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Auxotrophic Variants of *Pseudomonas aeruginosa* Are Selected from Prototrophic Wild-Type Strains in Respiratory Infections in Patients with Cystic Fibrosis

AFONSO L. BARTH AND TYRONE L. PITT*

Laboratory of Hospital Infection, Central Public Health Laboratory, London NW9 5HT, United Kingdom

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Twenty-four nutritionally dependent (auxotrophic) *Pseudomonas aeruginosa* strains were isolated from 20 cystic fibrosis (CF) patients and tested for their amino acid requirements. Two different methods were necessary to identify the nutritional status of all isolates. Methionine was the most common single amino acid required (9 of 24 isolates), followed by leucine and arginine or ornithine. In total, a requirement for 12 different compounds or combination of compounds was demonstrated. Auxotrophic and prototrophic pairs of isolates from the same patient were compared by macrorestriction analysis of DNA in pulsed-field gel electrophoresis. Thirteen of 18 pairs analyzed presented identical restriction fragment length polymorphism profiles following digestion of DNA with *Xba*I. Three of the remaining pairs showed percentage similarities of 77, 91, and 98%, and the profiles of two pairs could not be compared because of the excessive degradation of their DNA. These results suggest that auxotrophic and prototrophic *P. aeruginosa* isolates colonizing the same CF patient constitute an isogenic group and raise the possibility that auxotrophs are selected from the prototrophic population during the course of pulmonary infection in CF patients.

Pseudomonas aeruginosa frequently colonizes the respiratory tract of patients with cystic fibrosis (CF) and is associated with increased severity and mortality in these patients (8). During the course of colonization in CF, the organism expresses a variety of phenotypic modifications, including, among others, instability of colonial morphology and formation of an alginate exopolysaccharide (5), loss of cell wall lipopolysaccharide (16), sensitivity to serum (7), and hypersensitivity to semisynthetic penicillins (10).

Some strains of *P. aeruginosa* from CF also have specific nutritional requirements and are termed auxotrophic because of their inability to grow in a basal medium containing mineral salts and glucose as the sole carbon source (19). Auxotrophic strains are unable to synthesize a particular growth factor, which in the case of *P. aeruginosa* is usually a specific amino acid, most frequently, methionine. These strains are also sometimes found in patients with bronchiectasis but have not been isolated from patients with other conditions. A recent study has shown that auxotrophic *P. aeruginosa* strains are significantly associated with acute exacerbations of respiratory infection in CF patients (20), and these isolates are often more resistant to antipseudomonal agents than prototrophic wild-type strains (21).

Although most CF patients are infected by single strains of *P. aeruginosa*, as determined by genotype analysis (13), a minority of them sometimes harbor two or more distinct strains for prolonged periods (1). It was, therefore, necessary to determine whether auxotrophic variants of *P. aeruginosa* originated from wild-type prototrophs or were distinct from the latter strains. We have applied restriction fragment length polymorphism typing by using rare-cutting restriction endonuclease enzymes and pulsed-field gel electrophoresis (PFGE) to compare the genotypes of auxotrophic and prototrophic *P. aeruginosa* strains isolated from the same sputum specimens of patients.

* Corresponding author. Mailing address: Laboratory of Hospital Infection, Central Public Health Laboratory, 61 Colindale Ave., London NW9 5HT, United Kingdom. Phone: 081-200 4400. Fax: 081-200 7449.

MATERIALS AND METHODS

Bacterial isolates. Fifty-four CF patients were selected from in- and outpatients of the Department of Cystic Fibrosis of the Royal Brompton Hospital, Chelsea, London, United Kingdom.

Sputum samples were collected in sterile containers and homogenized by shaking with an equal volume of Ringer's solution and glass beads for approximately 3 min. The homogenate was diluted in 10-fold steps from 10^{-2} to 10^{-7}, and 0.1 ml of each dilution was spread evenly over the total surface of a King's A agar (KA) plate (12). After incubation at 37°C for 48 h, those plates which exhibited pseudomonas-like growth were replica plated (with the aid of a velvet pad) onto a minimal agar medium containing salts and 0.2% (wt/vol) glucose (3) (MAM) and a fresh KA plate. After incubation at 37°C for 48 h, isolates which failed to grow on the minimal medium but grew on KA were selected as probable auxotrophs.

The nutritional status of these colonies was confirmed as follows. Bacterial growth was suspended in sterile distilled water to give a reading of 12 to 14 with a densitometer ATB 1550 (API System S.A., Marcy, France), which corresponded to 10^8 CFU/ml. This suspension was diluted 10-fold and applied in 0.3-μl volumes to the surface of both KA and MAM agar media with the aid of a multipoint inoculator (Mast Laboratories Ltd., Merseyside, United Kingdom). Prototrophic colonies grew on both agar media, while the growth of auxotrophs was supported only by the complete medium.

All isolates were tested for oxidase production, oxidative reaction in Hugh and Leifson's medium, production of arginine dihydrolase and urease, and the formation of pyocyanin and/or fluorescein. Further confirmatory tests for *P. aeruginosa* included the pattern of utilization of sugars in ammonium salt medium (11).

Identification of amino acid requirement. Stock solutions of 23 amino acids, namely, alanine, arginine, asparagine, aspartic acid, cysteine, cystine, glutamic acid, glutamine, glycine, histidine, hydroxy-L-proline, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine (L forms; Sigma Chemical Co., St. Louis, Mo.) were prepared at a concentration of 2 mg/ml in either sterile distilled water or the solvent recommended by the manufacturer and filter sterilized.

Two basic approaches to the identification of amino acid requirement were used. In the first method, agar plates each containing a single amino acid were prepared by adding 0.2 ml of stock solution to 19.8 ml of molten MAM at 50°C. Plates were inoculated with 0.3 μl of the bacterial suspension diluted as described above and incubated at 37°C for 48 h. Control agars, KA and MAM without amino acids, were included in all tests. Growth on a specific amino acid(s) indicated a requirement for single or multiple compounds, and no growth was interpreted as a requirement for two or more amino acids in combination or

TABLE 1. Amino acid requirements of auxotrophic isolates of *P. aeruginosa* from CF patients

Amino acid requirement	Isolate no.	No. of isolates (n = 24)
Methionine	65,[a] 74, 80,[b] 132, 154, 156(A),[c] 186, 191, 193	9
Leucine	91, 121	2
Arginine[d]	104(B), 111	2
Histidine	102	1
Lysine	181(C)	1
Serine[e]	69	1
Tryptophan	145[f]	1
Isoleucine and valine	107(B), 124(D), 135	3
Isoleucine, valine, and leucine	125(D)	1
Methionine and leucine	157(A)	1
Methionine and lysine	179(C)	1
Arginine or aspartic acid or asparagine or proline	83	1

[a] Growth also promoted by cysteine or cystine.
[b] The strain grew weakly in the presence of other individual amino acids; however, methionine was the sole amino acid whose omission from the medium resulted in the absence of growth.
[c] Letters in parentheses denote isolates from same patient.
[d] Growth also promoted by ornithine.
[e] Growth also promoted by glycine.
[f] The strain grew weakly in the presence of phenylalanine.

some other growth factors. The latter was confirmed for some strains by a test on MAM containing each of the 23 amino acids.

The second method was adapted from Catlin (2) to complement the procedure described above. Here, all amino acids less one were combined at 20 μg/ml in agar plates. Isolates were spotted on each of these plates as described above, and a specific requirement for an amino acid or combination was evident if the isolate failed to grow on the agar not containing the compound.

The *P. aeruginosa* NCTC 50184 (*met*), NCTC 50181 (*trp*), and NCTC 50182 (*arg*) strains were used as controls.

Genotyping. The overnight bacterial growth from a KA plate was gently resuspended in 1 ml of SE buffer (75 mM NaCl, 25 mM EDTA [pH 7.5]), and the opacity was adjusted to 26 to 29 in the ATB 1550 densitometer. The suspension (500 μl) was mixed with an equal volume of 2% (wt/vol) low-gelling agarose (Seaplaque; FMC BioProducts, Rockland, Maine) in SE buffer at 56°C and dispensed into plastic molds (10 by 5 by 1 mm). After setting at 4°C, the agar blocks were covered with 2 ml of lysis buffer (1% [wt/vol] *N*-lauroylsarcosine, 0.5 M EDTA [pH 9.5]) and 20 μl of proteinase K enzyme (Sigma) to give a final concentration of 500 μg/ml. Following overnight incubation at 56°C, the lysis buffer was discarded and replaced, and the block was again incubated overnight with proteinase K enzyme. The block was washed thrice in TE buffer (10 mM Tris, 10 mM EDTA [pH 7.5]), each for 30 min, and stored at 4°C until required. Small portions (2.5 by 5 by 1 mm) of the blocks were equilibrated with 100 μl of the recommended reaction buffer in microcentrifuge tubes for 1 h at 4°C prior to digestion. After removal of the equilibration buffer, the DNA was digested with 20 U of *Xba*I (recognition sequence, TCTAGA) or *Dra*I (recognition sequence, AAATTT) (Gibco, Paisley, Scotland) in 100 μl of fresh buffer at 37°C for 6 h. DNA fragments were separated in 1.2% molecular biology-grade agarose by PFGE in a CHEF-DR II apparatus (Bio-Rad Laboratories Ltd., Hemel Hempested, United Kingdom) in 0.5 M TBE buffer (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA [pH 8.6]) for 36 h with initial and final pulse times of 5 and 25 s, respectively. Phage λ ladder DNA (Sigma) and *Escherichia coli* MG1665 digested with *Not*I were used as size markers. DNA fragments were stained with ethidium bromide (1 μg/ml) and photographed under UV illumination. Profiles were compared visually, and pairs of isolates which gave identical profiles were considered to represent the same strain. Pairs of isolates with similar but not identical profiles had their RFLP patterns compared, and their percentages of similarity were expressed with the use of the Dice coefficient (4), i.e., two times the number of shared bands divided by the sum of the number of bands in each profile.

RESULTS

Frequencies and amino acid requirements. *P. aeruginosa* was isolated from the sputa of 40 of 54 patients. Of the 40 patients, 20 yielded prototrophs alone and 18 harbored auxotrophs and prototrophs. Only two patients were found to be colonized exclusively by auxotrophic *P. aeruginosa*. The frequencies of pyocyanin production and expression of alginate (mucoid colonies) were 71 and 46%, respectively, for auxotrophic isolates and 55 and 53% for the prototrophs ($P < 1.0$).

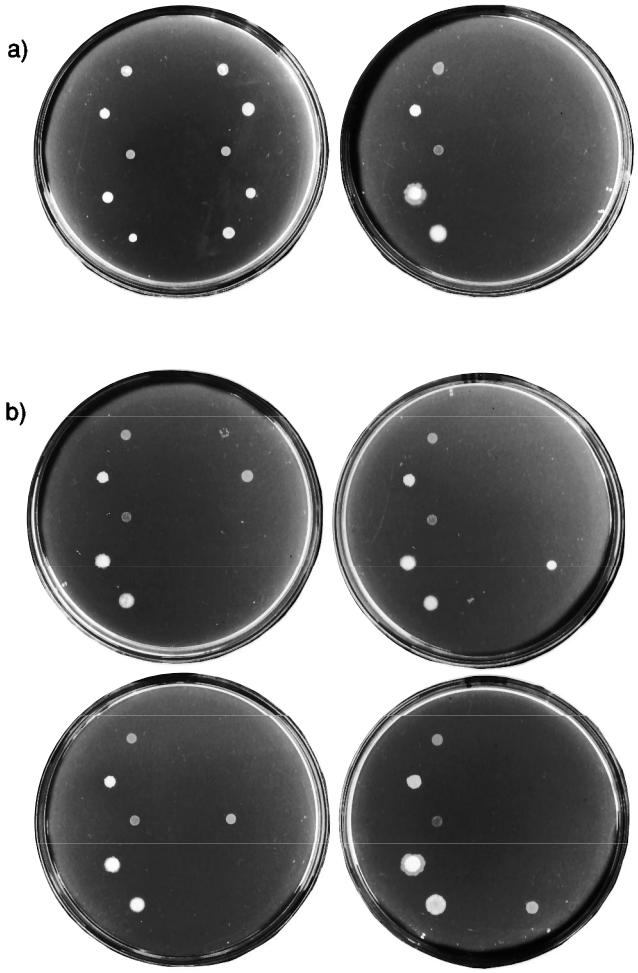

FIG. 1. (a) Growth on KA (left) and MAM (right) of 10 isolates from five CF patients. Prototrophic strains grew on both media. Auxotrophs grew only on KA (i.e., complex medium). (b) Growth of the same set of isolates on MAM with methionine (top left), MAM with tryptophan (top right), MAM with histidine (bottom left), and MAM with lysine (bottom right).

Table 1 shows that of the 24 auxotrophs identified, the most common single amino acid requirement was for methionine (nine isolates), followed by leucine and arginine or ornithine. Histidine, lysine, tryptophan, and serine or glycine were each required by a single strain (Fig. 1). Three isolates grew only on a combination of isoleucine and valine, and one isolate required leucine in addition. A requirement for methionine in combination with either leucine or lysine was identified in two isolates. The growth of one isolate was supported by any of four different amino acids. Pairs of isolates which exhibited different requirements were identified in four patients. For example, in patient 7, isolate 104 was *arg* or *orn* and isolate 107 was *ile* and *val* (Tables 1 and 2). In total, a requirement for 12 different compounds or combination of compounds was demonstrated.

Genotyping of isolates. An interpatient comparison of RFLP

TABLE 2. Genotyping of pairs of auxotrophic and prototrophic isolates of *P. aeruginosa* from the same CF patient

Patient no.	Strain no.	Phenotype[a]	Requirement	Genotype[b]	Dice coefficient (%)[c]
1	65	AUX	Met	A	
	63	PRO		A	
2	69	AUX	Ser or Gly	B	
	68	PRO		B	
3	74	AUX	Met	C	
	75	PRO		C	
4	80	AUX	Met	D	
	79	PRO		D	
5	91	AUX	Leu	E	
	89	PRO		E	
6	102	AUX	His	F	
	101	PRO		F	
7	104	AUX	Arg or Orn	G	
	106	PRO		G	
8	111	AUX	Arg or Orn	H	77
	109	PRO		H_1	
9	121	AUX	Leu	I	91
	123	PRO		I_1	
10	124	AUX	Ile and Val	J	
	126	PRO		J	
11	132	AUX	Met	K	
	133	PRO		K	
12	135	AUX	Ile and Val	L	
	137	PRO		L	
13	145	AUX	Trp	M	
	144	PRO		M	
14	154	AUX	Met	N	
	152	PRO		N	
15	156	AUX	Met	O	
	159	PRO		O	
16	186	AUX	Met	NT[d]	
	184	PRO		P	
17	193	AUX	Met	Q	
	196	PRO		Q_1	
18	83	AUX	Arg or Asp or Asn or Pro	NT	
	81	PRO		NT	

[a] AUX, auxotroph; PRO, prototroph.
[b] Arbitrary groups and subgroups.
[c] Dice coefficient of percentage similarity of PFGE profiles (100% unless stated).
[d] Not typeable because of excessive degradation of DNA.

profiles of *Xba*I-digested DNA indicated that each of the 18 patients who were colonized by auxotrophic and prototrophic *P. aeruginosa* isolates harbored genotypically distinct strains (Table 2). It was not possible to compare the RFLP profiles of two pairs of isolates because of excessive degradation of the DNA when incubated with the restriction endonuclease (Fig. 2, lane 11). Thirteen of the 18 pairs of auxotrophic and prototrophic isolates from the same patient gave identical RFLP profiles, as illustrated in Fig. 2. Of the remaining pairs, three showed differences in their profiles. In these cases, the percentage coefficients of similarity of banding patterns within an approximate molecular size range of 50 to 400 kb (21 to 29 bands) were 77, 91, and 98% for patients 8, 9, and 17, respectively (Table 2).

*Dra*I digestion of DNA from these three pairs showed that the isolates of the pair from patient 17 were identical, whereas those from patients 8 and 9 exhibited minor differences.

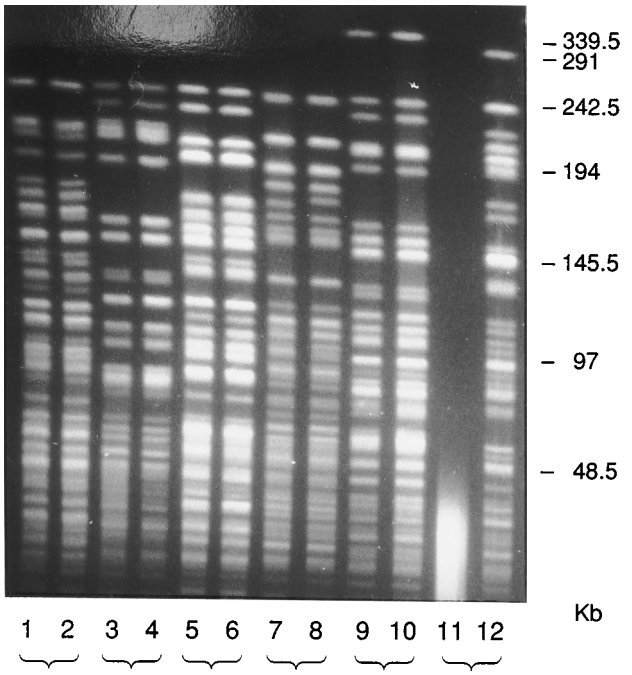

FIG. 2. DNA macrorestriction profiles of pairs of auxotrophic and prototrophic isolates from different patients (lanes 1 and 2, 3 and 4, 5 and 6, 7 and 8, 9 and 10, and 11 and 12).

DISCUSSION

It is noteworthy that in the earlier study of auxotrophy in *P. aeruginosa* (19), we had not been able to identify the requirements of all CF isolates. The probable reason for this was the use of a single method which relied upon random pools of combined amino acids (9). It is not possible by that method to identify isolates which require combinations of two amino acids separated in different pools. One of the aims of this investigation was to improve the identification of the specific amino acids required by CF strains of *P. aeruginosa*. To this end, we adopted a modified procedure of Catlin (2) which allowed not only the direct identification of single growth requirements but also the identification of multiple requirements by strains. However, the need for alternative amino acids by auxotrophs can be identified only by the use of single amino acids in a minimal medium. As a result, we found that all isolates exhibited requirements for either single amino acids such as methionine, a combination of two or more compounds (isoleucine and valine), or alternative energy sources (serine or glycine).

Auxotrophic mutants of *P. aeruginosa* can be selected in vitro from wild-type prototrophic strains by treatment with mutagenic agents (14, 15). The mutants obtained in this way may exhibit nutritional requirements similar to some of the apparently natural phenotypes in CF such as isoleucine and valine. The mechanism of selection in vivo remains to be investigated, but we have speculated that auxotrophic variants of *P. aeruginosa* emerge in CF as a mutational event from prototrophic wild-type strains. It was, therefore, necessary to demonstrate whether these varieties were genotypically related to the prototrophic strains coexisting in the same sputum specimen. It has been shown by various studies that macrorestriction analysis of chromosomal DNA of *P. aeruginosa* separated by PFGE provides an accurate and reproducible

means of identifying clonal relationships between strains (6, 18). We have used this approach to test our hypothesis.

Our results indicated that the strain populations among patients were highly heterogeneous and did not suggest cross-infection to any significant degree between the patients. Moreover, it was clear that in most cases the auxotrophic variant had a PFGE profile and, hence, genotype identical to those of the prototrophic forms when both varieties were isolated from the same patient. Where differences were seen, they did not exceed, with the exception of one pair, the parameters of similarity advocated by other studies (6, 18) for the distinction between similar and different strains. Thus, it is reasonable to assume that within an individual patient, both varieties had a common genetic lineage.

Most of the reports of the epidemiology of *P. aeruginosa* in CF as determined by gene probes support the view that a limited number of strains can occur together within a patient and that these may fluctuate in predominance over several months or years (17, 22). We are in the process of examining the genetic variability of auxotrophic variants isolated over a long period, and preliminary results suggest that these variants retain their genotype profiles as well as their nutritional status and amino acid requirements.

It has been suggested that acute exacerbations in patients are associated with an increase in the numbers of the auxotrophic population (20). This study has shown that populations of the same strain of *P. aeruginosa* with different nutritional requirements can coexist in the lungs of a CF patient. It is therefore necessary to examine the genetic basis of selection of auxotrophic variants, in vivo, and the role of therapy in their emergence in CF.

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