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EXPERIMENTAL ARTICLES

Clusterization of Halophilic and Halotolerant Eubacteria Using Whole-Cell Protein Electrophoresis Data

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Abstract—Total cell proteins of the nineteen halophilic and halotolerant eubacteria isolated from marine sediments and highly mineralized formation waters of oil fields were investigated by SDS gel electrophoresis. The microorganisms studied, phenotypically identified as belonging to the genera *Dietzia, Rhodococcus, Staphylococcus, Cytophaga, Brevibacterium,* and *Archangium*, were found to form clearly distinguishable clusters (20–33% similarity at the generic level) on the dendrogram derived from electrophoretic protein patterns. Protein similarity data confirmed the heterogeneity of *Rhodococcus maris* and its relatedness to the genus *Dietza.*

Key words: halotolerant eubacteria, gel electrophoresis of proteins, taxonomy

SDS gel electrophoresis is widely used for clusterization of bacteria and preliminary taxonomic identification of isolates anticipating the expensive genosystematic analysis. The taxonomic structure of the genera *Agrobacterium, Zigomonas, Propionibacterium, Alcaligenes, Pseudomonas, Achromobacter, Brucella, Yersinia, Bordetella,* and *Mycoplasmatales* analysed by this method correlated with the DNA–DNA hybridization data [1, 2]. In particular, protein electrophoresis confirmed the heterogeneity of the species *Pseudomonas paucimobilis*, derived from the analysis of the DNA–DNA homology data [3]. The grouping of 83 *Arcobacter* strains with respect to total cell proteins did not contradict the data of DNA–rRNA hybridization, DNA–DNA hybridization, and fatty acid composition analysis [4]. Although Vandamme *et al.* [5] noted that the gel electrophoresis of whole-cell proteins provides reproducible results only at the species and intraspecies levels. Other authors provided evidence of the applicability of this method to the identification of microorganisms at the generic level. Thus, the protein profiles of six species of the genus *Brucella* were reported to substantially differ from those of the members of the genus *Yersina* [6]. A numerical analysis of the 70 strains of methanotrophic bacteria confirmed the existence of five genera described earlier on the basis of physiological, cultural, and morphocytological characteristics [7]. Gel electrophoresis was also successfully used for the identification of extremely halophilic archaebacteria at the generic level and for their clusterization [8, 9].

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The present work was undertaken to study the possibility of applying the SDS gel electrophoresis of whole-cell cell proteins for the grouping of halophilic and halotolerant eubacteria.

MATERIALS AND METHODS

Bacteria and cultivation conditions. Halophilic and halotolerant eubacteria used in this work (Table 1) were isolated from the littoral bottom sediments of the Mediterranean Sea and from the brine of oil deposits in Tatarstan [10, 11]. The bacteria were identified by conventional methods of general and soil microbiology based on phenotypic characteristics [12–15].

The species *Dietzia maris, Rhodococcus erythropolis*, and *Brevibacterium lineus* were maintained on PYG agar medium containing (g/l) peptone, 10; glucose, 5; yeast extract, 5; and NaCl, 5 (pH 7.2–7.4). *Archangium* sp. strains were maintained on standard agar medium [10] containing 150 g/l NaCl. Cultivation was performed at 28°C in 50-ml flasks containing 20 ml medium with shaking (120 rpm) on a rotary shaker.

Preparation of samples for electrophoresis. Bacterial cells were twice washed with 0.15 M NaCl by centrifugation at 3600 *g*, after which 100 μl of 0.15 M NaCl was added to 100 mg of wet biomass, and cells were disrupted by sonication on a UZDN-2T disintegrator (22 kHz; 3–4 min; 4°C). Protein concentration in homogenates, determined by the method of Lowry, was brought to 12–14 μg/μl with 0.15 M NaCl. The homogenates were mixed with equal volumes of the sample buffer (3.1 g Tris, 20 ml 1 M NaCl, 4 g sodium dodecyl

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Table 1. Origin and halotolerance characteristics of the bacteria studied

Bacterium	Strain	Source	Mineralization, g/l	Tolerable NaCl concentration, %
Dietzia maris	2834	Oil-deposit brine	138	0–50
	7824b	The same	150	0–100
	2842r	Raw oil	301	0–100
	4-3	Oil-deposit brine	150	5–50
	7816r	The same	12	0–50
	4-2b	"	150	5–50
	367-5	"	272	5–50
	n-2(4)	Raw oil	163	0–50
Rhodococcus erythropolis	12049	Oil-deposit brine	12	0–50
	367-6	The same	272	0–50
	283	"	159	0–50
	sch	Oil- contaminated lake	–	0–50
Brevibacterium lineus	21830	Oil-deposit brine	291	0–150
Staphylococcus sp.	2839	The same	201	0–200
	3011wh	"	145	0–200
	2843	Raw oil	300	0–200
Cytophaga sp.	3011r	Oil-deposit brine	145	5–50
Archangium sp.	4z	Bottom sediments of the Mediterranean Sea	36	20–150
	5z	The same	36	20–150

Note: "–" stands for "not measured."

Table 2. Analysis of the electrophoretic protein patterns of the halophilic and halotolerant eubacteria studied

Strain	N*	3011r	21830	3011wh	2843	2839	283	sch	367-6	12049	4z	5z	7816r	n-2(4)	4-3	367-5	4-2b	2842r	7824b
3011r	49																		
21830	49	13																	
3011wh	45	15	14																
2843	48	17	12	40															
2839	48	15	17	41	47														
283	45	15	12	13	9	13													
sch	47	14	12	11	16	16	39												
367-6	47	17	13	17	16	16	37	40											
12049	45	15	16	20	13	19	27	25	34										
4z	38	15	10	11	11	11	14	7	8	6									
5z	46	7	14	11	10	10	12	11	17	14	24								
7816r	39	11	8	8	6	8	11	9	12	6	10	8							
n-2(4)	41	13	9	8	9	9	14	12	10	10	11	9	30						
4-3	45	13	11	9	10	8	11	11	12	11	8	8	32	32					
367-5	44	12	13	9	11	13	11	12	9	9	11	11	16	32	19				
4-2b	35	13	6	12	5	6	6	9	8	12	8	8	19	28	24	25			
2842r	38	14	13	6	12	12	13	7	10	6	9	7	25	23	30	17	21		
7824b	43	10	11	9	7	10	7	7	15	11	7	9	23	26	27	20	21	28	
2834	43	12	10	8	10	12	6	8	13	11	6	8	25	16	24	22	20	21	41

Note: *N** indicates the total number of protein bands in the electrophoretogram of a particular strain. Numerals in other columns indicate the number of similar protein bands in the electrophoretograms of the mutually compared strains.

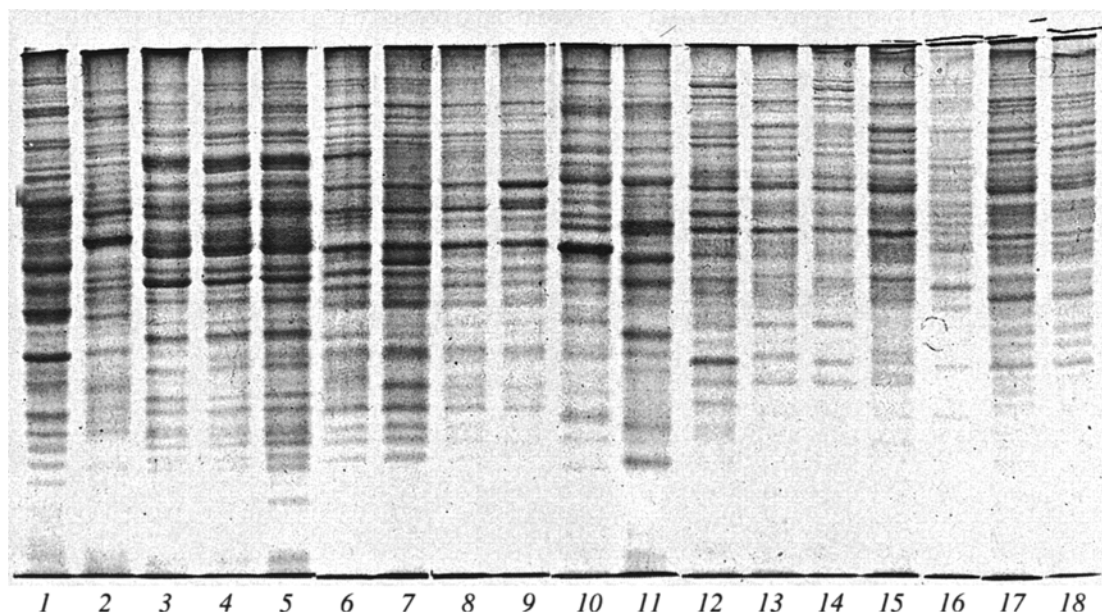

Fig. 1. Typical protein profiles of halotolerant bacteria: (*1*) *Cytophaga* sp. 3011r, (2) *Brevibacterium lineus* 21830, (*3*) *Staphylococcus* sp. 3011wh, (*4*) *Staphylococcus* sp. 2843, (*5*) *Staphylococcus* sp. 2839, (*6*) *Rhodococcus erythropolis* 283, (*7*) *R. erythropolis* sch, (*8*) *R. erythropolis* 367-6, (*9*) *R. erythropolis* 12049, (*10*) *Achangium* sp. 4z, (*11*) *Archangium* sp. 5z, (*12*) *Dietzia* (*Rhodococcus*) *maris* 4-2b, (*13*) *D.* (*R.*) *maris* 7816r, (*14*) *D.* (*R.*) *maris* 7824b, (*15*) *D.* (*R.*) *maris* 2834, (*16*) *D.* (*R.*) *maris* 2842r, (*17*) *D.* (*R.*) *maris* n-2(4), (*18*) *D.* (*R.*) *maris* 4-3.

sulfate (SDS), 10 ml glycerol, and distilled water to a total volume of 100 ml) and heated at 100°C for 10 min. The final protein concentration in samples was 6–7 μg/μl. If required, the samples were stored at –14°C.

Gel electrophoresis was carried out in polyacrylamide gel in the presence of SDS by the method of Laemmli with modifications of Gal'chenko and Nesterov [7].

Separating gel (10%) was prepared by mixing solution 1 (36.6 g Tris, 50 ml 1 N HCl, 0.46 ml TEMED, 0.8 g SDS, and distilled water to a total volume of 100 ml, pH 8.8), solution 2 (26.6 g acrylamide, 0.79 g bis-acrylamide, and distilled water to a total volume of 100 ml), and solution 3 (0.14 g ammonium persulfate and distilled water to a total volume of 50 ml). The gel length was 8 cm.

Stacking gel (4%) was prepared by mixing solution 1 (12.1 g Tris, 50 ml 1NHCl, 0.46 ml TEMED, and distilled water to a total volume of 100 ml, pH 6.8), solution 2 (13.5 g acrylamide, 2.5 g bis-acrylamide, and distilled water to a total volume of 100 ml), solution 3 (1.2 mg riboflavin and distilled water to a total volume of 25 ml), and solution 4 (0.1 g SDS and distilled water to a total volume of 50 ml). The gel length was 5 mm.

Gel electrophoresis was carried out in a vertical DESAGA chamber at a current of 40 mA when proteins migrated in stacking gel and 60 mA when they migrated in separating gel. Samples were introduced into wells in amounts of 6–15 μl. The molecular masses of proteins were estimated using albumin (67000 Da), ovalbumin (45000 Da), myoglobin (17800 Da), and cytochrome *c* (12300 Da) as the molecular weight markers. After the termination of electrophoresis, protein bands were fixed with 50% trichloroacetic acid for 18 h and then stained with a 0.01% Coomassie Brilliant Blue R-250 solution for 5 h. The stained gel slabs were then destained with 7% acetic acid, sandwiched between two transparent films, sealed, and examined in a scanner at a resolution of 600 dpi.

Electrophoretograms were analyzed with the aid of the Adobe PhotoShop software package at a 1- to 15-fold magnification for the total number of protein bands and the number of coincided proteins in each pair of the strains studied. Microsoft Excel was used to derive a matrix of the similarity coefficients calculated by the Dice formula [16]:

$$S = \frac{2m}{a+b}100\%,$$

where m is the number of protein bands common for a pair of strains and a and b are the numbers of the protein bands of the first and second strain of this pair.

The matrix was then transferred to the Feno program, which uses the unweighted mean relation algorithm [17], to generate dendrograms.

RESULTS AND DISCUSSION

Figure 1 presents typical electrophoretic protein profiles for the halotolerant bacteria under study (5–6 profiles were actually obtained for each strain). The number of proteins on electrophoretograms varied from 35 to 49

(Table 2). The R_f values of protein bands were between 0.024 and 0.98 (predominantly between 0.048 and 0.85).

On the protein phenogram shown in Fig. 2, the strains under study were grouped into six distinct clusters, *A, B, C, D, E*, and *F*, with the similarity levels (20–33%) which are intermediate between those of extremely halophilic archaea (20–25%) [9] and obligate methanotrophs (36–38%) [7]. Since analysis of the similarity coefficients of many physiological groups of microorganisms gives rather equivocal results, they are usually compared with the clustering data derived from other geno- and phenotypic characteristics. Thus, based on protein patterns, methanotrophic bacteria were grouped into five clusters in accordance with their generic affiliation. The validity of the generic classification based on the analysis of physiological, cultural, morphological, and cytological characteristics [7] was further confirmed by the results of DNA–DNA hybridization [18] and 5S and 16S ribosomal RNA sequencing [19, 20]. The phenotypic and genotypic classification of extremely halophilic archaea to the genera *Haloferax, Halobacterium, Halorubrum*, and *Halococcus* is also in good agreement with the protein-based clusterization of these bacteria [9].

Each of the six clusters of the halophilic and halotolerant bacteria under study included representatives of only one genus (Fig. 2). Members of *Dietzia* and *Rhodococcus* form separate clusters diverging at a level of 20%. These results are in agreement with the data of Rainey *et al.* [21], who separated the genus *Rhodococcus* into two genera, *Rhodococcus* and *Dietzia*, based on 16S rRNA sequencing data. As for *R. maris*, this species occupies an intermediate position between *Corynebacterium* and members of a vast cluster including the genera *Nocardia, Rhodococcus, Mycobacterium, Gordona*, and *Tsukamurella* [21]. Based on genotypic characteristics, Stackebrandt et al. [22] proposed a new class, *Actinobacteria*, in which the genera *Dietzia* and *Rhodococcus* belong to the families *Dietziaceae* and *Nocardiaceae*, respectively. Thus, the substantial difference between the protein patterns of eubacteria of the genera *Dietzia* and *Rhodococcus* revealed in this study is consistent with the conclusions of other authors about the genotypic dissimilarity of these two genera.

In some cases, gel electrophoresis may disclose some intricate relations between microorganisms. For example, in their morphological and physiological characteristics, bacterial strains of cluster *A* (Fig. 2) refer to the species *Dietzia maris* [10–12]. Meanwhile, the electrophoretic protein patterns of these strains substantially differ (Fig. 1), so that cluster *A* falls into two subclusters, a^1 and a^2, including the microorganisms whose coefficient of similarity (52%) corresponds to the interspecies similarity level (Fig. 2). By comparison, the interspecies (intrageneric) similarity of obligate methanotrophs ranges from 44 to 70% [7], and that of representatives of *Arcobacter*, from 60 to 75% [4].

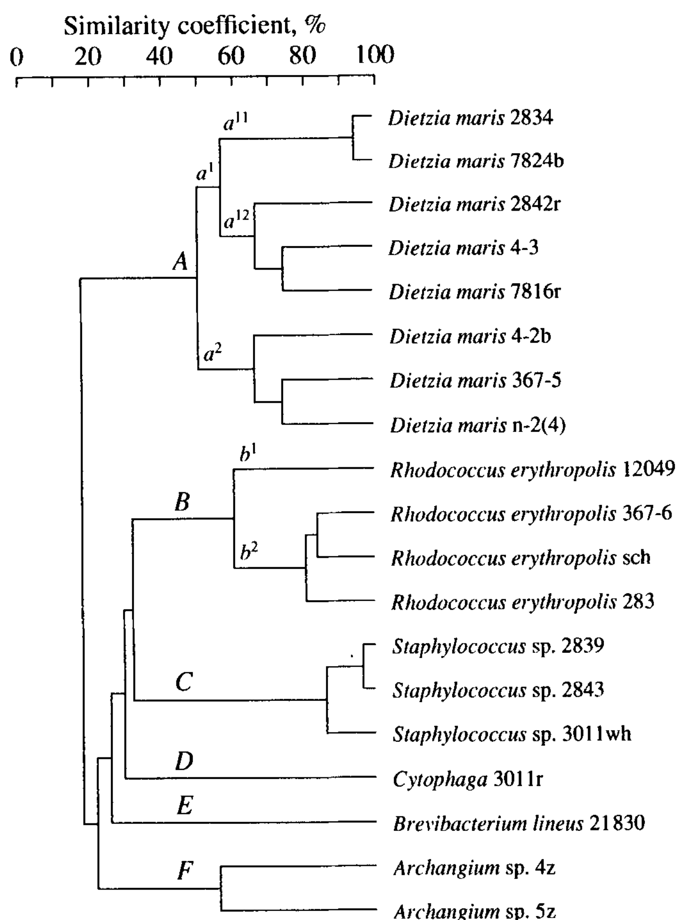

Fig. 2. Similarity phenogram of halotolerant eubacteria.

In turn, subcluster a^1 falls into two subsubclusters. The strains 2834 and 7824b of subsubcluster a^{11} with similarity coefficients of 95% considerably differ from the members of subsubcluster a^{12}, which have similarity coefficients of 58%. This suggests that strains 2834 and 7824b belong to one species. Furthermore, the high similarity coefficients of strains 4-3, 7816r, and 2842r (subcluster a^{12}) suggest that they also belong to a separate species. In our opinion, members of cluster *A* may, in fact, belong to three different species of the genus *Dietzia.* A similar situation existed with the strains of the extremely halophilic bacterium *Halorubrum distributum*: the analysis of their gel electrophoretic protein patterns showed that they comprised several clusters at a species level with similarity coefficients of 42–46% [9].

Cluster *B*, which includes the *Rhodococcus erythropolis* strains 12049, 367-6, sch, and 283, also falls into two subclusters, b^1 and b^2. The members of subcluster b^2, i.e., strains 367-6, sch, and 283, with similarity coefficients of 82–85% differ from strain 12049 (subcluster b^1), whose similarity coefficient is only 61%. This similarity value differs but little from the lower level of the intraspecies similarity between *Pseudomonas paucimobilis* strains, which comprises 67% [3]. On the other

hand, the lower levels of intraspecies similarity between methanotrophs [7] and *Arcobacter* species [4] are considerably higher (77–78%). Therefore, strain 12049 may also represent a separate species.

Cluster *C* includes strains 3011wh, 2843, and 2839, which are assigned to *Staphylococcus* sp. based on their morphological and physiological characteristics. The close values of the similarity coefficients of these species (97 and 87%) suggest that they are representatives of one species.

The halophilic myxobacteria of the genus *Archangium* (strains 5z and 4z) of cluster *F* (24% similarity level), greatly differ from the other bacteria studied. The low degree of similarity between strains 4z and 5z (57%) implies that they belong to different species.

Thus, clustering of halophilic and halotolerant bacteria at a generic level using electrophoretic protein patterns gives results which are in agreement with their classification by conventional methods. The strains belonging to the genera *Dietza, Rhodococcus, Staphylococcus, Cytophaga, Brevibacterium*, and *Archangium* comprise distinct clusters differing at a 20–33% similarity level. The data obtained in the present work confirm the validity of reclassification of *R. maris* to the genus *Dietzia* [21]. The results of the investigation of various groups of microorganisms testify to the existence of a direct correlation between electrophoretic protein patterns and DNA–DNA hybridization data [23]. The agreement between the data obtained by various method of polyphasic taxonomy, including gel electrophoresis, DNA–DNA hybridization, ribosomal RNA sequencing, and others has convincingly been demonstrated by Vandamme *et al.* [5] with respect to *Xanthomonas, Stenotrophomonas, Campylobacter, Aecobacter, Helicobacter, Lactobacillus, Comamonas*, and other bacteria.

Further evidence of the species independence of some of the halobacteria studied in this work can obviously be obtained in more thorough investigations of type strains with the use of various methods of polyphasic taxonomy. Comparison of our data with those accumulated by other authors shows that the information derived from the gel electrophoretic of whole-cell proteins can be used for the clustering of not only halobacteria and haloarchaea but also of other physiological groups of microorganisms.

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