Characterization and Identification of Marine Alteromonas nigrifaciens
Strains and Emendation of the Description

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Nine nonpigmented strains of gram-negative, aerobic, marine bacteria with polar flagella were isolated from the mussels Crenomytilus grayanus and Patinopecten jessoensis. These organisms were conspecific and exhibited relatively high levels of genetic relatedness (61 to 100%). The G+C contents of the DNAs of these strains were 38.5 to 40.2 mol%. The strains isolated from mussels were phenotypically distinct from previously described Alteromonas species that have similar DNA G+C contents (Alteromonas haloplanktis, Alteromonas tetraodonis, Alteromonas atlantica, and Alteromonas carrageenovora), and their DNAs exhibited only 12 to 41% similarity with the DNAs of the type strains of these species. DNA-DNA hybridization data revealed that the levels relatedness between the strains which we studied and the type strain of Alteromonas nigrifaciens were significant (66 to 70%). Production of a melanin-like pigment, which is characteristic of A. nigrifaciens, was observed only in tyrosine-containing media. The strains isolated from mussels were identified as A. nigrifaciens. We present an emended description of A. nigrifaciens that includes several phenotypic and chemotaxonomic characteristics.

The genus Alteromonas Baumann et al. 1972 includes both nonpigmented and pigment gram-negative, aerobic, polarly flagellated, marine bacterial species (4, 5, 7, 11), and there have been numerous proposals of new Alteromonas species recently (3, 10, 12, 16, 22). However, phenotypic discrimination of the species of this genus is problematic because of significant variations in their phenotypic traits. Phenotypic differences are frequently observed among even genetically closely related strains (2). Tests to determine utilization of specific carbon sources, which have been used successfully in Pseudomonas systematics (9), are less informative when they are used for differentiation of Alteromonas species. Some characteristics, such as requirements for growth factors and the production of pigments and some secondary metabolites, which were described as specific characteristics of certain species, have been shown to be strain-specific characteristics in recent studies. For example, one strain of Alteromonas tetraodonis produces tetrodotoxin, and recent investigations of the DNA relatedness of Alteromonas species have shown that A. tetraodonis is closely related genetically to Alteromonas haloplanktis, which never produces tetrodotoxin (10). Thus, genetic and chemotaxonomic methods appear to provide more reliable information than differential phenotypic characteristics in Alteromonas systematics.

We previously isolated several strains belonging to the genus Alteromonas from the Far Eastern mussels Crenomytilus grayanus and Patinopecten jessoensis (13). The purpose of this study was to characterize and identify these strains by using phenotypic, genetic, and chemotaxonomic methods in order to provide additional taxonomic data for Alteromonas species.

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MATERIALS AND METHODS

Bacterial strains and isolation. The strains which we used are listed in Table 1. Mussels (C. grayanus and P. jessoensis) were collected in 1989 and 1990 at the Pacific Institute Bio-Organic Chemistry Marine Experimental Station, Troitza Bay, Gulf of Peter the Great, Sea of Japan. The mussels were collected at a depth of 8 m (salinity, 33%; temperature, 12°C) and were prepared aseptically. Each sample of tissue (3 to 5 g) was homogenized in 10 to 15 ml of sterile seawater in a sterile glass homogenizer. Then 0.1 to 0.2 ml of the homogenate was spread onto plates containing marine agar 2216 (Difeo) (1) and plates containing medium B, which contained 0.2% (wt/vol) Bacto Peptone (Difco), 0.2% (wt/vol) casein hydrolysate (Merck), 0.2% (wt/vol) yeast extract (Difco), 0.1% (wt/vol) glucose, 0.02% (wt/vol) KH2PO4, 0.005% (wt/vol) MgSO4·7H2O, 1.5% (wt/vol) Bacto Agar (Difco), 50% (vol/vol) natural seawater, and 50% distilled water. The pH of the medium was adjusted to 7.8 with 1 M NaOH. All of the plates were incubated at 25 to 28°C. The strains were isolated from single colonies after 7 days of incubation, purified (14), and stored on the same semisolid medium in tubes under mineral oil at 4°C.

Phenotypic analysis. The phenotypic characteristics of each strain were determined by the methods described by Baumann et al. (4, 5) and Smibert and Krieg (23). The following physiological and biochemical properties were examined: oxidation or fermentation of glucose (18); arginine dihydrolase, catalase, urease, indole, and H2S production; and the ability to hydrolyze gelatin, starch, Tween 80, and chitin. The requirement for Na+ ions was determined on medium that contained 0.25% (wt/vol) yeast extract, 0.1% (wt/vol) glucose, 0.02% (wt/vol) KH2PO4, and 0.005% (wt/vol) MgSO4·7H2O (pH 7.8). The tests for utilization of various organic substrates as sole carbon sources (at a concentration of 0.1% [wt/vol]) were performed on synthetic solid BM medium (4). In some cases liquid BM medium (10 ml per tube) was used; the bacteria were grown with shaking on a rotary shaker at 160 rpm for 72 h at 24 to 26°C.

Susceptibility to antibiotics was determined by using the routine diffusion plate technique, plates containing solid medium B, and disks impregnated with lowing antibiotics: rifampin (15 μg), rifostin (150 μg), kanamycin (10 μg), ampicillin (10 μg), benzylpenicillin (10 μg), streptomycin (15 μg), neomycin (15 μg), erythromycin (15 μg), gentamicin (10 μg), oxacillin (20 μg), cephalaxin (10 μg), polymyxin B (50 μg), polymyxin E (10 μg), and oxfacin (10 μg).

Genetic analysis. DNA was isolated by the method of Marmur (20). The G+C content of each DNA was determined by the thermal denaturation method of Marmur and Doty (21). Levels of DNA-DNA hybridization were determined spectrophotometrically, and initial renaturation rates were determined as described by De Ley et al. (8) and Levano et al. (19).

Production and characterization of soluble pigment. Production of a soluble pigment was studied by using medium B and medium BT (medium B suppl-
A. nigrifaciens, Alteromonas undina, Alteromonas espejiana from guish them from the nonpigmented species that have similar DNA G+ C contents. The G+C contents of the DNAs ranged from 38.5 to 40.2 mol%. All of these data suggested the type strains of these species by utilizing mannose, fructose, sucrose, pyruvate, glycerol, and rhamnose. The strains which we studied had some phenotypic characteristics that differentiated them from the type strain of A. nigrifaciens. All of them produced amylase, and most of them grew at 35°C, utilized mannitol, adonitol, L-ornithine, and L-phenylalanine, and did not utilize citrate and glutarate.

**RESULTS AND DISCUSSION**

Phenotypic characteristics and G+C contents of DNAs. The bacteria isolated from Far Eastern mussels were motile gram-negative rods; each cell had a polar flagellum. Electron micrographs of the bacteria have been published elsewhere (13). Some cells were surrounded by a capsule. No strain accumulated poly-β-hydroxybutyrate as an intracellular reserve material and had an arginine dihydrolase system. All strains were strictly aerobic (negative in the oxidation-fermentation test) and oxidase positive and required NaF ions or seawater for growth. The strains which we studied differed from the type strain by exhibiting amylase activity and having a wider temperature range for growth. The G+C contents of the DNAs ranged from 38.5 to 40.2 mol%. All of these data suggested that the strains which we isolated belonged to the genus *Alteromonas*. The results of an investigation of the phenotypic properties of these organisms (Table 2) allowed us to distinguish them from the nonpigmented species that have similar DNA G+C contents (*A. haloplanktis, A. tetraodonis, Alteromonas atlantica, Alteromonas undina, Alteromonas espejiana*) and from *Alteromonas carrageenovora*. The differential characteristics of these species are shown in Table 3. Most of the nine strains isolated from mussels differed from members of other *Alteromonas* species by utilizing mannose, fructose, sucrose, pyruvate, glycerol, and rhamnose. The strains which we studied had some phenotypic characteristics that differentiated them from the type strain of *A. nigrifaciens*. All of them produced amylase, and most of them grew at 35°C, utilized mannitol, adonitol, L-ornithine, and L-phenylalanine, and did not utilize citrate and glutarate.

**DNA relatedness.** The results of our DNA-DNA hybridization experiments are shown in Table 4. The levels of DNA-DNA relatedness among the strains isolated from mussels ranged from 61 to 100%. These data indicate that the nine strains isolated from mussels belong to the same species. The levels of DNA-DNA similarity between these organisms and the type strains of *A. haloplanktis, A. haloplanktis* ranged from 12 to 41%. In contrast, the levels of DNA-DNA homology between the type strain *A. nigrifaciens* and the strains isolated from mussels were 66 to 70%. On the basis of the generally accepted criterion (27), we concluded that the strains isolated from mussels were *A. nigrifaciens* strains.

**Production of melanin-like soluble pigment.** A distinctive feature of *A. nigrifaciens* is its ability to produce a melanin-like pigment (as reflected by the name of the species). The strains which we isolated did not synthesize any pigment when they were grown on common media, such as marine agar 2216 or BAP. We also examined the formation of pigments on tyrosine-containing media that are optimal for melanogenesis by the type strain of *A. nigrifaciens* and on the same media in the presence of inhibitors of melanogenesis, including cysteine, EDTA, and ascorbic acid (Table 5). We found that all of the
**TABLE 2. Characteristics of nine strains of *A. nigrifaciens* isolated from marine mollusks and *A. nigrifaciens* IAM 13010\textsuperscript{T}**

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Susceptibility to:

- Rifampin (15 μg) + ND + + + + + ND ND ND +
- Ristomycin (150 μg) − ND − w + − w ND ND −
- Kanamycin (10 μg) w ND − w w − w ND ND −
- Ampicillin (10 μg) + ND + + + + + ND ND +
- Benzyl-penicillin (10 μg) − ND + − + + + ND ND +
- Streptomycin (15 μg) + ND w w w + + ND ND +
- Neomycin (15 μg) w ND − − − + + ND ND −
- Erythromycin (15 μg) + ND + + + + + ND ND +
- Gentamicin (10 μg) + ND + + + + + ND ND +
- Oxacillin (20 μg) − ND − − − − ND ND −
- Cephaloxin (10 μg) − ND − − − − ND ND −
- Polymyxin (50 μg) + ND + + + + + ND ND +
- Lincomycin (10 μg) − ND − − − − − ND ND −
- Ofloxacin (10 μg) + ND + + + + + ND ND −

Data from reference 6. ’ +, positive; −, negative; v, variable; ND, no data available; w, weakly susceptible.

nonpigmented strains isolated from mussels except strain 2 MC12 produced pigment on media containing tyrosine and were colorless on the media that contained the inhibitors. The pigment extracted from cells with a 0.5 N NaOH solution formed a brown solution. The alkaline pigment solution reacted qualitatively like melanin; it became colorless when H2O2 was added, and addition of KMnO4 resulted in a green solution. The absorption maximum of the pigment (225 nm) was close to the absorption maximum for synthetic melanin (Sigma) (223 nm). The data in Table 6 confirmed the melanin-like nature of the pigments tested.

The differences in pigment production and in utilization of some sources of carbon between the strains associated with mussels and other representatives of A. nigrifaciens might depend on the habitats of the strains. We found that bacterial associates of mussels had more active hydrolytic enzymes (amylases, gelatinases, lipases, alkaline phosphatases, α-galactosidase, etc.) than bacteria isolated from seawater. The most likely reason for these differences is symbiotrophic relationships between bacteria and mussels.

Antimicrobial agent susceptibility. The nine strains of A. nigrifaciens examined were susceptible to rifampin, ampicillin, erythromycin, gentamicin, polymyxin, and ofloxacin but were not susceptible to lincomycin, oxacillin, and cephalexin.

Description of A. nigrifaciens strains. Only one strain of A. nigrifaciens has been described previously (6). This strain was isolated by White (28) and was designated "Pseudomonas nigrifaciens," and it was later renamed because of its close relationship with species belonging to the A. haloplankis rRNA citron group (26). Detailed phenotypic, genetic, and chemo-

TABLE 3. Differential phenotypic characteristics of Alteromonas species

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<th>A. tetradonis IAM 14160&lt;sup&gt;T&lt;/sup&gt;</th>
<th>A. atlantica IAM 12975&lt;sup&gt;T&lt;/sup&gt;</th>
<th>A. carrageenovora IAM 12662&lt;sup&gt;T&lt;/sup&gt;</th>
<th>A. espejiana IAM 12640&lt;sup&gt;T&lt;/sup&gt;</th>
<th>A. undina IAM 12922&lt;sup&gt;T&lt;/sup&gt;</th>
<th>A. nigrifaciens</th>
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<sup>a</sup> Data from reference 3.
<sup>b</sup> +, positive; −, negative; d, 11 to 89% of the strains are positive.
taxonomic characteristics of Alteromonas nigrifaciens strains isolated from marine animals have not been described previously. The intraspecific variation of the species is discussed below, and an emended description of A. nigrifaciens is presented below.

There are no data about capsulated representatives of the genus Alteromonas available. Recently, we described the diversity of the polysaccharides produced by the Alteromonas strains that have been studied (13). The following monosaccharides of A. nigrifaciens capsules were identified: D-glucolactylic acid; l-gulosamine uronic acid; L-galactosamine uronic acid; L-gulosamine uronic acid; L-galactosamine uronic acid; bacillosamine, 4-aminoquinovose acylated with an alanine residue; 41°C.

The overall levels of polyamines in A. nigrifaciens were low, but putrescine, cadaverine, and spermidine were present (3a). Small quantities (0.2 to 0.6 μmol/g [dry weight]) of 2-hydroxyputrescine and spermine were detected in two strains. Svetashev et al. (24) reported that palmitoleic acid [16:1(n-7)], palmitic acid (16:0), heptadecenoic acid (17:1), and vaccenic acid [18:1(n-7)] were the most abundant fatty acids in strains of A. nigrifaciens. They also showed that low levels of the branched-chain fatty acids and certain hydroxy fatty acids and the absence of cyclopropane acids are characteristic features of these strains as well as of other Alteromonas species.

On the basis of the properties mentioned above, we present the following emended description of A. nigrifaciens.

**Emended description of Alteromonas nigrifaciens Baumann, Baumann, Bowditch, and Beaman 1984.** Alteromonas nigrifaciens (ni.gri.fa’ci.ens. L. adj. niger, black; L. v. facio, to make; M.L. part. adj. nigrifaciens, blackening). Cells are gram-negative, strictly aerobic, straight rods that are 0.8 to 1.2 μm wide and 1.8 to 2.3 μm long. They are motile by means of a single flagellum at one pole. Some cells are encapsulated. Cells grow at 4 to 30°C. Some strains grow at 35°C, but no strain grows at 41°C. A melanin-like dark pigment is produced by almost all strains in L-tyrosine-containing media, particularly at low temperatures. Produces oxidase and lipase. Sodium ions are required for growth. Growth factors are not required. Chitinase and arginine dihydrolase negative. Does not denitrify. Grows on D-glucose, D-galactose, D-lactate, pyruvate, D-sorbitol, L-threonine, and L-lysine. Pelargonate and aspartic acid are not used as sole carbon sources. Susceptible to rifampin, ampicillin, erythromycin, gentamicin, polymyxin, and ofloxacin but not susceptible to lincomycin, oxacillin, and cephalaxin.

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The type strain is strain KMM 661 (= LMG 2227 = IAM 13010 = ATCC 19375 = NCTC 10691).

### Table 4. Levels of DNA relatedness among strains

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### Table 5. Production of melanin-like dark pigments by A. nigrifaciens strains in different media

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<tr>
<th>Medium</th>
<th>KMM 160</th>
<th>KMM 159</th>
<th>KMM 158</th>
<th>KMM 161</th>
<th>KMM 155</th>
<th>KMM 153</th>
<th>KMM 156</th>
<th>KMM 294</th>
<th>A. nigrifaciens IAM 13010T</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>BT</td>
<td>w</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>ST</td>
<td>w</td>
<td>w</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td>+</td>
</tr>
<tr>
<td>BT + cysteine (1-10 mM)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
<td>+</td>
</tr>
<tr>
<td>BT + EDTA (1-10 mM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>BT + ascorbic acid (1-10 mM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
</tbody>
</table>

* = no pigment produced; +, pigment produced; w, weak brown pigment produced.
TABLE 6. Characterization of pigments produced by A. nigerfiaciens strains isolated from mollusks

<table>
<thead>
<tr>
<th>Organism</th>
<th>Color of pigment</th>
<th>Solubility of pigment in H2O</th>
<th>Precipitation of pigment</th>
<th>Tyrosinase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NaOH (0.1 M)</td>
<td>Blackberg-Wanger technique</td>
<td>From NaOH by HCl</td>
</tr>
<tr>
<td>A. nigerfiaciens IAM 13010T</td>
<td>Brown</td>
<td>I</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>KMM 160 (= 2MC41)</td>
<td>Brown</td>
<td>I</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>KMM 159 (= 2MC45)</td>
<td>Brown</td>
<td>I</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>KMM 158 (= 2MM6)</td>
<td>Brown</td>
<td>I</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>KMM 161 (= 3GM32)</td>
<td>Brown</td>
<td>I</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>KMM 156 (= 2ML26)</td>
<td>Brown</td>
<td>I</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>Aspergillus nidulans</td>
<td>Black</td>
<td>S</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sheawanna colwelliana</td>
<td>Brown</td>
<td>I</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

ACKNOWLEDGMENTS

This study was supported by grants from ISF and by a fellowship grant from the Foreign Researcher Invitation Program of the Agency of Industrial Science and Technology, Japan.

ADDENDUM

The members of the “A. haloplanktis” rRNA cluster and [Pseudomonas] piscicida have been reclassified as separate species of the genus Pseudoalteromonas (10a).

REFERENCES

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