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Use of 2-Aminoacetophenone Production in Identification of *Pseudomonas aeruginosa*

CHARLES D. COX* AND J. PARKER

Department of Microbiology, College of Medicine, The University of Iowa, Iowa City, Iowa 52242

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A grapelike odor is often of diagnostic importance in detecting the growth of *Pseudomonas aeruginosa* in culture and in burn wounds. The compound responsible for the odor has been identified as 2-aminoacetophenone by mass spectroscopy. Although the grape odor is sometimes difficult to detect in culture media, gas chromatographic, fluorometric, and colorimetric methods can be utilized to assay 2-aminoacetophenone production in a variety of media. Its synthesis occurs relatively early in the growth cycle. It has proved easy and convenient to detect 2-aminoacetophenone excretion by *P. aeruginosa* after 24 h of incubation on blood agar plates employing a fluorometric assay of ether extracts of the agar medium.

It is widely recognized that *Pseudomonas aeruginosa* growing in culture or in thermal injuries may be recognized as a sweet, grapelike odor. Personnel in diagnostic laboratories use the grape odor produced during growth on blood agar plates as an aid in quick identification of *P. aeruginosa*. The structure and origin of the compound responsible for the grape odor are known but have not been widely recognized. Mann (3) isolated a compound having a grape odor from 20-day-old plate cultures of *P. aeruginosa;* he identified the compound as 2-aminoacetophenone (2AA) by thin-layer chromatography. Mann presented evidence that the intensity of the grape odor can be enhanced by the addition of tryptophan to the medium and that 2AA is an intermediate in the biosynthetic pathway for quinazolines, a pathway branching from the tryptophan catabolic pathway (5). Evidence was also obtained suggesting that 2AA is more consistently produced by *P. aeruginosa* than pyocyanin (1).

We have confirmed that 2AA is the compound responsible for the grape odor. Early production of the compound during the growth cycle of *P. aeruginosa* makes it available for analysis after only 20 to 24 h of incubation. Production of 2AA would be a more useful diagnostic procedure if it could be recognized by some means in addition to odor. We present a rapid method of extraction and detection of 2AA which makes analysis less subjective and more sensitive than detection of odor.

MATERIALS AND METHODS

Bacteria and culture conditions. *P. aeruginosa* strain PAO-1 (ATCC 15692) was obtained from the American Type Culture Collection and was maintained on brain heart infusion agar (Difco) with monthly transfers. Clinical isolates from the Burn Unit of the University of Iowa Hospitals, designated IBU, were inoculated into media directly from the initial blood agar plates. These strains were stored at cell densities of approximately 10^9 bacteria per ml in 20% glycerol with 1% Trypticase soy broth at −20°C. Strains used repeatedly were maintained on brain heart infusion agar slants with monthly transfers. These strains were screened by the ALA pyocin typing procedure (2) to ensure the use of a diversity of strains in experiments rather than multiple isolates of the same strain. The pyocin typing strains were generously provided by J. J. Farmer.

Rich media were tryptone, peptone, Trypticase soy broth, brain heart infusion, meat infusion (Bacto-Beef; Difco), Casamino Acids, and yeast extract (Difco) media in 1.0% concentrations supplemented with 0.4 mM $MgCl_2$. Minimal media consisted of 10 mM concentrations of substrates, 0.4 mM $MgCl_2$, 0.1 mM K_2SO_4, 4.0 mM NH_4Cl, and 4.0 mM potassium phosphate buffer at pH 7.4. The medium used for the analysis of 2AA production consisted of 10 mM tryptophan, 0.1% yeast extract, 0.4 mM $MgCl_2$, and 4.0 mM potassium phosphate buffer at pH 7.4. Tryptophan was sterilized by filtration through a filter with 0.45-μm pore size (Millipore Corp.) and added to the yeast extract medium after autoclaving.

Blood agar plates consisted of blood agar base medium (Difco) plus 2% sheep erythrocytes (20 ml per plate). Chemicals were purchased from Fisher Scientific Co., and tryptophan was purchased from Sigma Chemical Co.

Identification of 2AA. Strain PAO-1 was grown in 1 liter of a medium containing 0.1% yeast extract, 4 mM potassium phosphate buffer (pH 7.5), 0.4 mM $MgCl_2$, and 10.0 mM tryptophan at 37°C with shaking for 24 h. The cells were centrifuged from the medium, and the supernatant fluid was extracted with 100 ml of ether in a separatory funnel. The ether layer was

removed and reduced to 0.5 ml by passing nitrogen gas over the surface. The ether solution was dried with anhydrous magnesium sulfate and injected onto a 10% SP1000 column in a Varian Aerograph series 2700 gas chromatograph equipped with a flame ionization detector. The elution of the grape odor from the column was matched to a peak on the recording by noting the time the odor was detected at the ejection port. The compound was then collected from subsequent injections by trapping the compound from the ejection port in a glass capillary tube chilled with Dry Ice. The trapped compound was dissolved in pentane and injected onto an SP1000 column in a gas chromatograph coupled to a Finnigan mass spectrometer.

Assay of 2AA. Three methods were used to assay 2AA in the ether extracts of culture media. The extraction procedure consisted of mixing 50 ml of culture medium which had been adjusted to pH 10.0 with 20 ml of ether in a 250-ml separatory funnel. The ether layer was removed and concentrated to between 1 and 4 ml by applying a vacuum (aspirator) to a desiccator containing the extracts.

Gas chromatographic assay of 2AA was conducted in a Varian Aerograph series 2700 gas-liquid chromatograph on columns of Carbowax 20M; quantitation was accomplished by injecting increasing concentrations of 2AA, cutting the peaks corresponding to 2AA from the chromatographs, and comparing the weights of the peaks with the quantities of authentic 2AA injected. The standard curve was linear from 0.5 to 10.0 μg.

Analysis of the fluorescing compounds produced by *P. aeruginosa* was accomplished by thin-layer chromatography on silicic acid layers (Eastman Kodak Co.) developed in isopropanol–25% aqueous NH_4OH–water (100:10:10) or in chloroform–acetic acid–ethanol (95:5:2). The R_f values of 2AA in these solvents were 0.82 and 0.87, respectively. Quinazolines are separable from 2AA on these chromatograms, but were never observed in the extracts used for these studies. Quantitation of 2AA was accomplished by scraping the silicic acid containing the fluorescent spots of 2AA, eluting the 2AA in ethanol, and analyzing the fluorescence of the dissolved 2AA. 2AA was quantified in ethanol dilutions of ether extracts of alkaline culture media because numerous chromatograms of fresh ether extracts of alkaline culture media demonstrated that 2AA was the only detectable fluorescent molecule in the ether phase, whereas the ether extracts of acidified media contained many fluorescent compounds. A standard curve of relative intensity of fluorescence versus 2AA concentration in ethanol was linear over the range of 0.1 to 10.0 nmol/ml, measuring emission at 430 nm and exciting the samples at 362 nm. Analyses were conducted in Teflon-stoppered cuvettes with an Aminco-Bowman Corrected Spectra SPF spectrofluorometer. Quinine sulfate (Aminco-Bowman) was used to calibrate the fluorometer.

Thin-layer chromatograms of authentic 2AA and ether extracts of alkaline culture media yielded single, yellow spots at the position of 2AA when the developed chromatograms were sprayed with Ehrlich reagent (1 g of *p*-dimethylaminobenzaldehyde in a solution containing 25 ml of HCl and 75 ml of methanol). Colorimetric assays for 2AA were conducted on 2.0-ml samples of ether extracts of alkaline culture media by adding 1.0 ml of 1% *p*-dimethylaminobenzaldehyde in methanol, followed by 2.0 ml of glacial acetic acid. After 10 min, the absorbance at 475 nm was compared with a standard curve of absorbance versus 2AA concentration. This curve was used in the range 1.0 to 8.0 μmol/ml of 2AA. The spectrum of the chromophore was determined in a Perkin Elmer model 124 spectrophotometer, and the routine colorimetric assays were conducted with a Gilford model 240 spectrophotometer.

The organic solvents were spectral-grade reagents from Baker Chemical Co., and *p*-dimethylaminobenzaldehyde and 2AA were purchased from Aldrich Chemical Co. 4-Methylquinazoline was synthesized by J. Cannon, University of Iowa, and 4-methylquinazoline and 2,4-dimethylquinazoline were gifts from W. L. F. Armarego, Australian National University, Canberra.

RESULTS

Identification of 2AA. Identification of the compound responsible for the grape odor of *P. aeruginosa* as 2AA was based upon three criteria. The compound from ether extracts of culture media migrated at the same location as authentic 2AA on thin-layer chromatograms, and this behavior could be correlated with 2AA as isolated and identified by Mann (4). Gas chromatographic analysis of authentic 2AA and the bacterial product on two chromatography support materials gave identical retention times (Table 1). Analysis of the bacterial grape odor compound by mass spectroscopy yielded a fragmentation pattern confirming its identity as 2AA (Fig. 1).

Time course of 2AA and quinazoline synthesis during culture. It was important for physiological and diagnostic reasons to know the time of synthesis of 2AA during the growth curve. *P. aeruginosa* strain PAO-1 was inoculated into 1 liter of a solution containing 0.1%

TABLE 1. *Gas chromatographic separation of 2AA and quinazolines*

Compound	Retention times (ml/min) with:	
	SP1000[a]	Carbowax 20M[b]
2-Aminoacetophenone	23.0	7.3
2,4-Dimethylquinazoline	16.0	19.8
4-Methylquinazoline	17.0	26.4

[a] The column was stainless steel, 6 feet by 0.125 inch (ca. 1.83 m by 3.2 mm), contained 10% SP1000, and was run at 200°F (ca. 93.3°C) at a flow rate of 40 ml/min.

[b] The column was stainless steel, 6 feet by 0.125 inch (ca. 183 m by 3.2 mm), contained Carbowax 20M, and was run at 180°F (ca. 82.2°C) at a flow rate of 40 ml/min.

yeast extract, 10 mM tryptophan, 4.0 mM potassium phosphate buffer (pH 7.4), 0.4 mM $MgCl_2$, and 10 μM $FeCl_3$ at a density of 10^3 bacteria per ml. Growth was measured by the optical density at 650 nm by using 1-cm cuvettes in a Gilford model 240 spectrophotometer. At various times during incubation, samples of 50 ml were withdrawn and extracted with 20 ml of ether. The ether layers were concentrated and analyzed by gas chromatography as described above.

The culture reached a maximum optical density after 30 h. The 2AA concentration reached its maximal level at approximately 22 h (Fig. 2). The concentration of 2AA then decreased rapidly to 40 μM by 80 h of culture. Repetitions of the experiment using both gas chromatographic and fluorometric analyses yielded the same pattern of 2AA prouction. Quinazolines were not detected early in culture. Only after 40 h was there sufficient 4-methylquinazoline to allow quantitation. 2,4-Dimethylquinazoline never accumulated in high concentrations, but a concentration of 1.0 μM 4-methylquinazoline was attained at 60 h of culture (Fig. 2).

Time course of 2AA synthesis during culture without tryptophan. To determine the time of synthesis of 2AA in a medium similar to the rich media used for clinical isolation, a 1% solution of brain heart infusion broth was prepared with 0.4 mM $MgCl_2$, and strain PAO-1 was inoculated at a density of 10^2 bacteria per ml. Growth was followed by the optical density of the culture at 600 nm, and 40-ml samples were removed at various times for extraction into

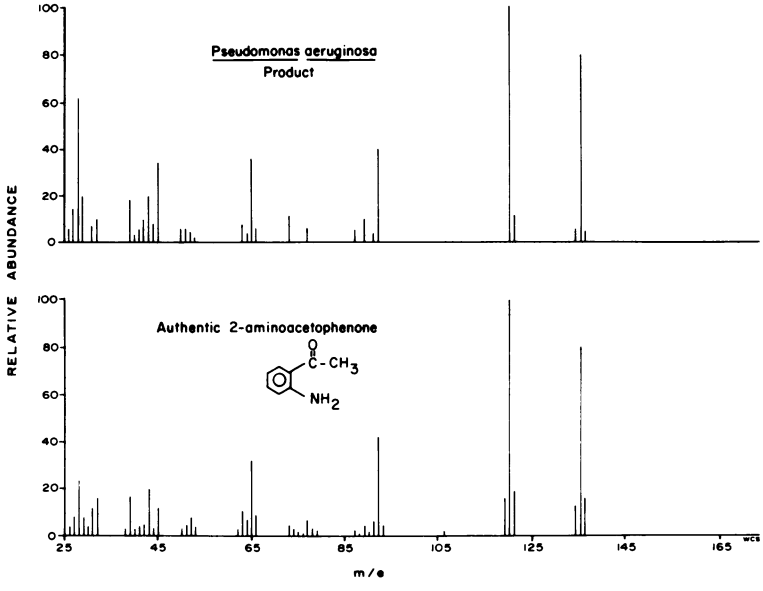

FIG. 1. *Mass spectra of the compound from P. aeruginosa possessing the grape odor and of 2AA.*

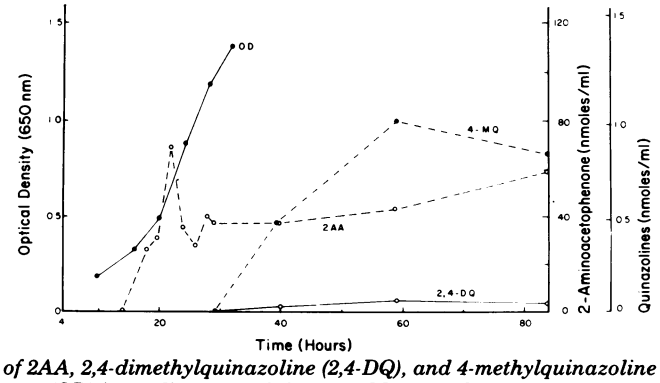

FIG. 2. *Synthesis of 2AA, 2,4-dimethylquinazoline (2,4-DQ), and 4-methylquinazoline (4-MQ) in relation to growth of P. aeruginosa (OD) in medium containing 10 mM tryptophan.*

ether. The ether layers were concentrated and processed for fluorometric quantitation of 2AA (see above). The culture reached the stationary phase after approximately 20 h of incubation, while the concentration of 2AA in the medium showed a burst of 2AA synthesis at 16 h, followed by a substantial increase in 2AA concentration as the culture entered the stationary phase (Fig. 3).

Production of 2AA in various media. The effects of different culture media on the production of 2AA were determined by inoculating 10^3 bacteria per ml of strain PAO-1 into 40 ml of 1% solutions of rich media in 250-ml flasks which were incubated with shaking at 37°C. After 20 h of incubation, the cultures were centrifuged to remove the cells, and the spent media were adjusted to pH 10.0 and extracted with 20-ml quantities of ether. The ether layers were concentrated and analyzed by fluorometric methods. The extracts were also chromatographed on silicic acid thin layers, and the fluorescent material migrating at the position of 2AA was eluted into ethanol and measured fluorometrically. Brain heart infusion and peptone media allowed the highest levels of 2AA production, yeast extract and meat infusion media allowed intermediate levels, and Casamino Acids, tryptone, and Trypticase soy broth media allowed low levels of 2AA accumulation (Table 2). Growth on tryptophan as a substrate allowed the greatest accumulation of 2AA of any of compounds tested in minimal medium, but growth on this substrate was extremely slow, and a 48-h incubation period was necessary to obtain maximal growth and a yield of 0.68 μM 2AA.

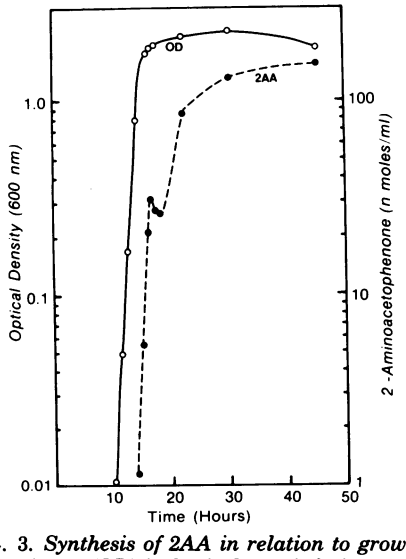

FIG. 3. *Synthesis of 2AA in relation to growth of P. aeruginosa (OD) in brain heart infusion medium without added tryptophan.*

TABLE 2. *Production of 2AA in various media*

Medium	Concn of 2AA (μM)	
	Without tryptophan	With tryptophan
Rich media		
Casamino Acids	0.21	21.78
Peptone	109.50	192.70
Brain heart infusion	73.50	186.42
Yeast extract	27.60	66.24
Meat infusion	20.25	129.60
Tryptone	1.80	40.96
Trypticase soy broth	0.76	25.31
Minimal media		
Glycerol	0.08	5.68
Gluconate	0.12	72.00
Acetate	0.12	89.32
Succinate	0.12	93.78
Tryptophan	0.68	

Because the biosynthetic pathway for 2AA branches from the tryptophan catabolic pathway (3), tryptophan was incorporated into various growth media in 10 mM concentrations. This evoked an increase in the 2AA concentration found in the cultures, even those in minimal media (Table 2).

Analysis of the production of 2AA on blood agar by clinical isolates. Blood agar is often used as a primary isolation medium for pathogenic bacteria and is constructed with a base medium containing a high concentration of beef heart infusion or meat infusion which, as shown in Table 2, allows considerable production of 2AA by *P. aeruginosa*. Strain PAO-1 and clinical isolates from the Burn Unit, University of Iowa Hospitals, including 11 different pyocin types, were individually streaked for isolation on blood agar plates. The plates were incubated at 37°C for 20 h and were extracted by flooding a plate with 5.0 ml of ether. The plate was swirled occasionally for 5 min, and then the ether was transferred by pipette to a test tube. This procedure can be used with plastic plates. No pH adjustment is required since blood agar after incubation with *P. aeruginosa* becomes alkaline. The most direct method of detecting 2AA in the extracts is by viewing the tubes under a hand-held ultraviolet lamp (360 nm). Most tubes demonstrate very strong fluorescence, visible even in a well-lighted room, whereas others show a more moderate fluorescence, still easily distinguishable in a darkened room. Tubes containing extracts from plates incubated with *Escherichia coli, Enterobacter cloacae, Pseudomonas fluorescens, Pseudomonas maltophilia,* and *Pseu-*

domonas cepacia displayed no fluorescence in a darkened room. Thin-layer chromatography of extracts of *P. aeruginosa* demonstated that fresh extracts from plates incubated for 20 h contained 2AA as the only detectable fluorescent compound. Other fluorescent compounds were detectable in minute amounts after aging the extracts or extracting plates which had been incubated for 48 h or longer.

Although extraction from blood agar was not considered highly efficient, quantitation of the extracted 2AA was attempted by fluorometry to determine the relative amounts of 2AA produced by different strains (Table 3). All strains of *P. aeruginosa* tested by this procedure demonstrated 2AA production, although the total amounts of 2AA produced by each strain on individual plates varied considerably.

DISCUSSION

Mass spectroscopy confirmed that 2AA is responsible for the grape odor of *P. aeruginosa*. Two other products, 4-methylquinazoline and 2,4-dimethylquinazoline, were separated and identified along with 2AA by gas chromatography. The quinazolines are produced in low concentrations late in the stationary phase of growth and do not interfere with the assay for 2AA. Growth studies indicate that 2AA is an early product during the growth cycle, accumulating in large amounts after 20 to 30 h of culture.

Although the amounts of 2AA produced at 20 to 24 h do not necessarily represent the maximal concentrations, this is an incubation period which is convenient for rapid analysis in clinical laboratories. Figure 3 demonstrates that there is considerable 2AA present by 18 to 24 h (in brain heart infusion), and this is a significant proportion of the final concentration. The appearance of a spike of 2AA production (Fig. 2 and 3) suggests that 2AA may be further utilized during growth. However, no clear demonstration of 2AA utilization has been observed, and we have not been able to grow strain PAO-1 on 2AA as a sole carbon source. Although addition of tryptophan to media increases the production of 2AA, it is not necessary for the production of large amounts in rich media.

TABLE 3. *Concentrations of 2AA extracted from blood agar plates*

Species or strain	Concn of 2AA (μmol/plate)[a]
P. aeruginosa	
IBU 1186	5.5
IBU 1180	6.0
IBU 1059	0.3
IBU 437	37.4
IBU 880	0.1
IBU 1061	1,311.0
IBU 1046	12.2
IBU 102	2.9
IBU 1058	2.5
IBU HW	48.5
PAO-1	16.6
P. cepacia	0
P. maltophilia	0
P. fluorescens	0
E. cloacae	0
E. coli	0

[a] 2AA concentrations were determined fluorometrically in ethanol dilutions of ether extracts.

Olfactory detection of 2AA is often used as a diagnostic aid in identifying *P. aeruginosa,* and the nose is usually sensitive to levels lower than 1 μg/ml. Olfactory sensation rapidly becomes saturated with 2AA, however, interfering with repeated analyses. There may also be many interfering odors in the media or other odors produced by the organisms. The variability in production of 2AA (Table 3) demonstrates why the grape odor of some strains (IBU 880 and IBU 1059) is difficult to detect. This report offers both fluorometric and colorimetric assays for rapid detection and quantitation of 2AA. The detection of fluorescence in the extracts with a hand-held ultraviolet lamp or by fluorometry is the most sensitive method. The colorimetric assay may be conducted on the entire ether extract to aid in the identification of the fluorescence as due to 2AA. The ability to measure 2AA in alkaline extracts depends upon the lack of charge on the molecule at basic pH values, which allows extraction of 2AA into the ether phase free from other fluorescing molecules. This rapid assay may be of use in early diagnosis of *P. aeruginosa* on plates used for primary isolation. It may also be of use on plates containing mixtures of microorganisms, but caution must be used because it is not known whether other bacteria produce fluorescent molecules of similar charge which could interfere with the assay. Coliforms and other species of *Pseudomonas* do not produce interfering compounds on blood agar plates in our experience, but we have not examined a diversity of strains of other species of bacteria. Plates containing mixtures of bacteria require treatment with 0.1 M NaOH to ensure an alkaline pH. Fluorescent pseudomonads do produce pyoverdin, which is an intensely fluorescent, water-soluble pigment. This compound presents confusion only if water is removed along with the ether layer; otherwise it does not appear in the ether extracts.

Habs and Mann (1) proposed that the grape-like odor due to the production of 2AA by *P.*

aeruginosa be included in the description of this species, and we call attention to their proposal. They were unable to detect 2AA in 5 of 50 apyocyanogenic strains, but this may have been due to the lack of sensitivity of using Erlich spray on paper chromatograms. Although we have not examined apyocyanogenic strains, we have used the more sensitive fluorometric assay and have found 2AA produced by all of the 60 different strains examined.

This paper presents information on 2AA production in culture and techniques for identification which may aid in analysis of the compound responsible for the grape odor of *P. aeruginosa*. 2AA may not be the only microbial product with the odor of grapes; hydroxyanthranilic acid also has an odor like that of grapes. Mann describes the production of 2AA by *P. fluorescens, Pseudomonas* (*Aeromonas*) *shigelloides, Bacillus cereus,* and *Sarcina* (*Micrococcus*) *lutea* in culture media incubated for 20 days (4). We were interested in bacterial products appearing within the initial 24 to 48 h of incubation, and we could not detect 2AA in culture media from *P. fluorescens, B. cereus, Bacillus subtilis,* or *Bacillus megaterium* in this time period. 2AA is generated from tryptophan during autoclaving, and uninoculated media yield varying amounts of 2AA from tryptophan depending upon aeration, temperature, and exposure to light during incubation. Controls must be included during short as well as prolonged incubations to assess the nonenzymatic degradation of tryptophan. Detection of 2AA and the grape odor is a supplement to other characteristics used to identify *P. aeruginosa*. Clearly much more information must be obtained about this compound and its production by other bacteria before an assessment of its importance in diagnosis may be made.

ACKNOWLEDGMENTS

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