Characteristics of Heavily Mucoid Bacterial Isolates from Fish Pen Slime

T. J. CHAI and R. E. LEVIN

Department of Food Science and Nutrition, University of Massachusetts, Amherst, Massachusetts 01002

Received for publication 14 April 1975

Weakly gram-positive pleomorphic rods isolated from the slime on the storage pen surfaces of fishing trawlers were found to produce extensive capsular material and intensely mucoid colonies. The isolates studied produced highly pleomorphic club-shaped cells indicative of coryneforms. Their DNA base composition ranged from 64.2 to 68.2 mol% guanine plus cytosine on the basis of melting point determinations.

The slime which accumulates on the surface of storage pens on fishing trawlers is salmon colored, viscus, and has an intensely offensive odor. The number of bacteria in such slime has been shown to uniformly exceed 10⁷ per gram of slime (10). The purpose of this study was to isolate and characterize intensely mucoid bacterial isolates from the slime obtained directly from the surfaces of fish storage pens on trawlers.

MATERIALS AND METHODS

Microorganisms. The six intensely mucoid cultures studied were isolated from the storage pen slime from three New England fishing trawlers, using trypic soy broth without dextrose (TSB, Difco) and Pseudomonas agar P (Difco). None of the samples of slime examined contained more than 2% of such organisms.

Biochemical tests and growth studies. All media contained 0.5% NaCl unless otherwise noted. The production of deoxyribonuclease activity and fluorescence were determined as previously described (3, 15). Lipase activity was detected by adding 10% tributyrin to nutrient agar prior to pouring into petri plates. Cultures were heavily spotted onto tributyrin agar plates and inspected for cleared zones of lipase activity after incubation for 2 weeks at 20 C. The production of trimethylamine (TMA) was determined by inoculating tubes containing 8.0 ml of nutrient broth and 0.1% TMA oxide (hydrate, K and K Laboratories) followed by incubation for 3 days at 20 C. Cultures were clarified by centrifugation and the method of Dyer (6) was used for the extraction and measurement of TMA using a standard curve prepared from TMA hydrochloride (K and K Laboratories). Cardinal growth temperatures were determined by inoculating tubes of nutrient broth followed by incubation from −10 to 45 C at intervals of 5 C. Tubes incubated below 0 C contained 5.0% glycerol and 2.0% NaCl which were found to have no deleterious effect on the rate of growth at 20 C. The extent of growth at incubation temperatures above 0 C was determined by recording absorbance values at 660 nm after 24, 48, and 72 h of incubation after vortex mixing of cultures. Absorbance values were determined directly with the culture tubes (14-mm path length) using a Bausch and Lomb Spectronic 20 colorimeter. Capsules were stained using a flagella stain (14) and all other tests were performed as previously described (14).

Construction of non-encapsulated mutants. Mutants were induced with N-methyl-N-nitroso-N'-nitroguanidine (NTG) according to the method of Adelberg et al. (1).

Purification of deoxyribonucleic acid (DNA). The method of Marmur (11) for DNA extraction and purification was used including the use of phenol. DNA samples purified according to the method of Marmur (11) and in single-strength saline citrate buffer were further purified at 2 C by the addition of an equal volume of 90% freshly distilled phenol saturated with 1 M phosphate buffer at pH 7.0. DNA was separated from phenol by centrifugation followed by ethanol precipitation. Residual phenol was removed by precipitating the DNA twice with isopropanol and by passage through a graded alcohol series.

Determination of DNA base composition. DNA melting point determinations were performed as previously described (9). The hypochromic spectral method of Felsenfeld (7) for the determination of DNA base composition was performed simultaneously with melting point (Tm) determinations.

RESULTS

Cytological and biochemical characteristics. A total of six highly encapsulated and intensely mucoid pen slime cultures were isolated. All were weakly gram positive, obligately aerobic rods, lacking flagella. Considerable pleomorphism was observed with young cul-
tures of these isolates. Swollen, club-shaped cells along with short coccobacilli as well as uniform rods were found to be simultaneously present in cultures (Fig. 1). Cells grown in TSB or agar without dextrose were found to be surrounded by an extensive capsule (Fig. 2).

**Biochemical characteristics.** Biochemical reactions (Table 1) indicated that isolates C1 and C4 were metabolically distinguishable from isolates C2, C3, C5, and C6. Each of the six isolates produced catalase and were negative with respect to cytochrome oxidase, gelatin liquefaction, H₂S production, fluorescence, and TMA production. None of the isolates brought about peptonization, reduction, or acidification of litmus milk. All six isolates exhibited lipase and urease activity. Metabolic distinctions among the isolates are presented in Table 1. None of the isolates produced acid from dextrose, galactose, rhamnose, ribose, xylose, sali-

| Test                        | C1 and C4 | C2, C3, C5, and C6 |
|-----------------------------|-----------|--------------------|
| Deoxyribonuclease           | +         | -                  |
| NO₂ reduction               | +         | -                  |
| Citrate                     | -         | +                  |
| Litmus milk (alkaline)      | -         | +                  |
| Tolerance to NaCl (%)       | 3         | 7                  |
| Cardinal growth temp        | 0         | 5                  |
| Minimum (C)                 | 25, a     | 20 b               |
| Optimum                     | 30        | 35                 |
| Maximum                     | 30        | 35                 |

* Isolate C1.  
* Isolate C4.

**Fig. 1.** Pleomorphic cells of culture C1. Dark phase microscopy. ×2,000.

**Fig. 2.** Capsules of cells from culture C1. Capsule stain. ×6,000.
cin, arabinose, lactose, maltose, raffinose, inulin, dextrin, lactate, starch, glycogen, adonitol, or dulcitol. Acid was produced by isolates C2, C3, C5, and C6 from fructose, sucrose, glycerol, sorbitol, mannitol, and inositol. Only isolates C2 and C3 produced acid from mannose. Isolates C1 and C4 failed to produce acid from all the above carbon sources which served to distinguish them from the remaining four. Isolates C1 and C4 grew at 0 C but not at -5 C, while the remaining four isolates grew at 5 C but not at 0 C.

Antibiotic sensitivity. The six isolates were all sensitive to the following antibiotics: bacitracin, 10 μg; erythromycin, 15 μg; furamazone, 100 μg; penicillin, 10 IU; oleandomycin, 15 μg; kanamycin, 5 μg; mandelamine, 3 mg; neomycin, 5 μg; streptomycin, 10 μg; terramycin, 5 μg; and vancomycin, 5 μg. All six isolates were resistant to the following antibiotics: colimycin, 10 μg; furacin, 100 μg; nalidixic acid, 5 μg; polymyxin B, 50 μg; furoxone, 100 μg; sonilyn, 0.25 mg; colistin, 10 μg; nystatin, 100 μg; oxacillin, 1 μg; madribon, 1 mg; gantricin, 2 mg; thiosulfil, 1 mg; sulfadiazine, 1 mg; nafcilin, 1 mg; and sulfamethoxypyridazine, 1 mg. Novobiocin distinguished isolate C1 from C4 (Table 2). Corlett et al. (4) used nine antibiotics to distinguish groups of organisms from foods. The antibiotic sensitivity spectrum of our six mucoid pen slime isolates resembled more closely those obtained by Corlett et al. (4) with bacilli, cytophaga, and micrococi than any of the other groups they studied. They did not include coryneforms in their study.

Extraction of DNA. The six mucoid isolates were found to be completely resistant to disruption by sodium lauryl sulfate and lysozyme. Prolonged ultrasonication also failed to disrupt the cells. An attempt was made to physically strip the capsular material from the cells by passing a viscous cell suspension in distilled water through a glass chromatogram sprayer several times as described by Juni and Heym (8). Cells treated in this manner were still resistant to ethylenediaminetetraacetate, sodium lauryl sulfate and lysozyme.

Rough colony mutants were readily obtained by NTG induction (Fig. 3). Such mutants, however, were still found to be resistant to ethylenediaminetetraacetate plus sodium lauryl sulfate, but were weakly sensitive to lysozyme. The cells of one such mutant, C2-m1, were treated with lysozyme at a final concentration of 0.05% in saline-ethylenediaminetetraacetate and held at 35 C for 12 h followed by a 3-day incubation period at 22 C. When sodium lauryl sulfate was added at this point, a considerable amount of DNA was released which was subsequently purified for Tm determinations. Rough mutants, however, tended to produce low yields of DNA due to the formation of extensive cell clumps, which rapidly settled out of suspension (Fig. 4) and which prevented high cell densities from being attained in shake cultures. The additional problem of weak sensitivity of rough mutants to lysozyme, which required several days of contact with this enzyme to achieve disruption with sodium lauryl sulfate, precluded further use of this approach.

The addition of penicillin at predetermined concentrations to TSB cultures effectively induced spheroplasts which were readily disrupted by ethylenediaminetetraacetate plus sodium lauryl sulfate. The optimum concentration of penicillin was found to vary considerably among the six mucoid isolates (Table 3). Concentrations of penicillin which exceeded the stated values (Table 4) resulted in excessive inhibition of growth without the development of spheroplasts. Concentrations below the stated values failed to sufficiently inhibit cell wall synthesis and resulted in extensive growth without spheroplast formation.

DNA base composition. The Tm and hypochromic spectral methods yielded reasonably close values of mol% of guanine plus cytosine (G+C). Tm values obtained from both penicillin-induced spheroplasts and a rough mutant of isolate C2 were very close (Table 3). No significant difference in Tm values was observed when DNA was purified according to the procedure of Marmur (11) and when samples of DNA were further purified by phenol extraction.

---

**Table 2. Differences in antibiotic sensitivity among mucoid pen slime isolates**

| Antibiotic       | C1 | C2 | C3 | C4 | C5 | C6 |
|------------------|----|----|----|----|----|----|
| Ampicillin, 100 μg | +  |   |   |   |    |    |
| Chloromycetin, 5 μg |   |   |   | + |    |    |
| Dihydrostreptomycin, 10 μg |   | + |   | + |    |    |
| Phenethicillin, 2 μg |   |   |   | - | + |    |
| Novobiocin, 5 μg |   |   |   |   | - | -  |
FIG. 3. Colony appearance of culture C1 on nutrient agar. (A) Wild-type colonies; (B) NTG-induced rough mutant colonies.

extraction, however, did result in consistently lower values of mol% G+C when compared to values obtained from DNA samples purified without phenol with the hypochromic spectral method. The mol% G+C from $T_m$ determinations ranged from 64.2 to 68.2 and from the hypochromic spectral method from 63.3 to 68.9. Both methods for determining DNA base composition yielded notably higher mol% G+C values (68.2 and 68.9%, respectively) with isolate C5.
The relatively rough mutant: the incidence of organisms yielding It on incubation of these isolates resulted in the growth of normal cells without spheroplast formation.

**DISCUSSION**

The isolates reported in this study represent a unique group of highly encapsulated mucoid organisms forming pleomorphic club-shaped cells, suggesting allocation to the genus *Corynebacterium*. Numerous workers have reported the incidence of coryneform bacteria on fish. The relatively low percentage (2%) of such organisms in pen slime does not readily allow them to be considered the major source of such slime. It is possible, however, that a higher percentage may actually exist which we have failed to detect with the methods employed. We used an incubation temperature of 20 °C for initial detection of these isolates. It is possible that an incubation temperature of 5°C may have yielded a higher percentage.

Pelroy et al. (12) found that coryneforms constituted the organisms of greatest abundance on fresh petrale sole. The pleomorphic appearance of our mucoid isolates closely resembles that of isolates studied by Vanderzant et al. (16). Da Silva and Holt (5) discussed the use and misuse of the term "coryneform" and its application to many widely divergent organisms having in common the formation of club-shaped cells. Bousfield (2) concluded that exclusive of the animal pathogens, many type species allocated to the genus *Corynebacterium* are widely divergent and are too dissimilar to the animal pathogenic members of the genus to be included with them.

Rogosa et al. (13) stated in the 8th edition of *Bergey's Manual of Determinative Bacteriology* a mol% G+C range of 57 to 60 for the base composition of DNA from human and animal pathogenic members of the genus *Corynebacterium* obtained from *T*<sub>m</sub> and buoyant density determinations, and a range of 65 to 75 mol%.

**Table 3. DNA base composition of mucoid pen slime isolates**

| Culture | Melting point method | Hypocromic spectral method |
|---------|----------------------|-----------------------------|
|         | *T*<sub>m</sub> (±)  | % G+C  | % G+C (±)  |
| C<sub>1</sub><sup>a</sup> | 95.7 ± 0.04 | 64.3 | 64.6 ± 0.3 |
| C<sub>1</sub><sup>c</sup> | 95.7 ± 0.06 | 64.2 | 63.3 ± 0.9 |
| C<sub>2</sub><sup>a</sup> | 96.4 ± 0.06 | 65.9 | 66.5 ± 1.2 |
| C<sub>2</sub><sup>c</sup> | 96.25 ± 0.18 | 65.6 | 65.6 ± 0.6 |
| C<sub>2</sub>-m<sub>1</sub><sup>c</sup> | 96.07 ± 0.16 | 65.2 | 64.9 ± 0.5 |
| C<sub>3</sub><sup>a</sup> | 96.0 ± 0.01 | 65.0 | 64.1 ± 0.5 |
| C<sub>4</sub><sup>a</sup> | 95.8 ± 0.11 | 64.5 | 66.4 ± 1.1 |
| C<sub>4</sub><sup>c</sup> | 95.9 ± 0.08 | 64.7 | 63.8 ± 0.6 |
| C<sub>5</sub><sup>a</sup> | 97.35 ± 0.43 | 68.2 | 68.9 ± 2.8 |
| C<sub>6</sub><sup>a</sup> | 96.67 ± 0.05 | 66.6 | 65.3 ± 0.4 |
| C<sub>6</sub><sup>c</sup> | 96.63 ± 0.03 | 66.5 | 64.6 ± 0.5 |

<sup>a</sup> Standard deviation (SD) obtained from four determinations with each method.
<sup>b</sup> DNA extracted by the method of Marmur (11).
<sup>c</sup> DNA extracted by the phenol method.
<sup>d</sup> DNA extracted from penicillin-induced spheroplasts.
<sup>e</sup> DNA extracted from NTG-induced rough colony mutant of isolate C2.

**Table 4. Optimum concentration of penicillin in TSB for the formation of spheroplasts from highly encapsulated fish pen slime isolates**

| Isolate | Final concn of penicillin (IU/mL) |
|---------|----------------------------------|
| C1      | 15                               |
| C2      | 310                              |
| C3      | 200                              |
| C4      | 12                               |
| C5      | 9                                |
| C6      | 20                               |
G+C for plant pathogenic isolates. No values are given for nonpathogenic members of the genus Corynebacterium. A range of 60 to 72 mol% G+C is given for the DNA base content of members of the genus Arthrobacter, also considered as coryneforms. On the basis of DNA base composition, pleomorphism, and their weak Gram-positive reaction, the pen slime isolates in this study appear to resemble members of the genera Corynebacterium and Arthrobacter. The range of 64.2 to 68.2 mol% G+C for the isolates in this study lends support for considering them as members of the family Corynebacteriaceae.

ACKNOWLEDGMENTS
This study was supported by Experiment Station Project no. 194 and by Public Health Service grant FD 00153-03 from the Food and Drug Administration.

LITERATURE CITED
1. Adelberg, E. S., M. Mandel, and G. C. C. Chen. 1965. Optimal conditions for mutagenesis by N-methyl-N'-nitrosoguanidine in Escherichia coli K12. Biochem. Biophys. Res. Commun. 18:788–795.
2. Bousfield, I. J. 1972. A taxonomic study of some coryneform bacteria. J. Gen. Microbiol. 71:441–455.
3. Chai, T., C. Chen, A. Rosen, and R. E. Levin. 1968. Detection and incidence of specific species of spoilage bacteria on fish. II. Relative incidence of Pseudomonas putrefaciens and fluorescent pseudomonads on haddock fillets. Appl. Microbiol. 16:1738–1741.
4. Corlett, D. A., Jr., J. S. Lee, and R. O. Sinnhuber. 1965. Application of replica plating and computer analysis for rapid identification of bacteria in some foods. I. Identification scheme. Appl. Microbiol. 15:808–817.
5. De Silva, G. A. N., and J. G. Holt. 1965. Numerical taxonomy of certain coryneform bacteria. J. Bacteriol. 96:921–927.
6. Dyer, W. J. 1945. Amines in fish muscle. I. Colorimetric determination of trimethylamine as the picrate salt. J. Fish. Res. Bd. Can. 6:351–358.
7. Felsenfeld, G. 1968. Ultraviolet spectral analysis of nucleic acids, p. 247–253. In L. Grossman and K. M. Davide (ed.), Methods in enzymology, vol. XII. Academic Press Inc., New York.
8. Juni, E., and G. A. Heym. 1964. Pathways for biosynthesis of a bacterial capsular polysaccharide. IV. Capsule resynthesis by decapsulated resting-cell suspensions. J. Bacteriol. 87:461–467.
9. Levin, R. E. 1972. Correlation of DNA base composition and metabolism of Pseudomonas putrefaciens isolates from food, human clinical specimens and other sources. Antonie van Leeuwenhoek J. Microbiol. Serol. 38:121–127.
10. Levin, R. E., F. M. Sawyer, and P. G. Scheurer. 1966. A limited study on the sanitation of fishing trawler holds. J. Milk Food Technol. 29:336–337.
11. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. 3:208–218.
12. Pelroy, G. A., J. P. Seman, Jr., and M. W. Eklund. 1967. Changes in the microflora of irradiated petral sole (Eopsetta jerdani) fillets stored aerobically at 0.5 C. Appl. Microbiol. 15:92–96.
13. Rogosa, M., C. S. Cummins, R. A. Lelliott, and R. M. Deddie. 1974. Coryneform bacteria, p. 599–632. In R. E. Buchanan and N. E. Gibbons (ed.), Bergey’s manual of determinative bacteriology, 7th ed. The Williams & Wilkins Co., Baltimore.
14. Rosen, A., and R. E. Levin. 1970. Vibrios from fish pen slime which mimic Escherichia coli on violet red bile agar. Appl. Microbiol. 20:107–112.
15. Sadovski, A. Y., and R. E. Levin. 1969. Extracellular nuclease activity of fish spoilage bacteria, fish pathogens and related species. Appl. Microbiol. 17:787–789.
16. Vanderzant, C., P. W. Judkins, R. Nickerson, and H. A. Fitzhugh, Jr. 1972. Numerical taxonomy of coryneform bacteria isolated from pond-reared shrimp (Penaeus aztecus) and pond water. Appl. Microbiol. 23:38–45.