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EXPERIMENTAL ARTICLES

Application of the Profiles of Amino Acid Utilization as the Sole Carbon and Nitrogen Sources for Pseudomonad Taxonomy

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Abstract—Profiles of utilization of 20 amino acids were determined for 218 strains of 10 *Pseudomonas* species (*P. aeruginosa, P. putida, P. fluorescens, P. stutzeri, P. alcaligenes, P. pseudoalcaligenes, P. luteola, P. oryzihabitans, P. mendocina,* and *P. chlororaphis*), including the type strains of these species. Amino acid utilization was determined on minimal salt agar with an amino acid as the sole source of nitrogen and carbon. All the investigated pseudomonad species had species-specific profiles of amino acid utilization. For the type strains of all species, Jaccard's coefficients of community were different (Sj = 0.31–0.82), in accordance with the interspecies differences. The similarity between the intraspecies variants of the profiles and that of the type strain was high; for 98% of *P. aeruginosa* strains, Sj = 0.85–1.0; for 100% of *P. putida, P. stutzeri*, and *P. alcaligenes* strains, Sj was 0.87–1.0, 0.90, and 0.86–1.0, respectively. Only for *P. fluorescens* and *P. pseudoalcaligenes* were low Sj of the intraspecies profiles revealed, in accordance with the known phenotypic heterogeneity of these species. These results agree with the known pseudomonad classification, and the method is therefore valid for identification of known species and for determination of the new members of the genus *Pseudomonas.*

Key words: amino acid utilization profiles, taxonomy, identification, *Pseudomonas.*

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Utilization of amino acids as sole carbon sources is a test used in pseudomonad taxonomy [1–3]. Identification of pseudomonad species involves a complex of phenotypic characteristics, including utilization of 17 amino acids present in proteins [3]. Some authors recommend utilization of 6 to 12 amino acids as a criterion for pseudomonad identification [1, 2]. Pseudomonads are also known to be able to utilize some amino acids as the sole source of nitrogen and carbon [4]. The importance of the profiles of utilization of all amino acids in the composition of proteins for pseudomonad classification has not been investigated. Refinement of the criteria of the genus *Pseudomonas* and its species composition by molecular techniques [3] and the unique pseudomonad marker of barium sensitivity [5–7] enabled more precise determination of the taxonomic value of the phenotypic characteristics.

The goal of the present work was determination of the taxonomic validity of utilization profiles for 20 amino acids incorporated in proteins (as sole nitrogen and carbon sources) for pseudomonad classification.

MATERIALS AND METHODS

The research involved 218 pseudomonad strains, including the type strains of 10 species. Type strains *P. putida* CIP 52191^T (ATCC 12633), *P. alcaligenes* CIP 101034^T (ATCC 14909), *P. luteola* CIP 102995^T (JCM 3352), and *P. oryzihabitans* CIP 102996^T (JCM 2952) were obtained from the collection of the Pasteur Institute (Paris); type strains of *P. fluorescens* IMV 4125 (ATCC 13525), *P. chlororaphis* subsp. *chlororaphis* IMV 4139 (ATCC 9446), *P. chlororaphis* subsp. *aureofaciens* IMV 4133 (ATCC 13985), *P. mendocina* IMV 4172 (ATCC 25411), *P. stutzeri* IMV 4136 (ATCC 17588), and *P. pseudoalcaligenes* IMV 4134 (ATCC 17440) were obtained from the culture collection of the Zabolotnii Institute of Microbiology and Virology (Kyiv, Ukraine); the type strain of *P. aeruginosa* GISK 190154 (ATCC 10145) was obtained from the bacterial collection of the Tarasevich State Research Institute for Standardization and Control of Medical Biological Preparations. The strains investigated included 150 strains of *P. aeruginosa*, 40 strains of *P. putida*, 10 strains of *P. fluorescens* (biovar I, IMV 4125; biovar II, 356-H; biovar III, 64-H, 176-H, 117-H, 240-K, and 817-K; biovar V, 128-H; 363-H; and 27481-K); 4 strains of *P. stutzeri* (INV 4133; 390-H; 12M-H; and 53-K), *P. alcaligenes* (CIP 101034^T; 18P-H; 19M-H; and 79-K), and *P. pseudoalcaligenes* (IMV 4134; 10-H; 16-H; and 6897-K); 2 *P. luteola* strains (CIP 102995^T; 22-K), 1 strain of *P. oryzihabitans, P. chlororaphis* subsp. *chlororaphis*, *P. chlororaphis* subsp. *aureofaciens*, and *P. mendocina*. Among these strains, 50 *P. aeruginosa*, 8 strains of *P. putida* biovar A (3-H, 4-H, 5-H, 7-H, 9-H, 11-H, 12-H, and 13-H), 2 strains of

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P. putida biovar B (2-H and 6-H), 6 strains of *P. fluorescens* (365-H, 64-H, 176-H, 117-H, 128-H, and 363-H), 2 strains of *P. stutzeri* (390-H and 12M-H), *P. alcaligenes* (18P-H and 19M-H), and *P. pseudoalcaligenes* (10-H and 16-H) were isolated by us from the Neva water. The remaining pseudomonad strains were isolated from the clinical material in the bacteriological laboratory of the Military Medical Academy (St. Petersburg). The strains were identified according to the methods and criteria described in [2, 3, 8]. Barium sensitivity was determined according to [6].

Amino acids (20) incorporated in proteins were manufactured by Reanal (Hungary), Merck (United States), ICN (United States), and Reakhim (Russia).

For cultivation of bacteria, the Nutrient Agar for Microbial Cultivation (GRM agar) of the Scientific Production Center for genetically engineered preparations was used (Obolensk, Russia). For determination of the profiles of utilization of amino acids as sole nitrogen and carbon sources, minimal salt agar was used containing the following (g/l distilled water): NaCl, 5; Na_2SO_4, 2; KH_2PO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.1; and bacteriological agar (Serva, Czech Republic; Difco, United States,; and Himedia, India), 15; pH was 7.2 ± 0.2. Each amino acid was introduced into 50 ml of got medium (0.1 g; 0.05 g for L-tryptophan); after pH adjustment, the medium was distributed into petri dishes. In the control variants, minimal salt agar without amino acids was used.

Determination of the profiles of amino acid utilization. For inoculum, bacteria were grown on GRM agar at 28°C for 18–24 h; a loopful ($d = 3$ mm) was collected and resuspended in 0.2 ml of sterile 0.85% NaCl in a well of a sterile polymer tray. The plates with amino acids and with the control media without amino acids were subdivided into eight sectors and marked according to the strain numbers. A loopful of the culture (1×10^7 CFU) was streaked on 20 sectors of petri dishes with amino acids and the control medium. The plates were incubated at 28°C for 3 days with daily examination. A pronounced bacterial lawn on the medium with an amino acid with no growth on the control medium without amino acids was considered the positive result of utilization of an amino acid as the sole carbon and nitrogen source. The experiment was repeated three times for the type strains and twice for the other strains.

The profiles of amino acid utilization by pseudomonad species were compared using Jaccard's coefficients of community [8]: $Sj = a/(a + b)$, where a is the number of the coinciding positive results and b is the number of not coinciding results revealed by comparison of the profiles of amino acid utilization for each profile compared to the profiles of the type strains.

RESULTS AND DISCUSSION

Among 218 strains of 10 *Pseudomonas* species, 35 variants were revealed for the profiles of utilization of 20 amino acids as sole nitrogen and carbon sources.

Within the species *P. aeruginosa* (150 strains), 13 variants of amino acid utilization profiles, nos. 1–13 were found (Table 1). Profile no. 1 was predominant; it was revealed in 45 strains (30%). Profile no. 1 was characteristic of the type strain *P. aeruginosa* ATCC 10145. All *P. aeruginosa* strains shared the following characteristics of amino acid utilization profiles: utilization of L-lysine, L-arginine, L-histidine, L-alanine, L-proline, L-asparagine, L-glutamine, L-aspartate, and L-glutamate; L-methionine, L-cysteine, and L-threonine were not utilized; characteristically, none of the strains utilized L-serine; glycine was utilized but rarely (1.4% of the strains). Utilization of other amino acids was variable. High similarity (Sj) was revealed between the intraspecies profiles and the profile no. 1 for the type strain *P. aeruginosa* ATCC 10145; for 98% of the strains, Sj = 0.85–1.0 (Table 1). Only three strains exhibited Sj = 0.7–0.8; they differed in pyomelanin production or glycine utilization.

In *P. putida* (40 strains), 5 variants of amino acid utilization profiles were revealed, nos. 14–18 (Table 2). Profile no. 14 was the most widespread; it was found in 28 strains (70%), including the type strain *P. putida* ATCC 12633 biovar A. All *P. putida* strains shared the following characteristics of amino acid utilization profiles: utilization of L-serine, L-lysine, L-arginine, L-histidine, L-alanine, L-asparagine, L-aspartate, L-glutamine, L-glutamate, L-tyrosine, L-leucine, L-isoleucine, L-valine, and L-proline; while L-methionine, L-cysteine, and L-threonine were not utilized. Two groups of profiles were revealed in glycine utilization; profiles nos. 14 and 15 with glycine utilization and profiles nos. 16–18 with no glycine utilization. In L-tryptophan utilization, biovar A (utilization) and biovar B (no utilization) differed. Both strains of *P. putida* biovar B (2-H and 6-H) were isolated from the water of the Neva river and were phenotypically similar. High similarity (Sj) was revealed between the intraspecies profiles of amino acid utilization by all *P. putida* strains and the profile no. 14 of the type strain *P. putida* ATCC 12633; for 100% of the strains, Sj = 0.87–1.0 (Table 2).

P. stutzeri (4 strains) exhibited two variants of amino acid utilization profiles, nos. 24 and 25, which differed in L-isoleucine utilization (Table 2). The profiles for all strains exhibited high similarity to profile no. 24 of the type strain *P. stutzeri* ATCC 17588; Sj = 0.90 (Table 2).

P. alcaligenes (4 strains) also exhibited two variants of amino acid utilization profiles, nos. 26 and 27 (Table 2); 3 strains had the profiles identical to that of the type strain *P. alcaligenes* ATCC 14909 (Sj = 1.0), while the utilization profile of one strain (*P. alcaligenes*

Table 1. Intraspecies profiles of amino acid utilization as the sole source of nitrogen and carbon for *P. aeruginosa* strains ($n = 150$)

Numbers of profile variants	Profiles of amino acid utilization																			Number of strains in the profile	S_j
	Ala	Gly	Asn	Asp	Gln	Glu	Lys	Arg	His	Met	Cys	Thr	Ser	Tyr	Try	Phe	Leu	Ile	Val	Pro	
1	+	–	+	+	+	+	+	+	+	–	–	–	–	+	+	–	–	+	+	+	45*	1.0
2	+	–	+	+	+	+	+	+	+	–	–	–	–	+	-	–	–	+	+	+	33	0.92
3	+	–	+	+	+	+	+	+	+	–	–	–	–	+	+	–	–	+	–	+	18	0.92
4	+	–	+	+	+	+	+	+	+	–	–	–	–	+	–	–	–	+	–	+	18	0.85
5	+	–	+	+	+	+	+	+	+	–	–	–	–	+	+	–	+	+	+	+	17	0.93
6	+	–	+	+	+	+	+	+	+	–	–	–	–	+	–	–	+	+	+	+	8	0.85
7	+	–	+	+	+	+	+	+	+	–	–	–	–	+	+	+	–	+	+	+	3	0.93
8	+	–	+	+	+	+	+	+	+	–	–	–	–	+	–	+	–	+	+	+	1	0.85
9	+	–	+	+	+	+	+	+	+	–	–	–	–	+	–	–	–	–	+	+	2	0.85
10	+	–	+	+	+	+	+	+	+	–	–	–	–	+	+	–	–	–	–	+	1	0.85
11	+	–	+	+	+	+	+	+	+	–	–	–	–	–	+	–	–	+	+	+	1	0.92
12	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	–	–	+	–	+	2	0.78
13	+	–	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	+	1	0.70

Note: +, indicates utilization of an amino acid; –, no utilization; *, including the type strain; S_j is the Jaccard's coefficients of community between the profile and that of the type strain; amino acin names, see Materials and Methods.

79-K) was similar to that of the type strain (Sj = 0.86); it differed in the presence of arginine dihydrolase.

For both investigated *P. luteola* strains, including the type strain *P. luteola* CIP 102995^T, the profiles of amino acid utilization (no. 32) were identical (Table 2).

In *P. fluorescens* (10 strains), 5 profiles of amino acid utilization (nos. 19–23) were revealed (Table 2). The type strain *P. fluorescens* ATCC 13525 biovar I had profile no. 19; one strain of biovar II, profile no. 20; five strains of biovar III, profile no. 21; and three strains of biovar V, profiles nos. 22 and 23. The strains with profiles nos. 20–23 exhibited low Sj similarity to profile no. 19 of the type strain of biovar I; Sj = 0.64–0.71 (Table 2). However, strains of biovars II and V, profiles nos. 20, 22, and 23, exhibited high similarity to profile no. 21 of biovar III (Sj = 0.85–0.92). These data indicate species heterogeneity of the *P. fluorescens* phenon, an isolated position of biovar I, insufficient information provided by the type strain of biovar I for the characterization of all the *P. fluorescens* phenon, and confirm the isolated position of the biovars according to the profiles of amino acid utilization and close relation between biovars II, III, and V.

The species *P. pseudoalcaligenes* (4 strains) exhibited diverse profiles of amino acid utilization, nos. 28–31 (Table 2). The profiles of all strains had low similarity to profile no. 28 of the type strain *P. pseudoalcaligenes* ATCC 17440 (Sj = 0.57–0.71); the strains with profiles nos. 29 and 31 (*P. pseudoalcaligenes* 10-H and 16-H), however, exhibited high similarity: Sj = 0.87. These results indicate the phenotypic heterogeneity of the species *P. pseudoalcaligenes*. Type strains *P. chlororaphis* subsp. *chlororaphis* ATCC 9446 and *P. chlororaphis* subsp. *aureofaciens* ATCC 13985 had identical profiles of amino acid utilization (no. 34), confirming their affiliation to one species (Table 2).

Type strains of *P. oryzihabitans* CIP 102996^T (JCM 2952) and *P. mendocina* IMV 4172 (ATCC 25411) had original profiles of amino acid utilization, nos. 33 and 35, respectively (Table 2).

The common generic characteristic of the profiles of amino acid utilization as the sole nitrogen and carbon source in the members of 10 *Pseudomonas* species are the following: L-alanine, L-glutamate, and L-aspartate are utilized; L-methionine, L-cysteine, and L-threonine are not utilized. Utilization of other amino acids is variable and has specific characteristics in different pseudomonad species. *P. putida* had the broadest spectrum of amino acid utilization (16 amino acids); and the most narrow spectrum (5 amino acids), was in

Table 2. Intraspecies profiles of amino acid utilization as the sole source of nitrogen and carbon for *Pseudomonas* spp.

Numbers of profile variants	Profiles of amino acid utilization																				Number of strains in the profile	S_j
	Ala	Gly	Asn	Asp	Gln	Glu	Lys	Arg	His	Met	Cys	Thr	Ser	Tyr	Try	Phe	Leu	Ile	Val	Pro		
P. putida (n = 40)																						
14	+	+	+	+	+	+	+	+	+	–	–	–	+	+	–	+	+	+	+	+	28*	1.0
15	+	+	+	+	+	+	+	+	+	–	–	–	+	+	–	–	+	+	+	+	1	0.93
16	+	–	+	+	+	+	+	+	+	–	–	–	+	+	–	–	+	+	+	+	6	0.87
17	+	–	+	+	+	+	+	+	+	–	–	–	+	+	–	+	+	+	+	+	3	0.93
18	+	–	+	+	+	+	+	+	+	–	–	–	+	+	+	+	+	+	+	+	2	0.88
P. fluorescens (n = 10)																						
19	+	–	+	+	+	+	–	–	+	–	–	–	+	+	+	–	+	–	+	+	1*	
20	+	–	+	+	+	+	–	+	+	–	–	–	+	+	–	+	–	+	+	+	1	0.66
21	+	–	+	+	+	+	–	+	–	–	–	–	+	+	–	–	–	+	+	+	5	0.64
22	+	–	+	+	+	+	–	+	–	–	–	–	+	+	–	–	+	+	+	+	2	0.71
23	+	–	+	+	+	+	–	+	–	–	–	–	+	+	–	+	+	+	+	+	1	0.66
P. stutzeri (n = 4)																						
24	+	+	+	+	+	+	–	–	–	–	–	–	–	+	–	–	–	–	–	+	1*	
25	+	+	+	+	+	+	–	–	–	–	–	–	–	+	–	–	–	+	–	+	3	0.90
P. alcaligenes (n = 4)																						
26	+	–	+	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	+	3*	1.0
27	+	–	+	+	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	+	1	0.86
P. pseudoalcaligenes (n = 4)																						
28	+	–	–	+	+	+	–	–	–	–	–	–	–	+	–	–	–	–	–	–	1*	
29	+	–	+	+	+	+	–	–	–	–	–	–	+	+	–	–	–	–	–	+	1	0.62
30	+	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	+	1	0.57
31	+	–	+	+	+	+	–	–	–	–	–	–	–	+	–	–	–	–	–	+	1	0.71
P. luteola (n = 2)																						
32	+	–	+	+	+	+	–	+	–	–	–	–	+	+	–	–	–	+	–	+	2*	1.0
P. oryzihabitans (n = 1)																						
33	+	–	+	+	+	+	–	–	–	–	–	–	+	–	–	–	–	–	–	+	1*	
P. chlororaphis subsp. c*hlororaphis* (n = 1)																						
34	+	–	+	+	+	+	–	+	+	–	–	–	+	+	+	+	+	+	+	+	1*	
P. chlororaphis subsp. *aureofaciens* (n = 1)																						
34	+	–	+	+	+	+	–	+	+	–	–	–	+	+	+	+	+	+	+	+	1*	
P. mendocina (n = 1)																						
35	+	–	+	+	+	+	–	+	+	–	–	–	+	+	–	+	–	–	+	+	1*	

Designations: see Table 1.

Table 3. Jaccard's coefficients of community (Sj) of the profiles of amino acid utilization as the sole carbon and nitrogen source for comparison of the profiles of the *Pseudomonas* type strains

Numbers of profile variants	Type strains of pseudomonads	Sj coefficients of the profiles of amino acid utilization for comparison of the profiles of the pseudomonad type strains									
		1	14	19	34	35	24	28	26	33	32
1	*P. aeruginosa* ATCC 10145	1									
14	*P. putida* ATCC 12633	0.70	1								
19	*P. fluorescens* ATCC 13525	0.66	0.64	1							
34	*P. chlororaphis* ATCC 9446	0.75	0.82	0.78	1						
35	*P. mendocina* ATCC 25411	0.66	0.75	0.73	0.80	1					
24	*P. stutzeri* ATCC 17588	0.50	0.50	0.53	0.43	0.53	1				
28	*P. pseudoalcaligenes* ATCC 17440	0.38	0.31	0.41	0.33	0.41	0.62	1			
26	*P. alcaligenes* ATCC 14909	0.53	0.43	0.46	0.58	0.58	0.66	0.50	1		
33	*P. oryzihabitans* CIP 102996^T	0.43	0.43	0.58	0.46	0.58	0.66	0.50	0.75	1	
32	*P. luteola* CIP 102995^T	0.64	0.55	0.57	0.66	0.71	0.63	0.50	0.70	0.70	1

P. pseudoalcaligenes. The absence of utilization of threonine, cysteine, and methionine is characteristic of the genus *Pseudomonas.* In this respect they differ, for example, from the genus *Burkholderia*; most of its members (*B. cepacia, B. pseudomallei*, etc.) utilize threonine and cysteine [9]. Since the genus *Pseudomonas* presently comprises 125 species and subspecies, these data should be verified using the information on all the species.

Comparison of the similarity (Sj coefficients) between the profiles of amino acid utilization for the type strains of 10 species revealed considerable differences between the species. The Sj values for *P. aeruginosa* were 0.33–0.75; for *P. putida*, 0.31–0.82; for *P. fluorescens*, 0.41–0.78; for *P. chlororaphis*, 0.33–0.82; for *P. mendocina*, 0.33–0.80; for *P. stutzeri*, 0.43–0.66; for *P. pseudoalcaligenes*, 0.31–0.62; for *P. alcaligenes*, 0.43–0.75; for *P. oryzihabitans*, 0.43–0.75; and for *P. luteola*, 0.50–0.71 (Table 3). Since within species the similarity (Sj) to the profiles of amino acid utilization for the type strain were 0.85–1.0 for 98% of *P. aeruginosa* strains and 0.87–1.0 for 100% of *P. putida* strains, the Sj value of 0.85 or higher probably indicates the species level of similarity for pseudomonads. Since for other species only a small number of strains were tested, this is a preliminary value.

Analysis of the utilization profiles for 20 amino acids (incorporated in proteins) as sole sources of nitrogen and carbon by 10 pseudomonad species revealed the species specificity of these profiles. This conclusion is supported by the following findings. First, the profiles of amino acid utilization were unique for all the strains studied, including the type strains. Among the 35 variants, each pseudomonad species exhibited variants not found in other species (Tables 1, 2). Type strains of *P. chlororaphis* subsp. *chlororaphis* and *P. chlororaphis* subsp. *aureofaciens*, which have been previously affiliated to different species, *P. chlororaphis* and *P. aureofaciens* [3], have the same profile of amino acid utilization (no. 34); this also confirms the species specificity of these profiles. Moreover, considerable differences between the species was revealed by comparison of the similarity (Sj coefficients) between the profiles of amino acid utilization for the type strains of 10 *Pseudomonas* species; Sj = 0.31–0.82 (Table 3). The Sj coefficients of the intraspecies variants of profiles of amino acid utilization and the profile of the type species revealed high similarity: for 98% of *P. aeruginosa* strains, Sj = 0.85–1.0; and for 100% of *P. putida, P. stutzeri*, and *P. alcaligenes* strains, Sj was 0.87–1.0, 0.90, and 0.86–1.0, respectively (Tables 1, 2). Only for *P. fluorescens* and *P. pseudoalcaligenes*, low similarity was found between the profiles of amino acid utiliza-

tion with the profiles of the type strains (Sj was 0.64–0.71 and 0.57–0.71, respectively); this is a reflection of the known phenotypic heterogeneity of these species which causes a discussion concerning the taxonomic level of these biovars [2, 3].

In the present work, apart from *P. aeruginosa* and *P. putida*, the profiles of amino acid utilization were determined for small numbers of strains of each species. Only some of the possible intraspecies profiles of amino acid utilization were therefore revealed. The Jaccard's coefficients of community between the profiles of the strain under study and the type strain of the species were important for differentiation of the pseudomonad species. Further accumulation of data on profiles of amino acid utilization is therefore required for the type strains of known species, as well as for the intraspecies profiles of pseudomonad species. It should be noted that profiles of utilization of 20 amino acids should be used for intrageneric identification of pseudomonads only for the strains with affiliation to the genus *Pseudomonas* determined by accepted methods and criteria, including the unique barium sensitivity marker.

The fact that the results obtained by determination of the utilization profiles of 20 amino acids as the sole source of nitrogen and carbon conform to the modern classification of pseudomonads confirms the validity of this method [3]. This approach may partially overcome one of the drawbacks of numeric taxonomy, namely, arbitrary choice of the phenotypic characteristics [10]. The method is based on an objective criterion for selection of the taxonomic characteristics, i.e., it utilizes universal and ubiquitous natural substrates, including all 20 amino acids present in proteins. The method may be useful for identification of the known pseudomonad species and for investigation of the taxonomic position of new members of the genus *Pseudomonas*.

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