Nucleic Acid Enzyme Studies of Nonfermentative Gram-Negative Bacteria Using Thin-Layer Chromatography

MARILYN M. KLEIN and DONNA J. BLAVEC

Department of Laboratory Medicine, University of Minnesota, Minneapolis, Minnesota 55455

Received for publication 10 September 1971

A rapid and easy to perform procedure for determining the nucleic acid enzyme reactions of intact bacterial cells was developed. Overnight organism growth on triple sugar agar was tested for nucleoside phosphotransferase, nucleosidase, and nucleotidase activity. Reaction products were detected by means of thin-layer chromatography and fluorescence. Characteristic patterns were seen with certain strains of nonfermentative gram-negative bacteria, which indicated that these tests could aid in classification and identification of E0-1 group, Pseudomonas multivorans, P. cepacia, P. maltophilia, certain other Pseudomonas species, and Herellea vagincola.

The taxonomic classification of many genera and species in the group of gram-negative, nonfermentative bacteria including Pseudomonas, Mima, Herellea, and Alcaligenes is still somewhat chaotic, and many of these strains isolated from human sources are difficult to identify (3, 17, 18). It was the purpose of this study to investigate and compare the nucleic acid enzyme reactions of intact cells of bacteria in this group by use of fluorescent thin-layer chromatography methods, and to analyze the potential usefulness of such methods as aids in the identification of these bacteria in the clinical laboratory.

MATERIALS AND METHODS

Strains. The 33 reference strains studied are listed in Table 1. An additional 72 nonfermentative strains isolated from clinical specimens were studied. These clinical isolates, identified according to methods described by King (8), were as follows: E0-1 group, 9; P. maltophilia, 17; P. aeruginosa, 14; brown-pigmented Pseudomonas (probable P. aeruginosa), 5; P. fluorescens, 2; P. putida, 13; P. stutzeri, 2; H. vagincola, 9; and group 1b, 1.

Nucleic acid enzyme tests. Guanosine 5'-monophosphate (5'-GMP) and a mixed isomer combination of 5'- and 2'-guanylic acid (5'(&2')-GMP), both obtained from P-L Biochemicals Inc., were dissolved in deionized water. Guanine and uric acid (Nutritional Biochemicals Corp.) and guanosine (P-L Biochemicals Inc.) were dissolved in 1 N NaOH and then diluted to volume with deionized water. Dilute solutions of guanosine, guanine, and uric acid were unstable after several days of storage, so working solutions were freshly prepared from 0.08 M stock solutions. All of the nucleic acid reagents were stored at 4 to 10 C.

The reaction mixture tube for each nucleoside phosphotransferase test contained 0.25 ml of 0.4 N acetate buffer (pH 4.7), 0.1 ml of 0.01 M CuSO₄, 0.25 ml of 0.02 M guanosine, and one tablet of Sigma 104 phosphatase substrate (Sigma Chemical Co.). The phosphatase substrate was equivalent to a final molar concentration of 90 amoles of p-nitrophenyl phosphate per ml. A strain of Serratia marcescens was used as a positive control for nucleoside phosphotransferase activity (4).

The reaction mixture tube for each 5'-nucleotidase test contained 0.125 ml of 0.4 N acetate buffer (pH 4.7), 0.05 ml of 0.01 M CuSO₄, and 0.125 ml of 0.02 M 5'-GMP. The reaction mixture tube for each 3'(5')-nucleotidase test and nucleosidase test contained 0.25 ml of 0.4 N acetate buffer (pH 4.7), 0.1 ml of 0.01 M CuSO₄, and 0.25 ml of the appropriate 0.02 M substrate.

Organisms were grown on triple sugar (TSI) agar slants for 18 to 24 hr at 35 C, except for P. fluorescens and P. fragilis, which both grew better at room temperature. One or two loopfuls of fresh growth were inoculated into each of the previously prepared substrate tubes to make a very dense suspension of organisms. An uninoculated substrate control tube and the inoculated tubes were incubated in a heating block at 37 C. After 3 hr, and again after about 24 hr, 2 µl of each incubation mixture was applied with a 5-µl syringe to the thin-layer sheet; 2-µl aliquots of the appropriate 0.002 M standard samples were also applied to each sheet. Plastic
Above to over Gabriel, nucleoside sheet front in panol photransferase stream, orescent pplied sheets 300/uv-254 Brinkmann Instruments, Inc. MN-Polygram Cel 300/uv-254 sheets with a fluorescent background were used for the nucleoside and nucleotidase tests, and MN-Polygram Cel 300 sheets without fluorescent indicator were used for the nucleoside phosphotransferase tests. After applying 1 μl, the applied spot was thoroughly dried with a cool air stream, and then the second 1 μl was applied and rapidly dried.

The sheets were developed at room temperature in a standard Desaga tank containing about 40 ml of saturated (NH₄)₂SO₄, 1 m sodium acetate-isopropanol (80:18:2, v/v). The chamber was saturated before the sheets were placed into it, and the solvent front was allowed to rise about 11.5 cm, which required about 90 min.

A procedure described by Randerath and Ranerath (20) was used to detect the products of the nucleoside phosphotransferase test. The developed sheet was exposed to concentrated HCl fumes for 4 to 5 min by placing the sheet cellulose side down over a dish, so that the sheet was about 10 to 15 cm above the HCl level. The sheets were then viewed with short-wave ultraviolet light, by use of a Chromato-vue box from Ultra-violet Products, Inc., San Gabriel, Calif. Individually applied standards of guanosine and GMP appeared as bright fluorescent blue spots against a purple background. The p-nitrophenyl phosphate substrate spot was dark purple. For the nucleosidase and nucleotidase tests, the sheets were viewed with simultaneous long- and short-wave ultraviolet light. Individually applied standards of guanine, uric acid, guanosine, and GMP appeared as dark bluish-purple spots against a bright yellow-green background.

### RESULTS

The results of all nucleic acid enzyme tests are summarized in Table 2. The synthesis of 5′-GMP was detected with all P. maltophilia strains within 3 to 7 hr. Under the test conditions used, all other organisms failed to synthesize GMP.

Nucleosidase, 3′(⁄2′)-nucleotidase, and 5′-nucleotidase activity was seen with all E01 group strains, with P. multivorans and P. cepacia, with two strains of P. fluorescens, and with P. pseudomallei and P. mallei. P. maltophilia, P. diminuta, and P. fragi degraded both nucleotides, but were negative for nucleoside. All but one strain of P. aeruginosa and all brown-pigmented Pseudomonas strains degraded the nucleoside; 10 of 15 P. aeruginosa strains and 1 of 5 brown-pigmented Pseudomonas strains degraded 5′-GMP. The group 1b strain and both group IVd strains degraded only 5′-GMP. H. vagincola weakly degraded guanosine, and some strains of P. putida also degraded only guanosine. Neither nucleotidase nor nucleosidase activity was detected with any other strains under the test conditions used. All but one E01 group strain, all P. multivorans strains, the P. cepacia strain, and four P. aeruginosa strains produced detectable uric acid in these tests. P. diminuta degraded only the 3′-GMP and left the 2′-GMP undegraded, but all other strains that were positive in the 3′(⁄2′)-nucleotidase test degraded both of the mixed isomers. When Cu²⁺ was omitted from the nucleosidase and nucleotidase test systems, no degradation of substrates occurred.

The products of nucleic acid enzyme activity were usually the same after 24 hr as they were after 3 hr of incubation. However, with many strains of P. maltophilia, 5′-GMP was no longer detected after 24 hr of incubation in the nucleoside phosphotransferase test because the nucleotidase apparently destroyed the 5′-GMP. Uric acid was sometimes detected after 3 hr and sometimes only after 24 hr of incubation. The 5′-nucleotidase activity was not seen with P. aeruginosa until after 24 hr, and nucleosidase activity was not seen with P. pseudomallei and P. mallei until after 24 hr.

### Table 1. Reference strains

| Organism                  | Designation | Source       |
|---------------------------|-------------|--------------|
| EO-1 group                | A474, A570, A723, A8619, B3720, 9492 | R. E. Weaver |
| *Pseudomonas multivorans* | ATCC 17460, 17616, 17759 | ATCC         |
|                           | NCTC KC84, KC965, B2985 | R. E. Weaver |
| *P. cepacia*              | ATCC 10855 | ATCC         |
| *P. maltophilia*          | ATCC 13637 | ATCC         |
| *P. pseudomallei*         | ATCC 22243 | ATCC         |
| *P. mallei*               | ATCC 15310 | ATCC         |
| *P. fluorescens*          | ATCC 13525 | ATCC         |
| *P. putida*               | ATCC 12633 | ATCC         |
| *P. fragi*                | ATCC 4973  | ATCC         |
| *P. diminuta*             | ATCC 11568 | ATCC         |
| *P. denitrificans*        | ATCC 19244 | ATCC         |
| *P. aeruginosa*           | ATCC 1615  | R. E. Weaver |
| *P. odorns*               | ATCC 15553 | ATCC         |
| *P. stutzeri*             | ATCC 11607 | ATCC         |
| *P. alcaligenes*          | ATCC 14909 | ATCC         |
| Group IVd                 | B1452, B1584-2 | R. E. Weaver |
| *Alcaligenes faecalis*    | ATCC 8750  | ATCC         |
| *Mima polymorpha*         | 7546        | R. E. Weaver |
| *M. polymorpha var. oxidans* | B1882 | R. E. Weaver |
| *Monacella osloensis*     | B1243       | R. E. Weaver |
| *Bordetella bronchiseptica* | ATCC 4617 | ATCC         |
DISCUSSION

Previous studies comparing the nucleoside phosphotransferase activity of many gram-negative bacteria have shown that *S. marcescens* (4, 6, 9–11, 14), many *Flavobacterium* strains (7, 15), *Erwinia herbicola* and some *E. carotovora* strains (10, 11), and most strains of *Enterobacter liquefaciens* (4) were distinctive in their ability to synthesize only 5'-nucleotide rather than 3'-(&2')-nucleotide. *Aeromonas* (14), *Escherichia coli* (6, 7, 9, 10), *Citrobacter* (9), *Enterobacter* (6, 9–11), *Proteus* (6, 9, 14), and *Salmonella* (9, 14) synthesized primarily 3'-(&2')-nucleotide and small amounts of 5'-nucleotide. The many different *Pseudomonas* species tested did not synthesize any nucleotides (14).

Incubation of intact bacterial cells with nucleotide substrates resulted in degradation of nucleotide to nucleoside and inorganic phosphate (14–16), and incubation with nucleoside substrates resulted in degradation of nucleoside to nucleic acid base and pentose (12; L. A. Manson and J. O. Lampen, Fed. Proc. 9:397, 1950). However, few studies have compared the degradative activity on nucleotides and nucleosides of different species or genera of nonfermentative, gram-negative bacteria.

Methods used for detection of nucleic acid products in bacteria have been column or paper chromatography, elution, and spectrophotometric absorption in ultraviolet light. However, thin-layer chromatography was found to be much faster and 50 to 100 times more sensitive than paper chromatography for separation and detection of nucleic acid compounds (21). The use of cellulose sheets with a fluorescent background was the basis of a simple method for detecting and identifying the product of bacterial nucleoside phosphotransferase (4). This method eliminated the elution and spectrophotometric procedures, making the study of these nucleic acid enzyme reactions of bacteria more technically feasible for clinical microbiology laboratories.

Co2+ and a pH higher than was used in our study were found to give optimal nucleotidase activity with the *Enterobacteriaceae* (15, 16), and Cu2+ was inhibitory (1). However, Mitsugi (13) had found that, at pH values less than 5.0, Cu2+ stimulated 5'-nucleotidase and that in

### TABLE 2. Nucleic acid enzyme reactions of 105 nonfermentative gram-negative strains

| Organism                        | No. of strains tested | Nucleoside phosphotransferase | 3'(4&2')-Nucleotidase | 5'-Nucleotidase | Nucleosidase | Uric acid product |
|---------------------------------|-----------------------|-------------------------------|----------------------|----------------|--------------|----------------|
| EO-1 group                      | 15                    | -                             | +                    | +              | +            | w+ (-)        |
| *Pseudomonas multivorans*       | 6                     | -                             | +                    | +              | +            | w+            |
| *P. cepacia*                    | 1                     | -                             | +                    | +              | w+           |               |
| *P. fluorescens*                | 3                     | -                             | + (-)                | +              | + (-)        | -             |
| *P. pseudomallei*               | 1                     | +                             | +                    | w+            | -            |               |
| *P. mallei*                     | 1                     | -                             | +                    | w+            | -            |               |
| *P. maltophilia*                | 19                    | +                             | +                    | 1              | -            |               |
| *P. diminuta*                   | 1                     | +                             | 1                    | -              | -            |               |
| *P. fragi*                      | 1                     | -                             | +                    | -              | -            |               |
| *P. aeruginosa* (brown-pigmented) | 15             | -                             | -                    | w+, -         | + (-)        | (w+)          |
| Group IVd                       | 5                     | -                             | w+,-                | -              | -            |               |
| Group Ib                        | 2                     | -                             | +                    | -              | -            |               |
| *Herellea vaginicola*           | 9                     | -                             | -                    | w+            | -            |               |
| *P. alcaligenes*                | 1                     | -                             | +                    | -              | -            |               |
| *P. odorans*                    | 1                     | -                             | -                    | -              | -            |               |
| *P. putida*                     | 14                    | -                             | -                    | +,-           | -            |               |
| *P. stutzeri*                   | 3                     | -                             | -                    | +             | -            |               |
| *P. denitrificans*              | 1                     | -                             | -                    | -             | -            |               |
| *Alcaligenes faecalis*          | 1                     | -                             | -                    | -             | -            |               |
| *Bordetella bronchiseptica*     | 1                     | -                             | -                    | -             | -            |               |
| *Mima polymorpha*               | 1                     | -                             | -                    | -             | -            |               |
| *M. polymorpha var. oxidans*    | 1                     | -                             | -                    | -             | -            |               |
| *Moraxella osloensis*           | 1                     | -                             | -                    | -             | -            |               |

* Key: - = negative, w = weak, + = positive, ( ) = reaction of an occasional strain.

* The 3'-nucleotide was degraded and the 2'-nucleotide was not degraded.
the presence of Cu\(^{2+}\) 3'&2')-nucleotidase activity was also stimulated, with an optimal pH of 5. Rapid degradation of 5' and 3'&2')-nucleotides occurred in our study with EO-1 group and P. maltophilia using Cu\(^{2+}\) and pH 4.7, and no degradation occurred without Cu\(^{2+}\). A comparative study of different organisms was done with the same experimental conditions. Since the 5'-nucleotidase activity of P. aeruginosa was weak, the experimental conditions used were probably not optimal for that species. This also appeared to be likely for the nucleosidase activity of H. vaginocola, P. pseudomallei, and P. mallei. It might be fruitful to determine optimal experimental conditions with each species for all of these nucleic acid enzyme reactions and to compare the reactions of the enzymes extracted from the cells with those of the intact cells.

In the present study, the lack of synthesis of nucleotide by many Pseudomonas species agreed with the results of Mitsugi et al. (14). The one P. maltophilia strain tested by them was negative, but this could be due to differences in methodology. The strong nucleotidase activity of P. maltophilia may have resulted in degradation of the 5'-GMP produced so that the nucleoside phosphotransferase test appeared negative after prolonged incubation.

The similar patterns obtained with EO-1 group, P. multivorans, and P. cepacia substantiate the work of Pickett and Pedersen (19) and Ballard et al. (2), who presented evidence that these three organisms should all be classified as P. cepacia.

With the few strains tested, the results indicated that nucleosidase activity of H. vaginocola could aid differentiation of this species. The nonmotile organisms called H. vaginocola in this study oxidized lactose and dextrose, but not mannitol. Therefore, they would be comparable to those organisms called Bacterium anitratrum by Hugh and Reese (5).

Although only single or few strains of some species were tested, when larger numbers of a particular organism were used, they showed quite uniform reactions. The results of previous studies, as well as those of the present study, indicated that the investigation of nucleic acid enzyme reactions of intact bacterial cells might be a potentially useful phenotypic taxonomic tool that has not been extensively explored. The results encourage further studies, using known organisms identified with reference methods. The thin-layer chromatography method is a simple method technically, with no need for expensive equipment. It can easily be performed in a routine clinical laboratory, and in many cases the results can be available 5 hr after growth has occurred on a TSI slant.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service training grant AHT 70-059 from the Department of Health, Education, and Welfare.

We thank R. E. Weaver of the Center for Disease Control, Atlanta, Ga., for sending some of the cultures used in this study.

LITERATURE CITED

1. Anraku, Y. 1964. A new cyclic phosphodiesterase having a 3'-nucleotidase activity from Escherichia coli B1. Purification and some properties of the enzyme. J. Biol. Chem. 239:3412–3419.

2. Ballard, R. W., et al. 1970. Taxonomy of the aerobic pseudomonas: Pseudomonas cepacia, P. marginata, P. aliticoa and P. carophylli. J. Gen. Microbiol. 16:199–214.

3. De Ley, J. 1964. Pseudomonas and related genera. Annu. Rev. Microbiol. 18:17–46.

4. Durand, A. M., and D. J. Blazevic. 1969. Differentiation of Serratia from Enterobacter on the basis of nucleoside phosphotransferase production. Appl. Microbiol. 19:134–157.

5. Hugh, R., and R. Reese. 1968. A comparison of 120 strains of Bacterium anitratrum Schaub and Hauber with the type strain of this species. Int. J. Syst. Bacteriol. 18:207–229.

6. Katagiri, H., et al. 1963. Microbial formation of nucleotides. Agr. Biol. Chem. 27:469–470.

7. Katagiri, H., et al. 1964. Bacterial synthesis of nucleotides. I. Nucleoside phosphotransferase of Escherichia coli. Agr. Biol. Chem. 28:577–585.

8. King, E. O. 1964. The identification of unusual pathogenic gram negative bacteria. Center for Disease Control, Atlanta, Ga.

9. Komagata, H., and Y. Tamagawa. 1966. Nucleoside phosphotransferase test as an aid to differentiation of Serratia marcescens from related bacteria. J. Gen. Appl. Microbiol. 12:191–193.

10. Komagata, K., Y. Tamagawa, and H. Iizuka. 1968. Characteristics of Erwinia herbicola. J. Gen. Appl. Microbiol. 14:19–37.

11. Komagata, K., Y. Tamagawa, and M. Kocur. 1968. Differentiation of Erwinia amylovora, Erwinia carotovora, and Erwinia herbicola. J. Gen. Appl. Microbiol. 14:39–46.

12. Lutzmann-Mann, C. 1936. CXCV. The decomposition of adenosine compounds by bacteria. Biochem. J. 30:1405–1412.

13. Mitsugi, K. 1964. Bacterial synthesis of nucleotides. IV. Effects of inhibitors and metallic ions on the nucleoside phosphotransferase. Agr. Biol. Chem. 28:669–677.

14. Mitsugi, K., et al. 1964. Bacterial synthesis of nucleotides. II. Distribution of nucleoside phosphotransferases in bacteria. Agr. Biol. Chem. 28:586–600.

15. Neu, H. C. 1968. The 5'-nucleotidases (uridine diphosphate sugar hydrolases) of the Enterobacteriaceae. Biochemistry 7:3766–3773.

16. Neu, H. C. 1968. The cyclic phosphodiestereses (3'-nucleotidases) of the Enterobacteriaceae. Biochemistry 7:3774–3780.

17. Pickett, M. J., and C. R. Mancsclark. 1970. Nonfermentative bacilli associated with man. I. Nomenclature. Amer. J. Clin. Pathol. 54:155–163.

18. Pickett, M. J., and M. M. Pederson. 1970. Nonfermenta-
tive bacilli associated with man. II. Detection and identification. Amer. J. Clin. Pathol. 54:164–177.
19. Pickett, M. J., and M. M. Pedersen. 1970. Characterization of saccharolytic nonfermentative bacteria associated with man. Can. J. Microbiol. 16:351–362.
20. Randerath, E., and K. Randerath. 1964. Resolution of complex nucleotide mixtures by two-dimensional anion-exchange thin-layer chromatography. J. Chromatogr. 16:126–129.
21. Randerath, K., and E. Randerath. 1964. Ion-exchange chromatography of nucleotides on poly (ethyleneimine)-cellulose thin layers. J. Chromatogr. 16:111–125.