FEMS Microbiology Letters 119 (1994) 303–308

Published by Elsevier

FEMSLE 06006

Comparison of phylogenetic relationships based on phospholipid fatty acid profiles and ribosomal RNA sequence similarities among dissimilatory sulfate-reducing bacteria

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(Received 22 March 1994; accepted 6 April 1994)

Abstract: Twenty-five isolates of dissimilatory sulfate-reducing bacteria were clustered based on similarity analysis of their phospholipid ester-linked fatty acids (PLFA). Of these, 22 showed that phylogenetic relationships based on the sequence similarity of their 16S rRNA directly paralleled the PLFA relationships. *Desulfobacter latus* and *Desulfobacter curvatus* grouped with the other *Desulfobacter* spp. by 16S rRNA comparison but not with the PLFA analysis as they contained significantly more monoenoic PLFA than the others. Similarly, *Desulfovibrio africanus* clustered with the *Desulfovibrio* spp. by 16S rRNA but not with them when analyzed by PLFA patterns because of higher monoenoic PLFA content. Otherwise, clustering obtained with either analysis was essentially congruent. The relationships defined by PLFA patterns appeared robust to shifts in nutrients and terminal electron acceptors. Additional analyses utilizing the lipopolysaccharide-lipid A hydroxy fatty acid patterns appeared not to shift the relationships based on PLFA significantly except when completely absent, as in Gram-positive bacteria. Phylogenetic relationships between isolates defined by 16S rRNA sequence divergence represent a selection clearly different from the multi-enzyme activities responsible for the PLFA patterns. Determination of bacterial relationships based on different selective pressures for various cellular components provides more clues to evolutionary history leading to a more rational nomenclature.

Key words: Fatty acid pattern; RNA sequence; Phylogenetic relationship; Bacterial evolution; Sulfate-reducing bacteria

Introduction

The purpose of this study was two-fold. First, to determine the relatedness of known dissimilatory sulfate-reducing bacteria (SRB) based on the patterns of phospholipid ester-linked fatty acids (PLFA) from isolates recovered from marine and

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SSDI 0378-1097(94)00152-H

freshwater environments. Second, to compare this phenotypic characterization of relatedness to phylogenetic relationships based on 16S rRNA sequence similarities. Data from both approaches can be utilized to rapidly identify specific strains after isolation and in many cases directly within environmental samples without prior isolation and cultivation. In a study of methane-oxidizing bacteria the relationships determined by similarity in PLFA profiles closely paralleled phylogentic relationships [1]. Since the relationships indicated by the highly conserved 16S rRNA and the presumably more broadly selected multi component systems responsible for the PLFA patterns showed such marked agreement, it became important to examine other groups of bacteria to determine the general applicability of this family. Insights into bacterial evolution could come from examination of differences between phylogenetic grouping and grouping based on phenotypic expressions of homologous bacterial pathways.

Materials and Methods

Bacterial strains

The species, sources, and origins of the SRB are listed in Table 1. All samples were received as frozen lyophilized pellets except *Desulfovibrio longus*, *Desulfobacter latus* and *Desulfobacter curvatus*. Growth conditions for the lyophilized cells have been previously reported (see Table 1 for sources). *D. longus* was received as a viable culture in media described by Magot et al. [2]. *D. latus* and *D. curvatus* were received from ATCC as viable cultures in ATCC media 1648. These bacteria were cultured anaerobically in ATCC media 1648 at 30°C, then harvested by centrifugation at the end of the exponential growth phase, for lipid extraction.

Extraction of phospholipids

The equivalent of 1–20 mg dry weight of bacterial cells was extracted in a Bligh and Dyer [3]

Table 1
Isolates, sources and origins of sulfate-reducing bacteria

	Key	Strain	Source	Origin
1.	Dv. afr.	*Desulfovibrio africanus*	ATCC 19996	Well water
2.	Dv. bac.	*Desulfovibrio baculatus*	DSM 1743	Culture
3.	Dv. des.	*Desulfovibrio desulfuricans*	DSM 642	Soil
4.	Dv. gig.	*Desulfovibrio gigas*	ATCC 19364	Freshwater
5.	Dv. long.	*Desulfovibrio longus*	Magot	Oil well
6.	Dv. pig.	*Desulfovibrio piger*	Widdel	Feces
7.	Dv. sal.	*Desulfovibrio salexigens*	Widdel	Mud
8.	Dv. vul.	*Desulfovibrio vulgaris*	Widdel	Soil
9.	Dr. baar.	*Desulfoarculus baarsii*	DSM 2075	Ditch mud
10.	Dt. sap.	*Desulfobotulus sapovorans*	ATCC 33892	Mud
11.	Dm. nigr.	*Desulfotomaculum nigrificans*	ATCC 7946	Freshwater
12.	Dbm. vac.	*Desulfotobacterium vacuolatum*	Widdel	Marine
13.	Dbm. nia.	*Desulfotobacterium niacini*	Widdel	Marine
14.	Dc. mult.	*Desulfococcus multivorans*	Widdel	Digester
15.	Dl. pro.	*Desulfobulbus propionicus*	Widdel	Fresh mud
16.	Ds. var.	*Desulfosarcina variabilis*	Widdel	Marine mud
17.	Dn. ace.	*Desulfuromonas acetoxidans*	Widdel	Marine
18.	Db. curv.	*Desulfobacter curvatus*	ATCC 43919	Marine mud
19.	Db. lat.	*Desulfobacter latus*	ATCC 43918	Marine mud
20.	Db. AcBa	*Desulfobacter* AcBa	Dowling	Oiltank
21.	Db. post.	*Desulfobacter postgatei* 2ac9	DSM 2034	Sediment
22.	Db. 4ac11	*Desulfobacter* sp. 4ac11	DSM 2057	Marine mud
23.	Db. hydr.	*Desulfobacter hydrogenophilus*	Dowling	Marine
24.	Df. tdj.	*Desulfomonile tiedjei*	DeWeerd	Sludge
25.	Geo. met.	*Geobacter metallireducens*	Lovely	Freshwater

ATCC, American Type Culture Collection, Rockville, MD; DSM, Deutche Sammlung von Mikroorganism, Braunscweig, FRG; DeWeerd et al. [16]; Dowling et al. [10]; Lovely et al. [14]; Magot et al. [2]; Widdel [17].

Table 2

Mole percents of major phospholipid ester-linked fatty acids of sulfate-reducing bacteria

PLFA	14:0	i15:0	a15:0	i16:1w6	i16:0	16:1w7c	16:0	i17:1w7	10Me16:0	a17:1w7	i17:0	a17:0	17:1w6	cy17:0	17:0	18:1w9c	18:1w7c	18:0	cy19:0	20:1w9c
Dv. afr.	4.0	3.7	6.6	3.1	1.7	12.3	15.4	5.1	0.0	5.6	0.4	0.9	0.9	0.0	0.8	1.4	28.1	4.8	0.0	0.0
Dv. bac.	0.0	7.9	5.0	0.7	0.4	8.6	7.6	19.3	0.0	4.0	6.9	5.3	0.0	0.0	0.4	5.5	14.2	10.6	0.0	0.0
Dv. des.	0.0	22.0	1.0	1.0	1.0	8.0	17.0	33.0	1.0	0.0	8.0	1.0	0.0	0.0	1.0	1.0	1.0	2.0	0.0	0.0
Dv. gig.	2.0	46.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	12.2	1.8	6.6	0.0	17.0
Dv. long.	0.2	9.2	15.7	0.0	0.8	0.9	10.4	13.6	0.0	2.3	22.9	17.5	0.0	0.0	0.6	0.7	0.8	1.5	0.0	0.0
Dv. pig.	0.3	31.2	1.0	0.8	1.1	3.2	13.8	22.4	0.0	0.7	24.3	0.3	0.0	0.0	0.0	0.0	0.0	0.8	0.0	0.0
Dv. sal.	0.5	22.9	3.4	0.1	0.2	5.3	19.7	24.9	0.0	2.4	11.2	1.9	0.0	0.0	0.2	0.2	4.0	1.5	0.0	0.0
Dv. vul.	0.1	6.7	5.4	9.1	9.2	2.5	6.0	20.7	0.0	5.7	6.1	6.3	0.5	0.0	1.6	0.1	6.9	6.2	0.0	0.0
Dr. baar.	26.9	2.4	6.6	0.0	2.6	0.6	5.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.4	1.1	0.0	0.0
Dt. sap.	2.4	0.0	0.0	0.0	0.0	11.8	26.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	14.2	35.5	2.3	0.0	0.0
Dm. nigr.	2.0	8.0	4.0	0.0	1.0	5.0	28.0	11.0	3.0	1.0	14.0	6.0	0.0	0.0	1.0	2.0	3.0	2.0	0.0	0.0
Dbm. vac.	6.6	0.0	0.6	0.0	0.0	42.4	23.4	0.0	0.0	0.0	0.0	0.8	5.7	0.0	3.3	0.6	4.0	0.8	0.0	0.0
Dbm. nia.	2.6	0.0	0.7	0.0	0.0	25.4	29.1	0.0	0.0	0.0	0.0	0.0	11.3	0.0	7.7	1.7	2.9	1.5	0.0	0.0
Dc. mult.	1.0	1.9	19.4	0.8	1.4	4.8	23.0	0.7	0.0	10.6	0.7	5.8	3.0	0.2	4.0	0.8	0.5	1.2	0.0	0.0
Dl. pro.	3.9	0.0	0.0	0.0	0.0	2.9	4.5	0.0	0.0	0.0	0.0	1.4	42.0	0.0	1.0	0.0	14.5	0.6	0.0	0.0
Ds. var.	1.6	1.3	34.1	0.6	0.7	9.2	26.8	1.5	0.0	4.2	0.2	1.3	1.9	0.0	4.0	0.3	0.6	1.2	0.0	0.0
Dn. ace.	3.6	5.4	1.1	0.0	0.0	40.6	42.2	0.3	0.0	0.0	0.7	0.2	0.0	0.0	0.2	0.8	0.9	1.2	0.0	0.0
Db. curv.	3.0	3.0	0.0	0.0	0.0	3.0	32.0	0.0	11.0	0.0	0.0	0.0	0.0	11.0	2.0	15.0	6.0	9.0	0.0	0.0
Db. lat.	3.0	0.0	0.0	0.0	0.0	10.0	24.0	0.0	2.0	0.0	0.0	0.0	0.0	2.0	2.0	31.0	12.0	10.0	1.0	0.0
Db. AcBa	31.4	1.6	0.0	0.0	0.4	5.9	18.6	0.1	9.4	0.0	0.0	0.0	0.0	27.3	0.0	0.0	0.1	0.1	0.2	0.0
Db. post.	9.5	1.1	0.0	0.0	0.1	4.9	30.7	0.1	16.5	0.0	0.2	0.2	0.0	27.0	0.0	0.2	0.6	0.3	1.1	0.0
Db. 4ac11	11.3	2.1	0.0	0.0	0.2	1.1	32.7	0.2	11.2	0.0	0.2	0.0	0.0	32.7	0.0	0.1	0.4	0.2	3.4	0.0
Db. hydr.	0.0	12.5	0.0	0.0	0.2	1.3	30.0	0.4	4.3	0.0	0.5	0.0	0.0	38.8	0.0	0.3	0.3	0.2	1.4	0.0
Df. tdj1 *	9.1	0.3	0.0	0.0	0.0	18.6	26.7	0.0	0.0	0.0	0.0	0.0	0.0	15.5	0.0	0.0	0.8	20.8	0.0	0.0
Df. tdj2 *	19.1	1.3	0.0	0.0	0.0	15.8	18.1	0.0	0.0	0.0	0.0	0.0	0.0	4.7	0.0	0.0	1.3	25.9	0.0	0.0
Df. tdj3 *	10.6	1.1	0.0	0.0	0.0	20.5	26.2	0.0	0.0	0.0	0.0	0.0	0.0	7.2	0.0	0.0	1.3	23.7	0.0	0.0
Geo. met1 *	3.7	10.5	0.1	0.0	0.0	45.1	32.2	0.0	0.7	0.0	0.9	0.0	0.0	0.0	0.0	0.0	3.7	0.7	0.0	0.0
Geo. met2 *	6.0	17.9	0.1	0.0	0.0	40.7	26.3	0.0	0.7	0.0	1.0	0.0	0.0	1.0	0.0	0.0	3.3	0.5	0.0	0.0

Dsm. tdj-1 grown with pyruvate and 3-chlorobenzoate; Dsm. tdj-2 grown with formate/acetate and 3-chlorobenzoate; Dsm. tdj-3 grown with isovanillate/acetate and sulfate; Geo. met-1 grown with ferric citrate; and Geo. met-2 grown with sodium nitrate.

single-phase solvent system modified to include phosphate buffer [4]. The total lipid extract was separated into lipid classes by silicic acid column chromatography as detailed previously [4].

The PLFA were prepared for gas chromatography (GC) analysis by a mild alkaline transesterification [4]. The resultant fatty acid methyl esters (FAME) were separated, quantified and identified as described [5]. Fatty acid double bond positions were confirmed by GC/MS (Hewlett-Packard 5995A) analysis of the dimethyl disulfide adducts of the mono-unsaturated FAME as described [6].

Fatty acid nomenclature

Fatty acids are designated as A : BwC, where A is the total number of carbon atoms, B is the number of double bonds, and C is the position of the double bond from the aliphatic (w) end of the molecule. Double bond geometry is indicated as 'c' for *cis* and 't' for *trans*. The prefixes 'i' and 'a' denote *iso*- and *anteiso*-methyl branching, respectively; 'cy', designates a cyclopropyl moiety.

16S rRNA sequencing

Details of reverse transcriptase 16S rRNA sequencing, phylogenetic analyses, and relationship rRNA sequence similarity to DNA relatedness were previously described [7,8].

Statistical analysis

The PLFA profiles of the bacteria were treated as multivariate data and analyzed using hierarchical cluster analysis. Dendrograms were constructed using an incremental sum of squares method with the PC-based software package, Ein * Sight (Infometrix; Seattle, WA). The dendrogram presented is essentially identical to others produced with other clustering algorithms available through Ein * Sight and the mainframe software package SPSSX (Version 3.0, Chicago, IL). PLFA representing < 0.1 mol% were excluded. Similarity values (1 = identical) were determined by the Ein * Sight program using modified Euclidean distances and shown as the length of the branches of the cluster.

Results and Discussion

PLFA data in terms of mol% of the component FAME from the extractable polar lipids is given in Table 2. Cluster analysis of the PLFA patterns agreed favorably with phylogenetic relationships inferred by 16S rRNA sequence comparisons (Fig. 1). The 16S rRNA relationships are based on the percent sequence similarity [7,8]. 70% DNA relatedeness (accepted DNA : DNA

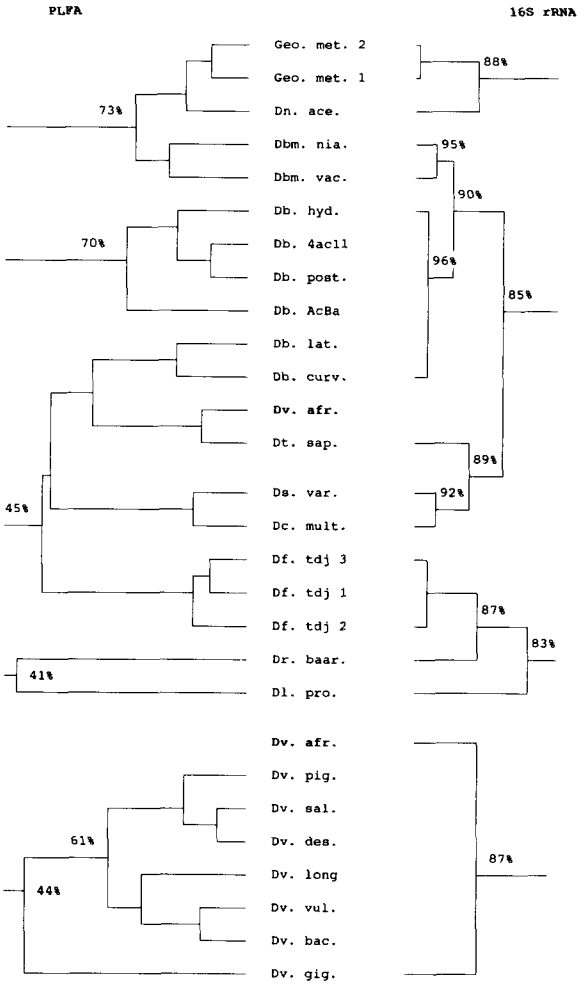

Fig. 1. Relationships between the sulfate-reducing bacteria based on hierarchical cluster analysis of the phospholipid ester-linked fatty acids with % similarity given (left side) and phylogenetic relationship based on 16S rRNA sequence similarity with % identical sequence given (right side).

homology value for a species) correlates with 98% 16S rRNA sequence similarity; and 20% DNA relatedness (genus level) correlates with 94% 16S rRNA sequence similarity [8]. The PLFA similarity values represent the Euclidean distances as determined by the Ein * Sight program. Any cluster having a similarity index ≤ 0.25 is considered dissimilar with respect to the other clusters [9].

Of the 25 isolates examined by PLFA analysis only three did not cluster as expected based on 16S rRNA sequence comparisons. These were *D. latus*, *D. curvatus*, and *D. africanus*.

D. latus and *D. curvatus* did not group with the other four *Desulfobacter* spp. by PLFA. Most *Desulfobacter* ssp. have been characterized by the presence of cy17:0, cy19:0, and 10me16:0 [10]. Although *D. latus* and *D. curvatus* contained 10me16:0 and low percentages of cy17:0 and cy19:0, they had much higher percentages of 18:0, 18:1*w*9c, and 18:1*w*7c. These differences were sufficient to separate them from the larger *Desulfobacter* spp. cluster and into a cluster with two *Desulfovibrio* spp. which had similar profiles.

D. africanus was placed within the Desulfovibrionaceae by 16S rRNA sequence comparison [11]. However, the PLFA profile of *D. africanus* clustered with *Desulfobotulus sapovorans* (formerly *Desulfovibrio*), *Desulfosarcina variabilis*, and *Desulfococcus multivorans* (Fig. 1). *D. africanus* failed to cluster with the *Desulfovibrio* spp. group due to high percentages of 16:1*w*7c and 18:1*w*7c and the presence of 17:1*w*8 and 17:1*w*6.

A principle components analysis revealed the *Desulfovibrio* spp. were separated due to high percentages of i15:0, i17:0, and i17:1*w*7. PLFA analysis showed that *Desulfoarculus baarsii* and *Desulfobutulus sapovorans* (both formerly *Desulfovibrio* spp.) failed to cluster with the *Desulfovibrio* spp. which was in agreement with the 16S rRNA results [12]. *D. baarsii* was the only SRB analyzed which contained the long chain natural saturates 22:0 and 24:0.

Desulfobulbus propionicus clustered with *Desulfoarculus baarsii* by PLFA and with *D. baarsii* and *Desulfomonile tiedjei* by 16S rRNA. *Desulfovibrio longus*, a recently described SRB, clustered with other *Desulfovibrio* spp. [2]. Magot et al. [2] showed *D. longus* clustering with *D. salexigens*, *D. vulgaris*, and *D. gigas* by 16S rRNA analyses.

Geobacter metallireducens grouped with *Desulfuromonas acetoxidans* as was reported [13].

Use of PLFA profiles for discerning phylogenetic relationships can be complicated by changes induced by shifts in nutrients. The PLFA patterns of two *Desulfobacter* species and *D. acetoxidans* when grown with acetate and volatile fatty acid supplements which they cannot oxidize, induced a shift toward odd-numbered and branched chains by apparent direct incorporation into the fatty acids as chain initiators [10]. These conditions are unlikely to occur in sediments where the volatile acids are acetate, a small amount of propionate and traces of higher fatty acids [14]. Vainshtein et al. [15] found shifting the medium components had little effect on the PLFA (plus ester-linked lipopolysaccharide hydroxy fatty acid (LPS-OHFA)) patterns of the *Desulfovibrio* spp. they examined. In Fig. 1 the dendrogram shows no differences in the PLFA patterns of *D. tiedjei* grown with three different carbon sources and terminal electron acceptors. There were no differences in PLFA patterns with *Geobacter metallireducens* grown with two different terminal electron acceptors.

As with the methane-oxidizing bacteria [1], the SRB showed a close agreement in relationships determined by PLFA patterns and 16S rRNA sequence similarities. The shifts in lipid composition induced by changes in growth conditions did not seriously affect the relationship analysis by PLFA.

Analysis of the lipopolysaccharide hydroxy fatty acid (LPS-OHFA) from the lipid A and the inclusion of the hydroxy fatty acids in the patterns should increase the precision of the analyses. Inclusion of the LPS-OHFA is important in eliminating an artifactual close relationship of the Gram-positive SRB with the Gram-negative SRB. This is evident for *Desulfotomaculum nigrificans*, a Gram-positive SRB which clustered closely with Gram-negative *Desulfovibrio longus* (data not shown). The fact that the Gram-positive bacteria have no LPS-OHFA indicates their obvious dif-

ferences which is reflected in the 16S rRNA. In general, inclusion of LPS-OHFA should add to the utility of phenotypic relationships based on similarities in fatty acid patterns but has a small effect as the proportions of LPS-OHFA are a relatively small part of the total. Not including LPS-OHFA (as many were not available) in the data used for Fig. 1 did not seriously affect the relatedness parallel to the 16S rRNA.

Acknowledgements

This work was supported in part by Grant DE-FG05-90ER60988 from the Subsurface Science Program of the Office of Health and Environmental Research, US Department of Energy, Grant RP-8011 from the Electric Power Research Institute, and Grant N00014-93-1-0326 from the Office of Naval Research. Use of trade names does not imply endorsement by the United States Environmental Protection Agency.

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