Amino Acid Catabolites of Gram-Negative Bacteria

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One hundred and two strains of aerobic, gram-negative bacteria, isolated mainly from drugs and cosmetics, were tested for catabolic products after incubation in liquid media containing single amino acids. Breakdown products from L-arginine, lysine, and ornithine were identified as their *N*-heptafluoryl butyl esters by gas-liquid chromatographic techniques. A Finnigan model 1015 D gas chromatograph/mass spectrometer/PDP-8E data system was used to identify trace amounts of catabolites after amino acid substrates were inoculated with bacteria. The derivatization reaction was monitored in like manner. The Finnigan data system may be used to identify catabolic compounds rapidly and thus provide a means for bacterial characterization and identification.

The arginine dihydrolase test is particularly valuable in differentiating between various species of pseudomonads and other carbohydrate-oxidizing bacteria (11, 13, 15, 18). Combination of this test with procedures reported by Moss et al. (15) and Lambert and Moss (12) for determining catabolic products of amino acids by gas-liquid chromatography (GLC) has resulted in a practical means of identifying bacteria. GLC has been utilized to differentiate the members of the family *Enterobacteriaceae* from the fermentative and oxidative bacteria. However, the decarboxylase reactions of the pseudomonads have not been studied as extensively as have those of the members of the family *Enterobacteriaceae*. We have evaluated the use of the GLC rapid test for differentiation of 102 strains of gram-negative bacteria. Both the saccharolytic and weakly saccharolytic, nonfermentative strains of *Pseudomonas* species as well as nonsaccharolytic nonfermentative bacteria, e.g., *Acinetobacter* (16), were examined.

The objectives of this study were to determine five catabolic metabolites produced by 102 various strains of gram-negative bacteria representing 12 species of *Pseudomonas* and 20 species of *Enterobacteriaceae* and to investigate the use of *N*-heptafluorobutyric anhydride as a GLC derivatizing reagent for the catabolites. This study was performed with the aid of a gas chromatograph-mass spectrometer-data system.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study were obtained from either the culture collection of the Drug Microbiology Branch, U.S. Food and Drug Administration, Washington, D.C., or the Ameri-

can Type Culture Collection, Rockville, Md. The strains used are listed in Table 1.

Methods. Identification of the bacteria was confirmed by established culture and biochemical procedures (4-6, 8, 9). The cultures were grown on brain heart infusion agar (Baltimore Biological Laboratories) slants for 16 to 24 h and then transferred to triple sugar iron agar (Difco) slants for an additional 36 to 48 h at 35°C. Growth from the top half of the triple sugar iron agar slants was used to inoculate all media. Cultures were tested for L-arginine dihydrolase and L-ornithine and L-lysine decarboxylase activities in two media: Moeller medium as described by Edwards and Ewing (5) and the medium described by Moss et al. (15) and Lambert and Moss (12).

GLC analysis for L-arginine catabolic products started with four standard bacteriological transfer loops (0.2 mm) of growth from the TSI slants inoculated into 1 ml of a 2.5 mM solution of L-arginine (435 µg; Sigma Chemical Co., St. Louis, Mo.) in deionized, distilled water (pH 6.8). This dense cell suspension in substrate was mixed and held at 35°C for 48 h. The arginine medium was overlaid with a thin layer of sterile mineral oil as described by Moss et al. (15). After incubation, the cells were removed by brief centrifugation; a portion of the supernatant which contained the L-amino acids arginine, citrulline, and ornithine, or other metabolic by-products, was transferred to a high-temperature glass vial (Applied Science Laboratories, Inc., State College, Pa., 3-ml size, catalog number 12994) and dried at 100°C under a stream of nitrogen. The dried residues were then converted to the butyl ester and *N*-heptafluorobutyl-*n*-propyl acetyl derivative for analysis by GLC (13). L-Ornithine and L-lysine (Sigma Chemical Co., St. Louis, Mo.) were added separately to 100-ml samples of the Lambert-Moss (12) medium to a final concentration of 0.25% of each amino acid. Media containing ornithine and lysine and a control medium containing no added amino acids were also prepared. After sterilization at 121°C

TABLE 1. Source of bacterial strains used in this study

Strain	Source ^a	Strain	Source ^a		
<i>Pseudomonas putida</i>	1-3-12	DMB	<i>Alcaligenes faecalis</i>	2-1-32	DMB
<i>Pseudomonas putida</i>	1-3-13	DMB	<i>Moraxella</i> sp.	AO II-6	DMB
<i>Pseudomonas putida</i>	1-3-14	DMB	<i>Moraxella</i> sp.	AO II-1	DMB
<i>Pseudomonas putida</i>	1-3-15	DMB	<i>Bacterium anitratum</i>	4-1-11	DMB
<i>Pseudomonas cepacia</i>	1-4-26	DMB	<i>Bacterium anitratum</i>	4-1-12	DMB
<i>Pseudomonas cepacia</i>	1-4-11	DMB	<i>Bacterium anitratum</i>	4-1-13	DMB
<i>Pseudomonas cepacia</i>	1-4-12	DMB	<i>Mima</i> sp.	II-2	DMB
<i>Pseudomonas pseudoalcaligenes</i>	1-14-1	DMB	<i>Mima</i> sp.	AO II-3	DMB
<i>Pseudomonas stutzeri</i>	1-5-1	DMB	<i>Mima</i> sp.	II-4	DMB
<i>Pseudomonas stutzeri</i>	1-5-11	DMB	<i>Mima</i> sp.	II-5	DMB
<i>Pseudomonas stutzeri</i>	1-5-12	DMB	<i>Herellea</i> sp.	AO II-4	DMB
<i>Pseudomonas stutzeri</i>	1-5-15	DMB	<i>Herellea</i> sp.	3-20-11	DMB
<i>Pseudomonas stutzeri</i>	1-5-16	DMB	<i>Herellea</i> sp.	AO III-5	DMB
<i>Pseudomonas stutzeri</i>	1-5-17	DMB	<i>Escherichia coli</i>	12-1-1	DMB
<i>Pseudomonas aeruginosa</i>	AO III-15	DMB	<i>Escherichia coli</i>	DAL-P-1	DAL
<i>Pseudomonas aeruginosa</i>	AO III-16	DMB	<i>Escherichia coli</i>	DAL-P-2	DAL
<i>Pseudomonas aeruginosa</i>	1-1-1	DMB	<i>Escherichia coli</i>	DAL-P-3	DAL
<i>Pseudomonas aeruginosa</i>	1-1-2	DMB	<i>Escherichia coli</i>	DAL-P-4	DAL
<i>Pseudomonas aeruginosa</i>	1-1-3	DMB	<i>Escherichia coli</i>	DAL-P-5	DAL
<i>Pseudomonas aeruginosa</i>	1-1-102	DMB	<i>Serratia marcescens</i>	13-1-11	DMB
<i>Pseudomonas aeruginosa</i>	1-1-103	DMB	<i>Enterobacter liquefaciens</i>	10-9-11	DMB
<i>Pseudomonas aeruginosa</i>	1-1-15	DMB	<i>Enterobacter liquefaciens</i>	P	DMB
<i>Pseudomonas aeruginosa</i>	1-1-35	DMB	<i>Enterobacter liquefaciens</i>	DM5370	DMB
<i>Pseudomonas aeruginosa</i>	1-1-150	DMB	<i>Enterobacter aerogenes</i>	DM1733	DMB
<i>Pseudomonas aeruginosa</i>	AO III-5	DMB	<i>Enterobacter aerogenes</i>	10-2-11	DMB
<i>Pseudomonas fluorescens</i>	13985	ATCC	<i>Enterobacter aerogenes</i>	10-1-12	DMB
<i>Pseudomonas fluorescens</i>	17386	ATCC	<i>Enterobacter aerogenes</i>	10-1-13	DMB
<i>Pseudomonas fluorescens</i>	1-2-14	DMB	<i>Enterobacter cloacae</i>	"B"-CDC	DMB
<i>Pseudomonas fluorescens</i>	1-2-1	DMB	<i>Enterobacter cloacae</i>	"A"DM-570	DMB
<i>Pseudomonas fluorescens</i>	1-2-11	DMB	<i>Enterobacter cloacae</i>	"P"	DMB
<i>Pseudomonas fluorescens</i>	1-2-12	DMB	<i>Enterobacter cloacae</i>	"A"DM5577	DMB
<i>Pseudomonas putida</i>	1-3-1	DMB	<i>Enterobacter cloacae</i>	10-5-11	DMB
<i>Pseudomonas putida</i>	1-3-11	DMB	<i>Enterobacter cloacae</i>	"A"10-6-11	DMB
<i>Pseudomonas maltophilia</i>	1-8-13	DMB	<i>Enterobacter cloacae</i>	"A" CDC	DMB
<i>Pseudomonas maltophilia</i>	1-8-14	DMB	<i>Enterobacter hafniae</i>	10-8-11	DMB
<i>Pseudomonas maltophilia</i>	1-8-15	DMB	<i>Hafnia</i> sp.	Ps-12	DMB
<i>Pseudomonas maltophilia</i>	1-8-11	DMB	<i>Erwinia</i> sp.	16-20-11	DMB
<i>Pseudomonas maltophilia</i>	1-8-12	DMB	<i>Erwinia</i> sp.	16-20-12	DMB
<i>Pseudomonas maltophilia</i>	1-8-13	DMB	<i>Erwinia</i> sp.	"Grula"	DMB
<i>Pseudomonas aureofaciens</i>	13985	ATCC	<i>Erwinia</i> sp.	CDC	DMB
<i>Pseudomonas striata</i>	1-12-11	DMB	<i>Klebsiella pneumoniae</i>	11-1-11	DMB
<i>Pseudomonas striata</i>	1-2-12	DMB	<i>Klebsiella</i> sp.	I-8	DMB
<i>Pseudomonas fragi</i>	4973	ATCC	<i>Klebsiella</i> sp.	11-2-11	DMB
<i>Pseudomonas</i> sp.	1-20-12	DMB	<i>Klebsiella</i> sp.	11-20-11	DMB
<i>Pseudomonas</i> sp.	1-20-13	DMB	<i>Klebsiella</i> sp.	11-20-12	DMB
<i>Pseudomonas testosteroni</i>	1-9-1	DMB	<i>Klebsiella</i> sp.	11-20-14	DMB
<i>Pseudomonas alcaligenes</i>	1-13-1	DMB	<i>Salmonella</i> sp.	DAL-D-1	DAL
<i>Alcaligenes faecalis</i>	2-1-11	DMB	<i>Salmonella</i> sp.	15-1-11	DMB
<i>Alcaligenes faecalis</i>	2-1-12	DMB	<i>Salmonella</i> sp.	15-2-11	DMB
<i>Alcaligenes faecalis</i>	2-1-24	DMB	<i>Salmonella</i> sp.	15-3-11	DMB
<i>Alcaligenes faecalis</i>	2-1-31	DMB	<i>Salmonella</i> sp.	DAL-D-2	DAL

^a Abbreviations: ATCC, American Type Culture Collection, Rockville, Md.; DAL, Dallas Collection; DMB, Drug Microbiology Branch.

for 15 min, all media were found to have a pH of approximately 6.0.

Two milliliters of each medium were aseptically dispensed to sterile, screw-capped test tubes (13 by 100 mm) and held at 4°C until used. The bacterial suspensions were agitated thoroughly and were placed in a 35°C incubator for 48 h. Each culture was then treated as by Lambert and Moss (12), and *N*-heptafluoryl butyl-*n*-propyl ester (*N*-HFB) derivatives were prepared for GLC analyses. The *N*-HFB derivatives of the amines and amino acids were dissolved in 100 μ l of ethyl acetate, and 3- to 5- μ l

portions were injected into the gas chromatograph.

Standards of putrescine (1,4-diamino butane), cadaverine (1,5-diaminopentane), citrulline (1-amino ureidovaleric acid), ornithine (2,5-diaminopentanoic acid), and agmatine (1-amino-4-guanidolbutane) (Aldrich Chemical Co., Milwaukee, Wis.) were prepared in chloroform. A 50- μ l portion of a 1:200 dilution of each standard was derivatized (12) and injected into the Barber-Colman model 5000 gas chromatograph to detect bacterial catabolites.

Gas-liquid chromatography. Catabolites from bacterial growth medium were analyzed as the *N*-

HFB derivatives with a Barber-Colman model 5000 gas chromatograph (Barber-Colman, Rockford, Ill.) equipped with a flame-ionization detector, as previously described by Fugate et al. (8). Two equivalent nonpolar columns were used: 3% OV-1 and 3% SE-30 on 80/100 mesh Chromosorb W (Applied Science Laboratories, State College, Pa.). The liquid phases were dissolved in CHCl_3 , the support was added, and the mixture was evaporated with dry air on a steam bath with gentle stirring. The free-flowing support was added to glass columns (1 m by 3 mm) and prepared by heating overnight at 250°C with a 50-ml/min N_2 flow. The columns were used for analysis by temperature programming from 150 to 250°C at 5°C/min with a 50-ml/min N_2 flow.

Both columns gave equivalent relative retention times for the N-HFB derivatives, and either column could be used for catabolite analysis. A 3- μl portion of each sample (1/100 of medium extract) was injected and analyzed against chromatograms of individual standard derivatives obtained under the same conditions.

A Finnigan model 1015 D gas chromatograph-mass spectrometer in the electron impact mode was used with a PDP-8E data system for qualitative analysis of several catabolites. Direct comparison analysis, in which standard and sample derivatives are examined under identical conditions, was used.

RESULTS

The average retention times in minutes, obtained with the N-HFB ester derivatives analyzed on the columns, were as follows: ornithine, 8.2; arginine, 11.8; citrulline, 6.0; agmatine, 9.3; cadaverine, 4.8; putrescine, 3.4; and lysine, 10.0. Retention times were comparable on both 3% SE-33 columns and 3% OV-1 columns.

The bacteria found positive for arginine dihydrolase converted arginine to ornithine exclusively in only two instances: one *Pseudomonas* sp. strain (1-20-13) and one *Hafnia* sp. strain (Ps-12).

The results from the use of Moeller medium are not presented since equivocal results and borderline indicator changes were obtained. Citrulline was not produced by two (1-1-35 and 1-1-150) of 11 strains of *Pseudomonas aeruginosa* and two (1-4-26 and 1-4-11) of three strains of *Pseudomonas cepacia*. Ornithine was produced by only one strain (1-2-1) of six *Pseudomonas fluorescens* strains tested. These results are shown in Table 2. Generally, these results were in agreement with Gilardi's findings (9), although the utilization of GLC techniques to obtain our data resulted in detectable amounts of chemical compounds in as little as 8 h compared to the conventional broth oil-overlay method requiring several days.

No ornithine decarboxylase activity was detected in any strain tested. Of the 45 strains of *Pseudomonas* listed in Table 2, none produced detectable amounts of putrescine.

Nineteen strains of nonoxidative nonfermentative bacteria were tested for production of amino acid metabolites. Only seven strains of two species showed activity on the applied substrates. One strain of *Pseudomonas testosteroni* and one of *Pseudomonas alcaligenes* were found to be inactive in all three substrates tested. Four of five strains of *Alcaligenes faecalis* (2-1-11, 2-1-24, 2-1-31, 2-1-32) produced only ornithine from arginine substrate, and all were inactive in lysine and ornithine broth media. The two *Moraxella* strains (AOII-1 AOII-6) catabolized arginine to both citrulline and ornithine but were inactive in lysine and ornithine broths. All strains of *Bacterium anitratum* (*Mima* and *Herellea*) tested were inactive in all amino acid substrate media.

Table 3 lists the results from 38 strains of *Enterobacteriaceae*. The six strains of *Escherichia coli* formed putrescine and agmatine from arginine substrate; putrescine was not

TABLE 2. Amino acid metabolites of oxidative and weakly oxidative bacteria detected by GLC

Species	Total no. of strains	Product from lysine: cadaverine	No. of strains producing the following products from L-arginine		
			Citrulline	Ornithine	Agmatine
<i>Pseudomonas aeruginosa</i>	11	0 ^a	9	11	0
<i>P. fluorescens</i>	6	0	6	1	0
<i>P. putida</i>	6	0	6	0	0
<i>P. cepacia</i>	3	3	1	3	0
<i>P. pseudoalcaligenes</i>	1	0	1	1	0
<i>P. stutzeri</i>	6	0	0	0	0
<i>P. maltophilia</i>	6	6	0	6	6
<i>P. aureofaciens</i> (ATCC #13985)	1	0	1	0	0
<i>P. striata</i>	2	0	0	0	0
<i>P. fragi</i> (ATCC #4973)	1	0	0	0	0
<i>Pseudomonas</i> sp.	2	0	0	1	0

^a Number of strains positive.

TABLE 3. Amino acid metabolites of members of the family Enterobacteriaceae detected by GLC

Species	Total no. of strains	Product from lysine: cadaverine	Product from ornithine: putrescine	No. of strains producing products from L-arginine			
				Putrescine	Citrulline	Ornithine	Agmatine
<i>Escherichia coli</i>	6	6 ^a	6 ^a	6	0	0	6
<i>Serratia marcescens</i>	1	1	1	0	0	0	0
<i>Enterobacter liquefaciens</i>	3	0	0	0	0	0	0
<i>Enterobacter aerogenes</i>	4	4	3	0	2	0	0
<i>Enterobacter cloacae</i>	7	0	7	3	2	2	1
<i>Enterobacter hafniae</i>	1	1	1	0	0	0	0
<i>Hafnia</i> sp. (Ps-12)	1	0	0	0	0	1	0
<i>Erwinia</i> sp.	4	0	0	0	0	0	0
<i>Klebsiella pneumoniae</i>	1	1	0	0	0	0	0
<i>Klebsiella</i> sp.	5	4	1	0	0	0	0
<i>Salmonella</i> sp.	1	1	1	0	0	0	0
<i>S. enteritidis</i> bioserotype paratyphi A (15-1-11)	1	1	1	1	0	1	0
<i>S. oranienberg</i> (15-3-11)	1	1	1	0	0	0	0
<i>S. manila</i> (15-2-11)	1	1	1	0	0	0	0
<i>S. enteritidis</i> serotype typhimurium	1	1	1	1	0	1	0

^a Number of strains positive.

produced by one (10-1-12) of four strains of *Enterobacter aerogenes*. Citrulline was not produced by two (10-1-11 and 10-1-12) of four strains of *Enterobacter aerogenes* tested in arginine broth. Cadavarine was not produced by one (11-20-12) of five strains of *Klebsiella* sp. tested in lysine broth. Only one strain (11-20-11) of *Klebsiella* produced detectable amounts of putrescine from ornithine medium.

Of the seven strains of *Enterobacter cloacae* examined by GLC for products from L-arginine, putrescine was produced only by strains "B" CDC, "P," and "A" (10-6-11) (see Table 4). These results emphasize the variability of metabolic products found within the species examined.

Figure 1 shows the chromatograms obtained with the gas-liquid chromatograph-mass spectrometer-data system. The upper trace represents approximately 10 ng from an extract, whereas the lower trace represents 50 ng of a standard N-HFB derivative. The electronic masses (*m/e*) of the fragments were identical except that the amplitude of the 169 fragment was approximately 5% higher for the standard derivative. This spectrum demonstrated that the trace component was in fact cadaverine.

Figure 2 illustrates a qualitative analysis of a trace amount of cadaverine formed by a strain of *Pseudomonas maltophilia* after incubation in lysine broth. The gas-liquid chromatograph-mass spectrometer-data system was programmed to use a mass-to-charge ratio (*m/e*) of 226 to identify cadaverine and an *m/e* of 281 to identify citrulline. These fragments apparently are characteristic for these derivatives. No molecular ions are observed for this type of derivative in the electron impact ionization mode.

TABLE 4. Amino acid metabolites from *Enterobacter cloacae* strains grown in arginine broth

<i>E. cloacae</i> strain	Products from L-arginine detected by GLC:			
	Putrescine	Citrulline	Ornithine	Agmatine
"B" CDC	+	+	-	-
"A" DM-570	-	-	-	-
"P"	+	-	+	-
"A" DM5577	-	-	-	+
(10-5-11)	-	-	+	-
"A" (10-6-11)	+	+	-	-
"A" CDC	-	-	-	-

Derivatives of standard catabolites and extracts were analyzed by identical comparison methods.

DISCUSSION

The data resulting from this study show the type of information that GLC analyses of bacterial metabolic products can provide. These analyses do not differ in intent or purpose from the estimation of identity afforded by methods where a change in a pH-sensitive indicator is the criterion for the presence or absence of an enzyme system (1, 2, 7, 10). However, it is apparent that the specificity and reproducibility of GLC procedures are distinct advantages in classifying those nonfermenting bacteria that produce little or no pH change.

Enzymatic systems possessed by pseudomonads are reflected in the production of specific catabolic products from the arginine substrate. The use of GLC analysis, combined with classical bacteriological testing, may assist in the specific identification of non-fermentative

FIG. 1. Chromatograms of cadaverine standard (50 ng of derivatized cadaverine) and of culture broth (2 ml of 48-h-old culture).

FIG. 2. Mass spectrogram of cadaverine (50 ng of derivatized cadaverine).

gram-negative bacteria.

The production of both ornithine and agmatine from L-arginine was distinctive for strains of *Pseudomonas maltophilia*.

The production of citrulline exclusively from arginine by *Pseudomonas putida* strains indicates that these organisms evidently have a very low ornithine transcarbamylase enzyme

system activity (carbamylphosphate: L-ornithine carbamoyltransferase, EC 2.K.3.3). The lack of this enzyme prevents the conversion of citrulline to ornithine (11, 17, 19).

In distinguishing the moraxellae (acinetobacters) from *Alcaligenes faecalis* and *Bacterium anitratum*, the available biochemical, serological, and morphological data are not con-

clusive (14). The data presented in this paper support the separation of *Bacterium anitratum*, *Moraxella*, and *Alcaligenes faecalis* into different groups on the basis of their enzyme patterns. *Bacterium anitratum* strains were unreactive in the three amino acid substrates tested whereas *Alcaligenes faecalis* strains produced ornithine from arginine broth, and *Moraxella* strains gave both citrulline and ornithine. The *Mima* strains are distinguished from moraxellae, but they are difficult to classify because of the limited number of biochemical reactions they exhibit (3, 16). The acinetobacters (*Mima*, *Herellea*) were found not to have any detectable activity in the amino acid broths tested.

When strains of *Escherichia coli* were incubated in arginine broth, all of the strains tested produced both agmatine and putrescine. This was a unique pattern revealed by strains of *E. coli*. Arginine catabolic products alone differentiated *E. coli* from the other members of *Enterobacteriaceae* (14).

The diversity of L-arginine catabolic systems among strains of *Enterobacter cloacae* is illustrated in Table 4. Two strains of *E. cloacae* type "A" did not catabolize arginine, whereas one strain of type "A" produced detectable amounts of agmatine and the other type "A" produced putrescine and citrulline in trace amounts. In contrast, all strains tested were unreactive in lysine broth but produced appreciable quantities of putrescine from ornithine broth.

The diverse metabolic products observed from these three substrates suggest that the analytical system may be a useful aid in the identification of gram-negative, non-fermenting bacteria. While products are occasionally obtained in only trace amounts, their identity is easily verified through existing Finnigan Data System research programs, as illustrated in Fig. 1.

Currently we are extending the data base to improve the usefulness of this identification system.

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