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Vibrio diabolicus sp. nov., a New Polysaccharide-Secreting Organism Isolated from a Deep-Sea Hydrothermal Vent Polychaete Annelid, *Alvinella pompejana*

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A deep-sea, facultatively anaerobic, heterotrophic, mesophilic new organism was isolated from the polychaete annelid *Alvinella pompejana* collected from a deep-sea hydrothermal field in the East Pacific Rise. On the basis of phenotypic characteristics, phylogenetic analyses, and DNA-DNA relatedness, this organism was identified as a new species of the genus *Vibrio*, for which the name *Vibrio diabolicus* is proposed. In batch cultures in the presence of glucose, this organism produced an innovative exopolysaccharide. This polymer had high contents of both uronic acids and hexosamines and was similar to other polysaccharides with interesting biological activities.

Because of the interesting properties of polymers secreted by marine bacteria to enhance their survival in their natural environments, many scientists have isolated such bacteria and have characterized the exopolysaccharides (EPS) produced (2, 4, 14, 16–18, 28). Since the discovery and exploration of many deep-sea hydrothermal ecosystems known for their extreme conditions, the search for EPS-secreting bacteria has been systematic (11, 23, 29). However, only a few EPS-secreting bacteria have been described (21, 22, 29, 30), and all strains isolated previously from deep-sea hydrothermal ecosystems have been assigned to the genus *Alteromonas*. In the present study we describe the first *Vibrio* strain isolated from a deep-sea hydrothermal vent and show that it is a member of a new species.

MATERIALS AND METHODS

Bacterial strains. During the French-American cruise HERO in October 1991, samples of vent fluids, chimney rocks, invertebrate tissues, and seawater were collected by the French-manned submersible *Nautile* at a depth of 2,600 m in a rift system of the East Pacific Rise (8, 12) (12°48.13'N, 103°56.30'W). Samples were inoculated into marine 2216 broth (Difco Laboratories, Detroit, Mich.) at pH 7.6 and incubated at 25°C for 24 to 48 h. Enrichment cultures were purified on marine 2216 agar (Difco) by using the same growth conditions. Pure cultures were maintained in 20% (vol/vol) glycerol at −20 and −70°C for use in future experiments and in liquid nitrogen for long-term storage. Three bacterial strains were isolated from the dorsal integument of the hydrothermal vent polychaete annelid *Alvinella pompejana* (6), which was collected from a dense population of annelids near the active hydrothermal vent ELSA. While we were searching for marine EPS-secreting bacteria, isolate HE800^T was selected because of its ability to exhibit the most interesting swarming mucoid phenotype on marine 2216 agar (Difco) supplemented with glucose (30 g/liter) after 3 days at 25°C.

Vibrio nereis LMG 3895^T and *Vibrio tubiashii* LMG 10936^T were obtained from the LMG Culture Collection (Ghent, Belgium). *Vibrio mytili* CECT 632^T was a gift from the Coleccion Espanola de Cultivos Tipo (Universidad de Valencia, Burjassot, Spain).

Cytological characterization. Gram staining was carried out as described by Conn et al. (5). Conventional transmission electron microscopy was used to observe strain HE800^T and to demonstrate the presence of flagella. Negative staining with 1% (wt/vol) uranyl acetate in which Formvar (Cu grid; 300-mesh)-carbon film (Oxford Instruments, Orsay, France) was used was done by a previously described procedure (19). The presence of pigment was determined by the method of King et al. (13), modified by adding NaCl (20 g/liter) to the medium. Polyhydroxybutyrate accumulation was tested by the method of Gauthier and Breittmayer (9) by using *V. nereis* as the positive control.

Physiological characteristics. Growth experiments were performed in 20-ml tubes containing 5 ml of marine 2216 broth. The tubes were inoculated (5%, vol/vol) with a primary bacterial culture in the exponential phase and were incubated inclined on a table rotary shaker (Infors HT, Bottmingen, Switzerland) with shaking at 200 oscillations per min. The turbidity in culture tubes was measured directly by spectrophotometry at 520 nm (Spectronic 401 instrument; Milton Roy, Rochester, N.Y.) at optical density values ranging 0.05 to 0.5. Growth rates were determined at temperatures ranging from 15 to 45°C. The effect of pH on the growth rate was determined at pH 5.5 to 8.5 by using 50 mM MES (morpholineethanesulfonic acid; pH 5.5 to 6.5), 50 mM MOPS [3-(*N*-morpholino)propanesulfonic acid; pH 6.5 to 8], and 50 mM AMPSO {3-1[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid; pH 8.5} buffers. Growth in the presence of different concentrations of Na^+ was examined by using NaCl concentrations between 10 and 70 g/liter. Three replicates were studied. Specific growth rates were calculated by performing a linear regression analysis with five to seven points along the logarithmic part of the resulting growth curves.

Biochemical characteristics. API 20E, API 20NE, API 50CH, API ZYM, and ATB 7 ANTIBIOGRAM strips (BioMérieux SA, Craponne, France) were used to detect metabolic properties and antibiotic susceptibilities according to the manufacturer's instructions. For these tests, the suspending medium was adjusted to an NaCl concentration of 20 g/liter, and the preparations were incubated at 25°C for 48 h. The same tests under the same conditions were performed with the three *Vibrio* species most closely related to strain HE800^T on the phylogenetic tree (*V. mytili*, *V. nereis*, and *V. tubiashii*) (see Fig. 3).

Sensitivity to vibriostatic agent 0/129 was determined by a disk method (150-μg disk; Diagnostics Pasteur, Marnes La Coquette, Paris, France) on marine 2216 agar. Production of chitinase was examined by using the well method with marine agar 2216 supplemented with 0.5% (wt/vol) purified chitin (Sigma).

DNA base composition. Genomic DNAs were isolated by the method described by Charbonnier et al. (3). Briefly, each culture was centrifuged (8,000 × *g* for 30 min at 4°C), and then the cell pellets were resuspended in 5 ml of TE-Na buffer (100 mM Tris-HCl, 0.50 mM EDTA, 100 mM NaCl; pH 8) and lysed at 40°C for 3 h after addition of 1% (wt/vol) *N*-lauryl sarcosine, 1% (wt/vol) sodium dodecyl sulfate, and proteinase K (final concentration, 0.4 mg/ml).

After centrifugation (5,000 × *g* for 10 min), the DNA was extracted with phenol-chloroform-isoamyl alcohol (24:24:1) (Sigma) and then treated with RNase (5 μg/ml) at 60°C for 1 h. This step was followed by one additional extraction with phenol-chloroform-isoamyl alcohol (24:24:1) and one extraction with chloroform. The DNA was precipitated by adding 2.5 volumes of cold pure ethanol, and the pellet was dried and suspended in TE buffer (10 mM Tris-HCl [pH 7.4], 2 mM EDTA [pH 8]). DNA concentration and purity were estimated at 230, 260, 280, and 320 nm with a GeneQuantII spectrophotometer (Pharmacia, Uppsala, Sweden).

The G+C content of the DNA was determined by thermal denaturation (15) by using a Kontron spectrophotometer (model UVIKON 940) and a type 9009 system disk that included the TM program (Kontron Instruments, St. Quentin-Yvelines, France). The spectrophotometer was equipped with a Huber cryothermostat (model Ministat HS 40), itself monitored with a Huber temperature

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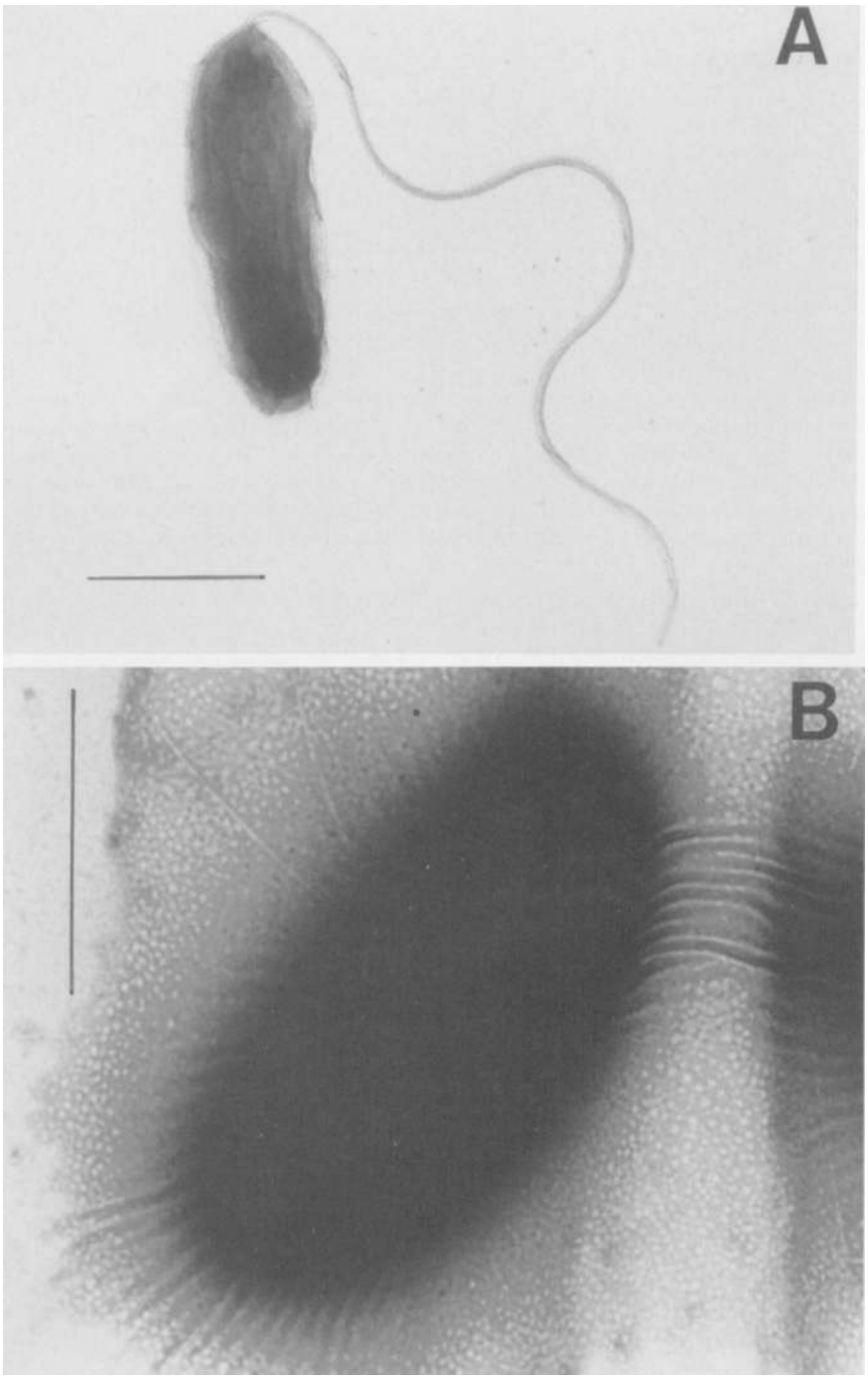

FIG. 1. Transmission electron micrographs of *V. diabolicus* grown in liquid medium (A) and on solid medium (B). Preparations were negatively stained with 1% uranyl acetate. Bars = 1 μm.

programmer (model PD415; Bioblock, Illkirch, France). A calibration curve was obtained by using ultrapure DNAs from *Escherichia coli* (57.0 mol% G+C), *Clostridium perfringens* (26.5 mol% G+C), and *Micrococcus lysodeikticus* (77.0 mol% G+C) (Sigma) as standards in TE buffer.

Sequencing of the 16S rRNA genes. (i) DNA amplification. The small-subunit rRNA gene was amplified by using two primers corresponding to positions 8 to 28 and 1,498 to 1,509 of the *E. coli* small-subunit rRNA sequence. The initial denaturation step consisted of heating the reaction mixture at 95°C for 180 s, and this was followed by an annealing step (52°C for 60 s) and an extension step (72°C for 90 s). The thermal profile then consisted of 25 cycles consisting of denaturation at 94°C for 30 s, annealing at 52°C for 60 s, and extension at 72°C for 90 s. The final extension step consisted of extension at 72°C for 5 min. This amplification reaction produced DNA molecules that were about 1.5 kb long. The PCR product was then directly sequenced by using a previously described protocol (24). Using conserved primers, we determined almost the entire small-subunit rRNA sequence (corresponding to positions 29 to 1,425 in the *E. coli* rRNA sequence).

(ii) Phylogenetic analysis and alignment: general procedure. For all phylogenetic analyses we combined the results of three clustering methods (neighbor joining, maximum parsimony, and maximum likelihood), and each topology was assessed for robustness by performing a bootstrap analysis. For the neighbor-joining analysis an algorithm developed by Saitou and Nei was used (25). For the maximum-parsimony analysis, the PAUP program (26) for Macintosh computers was used. All topologies were obtained first by using the heuristic option, fol-

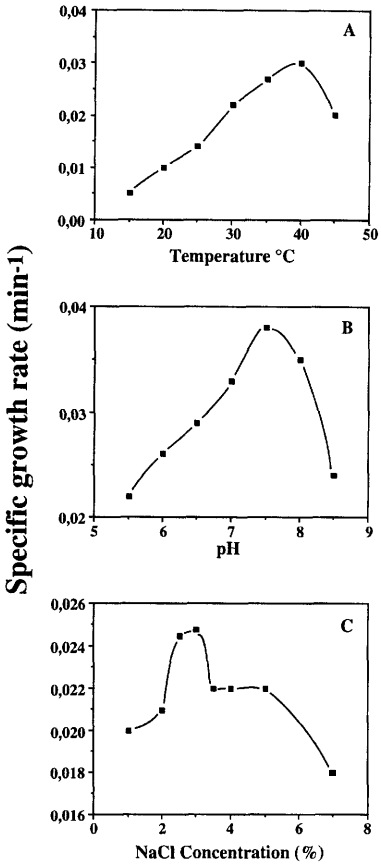

FIG. 2. Effects of temperature (A), pH (B), and NaCl concentration (C) on growth of strain HE800^T. Specific growth rates were calculated from the slopes of the growth curves.

lowed by a branch-and-bound search. When several most-parsimonious trees were obtained, a 100% consensus tree was constructed and treated as the most-parsimonious tree. For the maximum-likelihood analysis, the fDNAml program, derived from the DNAML program (7) and rewritten by G. J. Olsen (University of Illinois, Urbana), was recompiled on a Hewlett-Packard model 700 workstation. A bootstrap analysis was also performed (maximum parsimony, heuristic option, 100 replications). Finally, all trees were plotted by using a Macintosh computer and a program (njplot) developed by M. Gouy (CNRS, Université Claude Bernard, Villeurbanne, France) that allows transformation of a formal tree representation (Newick's format) into MacDraw drawings.

DNA reassociation. The percentages of DNA-DNA relatedness were determined from renaturation rates by the S1 nuclease method (10), as slightly modified by Popoff and Coynault (20). A DNA probe from strain HE800^T was labelled by using a Nick translation kit (Amersham, Buckinghamshire, England). The DNA-DNA hybridizations were performed under optimal conditions (25°C below the melting temperature) for 24 h.

Production of HE800^T polymer. Exopolymer production was analyzed at 25°C in a 2-liter fermentor (SGI-Inceltech, Toulouse, France) containing 1 liter of marine 2216 broth supplemented with glucose (750 ml of marine broth, 250 ml of filtered seawater, 40 g of glucose). The glucose solution was sterilized separately for 1 h at 105°C.

A batch culture was inoculated (10%, vol/vol) with a suspension of cells in the exponential phase. The pH was adjusted to 7.4 and maintained at this value by automatic addition of NaOH (0.25 N). Foaming was avoided by adding Pluronic-PE6100 oil (BASF, Levallois/Perret, France) at a concentration of 0.05% (vol/vol). The airflow was fixed at 30 liters/h, and the agitation rate ranged from 250 to 1,100 rpm to maintain a level of dissolved O_2 of 40%. Growth was determined by measuring the culture turbidity at 520 nm. Viscosity of the culture broth was controlled with a model DV II viscosimeter (Brookfield Engineering Laboratories, Stougthon, Mass.) equipped with a small adapter (model SC4-18/13R) at 25°C.

Extraction, purification, and characterization of the HE800^T polymer. Detailed procedures related to the extraction and purification of the HE800^T polymer (EPS800) have been reported elsewhere (27, 30). The total neutral carbohydrate, hexuronic acid, and hexosamine contents and the monosaccharide ratios of EPS800 were determined as previously described (21, 23).

Small-subunit rDNA sequence accession numbers. Two nucleotide sequences were determined in this study. They have been deposited in the EMBL database under the following accession numbers: *V. mytili* CECT 632^T, X99761; and strain HE800^T, X99762. The nucleotide sequence accession numbers for the closely related *Vibrio* strains used in the phylogenetic tree in this study are as follows: *Vibrio aestuarianus* ATCC 35048^T, X74689; *Vibrio alginolyticus* ATCC 17749^T, X74690; *V. alginolyticus* CIP 70.65^T, X74691; *V. alginolyticus* type strain (no designation), X56576; *Vibrio campbellii* ATCC 25920^T, X74692; *V. campbellii* type strain (no designation), X56575; *Vibrio carchariae* ATCC 35084^T, X74693; *Vibrio fluvialis* NCTC 11327^T, X76335; *Vibrio furnisii* ATCC 35016^T, X74704; *Vibrio gazogenes* ATCC 29988^T, X74705; *Vibrio harveyi* ATCC 14126^T, X74706; *V. harveyi* type strain (no designation), X56578; *Vibrio mediterranei* CIP 103203^T, X74710; *Vibrio natriegens* ATCC 14048^T, X74714; *V. natriegens* type strain (no designation), X56581; *Vibrio navarrensis* CIP 103381^T, X74715; *V. nereis* ATCC 25917^T, X74716; *Vibrio nigripulchritudo* ATCC 27043^T, X74717; *Vibrio orientalis*

TABLE 1. Differential characteristics of *V. diabolicus* HE800^T, *V. mytili*, *V. nereis*, and *V. tubiashii*[a]

API strips	Substrate, reaction, or enzyme	HE800^T	*V. mytili*	*V. nereis*	*V. tubiashii*
50CH	Glycerol	+	+	−	−
	L-Arabinose	−	+	−	−
	Ribose	+	+	+	+
	D-Xylose	−	+	−	−
	Galactose	+	+	−	+
	Glucose	+	+	+	+
	Fructose	+	+	+	+
	Mannose	+	+	+	+
	Mannitol	+	+	−	+
	N-Acetylglucosamine	+	+	+	+
	Amygdalin	+	+	−	+
	Arbutin	−	+	−	−
	Esculin	−	+	−	−
	Salicin	−	+	−	−
	Cellobiose	−	+	−	+
	Maltose	+	+	+	+
	Sucrose	+	+	+	+
	Trehalose	+	+	+	+
	Melizitose	−	+	−	−
	Starch	+	+	+	+
	Glycogen	+	+	−	+
	Gentiobiose	−	+	−	−
	D-Turanose	−	+	+	−
	Gluconate	+	+	+	+
20NE	Reduction of nitrates to nitrites	+	+	+	+
	Indole production	+	+	+	+
	Glucose acidification	+	+	+	+
	Arginine dihydrolase	−	+	−	+
	β-Galactosidase (PNPG)[b]	+	+	−	+
	Caprate assimilation	+	−	−	−
	Malate assimilation	+	+	+	+
	Citrate assimilation	+	+	+	−
20E	β-Galactosidase (ONPG)[c]	−	+	−	+
	Lysine decarboxylase	+	−	−	−
	Ornithine decarboxylase	+	−	−	−
	Tryptophan deaminase	+	+	+	+
	Indole production	+	+	+	+
	Gelatinase	+	−	−	+
	Glucose fermentation	+	+	+	+
	Mannitol fermentation	+	+	−	+
	Sucrose fermentation	+	+	+	+
	Amygdalin fermentation	+	+	−	+

[a] The characteristics were determined with API 50CH strips (carbohydrate assimilation) and API 20NE and API 20E strips (identification of gram-negative rods). Tests that were negative for all strains are mentioned in the text.

[b] PNPG, *p*-nitrophenyl-β-D-glucoside.

[c] ONPG, *o*-nitrophenyl-β-D-galactopyranoside.

TABLE 2. Differential characteristics of *V. diabolicus* HE800^T, *V. mytili*, *V. nereis*, and *V. tubiashii*[a]

API strips	Enzyme or antibiotic	HE800^T	*V. mytili*	*V. nereis*	*V. tubiashii*
ZYM	Phosphatase (alkaline)	+++	−	−	++
	Esterase (C_4)	++	++	+	+
	Esterase-lipase (C_8)	+++	+++	+	+++
	Lipase (C_{14})	+	+	−	+
	Leucine arylamidase	+++	+++	++	++
	Trypsin	++	+	−	−
	Chymotrypsin	++	−	−	−
	Phosphatase (acid)	+++	++	−	−
	N-Acetyl-β-glucosamidase	−	++	−	−
ATB 7	Amoxicillin (4–16 mg/liter)	R	S	S	S
	Cephalothin (8–32 mg/liter)	R	S	S	S
	Neomycin (8–16 mg/liter)	S	R	S	I
	Tobramycin (4–8 mg/liter)	I	R	S	R
	Gentamicin (4–8 mg/liter)	S	R	S	R
	Netilmicin (4–8 mg/liter)	S	R	S	I
	Chloramphenicol (8–16 mg/liter)	S	S	S	I
	Colistin (4 mg/liter)	R	R	S	S
	Bacitracin (2 mg/liter)	R	R	R	R

[a] The characteristics were determined with API ZYM strips (enzymatic activities) and ATB 7 strips (antibiotic susceptibilities). Tests that were negative for both strains are listed in the text. For API ZYM data: +, 5 nM hydrolyzed substrate; ++, 20 nM hydrolyzed substrate; +++, ≥40 nM hydrolyzed substrate (from API ZYM reading scale). For ATB 7 data: R, resistant; S, susceptible; I, intermediate.

ATCC 33934^T, X74719; *Vibrio parahaemolyticus* ATCC 17802^T, X74720; *V. parahaemolyticus* CIP 73.30^T, X74721; *V. parahaemolyticus* type strain (no designation), X56580 and M59161; *Vibrio pelagius* ATCC 25916^T, X74722; *Vibrio proteolyticus* ATCC 15338^T, X74723; *V. proteolyticus* type strain (no designation), X56579; *V. tubiashii* ATCC 19109^T, X74725; and *Vibrio vulnificus* ATCC 27562^T, X74726. Partial sequences of fish pathogens were also used to investigate possible relationships. The nucleotide sequence accession numbers for these *Vibrio* sp. strains are X70636, D21223, D21225, D21226, X70637, and U14582. In addition, a number of unpublished sequences for wild *Vibrio* strains characterized in our laboratory (3a) were also used. A total of 117 16S rDNA sequences of bacteria belonging to the family *Vibrionaceae* were used for the phylogenetic analyses.

RESULTS

Morphology. Strain HE800^T is a motile, facultative anaerobe that is capable of both fermentative and respiratory metabolism; it is a nonpigmented, nonluminescent, gram-negative, straight, rod-shaped organism whose cells are 0.8 μm wide and 2.2 μm long with single, sheathed, polar flagella when it is grown in liquid media (Fig. 1A). On solid media, cells are characterized by many lateral flagella associated with a capacity to swarm (Fig. 1B). After 3 days of growth, colonies on marine 2216 agar containing glucose are swarming and about 0.9 cm in diameter (they are 0.2 cm in diameter without sugar).

Cultural characteristics. The optimal temperature for growth was between 30 and 45°C (Fig. 2A), and the optimal pH was between 7 and 8 (Fig. 2B). The optimal ionic strength was between 20 and 50 g of NaCl per liter (Fig. 2C). A study of the growth rate under optimal conditions revealed doubling times ranging from 18 to 28 min.

Metabolic properties. Positive responses were obtained for catalase, cytochrome oxidase, and chitinase production. Reduction of nitrate to nitrite occurred. There was no accumulation of polyhydroxybutyrate. Sensitivity to vibriostatic agent 0/129 was observed. Other biochemical and nutritional characteristics are shown in Tables 1 and 2. Strain HE800^T was able to utilize a wide range of carbohydrate compounds as sole sources of carbon for energy and growth. Strain HE800^T was resistant to amoxicillin (4 to 16 mg/liter), cephalothin (8 to 32 mg/liter), tobramycin (4 mg/liter), colistin (4 mg/liter), and bacitracin (2 mg/liter).

A comparison between strain HE800^T and the three most closely related *Vibrio* species (Fig. 3) revealed many dissimilarities (Tables 1 and 2). The following tests were negative for all of the organisms: utilization of erythritol, D-arabinose, L-xylose, adonitol, β-methyl-D-xyloside, sorbose, rhamnose, dulcitol, inositol, sorbitol, α-methyl-D-mannoside, α-methyl-D-glucoside, lactose, melibiose, inulin, raffinose, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, 2-ketogluconate, and 5-ketogluconate, as determined with API 50CH strips; reduction of nitrates to nitrogen, urease activity, β-glucosidase activity, and utilization of adipate and phenylacetate, as determined with API 20NE strips; arginine dihydrolase activity, citrate utilization, H_2S production, urease activity, acetoin production, and fermentation of inositol, sorbitol, rhamnose, melibiose, and arabinose, as determined with API 20E tests; and cystine arylamidase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase activities, as determined with API ZYM strips. All of the organisms strains were susceptible to tetracycline (4 to 8 mg/liter), nalidixic acid (8 to 16 mg/liter), pefloxacin (1 to 4 mg/liter), rifampin (4 to 16 mg/liter), and cotrimoxazole (2 to 8 mg/liter), as determined with ATB 7 ANTIBIOGRAM strips.

DNA base composition. The G+C content of HE800^T was 49.6 ± 0.6 mol%.

Phylogenetic analyses. The 16S rDNA sequence of strain HE800^T was aligned with other sequences by comparing it to a database containing about 3,400 aligned eubacterial small-subunit rRNA sequences. Phylogenetic analyses that included representatives of all eubacterial phyla showed that strain HE800^T belonged to the gamma subdivision of the phylum *Proteobacteria* and that it should be included in the family *Vibrionaceae*. More detailed phylogenetic analyses were then performed by

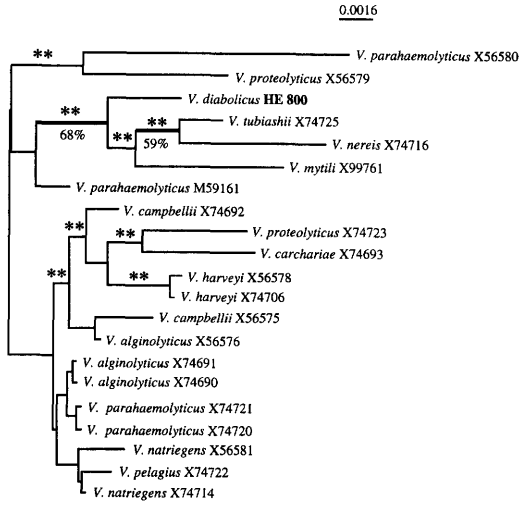

FIG. 3. Unrooted phylogenetic tree for a subset of the genus *Vibrio*. The topology shown was obtained by neighbor joining. Branches identified at $P < 0.01$ by maximum likelihood are indicated by asterisks. The thick lines show branches that were also retrieved in the most-parsimonious tree (percentages indicate how taxa were supported by a bootstrap analysis). *V. diabolicus* HE800^T belongs to a well-defined monophyletic unit that also includes *V. tubiashii*, *V. nereis*, and *V. mytili*. The numbers are the nucleotide sequence accession numbers in the EMBL database.

TABLE 3. Dissimilarity matrix based on small-subunit rDNA sequences[a]

Organism	% Dissimilarity or % homology[b]																		
	V. diabolicus HE800^T	*V. proteolyticus*	*V. pelagius*	*V. parahaemolyticus*	*V. natriegens*	*V. alginolyticus*	*V. carchariae*	*V. campbellii*	*V. harveyi*	*V. vulnificus*	*V. mediterranei*	*V. aestuarianus*	*V. navarrensis*	*V. mytili*	*V. nigripulchritudo*	*V. tubiashii*	*V. fluvialis*	*V. furnisii*	*V. nereis*
V. diabolicus HE800^T		0.022	0.011	0.012	0.013	0.011	0.022	0.015	0.017	0.045	0.020	0.039	0.043	0.011	0.030	0.016	0.033	0.030	0.015
V. proteolyticus	97.8		0.014	0.013	0.014	0.012	0.012	0.014	0.014	0.041	0.037	0.049	0.044	0.028	0.020	0.017	0.038	0.033	0.017
V. pelagius	98.9	98.6		0.004	0.001	0.003	0.013	0.007	0.009	0.041	0.029	0.036	0.038	0.017	0.029	0.016	0.037	0.031	0.021
V. parahaemolyticus	98.8	98.7	99.6		0.004	0.001	0.012	0.003	0.008	0.041	0.029	0.037	0.038	0.018	0.027	0.017	0.038	0.032	0.021
V. natriegens	98.7	98.6	99.9	99.6		0.003	0.014	0.007	0.009	0.041	0.031	0.038	0.039	0.017	0.030	0.017	0.038	0.032	0.023
V. alginolyticus	98.9	98.8	99.7	99.9	99.7		0.011	0.004	0.008	0.041	0.030	0.038	0.039	0.017	0.028	0.017	0.038	0.033	0.020
V. carchariae	97.8	98.8	98.7	98.8	98.6	98.9		0.009	0.008	0.050	0.039	0.047	0.047	0.027	0.020	0.023	0.047	0.041	0.026
V. campbellii	98.5	98.6	99.3	99.7	99.3	99.6	99.1		0.005	0.044	0.032	0.040	0.042	0.020	0.028	0.018	0.041	0.035	0.023
V. harveyi	98.3	98.6	99.1	99.2	99.1	99.2	99.2	99.5		0.044	0.035	0.043	0.045	0.023	0.028	0.019	0.041	0.037	0.022
V. vulnificus	95.5	95.9	95.9	95.9	95.9	95.9	95.0	95.6	95.6		0.042	0.034	0.016	0.048	0.047	0.034	0.018	0.035	0.044
V. mediterranei	98.0	96.3	97.1	97.1	96.9	97.0	96.1	96.8	96.5	95.8		0.034	0.043	0.026	0.037	0.025	0.042	0.037	0.029
V. aestuarianus	96.1	95.1	96.4	96.3	96.2	96.2	95.3	96.0	95.7	96.6	96.6		0.036	0.041	0.054	0.044	0.039	0.040	0.049
V. navarrensis	95.7	95.6	96.2	96.2	96.1	96.1	95.3	95.8	95.5	98.4	95.7	96.4		0.050	0.050	0.044	0.026	0.043	0.051
V. mytili	98.9	97.2	98.3	98.2	98.3	98.3	97.3	98.0	97.7	95.2	97.4	95.9	95.0		0.033	0.015	0.038	0.028	0.018
V. nigripulchritudo	97.0	98.0	97.1	97.3	97.0	97.2	98.0	97.2	97.2	95.3	96.3	94.6	95.0	96.7		0.023	0.037	0.032	0.026
V. tubiashii	98.4	98.3	98.4	98.3	98.3	98.3	97.7	98.2	98.1	96.6	97.5	95.6	95.6	98.5	97.7		0.026	0.021	0.011
V. fluvialis	96.7	96.2	96.3	96.2	96.2	96.2	95.3	95.9	95.9	98.2	95.8	96.1	97.4	96.2	96.3	97.4		0.019	0.033
V. furnisii	97.0	96.7	96.9	96.8	96.8	96.7	95.9	96.5	96.3	96.5	96.3	96.0	95.7	97.2	96.8	97.9	98.1		0.029
V. nereis	98.5	98.3	97.9	97.9	97.7	98.0	97.4	97.7	97.8	95.6	97.1	95.1	94.9	98.2	97.4	98.9	96.7	97.1	
V. orientalis	97.6	96.6	97.4	97.4	97.2	97.3	96.2	97.1	96.6	95.6	98.2	96.2	95.7	97.4	96.4	97.7	96.3	97.0	97.2
V. gazogenes	93.9	93.6	93.5	93.4	93.3	93.3	93.6	93.2	92.9	94.5	93.6	94.4	94.5	93.5	94.6	94.2	95.3	94.3	93.9

[a] The entire 16S rDNAs of the type strains were used to calculate homology values. The EMBL accession numbers of the sequences are given in the text.
[b] The values on the upper right are dissimilarity values, and the values on the lower left are homology values.

using all of the 16S rDNA sequences available for members of this family (117 sequences). These analyses demonstrated clearly by a congruence of all methods (neighbor-joining, maximum-likehood, and parsimony methods) and high bootstrap values that strain HE800^T should be included in a subset of *Vibrio* species. This subset was used in a final phylogenetic analysis (Fig. 2). Restriction of the phylogenetic analysis to this subset allowed us to use almost the entire 16S rDNA sequence for analysis, as the closely related sequences could be perfectly aligned along the entire molecule. These final analyses, performed by using three phylogenetic methods, gave identical results and suggested that HE800^T belonged to a clade (monophyletic taxon) that also included *V. mytili*, *V. nereis*, and *V. tubiashii*. When different domains were used for analysis, the position of the 16S rDNA sequence of strain HE800^T could vary slightly, but this sequence was always associated with the sequence of *V. mytili*. The percentages of dissimilarity between the 16S rDNA sequences used are shown in Table 3. The sequence of strain HE800^T differed by more than 1% from all other known *Vibrio* sequences.

DNA reassociation. Hybridization experiments were conducted with strain HE800^T and the most closely related *Vibrio* species, as deduced from the phylogenetic analyses (i.e., *V. mytili*, *V. nereis*, and *V. tubiashii*); strain HE800^T exhibited only low levels of similarity with these three *Vibrio* species (27, 15, and 5%, respectively).

Production and characterization of the EPS. During batch fermentation of strain HE800^T, the production of EPS began at the end of the exponential phase and continued throughout the stationary phase. Consequently, the broth became slightly viscous, and the viscosity increased up to 40 cP (at 60 rpm) at the end of the fermentation (48 h). The level of EPS production reached 2.5 g (dry weight) per liter.

Characterization of the EPS. The chemical properties of the EPS have been described previously (23). Briefly, a colorimetric analysis showed large proportions of uronic acids and hexosamines. Glucuronic and galacturonic acids were identified by gas chromatography along with glucosamine and galactosamine as amino sugars. The ^{13}C nuclear magnetic resonance spectrum of EPS800 showed that the occurrence of four sugars was supported by four anomeric carbons; it also contained signals for four carboxyl carbons. Other signals were assigned to the methyl of acetyl groups, indicating that there was *N*-acetylation of the hexosamines. Fourier-transform infrared spectroscopy indicated that no sulfate group was present.

DISCUSSION

The deep-sea, heterotrophic, mesophilic new organism that was isolated from a Pompeii worm tube collected from a dense population of *A. pompejana* has the general characteristics of the genus *Vibrio* (1). Gram-negative, polarly flagellated, fermentative rods, which required sodium chloride, produced catalase and oxidase, were sensitive to vibriostatic agent 0/129, and had a DNA G+C content of 49.6 mol%. Also, this organism clearly belongs to the genus *Vibrio* based on phylogenetic analyses of 16S rDNA sequences. 16S rDNA sequences are available for all type strains of the genus *Vibrio* (24), and the sequence of strain HE800^T differs by more than 1% from all previously described *Vibrio* sequences. Although this value alone suggests that strain HE800^T might be a member of a new species, it is not sufficient to warrant such recognition. Since phylogenetic analyses of 16S rDNA sequences clearly demonstrated that this bacterium formed a well-defined monophyletic cluster with three *Vibrio* species (*V. mytili*, *V. tubiashii*, and *V. nereis*), the levels of genomic DNA-DNA homology between strain HE800^T and these species were estimated. DNA-DNA homology values well below 50% were found, and it is appropriate to conclude that HE800^T is a member of a new *Vibrio* species. The name *Vibrio diabolicus* is therefore proposed for the new organism. This is the first description of a *Vibrio* species isolated from deep-sea hydrothermal sites.

As previously described (23), EPS800 can be compared to heparin; the similarity in chemical composition makes the EPS800 polymer very interesting.

Description of *Vibrio diabolicus* Raguénès and Christen sp. nov. *Vibrio diabolicus* (di.a.bo'li.cus. L. adj. *diabolicus*, devilish, diabolic). Cells are gram-negative rods (0.8 by 2.2 μm) that are straight and motile by means of a single sheathed polar flagellum in liquid medium. Cells swarm on solid medium by means of many lateral flagella. Not luminescent and not pigmented. Facultative anaerobe. Chemoorganotroph capable of fermentative and respiratory metabolism. Positive for catalase, oxidase, chitinase, and nitrate reduction to nitrite. Susceptible to vibriostatic agent 0/129. Growth occurs at 20 to 45°C but not at 4°C. No growth occurs without seawater base. Does not accumulate polyhydroxybutyrate. The G+C content is 49.6 mol%. Isolated from the dorsal integument of a Pompeii worm tube from a deep-sea hydrothermal vent on a rift system of the East Pacific Rise. Type strain HE800 has been deposited in the Collection Nationale de Culture de Microorganismes (Institut Pasteur, Paris, France) as strain CNCM I-1629.

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