrococcal 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 taxonomic 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 staphylolytic 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 lysostaphin 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 transformation 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 lysostaphin susceptibility by E. T. Thomas (Ph.D. Thesis, The University of Texas at Austin, 1964). The biochemical 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 (Tris)-chloride buffer (6) containing 0.145 M NaCl (Tris buffer) at *p*H 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 Tris 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 mμ with a DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). The relationship used between optical density (OD) at 260 mμ 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 Tris buffer plus 0.015 M trisodium citrate (TSC), *p*H 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-WI and *Sarcina lutea*) were disrupted by mechanical grinding with alumina at 4 C. *Micrococcus varians* and *Micrococcus lyso-deikticus* 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, *p*H 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 4 C.

Molecular weight determination by sedimentation coefficient. The sedimentation coefficient ($S_{20,w}$) was determined at 20 C in a model E analytical centrifuge (Spinco Div., Beckman Instruments. Inc., Palo Alto, Calif.) at 113,210 $\times$ *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 $S_{20,w}$ was found by extrapolating to infinite dilution the reciprocal of the $S_{20,w}$ 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 $S_{20,w}$ 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 Spinco model E analytical centrifuge at 145,656 $\times$ *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 mμ 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 2×10^8.

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
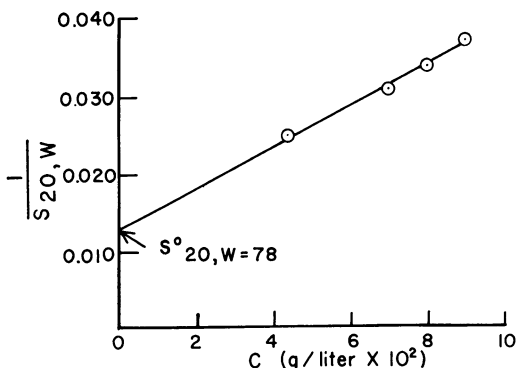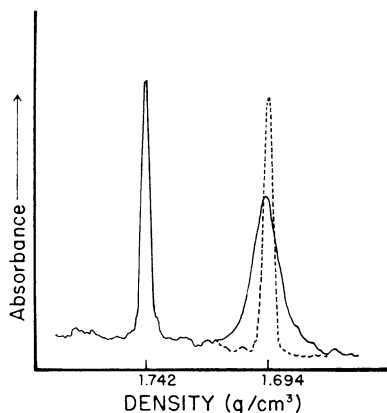

FIG. 2. *Ultraviolet absorbance tracing of DNA bands. The solid line peak at the left is that of the reference DNA, Bacillus subtilis SP 8 bacteriophage. The peaks at the right represent Staphylococcus aureus DNA liberated by mechanical grinding of the cells (solid line) and S. aureus DNA liberated by lysostaphin (dashed line).*

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. candicans*).

The six biochemically atypical strains and *M. candicans* 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. candicans*) 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

FIG. 1. *Reciprocal of the sedimentation coefficient ($S_{20,w}$) as a function of DNA concentration (C).*

TABLE 1. *DNA base ratios (GC) and lysostaphin susceptibility of selected strains of aerobic Micrococcaceae*

Culture no.	Original designation	Coag-ulase	Mole % GC	Lysis by lysostaphin[a]	Indicated genus
1	Staphylococcus aureus FDA 209P	+	35.7	>80	Staphylococcus
2	S. aureus SCL-I[b]	+	35.7	>80	Staphylococcus
3	S. aureus 63A1096	+	35.4	>80	Staphylococcus
4	S. aureus 63A459	+	34.7	>80	Staphylococcus
5	S. aureus 16851	+	35.7	>80	Staphylococcus
6	S. aureus PS 3C	+	35.4	>80	Staphylococcus
7	S. aureus 3189	+	35.7	>80	Staphylococcus
8	S. aureus BP 17	±	34.7	>80	Staphylococcus
9	Staphylococcus BP 1	−	34.7	79	Staphylococcus
10	Staphylococcus BP 9	−	34.7	69	Staphylococcus
11	S. albus Q 12[c]	−	34.7	>80	Staphylococcus
12	S. albus 442[c]	−	35.7	>80	Staphylococcus
13	S. albus UT 174	−	35.7	62	Staphylococcus
14	Staphylococcus BP 3	−	34.7	68	Staphylococcus
15	S. albus SX	−	34.7	24	Staphylococcus
16	M. violagabriellae	−	34.7	71	Staphylococcus
17	M. candicans	−	34.7	>80	Staphylococcus
18	Staphylococcus K-6-WI	−	35.7	0	Staphylococcus
19	M. varians B2158	−	72.4	0	Micrococcus
20	M. lysodeikticus	−	72.0	0	Micrococcus
21	Sarcina lutea 18FL	−	71.9	0	Sarcina

[a] Percentage of reduction in OD at 1 hr.

[b] The Smith encapsulated strain.

[c] 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-WI.

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 *p*H 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 *p*H 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 super-

natant 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 deproteinize 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. 2×10^8) appeared to be significantly higher than the molecular weight (8 to 12×10^6) 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 taxonometric 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. candicans* 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

LITERATURE CITED

1. AULETTA, A. E., AND E. R. KENNEDY. 1966. Deoxyribonucleic acid base composition of some members of the *Micrococcaceae*. J. Bacteriol. **92:**28–34.

2. BAIRD-PARKER, A. C. 1963. A classification of *Micrococci* and *Staphylococci* based on physiological and biochemical tests. J. Gen. Microbiol. **30:**409–427.

3. BAIRD-PARKER, A. C. 1965. *Staphylococci* and their classification. Ann. N.Y. Acad. Sci. **128:**4–25.

4. BERNS, K. I., AND C. A. THOMAS. 1965. Isolation of high molecular weight DNA from *Hemophilus influenzae*. J. Mol. Biol. **11:**476–489.

5. BLOBEL, H. 1961. Isolation and characterization of DNA from a strain of *Staphylococcus aureus*. J. Bacteriol. **82:**425–429.

6. COLOWICK, S. P., AND N. O. KAPLAN. 1955. Methods in enzymology, vol. 1. Academic Press, Inc., New York.

7. DOTY, P., B. B. McGILL, AND S. A. RICE. 1958. The properties of sonicated fragments of DNA. Proc. Natl. Acad. Sci. U.S. **44:**462–438.

8. HUGH, R., AND E. LEIFSON. 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram negative bacteria. J. Bacteriol. **66:**24–26.

9. JORDAN, D. O. 1960. The chemistry of nucleic acids. Butterworth and Co. Ltd., Washington, D.C.

10. KAY, E. L. M., N. S. SIMMONS, AND A. L. DOUNCE. 1952. An improved preparation of Na deoxyribonucleate. J. Am. Chem. Soc. **74:**1724–1726.

11. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.

12. MARMUR, J. 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. **3:**208–218.

13. MARMUR, J., AND P. DOTY. 1961. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J. Mol. Biol. **5:**109–118.

14. MASSIE, R., AND B. H. ZIMM. 1965. Molecular weight of the DNA in the chromosomes of *E. coli* and *B. subtilis*. Proc. Natl. Acad. Sci. U.S. **54:**1636–1641.

15. MASSIE, R., AND B. H. ZIMM. 1965. The use of hot phenol in preparing DNA. Proc. Natl. Acad. Sci. U.S. **54:**1641–1643.

16. SCHILDKRAUT, C. L., J. MARMUR, AND P. DOTY. 1962. Determination of the base composition of DNA from its buoyant density in CsCl. J. Mol. Biol. **4:**430–433.

17. SCHINDLER, C. A., AND V. T. SCHUHARDT. 1964. Lysostaphin: a new bacteriolytic agent for the *Staphylococcus*. Proc. Natl. Acad. Sci. U.S. **51:**414–421.

18. SCHINDLER, C. A., AND V. T. SCHUHARDT. 1965. Purification and properties of lysostaphin—a lytic agent for *Staphylococcus aureus*. Biochim. Biophys. Acta **97:**242–250.

19. SILVESTRI, L. C., AND L. R. HILL. 1965. Agreement between deoxyribonucleic acid base composition and taxometric classification of gram-positive cocci. J. Bacteriol. **90:**136–140.

20. WECHMAN, B. G., AND B. W. CATLIN. 1957. Deoxyribonuclease activity of micrococci from clinical sources. J. Bacteriol. **73:**747–753.