Differentiation of *Serratia* from *Enterobacter* on the Basis of Nucleoside Phosphotransferase Production

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Precoated cellulose thin-layer chromatograms were used to detect the production of guanosine 5' phosphate from guanosine and p nitrophenylphosphate by whole cell preparations. One hundred per cent of *Serratia* (163 strains) and 84% of *E. liquefaciens* (15 of 18 strains) produced the nucleotide. All other *Enterobacter* (23 strains), *Klebsiella* (10 strains), and *E. coli* (10 strains) were negative for the production of this nucleotide. The entire test procedure could be carried out in 4 hr. It is proposed that *E. liquefaciens* is more closely related to *Serratia* than *Enterobacter* and that reclassification of these organisms should be investigated.

In many diagnostic microbiology laboratories, the production of a pink to red pigment by *Serratia marcescens* has been used along with biochemical reactions to separate it from related genera (3, 19). However, most *Serratia* organisms isolated are nonpigmented (4, 6, 9). These strains present a particular problem in identification because they are biochemically and morphologically similar to other *Enterobacteriaceae* groups, namely *Klebsiella* and *Enterobacter*. The deoxyribonuclease (DNase) test has been suggested to differentiate *Serratia* (1, 18) and is satisfactory for separating *Klebsiella* and *Serratia*. However, one species of *Enterobacter*, *E. liquefaciens*, also produces DNase (12) and can have biochemical reactions similar to those of *S. marcescens* (1, 7). Therefore, the DNase test may not be specific enough to use as the differentiating characteristic for *E. liquefaciens* and *S. marcescens*. A method for separating *Serratia* from other bacteria on the basis of production of isomers of mononucleotides by bacterial nucleoside phosphotransferase activity has been suggested (13), and Komagata and Tamagawa (11) have used such an ability to differentiate *Serratia* from *E. liquefaciens*. Their experiments with paper electrophoresis to separate nucleotides produced by *Serratia* and several other organisms indicate that only *Serratia* produces the 5'-isomer (mononucleotide), whereas other *Enterobacteriaceae* produce the 3'-isomer. In their study of nucleoside phosphotransferases in plant and animal tissues, Brawermann and Chargaff (2) maintain that, in bacteria, only the 5'-nucleotides are formed.

Investigators have used various methods including paper electrophoresis (11), partition paper chromatography, ion-exchange chromatography and partition thin-layer chromatography (17) to separate nucleotides. Ion-exchange chromatography has not been shown effective for separation of isomeric nucleotides. Thin-layer chromatography gives sharper separation and requires a shorter time for development than either paper chromatography (16, 17) or paper electrophoresis (17) and is also more sensitive than paper chromatography or paper electrophoresis (17).

The principal objectives of the present study were to separate the nucleotides produced by bacteria by the use of thin-layer chromatography, to determine whether production of the 5'-nucleotide is specific for a large number of *Serratia* organisms, and to develop a fast, easy thin-layer chromatography method appropriate for use in the diagnostic laboratory.

**MATERIALS AND METHODS**

The *Serratia* organisms tested in this study were isolated from clinical specimens by the Diagnostic Microbiology Laboratory of the University of Minnesota Hospitals from 1966 to 1969 and had been maintained on sealed Trypticase Soy Agar (BBL) slants in the dark at room temperature. Eight were recent clinical isolates. The *Klebsiella*, *Enterobacter*, and *Escherichia coli* strains used were also recent isolates. Sheep blood-agar plates or eosin methylene blue plates were used for primary isolation. Triple Sugar Iron Agar (TSI) slants were inoculated with colonies from the primary plates, and biochemical

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tests were inoculated with growth from the TSI slants. In addition to these clinical isolates, nine E. liquefaciens were obtained from the National Communicable Disease Center in Atlanta, Ga., and one strain of E. liquefaciens ATCC 14460 was obtained from the American Type Culture Collection.

**Biochemical studies.** Cytochrome oxidase production was tested for with "PathTec-CO" pathogen differential test papers (General Diagnostics Division, Warner-Chilcott Laboratories, Morris Plains, N.J.). Citrate utilization was determined on Simmons' citrate agar (Difco). Motility, ornithine decarboxylase production, and indole production were determined using motility-indole-ornithine (MIO) medium. MIO medium contained 0.9% decarboxylase medium base (Difco), 0.5% peptone (Difco), 1.0% tryptone (Difco), 0.2% agar (Difco), and 0.5% L-ornithine hydrochloride (Nutritional Biochemicals Corp., Cleveland, Ohio) and was adjusted to pH 6.5 with 1 N HCl. The medium was dispensed in 5-ml amounts to tubes (13 by 100 mm) and was autoclaved for 15 min at 121 °C. The medium was stabbed to the bottom and incubated for 18 to 24 hr at 35 °C. Motility (indicated by turbidity) and ornithine decarboxylase production (indicated by purple color of tube) were read. Kovacs' reagent was added, and the indole reaction was read. Lysine decarboxylase and arginine dihydrolase production were determined by using decarboxylase medium base with 0.5% of each of these amino acids. After inoculation the tubes were overlaid with melted paraffin. A medium control with no amino acid was included for each organism. Purple broth base (Difco) containing various sugars, previously sterilized by filtration, in a concentration of 0.5% was used for fermentation determinations. Tubes were incubated at 35 °C for 10 days. Gelatin liquefaction was determined by using nutrient gelatin prepared by the method of Edwards and Ewing (5); after inoculation the tests were incubated at 25 °C. Deoxyribonuclease production was determined on DNase test base agar (Difco). Twelve organisms were inoculated on one plate. Plates were flooded with 1 N HCl after 18 to 24 hr of incubation at 35 °C. A clear zone indicated DNase production.

**Nucleoside phosphotransferase determination.** Guanosine (anhydrous crystalline), guanosine 5'-phosphate (sodium salt), and guanosine 3' (and 2')-phosphate (sodium salt) were obtained from P-L Biochemicals, Inc., Milwaukee, Wis.; p-nitrophosphorylphosphate (disodium salt) was purchased from Nutritional Biochemicals Corp.

A tube containing 0.3 ml of a 0.2 M solution of p-nitrophosphophosphate in distilled water (prepared daily, 0.5 ml of a 0.08 M solution of guanosine in distilled water (stored at 4 to 10 °C), 0.2 ml of 0.01 N CuSO_{4}, and 1.0 ml of 0.4 N acetate buffer (pH 4.7), prepared by the method of Henry (8), was inoculated with a generous loopful of organism, at an 18- to 24-hr culture grown on a Trypticase Soy Agar slant at 35 °C. The mixture was then incubated at 35 °C for 18 to 24 hr to allow synthesis of the mononucleotide by the phosphotransferase to take place.

In a shortened method, the same reagents were used, but volumes were reduced by one-half. The tubes were inoculated with overnight growth taken from TSI slants. Mixtures were incubated at 35 °C and samples were removed for detection of nucleotide at 30-min intervals up to 4 hr to determine minimal incubation time.

Detection and identification of the nucleotides produced were accomplished by thin-layer chromatography. A 2-ml amount of the above incubation mixtures was applied to a cellulose precoated plastic sheet containing a fluorescent indicator (Polygam Cel 300/UV-254 from Brinkmann Instruments, Inc., Westbury, N.Y.) with a 5- or 10-ml syringe (Hamilton Co., Inc., Whittier, Calif.) in 1-ml portions. The first 1-ml portion was applied and dried with a stream of cold air, and then the second portion was applied and dried. The syringe was rinsed several times with distilled water and then used again to apply ensuing mixtures. The mixtures were applied to a starting line 1.5 cm from the lower edge of the thin layer at 1.8-cm intervals. On each chromatogram, 2-ml portions of a standard mixture of 0.001 M guanosine 5'-phosphate and guanosine 3' (and 2')-phosphate (stored at 4 to 10 °C) was applied. A previously determined positive organism and an uninoculated reagent mixture were also included on each layer. The solvent recommended by Randerath (17) for separation of isomeric nucleotides was used: saturated ammonium sulfate-1 M sodium acetate-isopropanol (80:18:2; v/v/v). A standard Desaga development tank was filled to a depth of approximately 0.7 cm, and the atmosphere in the tank was made fully saturated by covering its walls with filter paper. Development distance was 10 cm in approximately 90 min. Layers were allowed to dry and then were observed under an ultraviolet (UV) light source ("Chromato-vue," Ultraviolet Products, Inc., San Gabriel, Calif.) with long- and short-wave UV simultaneously.

The entire nucleotide determination was repeated on all negatives and questionable positives.

**RESULTS**

The transfer of the phosphate group from p-nitrophosphophosphate resulted in the formation of p-nitrophenol, which formed a spot on the chromatogram along with the two substrates and the mononucleotides. The two isomers of guanosine monophosphate were well-separated with the thin-layer method used. The average $R_f$ values for each substance spotted on the chromatogram were as follows: guanosine 3' (and 2')-phosphate, 0.46; guanosine 5'-phosphate, 0.58; guanosine, 0.38; p-nitrophosphophosphate, 0.53; and p-nitrophenol, 0.11. The mononucleotides could only be observed under the short-wave ultraviolet light, but when both the long and short wave were used simultaneously the p-nitrophosphophosphate appeared green, whereas the guanosine 5'-phosphate appeared light purple. This made the detection of the 5'-mononucleotide
easier, since it was only separated from the p-nitrophenylphosphate by 5 mm.

Organisms were identified as Serratia if they liquefied gelatin within two days and failed to ferment arabinose, raffinose, and rhamnose (5). All 163 strains positively identified as Serratia were indole-negative, grew on Simmons' citrate agar, were lactose-negative, produced an acid reaction throughout TSI slants with little or no gas, were motile, produced DNase, and were cytochrome oxidase-negative. Only 19 of the 163 strains were pigmented on Trypticase Soy Agar, and 7 were ornithine decarboxylase-negative. The 5'-nucleotide was produced by all 163 strains.

Eight other organisms gave biochemical reactions similar to the Serratia organisms, except that two fermented arabinose, raffinose, and rhamnose, five fermented arabinose and raffinose, and one fermented only arabinose, but had a red pigment typical of Serratia. Those strains which fermented all three sugars did not produce the 5'-nucleotide, whereas the other six strains did produce the 5'-nucleotide.

All ten E. liquifaciens strains were DNase-positive. Eight fermented arabinose and raffinose, and two fermented arabinose, raffinose, and rhamnose. Nine produced the 5'-nucleotide; one of the two that fermented all three sugars did not produce the 5'-nucleotide. The ATCC 14460 strain was one of those which produced the 5'-nucleotide.

Strains were identified as Klebsiella if they were indole-negative, citrate-positive, ornithine decarboxylase-negative, nonmotile, and DNase-negative. Enterobacter species, not E. liquifaciens, were indole-negative, citrate-positive, ornithine-positive or negative, motile, and DNase-negative. Differentiation into species was according to Ewing (7). Strains were identified as E. coli on the basis of indole production and failure to grow on citrate agar. All these organisms produced an acid reaction on TSI and were oxidase-negative. The 23 Enterobacter organisms (14 E. cloacae, 8 E. aerogenes, and 1 E. hafniae), 10 Klebsiella strains, and 10 E. coli organisms tested failed to produce the 5'-nucleotide. Slight 3'-nucleotide production was detected in some of these strains.

The 10 strains of Serratia tested by the short method were all positive for the 5'-nucleotide after 120 min of incubation. Two strains from the same patient were negative when incubated for 18 to 24 hr; however, when incubated for 2 hr, they were both positive. Thin-layer chromatography of these 18- to 24-hr mixtures gave unusual spots; no excess p-nitrophenylphosphate was present, whereas all other mixtures which had been called negative contained some p-nitrophenylphosphate at the end of the incubation period. Experiments in which these two strains were incubated with p-nitrophenylphosphate and with the standard 5'-nucleotide under the same conditions as the original test showed that breakdown of both of these substances occur.

Of Enterobacter, 23 strains were negative for the 5'-nucleotide after 2 hr of incubation with the reagent mixture.

When strains of Serratia emulsified in water were applied to the chromatogram, no spots at all were visible.

DISCUSSION

Determination of specific nucleoside phosphotransferase activity by thin layer chromatography is quite simple. Results are easy to read and with the shortened incubation time, differentiation of Serratia from Enterobacter can be achieved within 4 hr. In fact, the shortened method may be advantageous in detecting 5'-nucleotide producers that appear negative after 18 to 24 hr due to the presence of other enzymes such as nucleotidases (14, 15) and phosphatases (15) that use guanosine monophosphates as substrates or compete with the phosphotransferase for p-nitrophenylphosphate. These results indicate the importance of using a shorter incubation time in the investigation of other organisms, particularly Enterobacter. It appears that 5'-nucleotide production rather than sugar fermentation may be a better way to identify Serratia, since one strain with a typical pigment fermented arabinose.

The five organisms which produced the 5'-nucleotide, liquefied gelatin rapidly, produced DNase, and fermented arabinose as well as raffinose would be classified as E. liquifaciens by Edwards and Ewing (5). These strains as well as the eight rhamnose-negative, 5'-nucleotide-positive E. liquifaciens are perhaps more closely related to the Serratia group than to the Enterobacter group. The one rhamnose-positive E. liquifaciens that was positive for 5'-nucleotide production remains a mystery. Except for this one organism, rhamnose negativity seems to correlate with 5'-nucleotide production, and the relationship between these two with regard to Serratia and Enterobacter would merit investigation.

Results obtained in the present study do not agree with those of Brawermann and Chargaff (2). Their experiments indicate that both E. coli and Serratia produce 5'-nucleotides. Data obtained by Katagiri et al. (10, 13) indicate that Serratia is the only genus of the Enterobacteriaceae that produces primarily 5'-nucleotides.
The present study has yielded similar results as have the studies of Komagata and Tamagawa (11). They tested one strain of E. liquefaciens which did not produce the 5'-nucleotide; however, they do not indicate on what biochemical reactions they base classification of their organisms as Serratia and E. liquefaciens. Therefore, it is difficult to evaluate their results in relation to differentiating the two organisms.

One possible explanation for the discrepant results between the above two groups of investigators is that Brawermann and Chargaff examined disrupted cells, whereas the others studied whole-cell preparations, as did we in this study. It may be that Serratia cells are permeable to the substrates, whereas the other organisms are not. So perhaps we have really been determining this permeability rather than the ability of the microorganisms to produce the 5'-nucleotide. Another possibility would be that the phosphotransferase of Serratia is extracellular, whereas those of the other organisms are intracellular. Investigations into these possibilities are presently being carried out.

Production of the 5'-nucleotide by the techniques used in this study correlates well with DNase production, since 98% of the DNase-producers also produced the 5'-nucleotide. This evidence suggests that DNase production is a good criterion on which to base identification of an organism as Serratia in the diagnostic laboratory.

The use of the test for phosphotransferase production described shows the close relationship between S. marcescens and E. liquefaciens and provides another tool for more investigations into their classification. We feel that the production of a nuclease and nucleoside phosphotransferase is a more sound basis for determining the relationship of these two groups of microorganisms than sugar fermentation patterns.

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