

## Headspace Analysis of Volatile Metabolites of *Pseudomonas aeruginosa* and Related Species by Gas Chromatography-Mass Spectrometry

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Gas chromatographic-mass spectrometric analysis of headspace volatiles was performed on cultures of 11 strains of *Pseudomonas aeruginosa* and 1 strain each of *Pseudomonas cepacia*, *Pseudomonas putida*, *Pseudomonas putrefaciens*, *Pseudomonas fluorescens*, and *Pseudomonas maltophilia*. All strains of *Pseudomonas aeruginosa* produced a distinctive series of odd-carbon methyl ketones, particularly 2-nonanone and 2-undecanone, and 2-aminoacetophenone. The other strains failed to produce 2-aminoacetophenone. Two sulfur compounds, dimethyldisulfide and dimethyltrisulfide, were present in all strains of *P. aeruginosa* and in variable amounts in other species. Butanol, 2-butanone, 1-undecene, and isopentanol were also detected in *P. aeruginosa* cultures.

The application of gas chromatography (GC) to the identification of microorganisms has received widespread attention (27). The techniques which have been developed are based on an analysis of either the unique metabolites of a given organism or on its individual structural components. Culture extracts have revealed specific amines for clostridia (5), hydroxy acids and fatty acids for *Neisseria* (4, 5), and aliphatic acids for anaerobes (12, 13). Bacterial cell wall preparations examined for unique fatty acid profiles have included pseudomonads (29, 37). Pyrolysis-GC of whole cells of clostridial bacteria gives identifiable differences in the observed fragmentation patterns (33).

More recently, headspace analysis techniques have been developed to sample directly the volatile metabolites produced in culture. These techniques either have involved sampling the culture headspace directly, as in the case of aliphatic acids (2, 21) and amines (21) for various anaerobes and sulfides for *Proteus* (14), or have made use of volatile concentration methods for *Pityrosporum* and pseudomonads (18, 22, 28). Headspace analysis has also been applied to samples of human body fluids including saliva (17), urine (25, 39), and blood serum (24).

Whereas anaerobic organisms appear to be limited in their volatile metabolic products (13, 32), aerobic organisms have the potential for generating a wide variety of unique metabolites. Various odors have been associated with some of these organisms, and in a few cases unique chemicals or groups of chemicals have been identified. Pyrazines (*Pseudomonas taetrolens*

and *P. perolens*), 2-aminoacetophenone (*Pseudomonas aeruginosa*),  $\gamma$ -lactones (*Pityrosporum ovale* and *Sporobolomyces odorosus*),  $\delta$ -lactone (*Trichoderma viride*), esters (*Saccharomyces cerevisiae*), and monoterpenes have been associated with aerobic bacteria, yeasts, and fungi (9, 10, 18, 19, 28, 31, 35).

We have applied headspace analysis to cultures of *P. aeruginosa*, a pathogen which has been directly implicated in bacterial infections of burn patients and in lung infections of individuals with cystic fibrosis (20, 30). The pathogen is a transient skin resident in some individuals and is readily isolated in hospital environments (6, 30). The total volatile profiles of various strains of *P. aeruginosa* have been examined and compared with the profiles of other pseudomonads in the hope of developing a rapid and sensitive detection system of this species in bacterial infections of body fluids.

### MATERIALS AND METHODS

**Microorganisms and culture media.** *P. aeruginosa* American Type Culture Collection (ATCC) strains 19660, 7700, 17423, 27313, 27312, 27316, 17429, 17423, 7701; Center for Disease Control (CDC) strains 9104, 9171; *Pseudomonas putrefaciens* ATCC strain 8073; *Pseudomonas maltophilia* ATCC strain 13673; *Pseudomonas fluorescens* ATCC strain 13525; *Pseudomonas cepacia* ATCC strain 17759; and *Pseudomonas putida* ATCC strain 12633 were grown on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) in single-neck, round-bottom flasks enclosed with cotton plugs and incubated at 37°C for 24 h.

**Sampling and analysis procedure.** Round-bottom flasks (250 ml) were fitted with a nitrogen inlet

tube and an outlet tube which was attached to a stainless steel tube (6 by 1/8 in. [ca. 15.2 by 0.31 cm]) containing 70 mg of Tenax GC (Applied Science), a porous polymer with good absorption properties and thermal stability. The headspace of the culture was swept with nitrogen at a flow rate of 80 ml per min for 2 h at 37°C. The collected volatiles were then back-flushed with heating (220°C for 10 min) onto the first 15 cm of the GC column, which was cooled with dry ice. The volatiles were then separated and identified by combined GC-mass spectrometry (GC-MS). The GC column (10 ft. [ca. 3 m] by 2 mm) was Pyrex 20 M Carbowax on 80/100 Gas-Chrom Q programmed at 70°C (4 min) and 70 to 220°C (4°C per min). The GC-MS system was a Perkin-Elmer 990 GC interfaced with a Watson-Biemann separator to a Hitachi/Perkin-Elmer RMU-6L mass spectrometer. The mass spectrometer conditions included an ionizing voltage of 70 eV; a source temperature of 200°C, and a temperature of interface of 260°C. The mass spectra were recorded on a B and F model 3006 oscillographic recorder and interpreted manually. Individual components were identified by comparison of their fatty acid ethyl ester retention indexes on a Carbowax column with those previously reported (36, 38) and by comparison of retention times and mass spectral data with authentic samples.

The procedure for the direct headspace analysis of cultures for volatile sulfur metabolites employed a GC equipped with a flame photometric detector. It is essentially the same system as that described by Tonzetich, except that a Perkin-Elmer 3920B GC with a 6-port injector valve (Valco Instruments Co., Houston, Tex.) and all Teflon lines were used (34).

## RESULTS

The volatile profile of *P. aeruginosa* is shown in Fig. 1. The distinctive feature in the profile of all strains of *P. aeruginosa* is the presence of a series of odd-carbon-numbered methyl ketones, particularly 2-nonanone and 2-undecanone. Table 1 shows the intensity of the m/z 58 peak in

the mass spectra of these two components for various strains of *P. aeruginosa*. The characteristic m/z 58 ion in the mass spectra of methyl ketones is formed by the well-known "McLafferty rearrangement" (7). Consequently, increased sensitivity and selectivity for the presence of methyl ketones in pure and mixed cultures can be obtained by single-ion monitoring of the GC effluent at this mass in combination with fatty acid ethyl ester retention indexes (Fig. 2). *P. maltophilia* and *P. putida* showed reduced amounts of 2-nonanone and trace levels of 2-undecanone.

Two sulfur metabolites, dimethyldisulfide (DMDS) and dimethyltrisulfide (DMTS), are present in all strains of *P. aeruginosa* and in variable amounts in the other species. Though the peak for DMTS was overlapped with that for 2-nonanone, its presence could be determined from its unique mass spectrum (m/z 126, 94, 79, 64, 61). In addition, the presence of methyl mercaptan could be shown by direct headspace sampling (10 ml) of the cultures (Fig. 3). No DMTS and only trace amounts of DMDS could be found without headspace concentration. A previous report indicates the presence of DMDS, but not methyl mercaptan, in cultures of *P. aeruginosa* (15).

2-Aminoacetophenone (2-AA) was previously reported to be present in ether extracts of *P. aeruginosa* cultures (10). This compound was routinely detected by the headspace sampling technique reported here for all strains of *P. aeruginosa*.

Butanol, toluene, 2-butanone, 1-undecene, and isopentanol are the other components routinely observed in the *Pseudomonas* cultures. Long incubations (7 days) and collection times (17 h) showed basically the same profile for *P.*

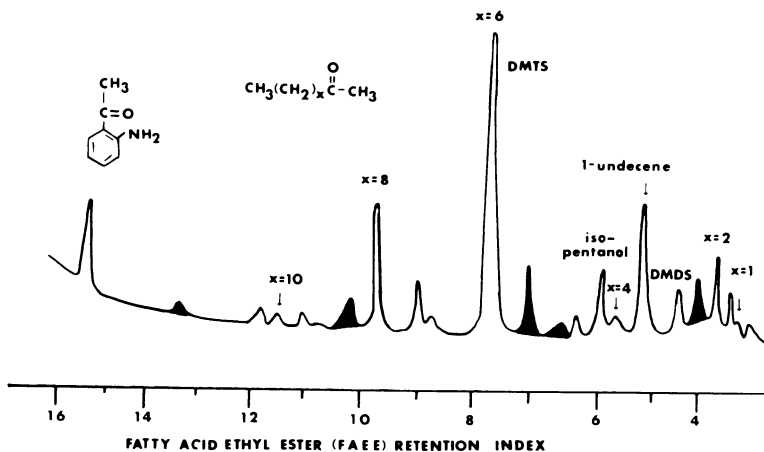


FIG. 1. Volatile profile of *P. aeruginosa*.

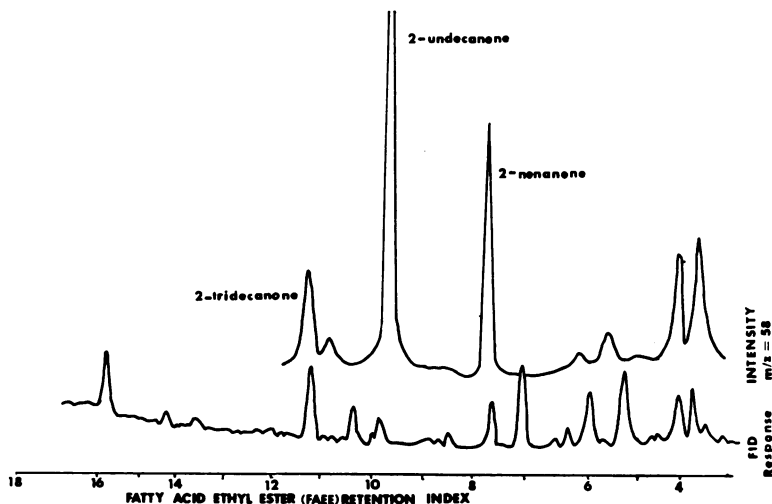


FIG. 2. Volatile profile of *P. aeruginosa* with single-ion monitoring.

TABLE 1. Concentration of methyl ketones in volatile profile of *P. aeruginosa*

<i>P. aeruginosa</i> strain	Nonanone <sup>a</sup>	Undecanone <sup>a</sup>
ATCC 17429	0.13	0.19
ATCC 17423	0.14	0.05
ATCC 27313	0.30	0.15
ATCC 27312 <sup>b</sup>	0.12	0.14
ATCC 27316 <sup>b</sup>	0.25	0.23
ATCC 7701	0.20	0.15
CDC 9171	0.11	0.19
CDC 9104	0.11	0.13

<sup>a</sup> Concentration in micrograms based on comparison of intensity of mass spectrum peak  $m/z$  58 with known amounts of 2-nonanone (38 mm/0.1  $\mu$ g) and 2-undecanone (36 mm/0.1  $\mu$ g).

<sup>b</sup> Average of two runs.

*aeruginosa* with increased peak intensities for 2-AA and the presence of 4-methyl-quinazoline, a reaction product of 2-AA (10). Table 2 summarizes the relative peak intensities for the strains of *P. aeruginosa* and for the other pseudomonads studied. In all samples reported here mass spectral data were used to confirm the identity of the GC peaks. Uninoculated culture media showed several volatile components including methyl-, dimethyl- and trimethylpyrazines, phenol, benzaldehyde, acetophenone, 2-ethyl-1-hexanol, and alkyl benzenes. Dimethylpyrazine was present in all cultures at an average GC peak intensity of 33 cm ( $m/z$  108, 68 mm).

## DISCUSSION

Various *Pseudomonas* species have been implicated in food spoilage through the detection of their odorous metabolites. *Pseudomonas fragi* hydrolyzes and esterifies the short-chain

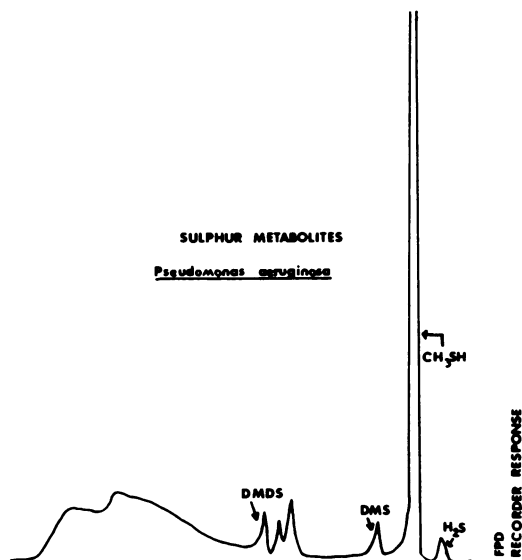


FIG. 3. Profile of sulfur metabolites of *P. aeruginosa* by direct headspace analysis. DMS, dimethyl sulfide.

acids in milk fat to give a fruity aroma to milk products (ethyl butyrate and ethyl hexanoate) (28). *Pseudomonas taetrolens* when incubated on milk culture forms 2,5-dimethylpyrazine and 2-methoxy-3-isopropyl-pyrazine. The latter possesses a potent potato-like aroma (28). Similarly, two alkyl pyrazines, 2-methoxy-3-sec-butyl-pyrazine and 2-methoxy-3-isopropyl-pyrazine are produced by *Pseudomonas perolens* when incubated on fish tissue and are responsible for potato mustiness of refrigerated fish (28). A va-

TABLE 2. *Relative intensities of volatile components of pseudomonads*<sup>a</sup>

Strain	DMDS	Butanol + undecene	Isopentanol	DMTS + 2-nonanone	2-Undecanone	2-AA
<i>P. aeruginosa</i>						
ATCC 19660	6	50	16	17	20	14
ATCC 7700	tr	30	80	42	30	6
ATCC 27312 <sup>b</sup>	10	40	20	45	20	24
ATCC 27316 <sup>b</sup>	21	45	22	29	16	17
ATCC 27313	6	15	50	40	9	?
ATCC 7701	6	12	31	25	13	3
ATCC 17429	22	55	100	85	17	50
CDC 9171	26	40	50	40	23	40
CDC 9104	3	5	7	5	3	14
<i>P. cepacia</i> ATCC 17759	12			4 <sup>d</sup>		
<i>P. putrefaciens</i> ATCC 8073	60	60 <sup>c</sup>	960	4 <sup>d</sup>		
<i>P. maltophilia</i> <sup>b</sup> ATCC 13637	tr	50 <sup>c</sup>	1,200	4 <sup>d</sup>	3	
<i>P. fluorescens</i> ATCC 13525		50 <sup>c</sup>	640	8 <sup>d</sup>		
<i>P. putida</i> ATCC 12633	120	10 <sup>c</sup>	20	27 <sup>d</sup>	2	

<sup>a</sup> Values are relative GC peak intensities (centimeters) on  $1 \times 10^{-10}$ , with half the material going to the mass spectrometer. Volatiles were concentrated on Tenax (2 h; 80 ml/min) and transferred with heating to the gas chromatograph before analysis.

<sup>b</sup> Average of two runs.

<sup>c</sup> Only butanol.

<sup>d</sup> Mainly DMTS.

riety of volatile compounds are also formed by *P. putrefaciens* and *P. fluorescens* when incubated on sterile fish muscle and include the following: methyl mercaptan, DMDS, DMTS, and isopentanol (26). A detailed study of volatiles produced by *P. putida* and *P. fluorescens* isolated from spoiled, uncooked chicken and incubated on Trypticase soy agar identified 20 different compounds including DMDS, DMTS, 1-undecene, 2-butanone, and 2-nonanone, which were also identified in this study (22).

*P. aeruginosa* when grown in culture produces a compound that imparts a grapelike odor to the culture and aids in the identification of this organism. Recent studies have confirmed that 2-AA is responsible for this observed odor and is maximized at 20 to 24 h of incubation (10). Analysis of cultures for 2-AA either by fluorescence or by GC is thus potentially useful for identification. Our data confirm the ubiquitous production of 2-AA in *P. aeruginosa* and show that other clinically relevant pseudomonads fail to produce 2-AA.

A series of odd-carbon-numbered methyl ketones as reported here for *P. aeruginosa* can be formed from the  $\beta$ -oxidation and decarboxylation of even-carbon-numbered fatty acids (11). A similar ketone series has been observed in other biological systems. The odor of the pedal gland of the bontebok was reported to be due to 2-heptanone, 2-nonanone, 2-undecanone, and principally 5-undecen-2-one (8). Whether these compounds are products of bacterial decomposition, as has been reported to be the case in

pheromonal production in other mammals, is not known (1). A report on the bacterial production of the ovipositional attractant for mosquitoes has implicated *P. aeruginosa*. The active compound(s) is produced from the action of this organism on fatty acid substrates, particularly decanoic acid (16). *Penicillium roqueforti* readily transforms fatty acids in lipolyzed milk fat to 2-pentanone, 2-heptanone, 2-nonanone, and 2-undecanone, which contribute to the blue cheese odor (11). A mechanism based on the deacylation of  $\beta$ -oxo-acylthiolester was suggested for ketone formation.

A recent report on the discrimination of *Pseudomonas* species by cellular fatty acids indicates a difference in the presence and amount of 3-hydroxy-dodecanoic and 3-hydroxy-decanoic acids (29), which could serve as direct precursors to 2-undecanone and 2-nonanone, respectively. *P. fluorescens*, *P. aeruginosa*, and *P. putida* show similar levels of these acids, whereas the latter two produce both ketones and *P. fluorescens* produces 2-nonanone (Table 2). Additional strains of these species as well as other pseudomonads will have to be examined to determine whether a correlation exists between cellular hydroxy acids and volatile ketones.

Secondary metabolites of *P. aeruginosa* such as pyolipids and rhamnolipids contain  $\beta$ -keto-decanoic acid. The pyolipids are condensation products of anthranilic acid and  $\beta$ -keto acids, principally  $\beta$ -keto-decanoic acid. Similarly rhamnolipids, containing  $\beta$ -hydroxy-decanoic acid and decanoic acid, have been identified as

constituents of the lipopolysaccharide component of the outer membrane of certain gram-negative bacteria including pseudomonads (23). It is thus not unexpected that 2-nonanone, a metabolite of these acids, is found in cultures of the pseudomonads.

Additional work is needed to determine whether a combination of volatile metabolites, such as 2-AA, methyl ketones, and sulfides, is sufficient for identification of *P. aeruginosa* and to determine whether these same metabolites are found in mixed cultures with other organisms on substrates of clinical interest such as saliva, serum, skin secretions, and lung fluid. The use of multiple-ion detection would enable these metabolites to be profiled even in complex matrices. The detection of *P. aeruginosa* by a specific volatile profile in clinical material could substantially aid the rapid administration of effective therapy.

#### ACKNOWLEDGMENTS

We thank Annette Rothermel for technical assistance and James Kostelc for use of the instrumentation for direct analysis of the sulfur metabolites.

This research was supported in part by Public Health Service grant 2 ROI AM 26195-02 from the National Institute of Arthritis, Metabolism, and Digestive Diseases.

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