Contribution of the Electrophoretic Pattern of Cell Envelope Protein to the Taxonomy of *Hyphomonas* spp.

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The membrane protein profiles of marine budding bacteria belonging to the genus *Hyphomonas* were determined and analyzed. *Hyphomonas* spp. synthesized an unusually large number (four or more) of high-apparent-molecular-weight proteins (apparent molecular weight, \( \geq 64,000 \)). Species were compared by using a membrane protein profile index developed for this study. Our results supported the division of extant members of this genus into five species. The membrane protein profile index for *Hyphomonas hirsutiana* and *Hyphomonas neptunium* was higher than suggested by other phenetic characteristics.

When nutrient concentration (6), osmolarity (2, 10), oxygen tension (29, 33), temperature (35), and other growth parameters have been defined (1; J. S. Poindexter, Abstr. Annu. Meet. Amer. Soc. Microbiol. 1985, p. 264, 5); membrane protein composition of bacteria has been shown to be characteristic for each species (5, 25, 26). This has made it possible to use comparisons of membrane protein profiles as phenetic criteria. Conventionally, the membrane proteins of terrestrial and freshwater procaryotes have been isolated by using moderate concentrations of ionic (3, 7) or nonionic (4) detergents, and they have been adequately separated, for purposes of comparative analysis, by using one-dimensional electrophoresis (27).

Marine budding bacteria belonging to the genus *Hyphomonas* have recently been characterized and placed within proposed species on the basis of morphological, biochemical, and growth characteristics (34). In this paper we describe a method for isolation of membrane proteins from marine bacteria, characterization of the molecular weights of the membrane proteins of *Hyphomonas* spp., and analysis (18) of the resulting data, which support proposed species placement.

(A portion of this work was conducted by L. Dagasan in partial fulfillment of the requirements for a Ph.D. degree from the University of Maryland, College Park, and preliminary findings from part of this work have been presented previously [Dagasan and Weiner, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, R31, p. 215].)

**MATERIALS AND METHODS**

**Growth conditions and media.** The *Hyphomonas* spp. strains from which membrane proteins were extracted are listed in Table 1. All of the strains currently classified as members of the genus *Hyphomonas* were included in this study. Stock cultures were maintained at 31°C. All strains except strains SCH-89 and VP-5 were grown with aeration on a rotary shaker (New Brunswick Scientific Co., Inc., Edison, N.J.) at 160 rpm in 300-ml sidearm flasks containing 50 ml of marine broth 2216 (Difco Laboratories, Detroit, Mich.). All strains were harvested by centrifugation at 10,000 rpm for 10 min in a model RC-5B centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) equipped with a model SA600 fixed-angle head (Sorvall), suspended, and washed twice in 20 ml of 50 mM tris(hydroxymethyl)aminomethane (Tris) hydrochloride buffer (pH 7.8) (Tris buffer) at 4°C. They were disrupted by using a sonic probe (Biosonic disrupter equipped with a Biosonic 2 probe; Brownwill Scientific Inc., Rochester, N.Y.) at the maximum setting and 30-s on-off pulses for 9 min in an ice bath. The crude extract was centrifuged at 10,000 rpm for 10 min, and the pellet, which consisted mainly of broken cells, was discarded. The supernatant was centrifuged at 50,000 rpm for 1 h with a model L8-80 ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.) equipped with a type Ti70.1 fixed-angle head and washed twice in Tris buffer. The resulting pellet, which was enriched for cell envelopes, was suspended in Tris buffer at a final protein concentration of 5 to 6 mg/ml (21). The suspension was dialyzed for 24 h against two changes of distilled water (4°C) and stored at \(-20^\circ C\).

A 30-µl portion of the cell envelope preparation, containing 5 to 6 mg of protein, was pipetted into an Eppendorf centrifuge tube (capacity, 1.5 ml) and mixed with 30 µl of sample buffer (10% SDS, 10% 2-mercaptoethanol, 10% glycerol, 1% urea, 0.08% bromphenol blue). This mixture was boiled for 5 min at 100°C, cooled to 20°C, and then centrifuged at 15,000 rpm for 10 min in a model 5414 Eppendorf microcentrifuge. The percentage of solubilization was estimated by dividing the amount of protein in the supernatant by the amount of total protein in the tube (pellet plus supernatant). The supernatant, which contained the solubilized proteins, was removed with a micropipette, and within 24 h of preparation 15-µl samples were loaded into gels and run under constant current until the dye front reached the bottom of the gel.

**SDS-polyacrylamide gel electrophoresis.** Proteins were separated and analyzed on a 1% SDS–polyacrylamide gel (1.5 mm) by using the discontinuous buffer system of Laemml (19). The vertical slab gel (type 2001 [LKB Bromma, Gaithersburg, Md.] or type SE600 [Hoeffer Scientific, San Francisco, Calif.]) consisted of an 11.5% resolving gel (0.374 Tris) and a 5% stacking gel (0.124 M Tris). Electrophore-
sis was performed at a constant current of 30 mA in a Tris-glycine buffer system (pH 8.3).

The finished gels were immediately fixed and stained overnight in 100 ml of 10% acetic acid and 100 ml of stain concentrate (0.25 g of Coomassie brilliant blue R-250 in 100 ml of ethanol). They were destained for 1 to 2 h in a solution containing 200 ml of 95% ethanol and 300 ml of acetic acid, followed by another 1 to 2 h in a solution containing 150 ml of 95% ethanol and 350 ml of 5% acetic acid. If this procedure did not sufficiently destain the gels, they were placed in a solution consisting of 100 ml of 95% ethanol plus 400 ml of 5% acetic acid for 24 h.

Before staining with Coomassie brilliant blue, the gels were frequently stained with silver stain by using a modification of a new procedure (22; C. R. Merrill personal communication). The gels were soaked in 200 ml of fixative I containing 50% methanol, 40% distilled water, and 10% acetic acid for 1 to 24 h. The fixing solution was discarded, and the gels were placed in 200 ml of fresh fixative II (fixative I containing 0.4 g of NaCl [0.2%]) for 15 min with slow agitation. The gels were rinsed two times with fixative I and soaked in 200 ml of silver stain (2.4 g of silver nitrate in 200 ml of fixative I) for 20 min with slow agitation. They were again rinsed two times with fixative I, placed on a light box for 1 to 1.5 h, soaked in 10% acetic acid to stop development, and stored in water. (Artifacts caused by pressure and surface drying were minimized by careful handling.)

Analysis of the SDS-polyacrylamide gels. The destained

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### TABLE 1. Hyphomonas spp. strains and sources

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. jannaschiana</em></td>
<td>VP-1 (= ATCC 33882)</td>
<td>Seawater</td>
<td>15</td>
</tr>
<tr>
<td><em>H. jannaschiana</em></td>
<td>VP-2 (= ATCC 33883)</td>
<td>Seawater</td>
<td>15</td>
</tr>
<tr>
<td><em>H. jannaschiana</em></td>
<td>VP-3 (= ATCC 33884)</td>
<td>Seawater</td>
<td>15</td>
</tr>
<tr>
<td><em>H. jannaschiana</em></td>
<td>VP-4 (= ATCC 33885)</td>
<td>Seawater</td>
<td>15</td>
</tr>
<tr>
<td><em>H. jannaschiana</em></td>
<td>VP-5 (= ATCC 33886)</td>
<td>Seawater</td>
<td>15</td>
</tr>
<tr>
<td><em>H. neptunium</em></td>
<td>LE-670 (= ATCC 15444)</td>
<td>Seawater</td>
<td>20</td>
</tr>
<tr>
<td><em>H. polymorpha</em></td>
<td>PR-727 (= ATCC 33880)</td>
<td>Human sinus</td>
<td>24</td>
</tr>
<tr>
<td><em>H. polymorpha</em></td>
<td>PR-728 (= ATCC 33881)</td>
<td>Human sinus</td>
<td>24</td>
</tr>
<tr>
<td><em>Hyphomonas oceanitis</em></td>
<td>SCH-89 (= ATCC 33879)</td>
<td>Brackish water sediment</td>
<td>14</td>
</tr>
</tbody>
</table>

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**FIG. 1.** SDS-polyacrylamide gel electrophoresis of membrane proteins of nine *Hyphomonas* spp. strains. Membranes were isolated and proteins were solubilized as described in Materials and Methods. The gels were stained with Coomassie brilliant blue R-250. Lane 1, strain VP-1; lane 2, strain VP-2; lane 3, strain VP-3; lane 4, strain VP-4; lane 5, strain VP-5; lane 6, strain LE-670; lane 7, strain PR-727; lane 8, strain PR-728; lane 9, strain SCH-89. The arrowheads in lane 1 indicate proteins common to each of the nine strains of *Hyphomonas* spp., with (from top to bottom) apparent molecular weights of 95,000, 90,000, 77,000, 48,000, 36,000, 31,000, and 14,000. The arrowhead in lane 2 indicates the heat-induced protein with an apparent molecular weight of 64,000. The arrowheads in lane 6 indicate proteins with apparent molecular weights of 105,000, 100,000, 86,000, and 18,000 which were unique to strains LE-670 and VP-5. Lane 10 contained molecular weight standards.
Gels were scanned and analyzed by using a computer-assisted laser densitometer (LKB Bromma model 2202 ultrascan laser densitometer) with 3-nm resolution. The gels were also photographed by using a Canon type AE camera at an f-stop setting of 16, a 10-s exposure, and Kodak ASA 1 line film.

To compare the membrane protein profiles of each *Hyphomonas* sp. strain empirically with the profiles of every other strain, the total number of molecular weight matches divided by the total number of isolated proteins of the reference strain was calculated as follows: overall similarity membrane protein profile index (MPPI) = (number of molecular weight matches of experimental and reference strains)/(total number of proteins of reference strain).

When there was apparent molecular weight identity but a difference of ≥10% in the total membrane protein concentration (determined with a laser densitometer integrator), the results were recorded as a mismatch. Overall similarities were recorded as averages of the results of the reciprocal crosses of the reference strains.

**RESULTS AND DISCUSSION**

*Hyphomonas* spp. strains, particularly the deep-sea VP strains, had envelopes that were resistant to disruption (34) and solubilization. A new procedure for extraction of membrane proteins (see Materials and Methods) devised for this study yielded 75% solubilization, compared with 15 to 21% when conventional methods were used (8, 12, 13, 17).

One-dimensional polyacrylamide gel electrophoresis normally yields ca. 30 protein bands (16). Although not as sensitive as two-dimensional analyses (23), one-dimensional polyacrylamide gel electrophoresis can be used to resolve serovars within species (11, 31, 32); it is rapid, uncomplicated and reproducible. In the case of *Hyphomonas* spp., adding a second dimension is especially impractical because of the difficulty of solubilizing the membrane proteins with nonionic detergents.

Silver staining with 100-ng resolution revealed an average of six additional membrane proteins per strain in addition to the proteins identified by using Coomassie brilliant blue R-250, which has 10-μg resolution. However, the species placements were not altered when these minor proteins were considered.

All nine strains of *Hyphomonas* spp. had membrane proteins with apparent molecular weights of 95,000, 90,000, 77,000, 48,000, 36,000, 31,000 and 14,000 (Fig. 1 and 2). A protein with an apparent molecular weight of 64,000 was also synthesized by all strains and was most readily detected in extracts of cells cultivated near the maximum growth temperature. Two species, *Hyphomonas hirsichiana* (strain VP-5) and *Hyphomonas neptunium* (strain LE-670), which were previously categorized as distantly related (9), shared proteins that were not found in other species with apparent molecular weights of 105,000, 100,000, 86,000 and 18,000. The two strains of *Hyphomonas polymorpha*, strains PS and PR, which are thought to be different phenotypic manifestations of the same genotype (24), did in fact have identical membrane protein profiles. In addition to the seven proteins common to each member of the genus, *H. polymorpha* had five proteins in common with *Hyphomonas jannaschiana* VP-1, VP-2, VP-3, and VP-4. *H. hirsichiana* had fewer proteins in common with *H. jannaschiana*, sharing three unique protein bands.

Several factors undoubtedly influenced the overall percentages of similarity of the membrane protein profiles. Two or more proteins with molecular weight differences of ≤500 would not have been resolved by SDS-polyacrylamide gel electrophoresis. Proteins in the ≤100-ng range would not have been detected by the staining procedures used in this study. A few proteins may have been modulated, degraded, or complexed in vivo or in vitro, possibly appearing in two or more bands in the gels. Other proteins may not have been solubilized or may have been lost during isolation. Thus, the MPPI approximates actual differences in membrane protein content and is an empirical parameter. However, we think that it is both reliable and meaningful, provided cell growth

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**FIG. 2.** Laser densitometer tracings of the electrophoretic patterns shown in Fig. 1. The direction of migration was from left to right. The settings were maximum at an optical density (O.D.) of 2.0; the chart speed was 6.0 cm/min; the attenuation was 7.0; the peak width was 0.04; and the scan speed was 50. Tracing 1, strain VP-1; tracing 2, strain VP-2; tracing 3, strain VP-3; tracing 4, strain VP-4; tracing 5, strain VP-5; tracing 6, strain LE-670; tracing 7, strain PR-727; tracing 8, strain PR-728; tracing 9, strain SCH-89.
conditions and membrane protein isolation procedures are standardized.

When they were compared with strains from three other genera (Escherichia, Aeromonas, and Rhodomicrobium) (data not shown), the MPPIs of the Hypomonas spp. were ≥30%. Within the genus Hypomonas, the MPPIs fully supported existing taxonomic divisions (Table 2). Different Hypomonas species were related at levels of 33 to 78%, depending on the strains examined. Strains of the same Hypomonas species were related at levels of ≥80%. These results were entirely consistent with biochemical, genetic, and serological evidence used to assign extant strains of Hypomonas to five species (30) (Table 1).

An unexpected finding was that H. jannaschiana was less closely related to H. hirschiitata, which was also isolated from thermal vents, than to H. neptunium. We also did not predict that the MPPI would be as high as 78% for H. hirschiitata and H. neptunium, since the level of deoxyribonucleic acid homology of these two species is <5% (9).

Most interestingly, an uncommonly large number of the major proteins of all Hypomonas spp. strains have high molecular weights (≥64,000) (Fig. 1). Caulobacter is among other procaroyctic genera that also synthesize so many high-molecular-weight membrane proteins (1); (J. Poinexter, personal communication). Some of these proteins have an isoelectric point of pI 4, and one or two of them could be RS-layer proteins (28). It remains to be determined whether others are involved in prosthecA formation or activity.

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LITERATURE CITED


