APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1985, p. 83–90
0099-2240/85/010083-08$02.00/0

Vol. 49, No. 1

Characterization of a Marine Bacterium Associated with *Crassostrea virginica* (the Eastern Oyster)

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Received 15 May 1984/Accepted 2 October 1984

A gram-negative bacterium found to be closely associated with oysters has been isolated and characterized. The organism, designated LST, has a generation time of 106 min in Marine broth under optimal growth conditions at 25°C. During the decline phase of growth, it exhibits a morphological transition from a motile rod (ca. 1 μm in length) to an elongated, 3- to 40-μm, nonmotile, tightly coiled helix. LST synthesizes and releases a pigment in the stationary and decline phases of growth. Identified as melanin on the basis of chemical properties and UV absorbance maxima, the pigment comprises polymers of heterogeneous molecular weights, ranging from 12,000 to 120,000. The guanosine-plus-cytosine content of the LST DNA is 46%, and results of phenetic analysis and DNA-DNA hybridization indicate that this bacterium represents a new species. LST adheres to a variety of surfaces, including glass, plastics, and oyster shell, and has been shown to promote the settlement of oyster larvae.

Microbial films on marine surfaces are important for subsequent attachment and development of many invertebrate larvae (17, 18, 20, 37, 38). In the initial phase of periphytic succession, the surface is coated by organic matter (32). Bacteria attach, grow, and form microcolonies within several hours (15, 19, 25). Subsequently, diatoms, fungi, protozoans, microalgae, and other microorganisms attach to form a slime layer (29, 48). Such microbial colonization normally precedes the final stage of succession, during which macroorganisms, viz., invertebrates, attach and grow on the surface (16, 17).

Specific bacterial inducers of invertebrate attachment have been identified (13). The factor that triggers settlement and metamorphosis of *Lytechinus pictus* has a molecular weight of less than 5,000 (11). Planulae larvae of another invertebrate, *Hydractinia*, metamorphose in response to a specific molecule synthesized at the end of the exponential phase of growth of an unidentified, gram-negative marine bacterium (35). Lectins synthesized by *Pseudomonas marina* mediate settlement and metamorphosis of *Janua* (*Dexiospira*) *brasiliensis* Grube (30). Yet another strain of a marine pseudomonad has been shown to attract oyster larvae (56).

L-β-3,4-Dihydroxyphenylalanine (L-DOPA), a melanin precursor, has been detected in marine surface films and cements (53, 54). Melanin and melanin precursors are synthesized by a variety of fungi (9, 23, 41), bacteria (1–4, 40, 43, 47, 52), and marine invertebrates (24, 49, 53, 54) via a variation of the Mason-Raper pathway (39, 51), involving the action of a tyrosinase (31), oxidase (41), or catecholase (52).

We have repeatedly isolated a pigment-producing marine bacterium, designated LST, from oysters and oyster habitats and have shown it to be involved in the formation of pioneer films. In this report we characterize LST and describe its role in the settlement and metamorphosis of larvae of *Crassostrea virginica* (the Eastern Oyster).

MATERIALS AND METHODS

Organism and culture conditions. The bacterium, designated LST, was originally isolated from oysters and holding tanks for oyster spat at the Mariculture Unit at Lewes, Del. It was purified and maintained on Marine agar 2216 (57). Conditions of growth and storage have been previously described (27), except that the temperature of propagation was 25°C. For maximum capsule production, LST was grown on brain heart infusion broth amended with 3% (wt/vol) NaCl.

Morphology. Morphological transitions of LST were directly observed on Marine agar by a modification (7) of a technique described by Casida (12). Flagella stains were prepared by the procedures of Mayfield and Innis (36).

LST, grown in synthetic medium, was prepared for scanning electron microscopy by a previously reported procedure (5) modified as follows. Centrifugation was at 2,500 × *g* for 10 min, and the washing buffer was phosphate-buffered saline (PBS) (27). Cells were fixed with glutaraldehyde (Polysciences, Inc., Warrington, Pa.) for 12 h at 4°C. After fixation, cells were dehydrated in a seven-step series in which 5 ml of EtOH in sequential concentrations of 30, 50, 70, 90, and 3 × 100% were slowly passed through 0.2-μm (pore size) membrane filters (13 mm in diameter; Nuclepore Corp., Pleasanton, Calif.). The filters were critical point dried, and to reduce charging of the specimen, small drops of silver paint were placed on four corners of the stub connecting the filter surface to the stub metal. The stubs were coated with Ag-Pd metal alloy in a sputter coater and stored in a desiccated environment.

Transmission micrographs and thin-sectioned LST were obtained by the method of Cole and Popkin (14).

Phenetic and molecular genetic analyses. Unless otherwise specified, routine tests were done as described by Smibert and Krieg (50). Dehydrated media (Difco Laboratories, Detroit, Mich.) were prepared as prescribed by the manufacturer, and sodium chloride was added to a final concentration of 3.5%. Unless otherwise noted, the pH was always adjusted to 7.4.

DNA was isolated by a modification of the method of Marmur and Doty (34). Bacteria were harvested from 2-liter cultures (6,500 × *g*, 15 min, 5°C), washed twice in TES buffer (0.1 M NaCl, 0.05 M Tris base, 0.05 M EDTA, pH 8.1) resuspended in TES buffer, and incubated with 1 mg of lysozyme (Sigma Chemical Co., St. Louis, Mo.) and 50 g of pronase (Calbiochem-Behring Corp., La Jolla, Calif.) per ml for 30 min at room temperature. Cells were lysed with 6 mg

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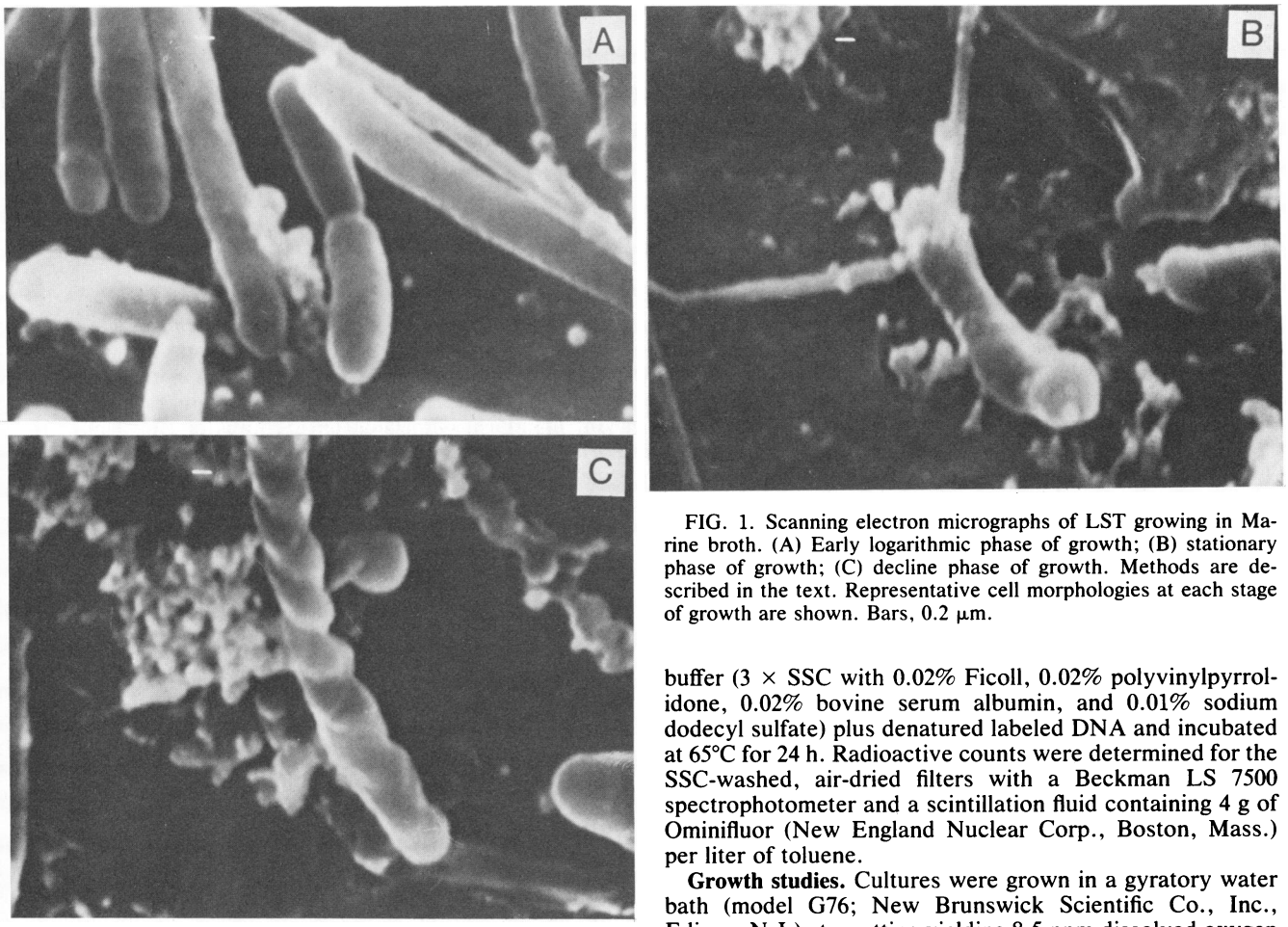

FIG. 1. Scanning electron micrographs of LST growing in Marine broth. (A) Early logarithmic phase of growth; (B) stationary phase of growth; (C) decline phase of growth. Methods are described in the text. Representative cell morphologies at each stage of growth are shown. Bars, 0.2 μm.

of sodium dodecyl sulfate per ml. The lysate was deproteinized with phenol-chloroform-isoamyl alcohol, and the RNA was digested with pancreatic RNase (Sigma). The phenol extraction was repeated and followed by three to five chloroform-isoamyl alcohol extractions and a final precipitation (while spooling) by a 0.1 volume of 2.2 M sodium acetate–0.01 M EDTA (pH 7.2) and a 0.6 volume of isopropyl alcohol (−20°C). The purity of the DNA was determined spectrophotometrically with values of 2.0 for the OD ratios 260/230 nm and 1.75 to 1.85 for the ratio 260/280 nm. The mean guanine-plus-cytosine content of DNA was determined by the thermal melting point method (33) with a Gilford 2400-S spectrophotometer. Control DNA was prepared from *Escherichia coli* 11775^T.

For DNA-DNA hybridization analysis, unlabeled base denatured DNA was gravity-filtered through nitrocellulose filters (25 mm in diameter, 0.45 μm pore size, type BA 85; Schleicher & Schuell, Dassel, West Germany). The amount of filter-bound DNA was estimated by spectrophotometric determination of DNA in the filtrate (26). The filters were washed with 10 ml of 3 × SSC (1 × SSC = 0.15 M NaCl plus 0.015 M sodium citrate), dried overnight at 20°C, and vacuum-dried for 2 h at 80°C and at a pressure of 15 lb/in^2.

In vitro radioactive labeling of DNA was achieved by the nick-translation method of Rigby et al. (44). Alkaline-denatured, labeled DNA was hybridized with nitrocellulose-fixed, unlabeled DNA in a system in which filters were placed in preheated (65°C), stoppered vials containing PM buffer (3 × SSC with 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, and 0.01% sodium dodecyl sulfate) plus denatured labeled DNA and incubated at 65°C for 24 h. Radioactive counts were determined for the SSC-washed, air-dried filters with a Beckman LS 7500 spectrophotometer and a scintillation fluid containing 4 g of Ominifluor (New England Nuclear Corp., Boston, Mass.) per liter of toluene.

Growth studies. Cultures were grown in a gyratory water bath (model G76; New Brunswick Scientific Co., Inc., Edison, N.J.) at a setting yielding 8.5 ppm dissolved oxygen at 25°C. The media employed were Marine broth (2216; Difco) and aspartic acid-glutamic acid-methionine-serine synthetic medium (27), with a 3% final concentration of NaCl and minimal synthetic medium containing only aspartic acid and glutamic acid as carbon sources (AG medium). Amino acid combinations were tested at concentrations of 125 mM for each amino acid. Growth was measured with a Klett-Summerson colorimeter fitted with a green filter and by direct count or plate count or both (27).

Mutagenesis. To test the hypothesis that LST pigment or its precursor, viz., L-DOPA, can attract oyster larvae, pigmentless or pigment-poor variants were required as controls. Consequently, LST was mutagenized with ethylmethane sulfonate (Sigma) by a procedure used previously (B. A. McCardell, Ph.D. thesis, University of Maryland, College Park, 1979). Logarithmically growing cultures of LST were suspended in 0.066 M PBS to an approximate concentration of 2 × 10^9 cells per ml. Ethylmethane sulfonate was added to 1-ml samples of culture to yield final concentrations ranging from 10 to 30 g/ml (5-μg intervals). These suspensions were incubated for 1.0 or 1.5 h, diluted 1:10 in PBS, centrifuged, washed with 5 ml of PBS, and resuspended in 3 ml of PBS. Two milliliters of the final suspension was inoculated into AG broth and incubated for 2 to 5 days. After this adaptation period, the mutagenized culture was spread on Marine agar. The remaining 1 ml of treated suspension was used to directly inoculate AG and Marine agars, on which colonies were screened for pigment production.

Alternatively, ICR 191 was tested in a procedure modified slightly from that described above and from that of Roth (45). The reaction mixture consisted of AG minimal medium containing 10% Marine broth, 3×10^8 to 6×10^8 LST per ml, and 10 μg of ICR 191 per ml. Cells were incubated at 30°C in the reaction mixture for 12 h and diluted 1:100 into fresh Marine broth to provide an adaption period of 12 to 72 h. Mutants were screened on Marine and AG agars and on AG medium plus 1 mM tyrosine.

Pigment isolation and characterization. Crude pigment was extracted from broth cultures grown for at least 48 to 72 h, i.e., to the stationary phase of growth in either Marine or AG broth. Spent medium was centrifuged at 2,500 × *g* for 15 min to remove the cells, and the supernatants were dialyzed (8,000-molecular-weight exclusion) against distilled water for 24 h and subjected to gel filtration.

Sephadex G-50, G-75, and G-150 columns (Pharmacia Fine Chemicals, Piscataway, N.J.), in which the dextran beads were swollen in distilled water with 0.02% sodium azide (J. T. Baker Chemical Co., Phillipsburg, N.J.) were calibrated with lysozyme, tripsinogen, egg albumin, bovine albumin, and yeast alcohol dehydrogenase standards (Pharmacia). Running buffer consisted of distilled water with 0.02% sodium azide adjusted to pH 8.5. Void volume was determined with dextran blue 2000, and the fractions were monitored at 280 nm. Pigment fractionation and identification was achieved on a model 328 fraction collector (ISCO, Lincoln, Neb.) with an ISCO type 6 optical unit and an ISCO model UA-5 absorbance-fluorescence monitor.

The optical densities of the Sephadex fractions were analyzed with a model 25 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) in the scan mode (200 through 750 nm). In general, melanin showed a much lower extinction coefficient in the visible range than in the UV range, making dilution of samples necessary for analysis in the range of 200 to 350 nm. The absorption spectra of glutaraldehyde-treated cultures were also determined. By another method, crystallized pigment was extracted, the liquid phase of a culture supernatant was heat evaporated, and the residue was dried.

Pigment solubilities were tested with 0.5 ml of culture supernatant in 2.5 ml of solvent. Solutions were agitated and maintained for at least 30 min, after which they were centrifuged to separate the precipitate. The criteria of Zussman et al. (58) were adopted to describe the solubility of the pigment in solvents. Pigments were designated soluble if they dissolved in the solvent, slightly soluble if the solvent became colored but the pigment did not dissolve, and insoluble if no color was imparted to the solvent. Solvent-pigment combinations were also examined spectrophotometrically.

Shellfish set. In situ studies were carried out at the Mariculture Unit of the University of Delaware, Lewes. Setting tanks for oyster larvae were filled with triple sand-filtered seawater from the Indian River Inlet, Md. Approximately 3000 eyed larvae (250 μM in diameter) were introduced into each 20-gallon (75.7-liter) tank. Light, temperature, and aeration were uniformly maintained. Acid-cleaned, sterilized, sample treated slides (3 by 1 inch; 7.62 by 2.54 cm) were randomly distributed at the base of each of the tanks. Composite results for three such tank experiments are reported here. During the 24-h data collection period, approximately 50% of the larvae set, as determined by the larvae cementing themselves to a glass surface.

The various slide sample treatment groups were prepared for 24 h before immersion in the setting tanks: (i) controls (no treatment or immersion in Marine broth); (ii) prefouled by immersion in the Mariculture Unit holding tank, which contained 10^5 CFU of bacteria per ml; (iii) *Hyphomonas neptunium* coated by immersion in Marine broth containing 5×10^8 *H. neptunium* per ml; (iv) LST coated by immersion in Marine broth containing 5×10^8 LST per ml; (v) culture pigment coated, i.e., immersed in pigment prepared from an LST culture in the late stationary phase of growth in Marine broth at 25°C which was centrifuged at 2,500 × *g* for 10 min and filtered through a 1.2-μm membrane filter (Millipore Corp., Bedford, Mass.) to remove cells (the slides were immersed in cell-free filtrate for 24 h, by which time thin film formed); (vi) coated with UV-killed LST, i.e., immersion in a 1-cm film of 10^5 LST per ml of PBS exposed to UV light at a dose of 800 ergs mm^{-2} (55); (vii) coated with a hypopigment producer (LST-O), i.e., immersed in 7×10^8 LST-O per ml of Marine broth; and (viii) coated with a hyper

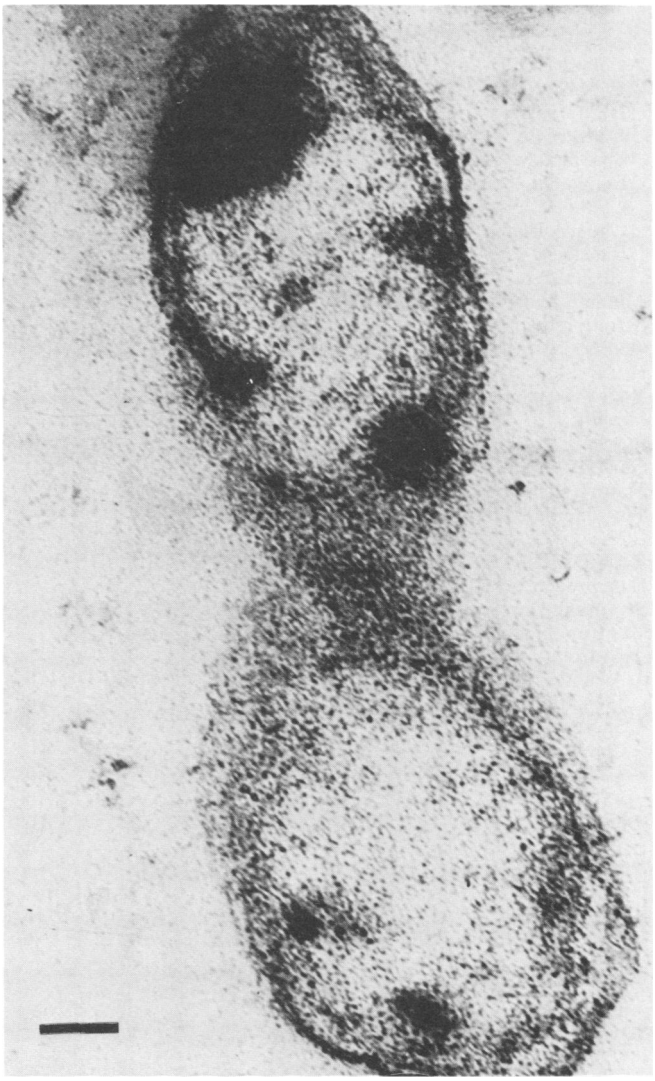

FIG. 2. Transmission electron micrography showing septum and spiral nature of LST in stationary phase of growth in Marine broth. Cells were fixed for 3 h at room temperature in a final concentration of 0.1 M sodium cacodylate buffer plus 1.25% glutaraldehyde, pH 7.4, followed by washing and addition of 1% osmium tetroxide. Cells were stained with 1% (wt/vol in water) uranyl acetate. Bar, 0.2 μm.

TABLE 1. Selected physiological characteristics and tolerances of LST[a]

Test	Results
Growth at:	
NaCl	
0%	−
3%	+
6%	−
8%	−
10%	−
42°C	−
pH 4.5	−
pH 10.0	+
Enzyme activity	
Oxidase	+
Urease	−
Lysine decarboxylase	−
Ornithine decarboxylase	−
Arginine decarboxylase	−
Catalase	+
Amylase	+
Gelatinase	+
Elastase	−
Lecithinase	−
DNase	+
Phosphatase	+
Sulphatase	−
Chitinase	−
Chondroitinase	−
Caseinase	−
Alginase	−
Albumin hydrolysis	−
Nitrate reduction	+
ONPG	−
H · S	−
Voges-Proskauer	−
Motility	+
Indole	+
Gluconate oxidation	−
Thornley argine dihydrolase	−
Tyrosine degradation	−
CCA	−
Hemolysis	−
Luminescence	−
Swarming	−
Gas from glucose	−
Acid from:	
Arbutin	Alk
Trehalose	Alk
Sucrose	Alk
Cellobiose	Alk
Mannose	Alk
Inositol	Alk
Mannitol	Alk
Sorbitol	Alk
Salicin	Alk
Arabinose	Alk
Hydrolysis of:	
Tween 20	+
Tween 40	+
Tween 60	+
Tween 80	+
Sensitivity to:	
Ox bile	
0.5%	R
1.0%	R
0/129	
10 g	R
50 g	R
150 g	R
Neutral red	
0.002%	R
0.004%	R
Brilliant green	
0.001%	S
0.002%	S
Teepol	
0.4%	R
0.6%	S
0.8%	S
SLS	
0.001%	R
0.005%	R
0.01%	R
Pyronin Y	
0.002%	S
0.004%	S
Crystal violet	
0.002%	S
0.001%	S
Methylene blue	
0.001%	S
Methylene violet	
0.0002%	S
0.0004%	S
Potassium tellurite	
0.0005%	R

[a] Abbreviations: ONPG, *o*-nitrophenyl-β-D-galactopyranoside; CCA, chicken cell agglutination; Alk, alkaline; R, resistant; S, sensitive; 0/129, 2,4-diamino-6,7-diisopropylpteridine phosphate; SLS, sodium lauryl sulfate.

pigment producer (LST-H), i.e., immersed in a culture of 10^8 LST-H per ml of Marine broth.

After immersion for 24 h in the setting tanks, all of the treated slides and plates were removed, and spat attached to the slides were counted with a stereoscope (10×; Bausch & Lomb, Inc., Rochester, N.Y.). Coating of and attachment to the slides was verified by scanning electron microscopy.

RESULTS

Morphology and morphogenesis. Motile cells of LST in the logarithmic phase of growth were gram-negative, polarly flagellated, capsulated, and 1 to 2 μm in length and 0.5 μm in width (Fig. 1A). Cells of the stationary and decline phases of growth adhered to surfaces, including glass, copper, Teflon, and polycarbonate. During the late stationary and decline phases of growth, the cells of LST became elongated and subsequently helical (Fig. 1). This transition occurred in laboratory culture and when LST was inoculated in situ into Mariculture Unit tanks and was also documented for isolated cells imbedded in agar. The helical indentations were found to be septa (Fig. 2) of cells that did not complete division and consequently did not separate as individual cells to colonies. However, these cells remained viable, producing the characteristic brown-black pigment.

Phenetic and molecular genetic characteristics. LST was aerobic, did not utilize the carbohydrates tested, and could not be identified on the basis of the pattern of biochemical reactions and physiological tolerances recorded (Table 1). The moles percent guanosine-plus-cytosine content was 46%. LST did not share significant DNA homology with any of the species examined in this study, including *Vibrio* (12 species), *Aeromonas*, *Escherichia*, and *Pseudomonas* spp. (Table 2).

Growth in complex and synthetic media. LST demonstrated a generation time of 106 min in Marine broth and 414 min in AG synthetic medium (Fig. 3). Of the amino acids tested, aspartic acid, glutamic acid, and serine supported a

relatively rapid rate of growth and the highest titers in minimal media (Table 3).

LST produced less pigment in a synthetic medium than in the complex media. Addition of 1.0 mM tyrosine and 1.0 μm L-DOPA did not stimulate pigment production in AG medium.

Mutagenesis with ethylmethane sulfonate. A 1.0-h exposure to ethylmethane sulfonate reduced the viability of LST by two- to threefold, as determined by spread plate counts on Marine agar. No colonies formed on AG agar when LST was plated directly after mutagenesis. Mutagenized suspensions, after suspension for 2 to 5 days in AG broth, were streaked on Marine and AG agars, with counts on Marine agar ranging from 1.3×10^9 to 6.6×10^9 and approximately 2 logs lower on AG agar, i.e., 1.4×10^7 to 6.5×10^7.

Cells treated with ethylmethane sulfonate (all concentrations) for 1.5 h did not form colonies, regardless of whether they were tested by direct plating (Marine or AG agars) or after a holding period in AG broth. No pigmentless mutants were detected on either undefined or minimal media among the approximately 5,000 colonies screened.

ICR 191. A total of 24,803 colonies were screened, of which 39 colonies showed variation in pigmentation. Seven were not pigmented (hypo) and two were darker (hyper). Two colonies were light tan, 27 were various shades of red, and one was yellow. An LST-O and an LST-H were selected for experiments testing attraction of *C. virginica* larvae.

The seven hypo "mutations" proved to be unstable, reverting, on the average, about 1 in 5 to 50 generations. LST-O was the most stable of the group. Viability on AG agar was 83% of that on Marine agar, suggesting that a considerable fraction was the result of auxotrophic mutation. For AG agar plus 1 mM tyrosine, the differential was even greater, as only 66% of the count on Marine agar titers was achieved.

Characterization of the pigment. When Marine broth cultures of LST reached the stationary phase of growth, a soluble pigment, ranging from reddish brown to dark brown, was produced. The pigment was retained in dialysis (pore size, 8,000 molecular weight) and precipitated in water when the pH was adjusted to 3.0. It was soluble in water at pH 10.0, but only slightly soluble in ethanol and methanol and insoluble in acetone, chloroform, cyclohexane, and ethylene dichloride.

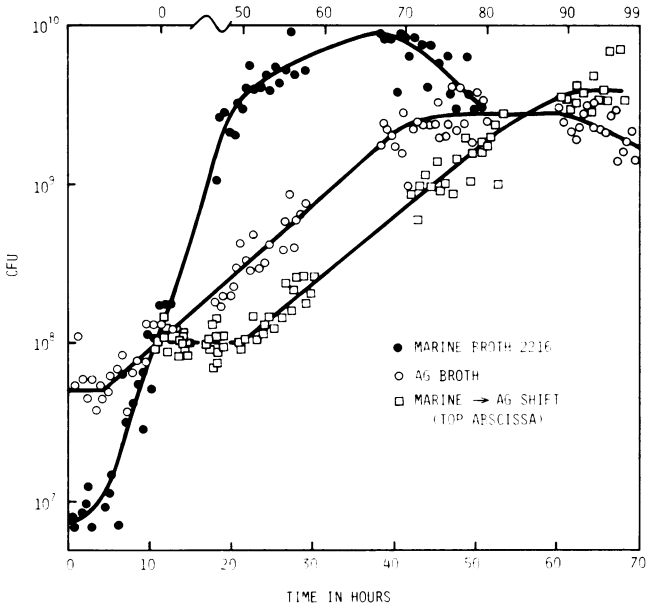

FIG. 3. Growth of LST in synthetic and nonsynthetic media. The response of LST to shift-down conditions, consisting of an extended lag in cell division (top abcissa) is shown in an experiment in which LST, in the logarithmic phase in Marine broth, was reinoculated into AG medium.

The chemical properties and absorbance maxima of the LST pigment were consistent with those reported for melanins (Table 4). The major pigment fraction, resolved with Sephadex G-150 columns, corresponded to a molecular weight of 120,000. Minor peaks were noted at molecular weights of 61,000 and 12,000.

Attraction of *C. virginica*. Slides exposed to oyster waters (prefouled) developed a film of autochthonous microorganisms within 24 h. In other respective samples, confluent films of *H. neptunium*, LST, LST-O, and LST-H were observed by phase-contrast microscopy. UV-treated LST adhered to

TABLE 2. Results of DNA-DNA hybridization of LST DNA with DNA prepared from selected species

Strain	% DNA homology with reference DNA of LST[a]
LST	100.0
Vibrio anguillarum 19264[b]	16.3
V. harveyi 14126	6.9
V. fischeri 7744	7.6
V. cholerae 14035	6.2
V. algosus 14390	8.7
V. parahaemolyticus 17802	3.2
V. albensis 14547	5.2
V. alginolyticus 19949	4.1
V. splendidus B-379	9.9
V. marinovulga 14749	7.0
V. furnissii VL 2386[c]	16.1
V. cholerae 688 non-O1	7.4
Aeromonus hydrophila 14048	5.3
Escherichia coli WP-2	4.5
Pseudomonas aeruginosa	15.4

[a] Percent homology with reference DNA was normalized to 100%.
[b] ^{32}P-labeled *V. anguillarum* 19264 hybridized with unlabeled LST DNA resulted in 12.8% hybridization.
[c] Formerly *V. fluvialis* biotype II.

TABLE 3. Generation times calculated for LST in various synthetic media

Medium[a]	Generation time (h)	*n*	SD[b]	Final titer (cells per ml)
Asp, Glu, Ser, Met	7	2	2	4×10^9
Asp, Glu, Met	7	3	1	3×10^9
Asp, Met, Ser	8	2	0	—[c]
Glu, Met, Ser	8	3	3	3×10^9
Asp, Ser	7	2	0	1×10^9
Asp, Glu	7	5	3	4×10^9
Ser, Glu	10	2	0	—
Glu, Met	11	3	2	6×10^8
Asp, Met	13	3	7	1×10^9
Ser	8	2	—	—

[a] Salts base (27) supplemented as shown. Asp, Aspartic acid; Glu, glutamic acid; Ser, serine; Met, methionine.
[b] *n* weighting.
[c] —, No data.

TABLE 4. Properties of LST pigment compared with those of melanin produced by other microorganisms

Organism	Color	Solubility in[a]:		Blackberg-Wanger test	Precipitation from:		Reduction (glutathione)	Reoxidation	Absorption peaks[b]	Water bleaching	Mol wt (10^3)
		Water (pH 7)	0.1 N NaOH		$FeCl_3$	Acid					
Aeromonas liquefaciens[c]	Brown-black	I	S	PPT[d]	PPT	PPT	+[e]	+	Diffuse	ND[f]	ND
Vibrio cholerae[g]	Brown	ND	S	PPT	PPT	PPT	+	ND	345, 480	+	ND
Aspergillus nidulans[h]	Black	ND	S	ND	PPT	PPT	+	+	480, 535	+	29–2,000
LST[i]	Brown	SS	S	PPT	PPT	PPT	+	+	223, 264, 407	+	12–120

[a] I, Insoluble; S, soluble; SS, slightly soluble.
[b] Synthetic melanin (Sigma) absorption peaks: 223, 269.
[c] Aurstad and Dahle (3).
[d] PPT, Precipitated.
[e] +, Positive.
[f] ND, No data.
[g] Ivins and Holmes (28).
[h] Swan (51).
[i] Present study.

glass slides, even though they were not viable. LST-H produced more pigment, turning the culture medium black within 24 h, whereas LST-O produced significantly less pigment than LST in culture, with the medium remaining clear.

Prefouled, LST-coated, and pigment-coated samples attracted more larvae than the controls as measured by counting the number of attached and metamorphosing oysters (Table 5). Furthermore, LST-H attracted more larvae than prefouled or LST-coated samples. The nonpigmented bacteria neither attracted nor repelled larvae.

DISCUSSION

LST was not identified to either genus or species by conventional criteria (10). It is differentiated from *Pseudomonas* by its helical stage of growth and overall DNA base composition (guanosine plus cytosine) of 46%. It is resistant to 2,4-diamino-6,7-diisopropylpteridine phosphate (0/129) and appears to be a strict aerobe. These characteristics rule out the genera *Vibrio*, *Aeromonas*, and *Flavobacterium*. It is motile and does not produce acid from sugars, ruling out the genera *Moraxella* and *Acinetobacter*. Although LST is morphologically similar to the genera *Anaerobiospirillum* (21) and *Seliberia* (46), it is metabolically different. On the basis of these and other considerations, we conclude that LST represents a new species of marine bacteria.

TABLE 5. Attraction of *C. virginica* larvae to surfaces precoated with LST

Type of treatment[a]	No. of samples tested[b]	Mean no. of spat at 24 h[c]	95% Confidence interval[d]
Control	16	3.8 ± 1	1< >7
Prefouled[e]	27	24.8 ± 7	12< >38[f]
Hyphomonas neptunium[g]	14	5.0 ± 2	2< >8
LST[g]	16	17.5 ± 3	12< >23*
Pigment exopolymer from LST	10	30.0 ± 14	2< >58
UV-killed LST[g]	16	1.8 ± 1	0< >4
LST-O[g]	8	2.0 ± 1	1< >4
LST-H[g]	8	29.3 ± 8	13< >46*
Control[h]	5	2.4 ± 1	2< >3
LST	14	9.7 ± 1	7< >12*

[a] All slides were tested at the oyster mariculture unit in Lewes, Del., with filtered estuarine water (see text).
[b] Chemically cleaned and sterilized microscope slides (see text).
[c] Larval settlement or attachment per slide X 10. Standard error is given.
[d] Significant deviation from control.
[e] Slides were placed in the Mariculture Unit holding tank (10^5 viable bacteria per ml) before immersion in the oyster larvae tank.
[f] Slides were placed in Marine medium for 24 h before immersion in oyster tank.
[g] Slides were coated before immersion in the oyster larvae tank.
[h] Another set of slides tested simultaneously but independently of the above group and included to allow estimate of the experimental variability.

The LST pigment is believed to be a melanin, which refers to a class of heteropolymers, the structures and molecular weights of which are determined by availability of monomeric precursors (51) and reaction conditions (49). Cognizant of the obvious difficulties in identification and characterization that these parameters impose, we conclude that the pigment of LST shares physical and chemical properties of pigments identified as melanins (6, 28, 58).

Although LST may synthesize melanin during early phases of growth, it was detected only at the later stages, suggesting that melanogenesis in LST may be switched on during the later growth stages. This is plausible when it is considered that catalytic quantities of L-DOPA are required to prime the initial reaction of melanogenesis, which is the conversion of tyrosine to L-DOPA (32), and in *Trychophyta rubrum*, the production of melanin in late growth is attributed to a lag caused by synthesis of catalytic amounts of L-DOPA by an alternate pathway (58).

LST pigmentless mutants are acquired at a very low frequency, suggesting that a single enzyme (small target) catalyzes pigmentation, that the enzyme(s) may have isoenzymes (genetic redundancy), or that viability and pigment synthesis may be linked. Observed mutation rates of 0.03% are consistent with observations on melanogenesis (51), which requires a single enzyme, a phenoloxidase or tyrosinase (31). Under these circumstances, it is also likely that LST-O variants would have additional lesions, some of them being lethal or conditionally lethal, as suggested by reduced viability of mutagenized cells on synthetic medium compared with counts obtained on the Marine medium. Organisms as diverse as the genera *Agaricus* (31) and *Vibrio* (42) synthesize isoenzymes that catalyze melanin biosynthesis. Genetic redundancy could also be invoked to explain the low LST pigment mutation rates and high LST-O reversion rates.

Pigment-producing strains of LST attracted oyster larvae and LST-O did not. Surfaces coated with UV-irradiated LST attracted slightly fewer larvae than the controls (Table 5), possibly because the melanin was photooxidatively degraded (8).

We hypothesize the following mechanism for the interaction of LST and oyster larvae. In situ, LST coheres to surfaces by synthesizing an acetic polysaccharide exopolymer (22). LST also produces L-DOPA, other melanin precursors, and melanin itself. These compounds enter the water column. L-DOPA and its polymers act to attract *C. virginica* larvae, which then settle on films of LST formed on the surface. The larvae metamorphose into adults, and the bacteria flourish in the periphytic community. Clearly, LST and the oyster larvae provide an interesting macro- and microbiotic interaction in the estuarine ecosystem.

ACKNOWLEDGMENTS

This work was supported by grant NOAA-NA810AA-D-00040.

We thank H. Hada for assistance with the molecular genetic analysis, P. West and P. Brayton for the phenotypic characterization, and P. Sorando for the electron microscopy.

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