

## Short-Chain Acids of *Pseudomonas* Species Encountered in Clinical Specimens

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Received for publication 16 November 1973

The short-chain acids of 36 strains of *Pseudomonas* grown on Trypticase soy agar were determined by gas-liquid chromatography. Distinct acid profiles were observed for each of the eight species tested. Propionic, isobutyric, and isovaleric acids were the principal acids detected in media extracts of *P. maltophilia*, *P. cepacia*, *P. pseudoalcaligenes*, *P. diminuta*, and *P. vesiculare*. The presence and relative amounts of the isobutyric and isovaleric acids clearly distinguished *P. maltophilia*, *P. pseudoalcaligenes*, and *P. cepacia* from other species. *P. diminuta* could be distinguished from *P. vesiculare* by the production of glutaric acid; *P. testosteroni* was the only species tested which produced relatively large amounts of phenylacetic acid.

The increasing importance of *Pseudomonas* species in human infections has emphasized the need for accurate and reliable criteria for their identification (5, 6). The extensive battery of biochemical tests presently used for the identification of clinical isolates is cumbersome, and the results are often ambiguous (3, 4, 13, 14). Although tests such as substrate utilization (1, 16), DNA homology (12), and nucleic acid enzyme assays (7) are available for characterization of pseudomonads, their application in a routine laboratory is time consuming and awkward for handling a large number of isolates. For these reasons, a search for additional criteria which might lead to the development of rapid, specific, and reliable tests for distinguishing the various pseudomonads is warranted.

We have investigated differences in the cellular components and metabolic products of various *Pseudomonas* species by gas-liquid chromatography (GLC). Our data show that some of the clinically important species of *Pseudomonas* may be identified by the fatty acid content of the cells (10, 11, 15). The following report contains evidence that species of *Pseudomonas* may also be identified by differences in short-chain acid products. These differences are distinct, even among pseudomonads which are difficult to identify by conventional tests. An accurate, rapid, and sensitive GLC procedure was used to measure the short-chain acids.

### MATERIALS AND METHODS

**Cultures.** Eight ATCC strains and 28 clinical isolates of *Pseudomonas* were analyzed for short-chain acid products. The ATCC strains were gifts

from R. Y. Stanier (16) and N. J. Palleroni (12), University of California, Berkeley, and are designated as follows: *P. alcaligenes*, ATCC 14909; *P. acidovorans*, ATCC 15668; *P. testosteroni*, ATCC 11996, ATCC 17510; *P. maltophilia*, ATCC 13637; *P. cepacia*, ATCC 17759; *P. diminuta*, ATCC 11568; and *P. vesiculare*, ATCC 11426. The clinical isolates, which were obtained from the Clinical Microbiology Laboratory, Center for Disease Control, originated from different geographical locations where they were cultured from a variety of clinical materials (17).

**Growth of cells and extraction of acids.** For short-chain acid analysis, cells were grown in Trypticase soy broth (Baltimore Biological Laboratories, Cockeysville, Md.) for 24 h at 37 C. After incubation, approximately 0.1 ml of the broth culture was spread over the surface of a 15- by 85-mm plate containing 10 ml of Trypticase soy agar (TSA; BBL). Duplicate plates of each culture were prepared and processed. Plates were incubated overnight at 37 C, and then acidified with 1.0 ml of 50% H<sub>2</sub>SO<sub>4</sub> (vol/vol). After 10 min, the agar was cut into small pieces, transferred to a 50-ml screw-cap test tube, and heated for 1 h at 80 C. The cooled agar was then divided into equal portions, and each portion was extracted with 20 ml of diethyl ether (Fisher Scientific, Inc., Fair Lawn, N.Y.). The ether layer was transferred to a small beaker and evaporated to 0.4 ml under a gentle stream of nitrogen gas. Anhydrous sodium sulfate crystals were added to remove any traces of water. The concentrated extract was transferred to a 13- by 100-mm screw-cap tube and further evaporated to 0.2 ml. One of the concentrated extracts was derivatized and analyzed by GLC on a relatively nonpolar column (Dexsil); the second extract was analyzed directly by GLC on a polar column (Resoflex) without derivatization. Each culture was tested in duplicate with two TSA plates; an uninoculated TSA plate, which was processed in a manner identical to that used for the cultures, served as a control.

**Derivative formation.** To esterify acids extracted from the agar, we added approximately 0.1 ml of nanograde quality chloroform (Matheson, Coleman, and Bell, Rutherford, N.J.) and 0.1 ml of 14% (wt/vol) Borontrifluoride Butanol reagent (BF<sub>3</sub> butanol, Applied Science, State College, Pa.) to the concentrated ether extract. This mixture was allowed to stand in an open tube until the ether had completely evaporated. The tube was then closed with a Teflon-lined cap and heated for 4 min at 100 C. The resulting butyl esters were cooled to room temperature and acetylated with 0.1 ml of trifluoroacetic anhydride (Pierce Chemical Co., Rockford, Ill.) in the presence of 5  $\mu$ liters of pyridine, which was used as a catalyst. The reaction mixture was then heated for 6 min at 100 C and cooled; then 0.2 ml of distilled water was added. The tube was shaken vigorously and then allowed to stand approximately 30 min. The chloroform layer was carefully removed with a Pasteur pipette to a clean, dry tube, and the washing procedure was repeated. The water layer was removed immediately, and the chloroform layer was added to that of the first extraction. The combined chloroform fraction was evaporated to near dryness under N<sub>2</sub>. A final volume of 0.2 ml of chloroform was carefully measured into the tube in order to achieve equivalent volumes for each sample. For routine GLC analysis, 1  $\mu$ liter of the chloroform layer was injected onto the GLC column.

**GLC.** The analysis of butyl esters was accomplished on a gas chromatograph (model 990, Perkin-Elmer, Norwalk, Conn.) equipped with a hydrogen flame detector and a disk integrator recorder. The instrument contained a 3.66-m (12-ft) coiled glass column (inside diameter 4.03 mm) that was packed with 15% Dexsil 300 GC coated on 80/100 mesh, acid-washed, DMCS-treated Chromosorb W (Analabs, North Haven, Conn.). After the sample was injected, the column was held at 90 C for 6 min and then temperature programmed to 265 C at a rate of 6 C/min. The time interval (32 min) permitted the elution of the butyl ester derivatives of acids ranging from formic (C<sub>1</sub>) to heptanoic (C<sub>7</sub>). Lactic, succinic, phenylacetic, and glutaric acids also eluted within 32 min.

Samples were also analyzed on a 4.06-mm (inside diameter) by 2.4-m (8-ft) column of Resoflex Lac-1-R-296 (Burrell Corp., Pittsburgh, Pa.), a polar phase which has been used by other investigators for the identification of short-chain acids produced by microorganisms (9). The column temperature was held at 120 C for 3 min and then increased to 155 C at a rate of 5 C/min. The identification of short-chain acids was achieved by comparing retention times of both free and esterified acids in the sample to those of highly purified acid standards (Chemical Service, Inc., Media, Pa.; Eastman Organic Chemicals, Rochester, N.Y.). Retention time comparisons were made on both columns.

## RESULTS AND DISCUSSION

Identification of some of the species used in this study by conventional tests was difficult even though 40 or more tests were used in the

identification scheme (17). This difficulty arises from the fact that some species are quite unreactive in many of these tests. In addition, weak or variable reactions are often encountered. Analysis of short-chain acids produced during growth on TSA, however, showed clear, distinct differences among the various species. An example of these differences is illustrated in the chromatograms in Fig. 1. The bottom chromatogram shows that isobutyric and isovaleric acids are the major products produced by *P. pseudoalcaligenes*, whereas these two acids were absent or present in only trace amounts in *P. alcaligenes* (center). An additional difference between the two species was the presence of an unidentified peak at 23.5 min in *P. pseudoalcaligenes* which was consistently absent in *P. alcaligenes*. Both species produced relatively small amounts of phenylacetic acid.

The top chromatogram is a sample of the uninoculated control TSA medium and shows three major peaks labeled C2, M1, and M2. The relative size of these peaks remained essentially constant among different plates of control medium which were processed daily along with cultures. The identity of the C2 peak was established by GLC and by mass spectrometry as acetic acid. The M1 and M2 peaks were not identified, but on the basis of their solubility characteristics and their conversion to esters under normal esterification procedures, they also appear to be acids. Whatever their nature, it is interesting to note that certain species apparently were able to use these components as evidenced by a marked decrease in peak areas compared with those of controls. For example, the size of the M2 peak was comparable in the media control and the *P. alcaligenes* culture, but the peak was completely absent in the *P. pseudoalcaligenes* cultures (Fig. 1). The acetic acid peak was also markedly reduced by several species including *P. alcaligenes* and *P. pseudoalcaligenes* (Fig. 1). The use of acetate as the sole source of carbon and energy has been reported for various pseudomonads (3, 16).

The short-chain acids of other *Pseudomonas* species are listed in Table 1. No acids were detected from strains of *P. acidovorans*. Only phenylacetic acid was produced by *P. testosteroni* and *P. alcaligenes*. These two species could be distinguished from each other, however, on the basis of relatively large amounts of phenylacetate from *P. testosteroni* and small amounts from *P. alcaligenes*. These data are consistent with earlier studies which showed phenylacetate as the major acid from *P. testosteroni* after growth on heart infusion agar (2). The distinguishing feature of *P. maltophilia*

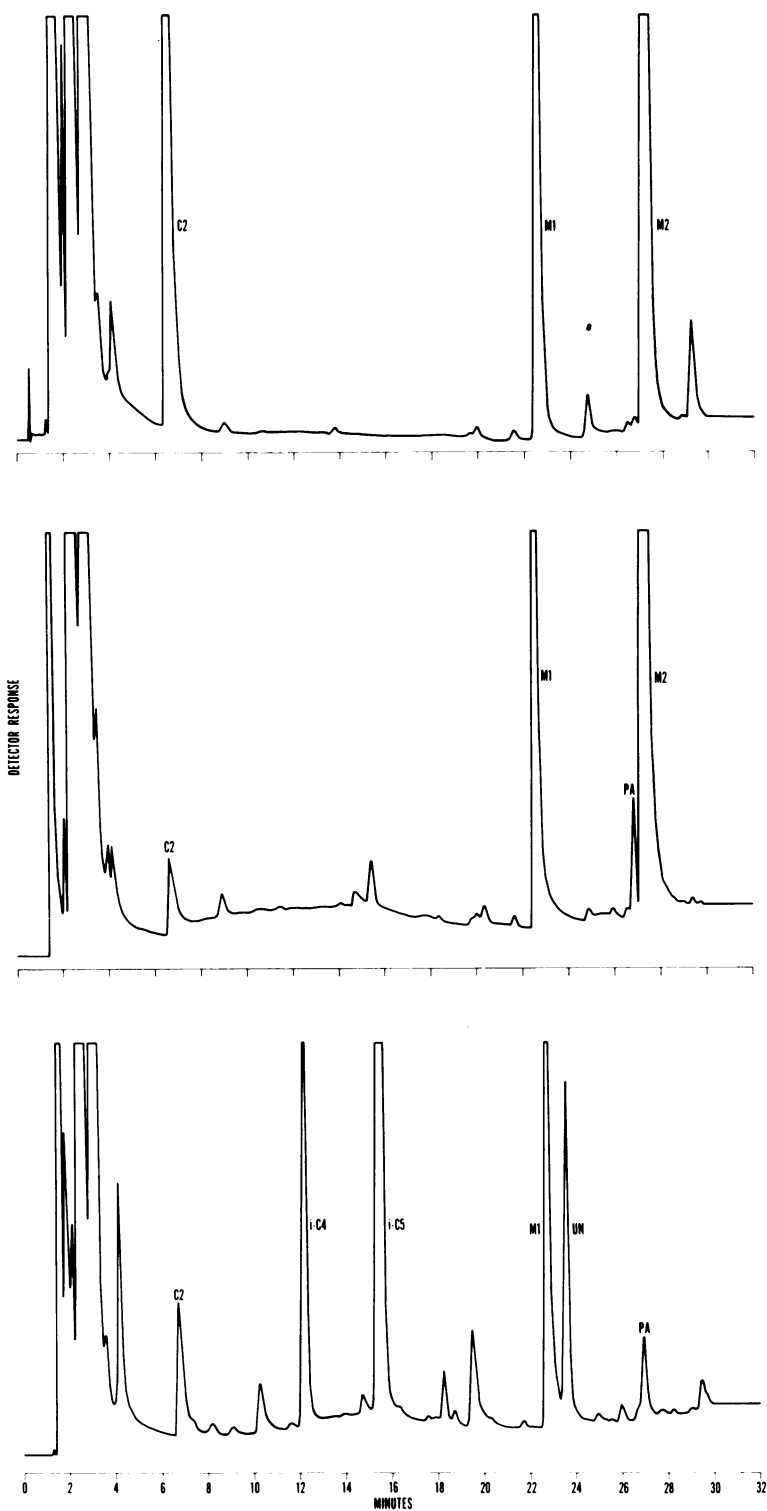


FIG. 1. Gas chromatograms of esterified short-chain acids from *P. pseudoalcaligenes* (bottom), *P. alcaligenes* (center), and uninoculated Trypticase soy agar (top) run on a 15% Dexsil 300 GC column. C2, iC4, iC5, M1, M2, UN, and PA refer to acetic acid, isobutyric acid, isovaleric acid, three unidentified compounds, and phenylacetic acid, respectively.

TABLE 1. Short-chain acids of *Pseudomonas* species isolated from clinical specimens

Organism	Acid <sup>a</sup>						
	C <sub>2</sub>	C <sub>3</sub>	iC <sub>4</sub>	iC <sub>5</sub>	Lactic	Phenylacetic	Glutaric
<i>Pseudomonas</i>							
<i>acidovorans</i> (5) <sup>b</sup>	1 <sup>c</sup>	—	—	—	—	—	—
<i>testosteroni</i> (5)	2	—	—	—	—	4	—
<i>alcaligenes</i> (5)	1	—	—	—	—	1	—
<i>pseudoalcaligenes</i> (5)	1	—	4	12	—	1	—
<i>maltophilia</i> (3)	8	—	—	4	—	1	—
<i>cepacia</i> (3)	8	—	3	—	3	T	—
<i>diminuta</i> (5)	12	12	12	12	—	1	4
<i>vesiculare</i> (5)	12	12	12	12	—	T	—
Uninoculated TSA <sup>d</sup> medium	12	—	—	—	—	—	—

<sup>a</sup> C<sub>2</sub>, acetic acid; C<sub>3</sub>, propionic acid; iC<sub>4</sub>, isobutyric acid; iC<sub>5</sub>, isovaleric or 2-methylbutyric.

<sup>b</sup> Number in parenthesis is number of strains.

<sup>c</sup> Numbers refer to relative areas of peaks. T, Peak with less than 10% of full scale deflection; 1, 10 to 39%; 2, 40 to 69%; 3, 70 to 90%; 4, peak with full-scale deflection or greater; 8, peak with twice the area of 4; 12, peak with three times the area of 4; and —, acid not detected.

<sup>d</sup> TSA, Trypticase soy agar. Routine GLC analysis of butyl esters was made by using a 15% Dexsil column. The identity of the acids was confirmed with a Resoflex column.

was the presence of relatively large amounts of isovaleric acid and the absence of other acids, except for small amounts of phenylacetate. *P. cepacia* was the only species which produced detectable amounts of lactic acid. Relatively large amounts of isobutyric and isovaleric acids were produced by *P. pseudoalcaligenes*, *P. diminuta*, and *P. vesiculare*. *P. pseudoalcaligenes* could be distinguished from the latter two species by its failure to produce propionic acid. *P. diminuta* was easily distinguished from *P. vesiculare* and from other species by the production of glutaric acid. Identification of this acid by GLC and by mass spectrometry is detailed in another report. The acetate which was present in TSA medium was used to varying degrees by all species except *P. diminuta* and *P. vesiculare* (Table 1).

The above data indicate that analysis of short-chain acids is a useful additional means for identification of various pseudomonads. All strains within a species produced similar short chain acid profiles. Moreover, each strain when tested two or more times through the entire procedure (growth, extraction, GLC) gave similar results. The extraction, derivatization, and GLC steps can be accomplished without difficulty by technical personnel. A complex mixture of 12 or more acids can be determined simultaneously within 37 min on a 15% Dexsil column (8). The excellent temperature stability of Dexsil indicates its usefulness for multiple analysis of short-chain acids from microorganisms.

#### ADDENDUM IN PROOF

Since this manuscript was submitted for publication, additional strains of each species have been tested for short-chain acids. The results obtained were identical to those presented in Table 1, except that two of five clinical isolates of *P. cepacia* produced only trace amounts of lactic and isobutyric acids. Studies are in progress to determine if these results are due to strain variation, media composition, or slight environmental changes.

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