

Additional Evidence that Juvenile Oyster Disease Is Caused by a Member of the *Roseobacter* Group and Colonization of Nonaffected Animals by *Stappia stellulata*-Like Strains†

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Juvenile oyster disease (JOD) causes significant annual mortalities of hatchery-produced Eastern oysters, *Crassostrea virginica*, cultured in the Northeast. We have reported that a novel species of the α -proteobacteria *Roseobacter* group (designated CVSP) was numerically dominant in JOD-affected animals sampled during the 1997 epizootic on the Damariscotta River, Maine. In this study we report the isolation of CVSP bacteria from JOD-affected oysters during three separate epizootics in 1998. These bacteria were not detected in nonaffected oysters at the enzootic site, nor in animals raised at a JOD-free site. Animals raised at the JOD enzootic site that were unaffected by JOD were stably and persistently colonized by *Stappia stellulata*-like strains. These isolates (designated M1) inhibited the growth of CVSP bacteria in a disk-diffusion assay and thus may have prevented colonization of these animals by CVSP bacteria in situ. Laboratory-maintained *C. virginica* injected with CVSP bacteria experienced statistically significant elevated mortalities compared to controls, and CVSP bacteria were recovered from these animals during the mortality events. Together, these results provide additional evidence that CVSP bacteria are the etiological agent of JOD. Further, there are no other descriptions of specific marine α -proteobacteria that have been successfully cultivated from a defined animal host. Thus, this system presents an opportunity to investigate both bacterial and host factors involved in the establishment of such associations and the role of the invertebrate host in the ecology of these marine α -proteobacteria.

Juvenile oyster disease (JOD) refers to a syndrome of unknown origin that results in seasonal mortalities of hatchery-produced juvenile *Crassostrea virginica* raised in the northeastern United States (9, 11, 17). While the severity of the annual epizootics has been variable since they first appeared in the late 1980s, mortalities in some years have exceeded 90% of total production at JOD enzootic sites in Maine, Massachusetts, and New York (9, 11, 39, 40). Typical external signs of JOD include a reduction in growth rate, the development of fragile and uneven shell margins, and cupping of the left valve. Internally, signs of JOD usually include mantle retraction and lesions and proteinaceous deposits (conchiolin) on the inner shell surfaces (9, 11, 17). Such signs usually appear within 4 to 6 weeks after deployment of seed at enzootic sites, and they immediately precede mortality events during which losses may exceed 50% of total production in a single week (3, 7).

Several hypotheses concerning the etiology of JOD have been explored, and evidence indicates that the disease is infectious rather than due to nutritional and/or abiotic factors (9). Although no obvious agent has been identified in histological samples (9, 15, 39, 40), the pathology and correlating environmental factors (e.g., warm temperatures and moderate salinity) have led to investigations of a possible bacterial (9, 18, 31) or protistan etiology (14, 33, 44). *Vibrio* spp., in particular, have been investigated (18, 31) because of the similarities

between the signs of JOD and those of brown ring disease of manila clams, caused by *Vibrio tapetis* (8, 16, 36, 37). JOD has been reproduced in animals both by injection with homogenates from affected animals (38) and by proximity to JOD-affected animals in experimental aquaria (34). These experiments further support the involvement of an infectious agent such as a bacterium or protozoan.

In 1997, for the purpose of further elucidating the etiology of JOD, we tested the effect of two antibacterial antibiotics (norfloxacin and sulfadimethoxine-ormetoprim) on JOD mortalities of cultured juvenile *C. virginica* (7). Repeated immersion in either antibacterial solution resulted in a delay in the onset of JOD mortalities in treated animals, reduced weekly mortality rates, and a statistically significant reduction in cumulative mortalities compared to that of controls. Bacteriological analyses revealed that healthy oysters generally harbored low numbers of phenotypically diverse bacteria. In contrast, JOD-affected animals were found to be extensively colonized by a previously undescribed species of the marine α -proteobacteria, *Roseobacter* group. A role for this bacterium (designated CVSP) as either the primary etiological agent or an efficient colonizer of JOD-affected animals was further supported by the fact that they either were not recovered from or were present at very low levels (<1% of total CFU) in animals that survived the JOD epizootic.

To date, the CVSP α -proteobacterium represents the only organism that has been definitively correlated with any JOD epizootic. The objectives of this study were to determine (i) if the presence of CVSP bacteria was a consistent feature of subsequent epizootics, (ii) if CVSP bacteria were present in oysters raised at a site where JOD is not enzootic, and (iii) if JOD could be reproduced under laboratory conditions by exposure of juvenile *C. virginica* to a CVSP isolate.

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† This is Maine Agriculture and Forestry Experiment Station Publication number 2434.

MATERIALS AND METHODS

Bacterial cultures. Bacteria were isolated and maintained on a seawater-based complex medium (SWT) (6). CVSP strain CV919-312 was isolated from a JOD-affected animal during the 1997 epizootic (7). All other bacterial isolates originated from this study.

Oyster deployments. Approximately 6,000 juvenile *C. virginica* (2.5-mm shell height) were obtained from a local hatchery on 26 June 1998. Ten groups of 500 animals each were placed into mesh bags constructed of fiberglass window screen at a density of 5,000 m⁻². Each bag was then attached to a rectangular frame constructed of 0.5-in. polyvinyl chloride pipe and was placed into buoyant polyethylene trays. Three trays were deployed at a commercial shellfish nursery on the Damariscotta River, Maine (44°1'N, 69°32'W), and the remaining seven trays were deployed at a commercial nursery on Maquoit Bay, Maine (43°50'N, 70°03'W). Animals in four of the trays at Maquoit Bay were removed after 1 month and used for bacterial challenge experiments (see below). A deployment of juvenile oysters from another local hatchery was performed on 12 August 1998. A total of 2,000 animals were distributed into each of four bags as described above. Three of these were deployed in trays at the Damariscotta River site and were monitored and sampled as described below. The fourth bag was deployed at Maquoit Bay for the purpose of comparing the bacteriology of these animals with those raised on the Damariscotta.

Sampling protocols. Animals were examined weekly for JOD signs and mortality, and sampling for bacteriological analyses was conducted at least biweekly. A minimum of three animals were removed from each tray, sealed in waterproof baggies, and packed in ice. Animals from any given tray were processed together, and unless otherwise noted, the entire meats were sampled. After being rinsed with filter-sterilized seawater, the hinge ligament and adductor muscle of each oyster were severed using a flame-sterilized scalpel and forceps. In every instance, we verified that animals included in the bacteriological analyses were alive. This was done by observing the heartbeat under the dissecting microscope and testing for contraction of the tissue and mantle in response to pressure. The tissues were then combined in a preweighed microcentrifuge tube containing 0.35 ml of sterile 70% seawater (natural seawater diluted to 70% in double-distilled water [ddH₂O]) and were homogenized using a sterile pellet pestle. After being reweighed, the homogenates were serially diluted in sterile 70% seawater, and 20 µl of each dilution was spread onto SWT agar.

After 12 August, animals originating from the first deployment were sufficiently large (15 to 20 mm) that sampling of entire meats was discontinued, and samples were instead collected using a sterile swab. After collecting the sample from the external tissues and inner shell surfaces of an aseptically dissected animal, the swab was vortexed in 1 ml of sterile 70% seawater. The suspension was then diluted and plated as described above. This method was also used to analyze JOD-affected animals originating from two different epizootics on Damariscotta River commercial leases in the fall of 1998.

All plates were incubated for 7 days at 23°C before enumeration of CFU to estimate total recoverable bacteria. The culture plates were also inspected visually for the presence of numerically dominant colony morphologies. Dominant types were enumerated and the relative abundance was calculated as a percentage of total recoverable CFU. Selected representatives of these groups were transferred to fresh media for further characterization.

Exposure of juvenile oysters to CVSP bacteria by immersion. Four trays of animals (approximately 2,000 animals total) were removed from Maquoit Bay on 23 July 1998. The animals (mean shell height, 8.4 mm) were divided into 9 groups of 200 animals each and placed into each of 9 randomly assigned 5.5-gal aquaria containing aerated, UV-sterilized artificial seawater (Crystal Sea Marinemix; Marine Enterprises Intl., Baltimore, Md.) at a salinity of 31 ppt. Oysters were maintained on algal spat formula (Innovative Aquaculture Ltd., New Brunswick, Canada), and standard aquarium heaters were used to maintain water temperatures between 19 and 21°C. After a 1-week acclimation period, a culture of CVSP strain CV919-312 (grown to an optical density at 600 nm [OD₆₀₀] of 1.0) was added to three aquaria at a final concentration of 10⁶ CFU/ml. Another three aquaria each received a diluted inoculum of CV919-312 (10³ CFU/ml final concentration), and the remaining three aquaria each received an equivalent volume (40 ml) of SWT medium only. Thereafter, CVSP bacteria (at either 10³ or 10⁶ CFU/ml, final concentration) or medium alone was added to the assigned aquarium three times weekly for 5 months. Animals were fed daily and monitored weekly for signs of JOD. One month after the start of the experiment, two animals from a tank receiving the 10⁶ CFU/ml dose of CV919-312 and two animals from a control tank were removed for bacteriological analysis. Each animal was processed individually by removing the tissues, homogenizing, diluting in 70% filter-sterilized seawater, and plating onto agar media as described above.

Exposure of juvenile oysters to CVSP bacteria by injection. Juvenile oysters (*C. virginica*) (mean shell height, 36 mm) were obtained from Middle Peninsula Aquaculture (North, Va.) in late September 1999. They were acclimated for 2 weeks in laboratory aquaria containing aerated artificial seawater at ambient room temperature (20 to 22°C), and were fed a maintenance diet of algal paste. An inoculum of CVSP strain CV919-312 was prepared by diluting an overnight broth culture 1:100 in fresh SWT broth and incubating with shaking (250 rpm) at 25°C until the OD₆₀₀ was 1.5 (approximately 7 h). The animals were then randomly assigned to either the treatment or control group and were notched on

the right valve margin to facilitate delivery of the inoculum. One hundred microliters of the CVSP culture (approximately 3.3 × 10⁷ cells) was injected through a 23-gauge needle on a repeating pipettor into the mantle cavity of each animal in the treatment group (*n* = 121). Each animal in the control group (*n* = 122) was similarly injected through the notch on the valve margin with 100 µl of sterile SWT medium. In all cases, the needle was positioned so that the inoculum was delivered directly into the mantle cavity and not into the soft tissues of the animals. After injection, oysters were kept out of the water for 4 h and then were distributed into six (randomly assigned) aquaria; three held treatment animals and three contained the control animals (*n* = 44 ± 1 per aquarium). The animals were fed every other day and monitored weekly for JOD signs and deaths. During mortality events in treatment aquaria, two animals (one each from the first and the last treatment aquarium to show mortalities) were removed for bacteriological analyses.

Statistical analysis. Percent cumulative mortality was calculated as previously described (7). The data were subjected to the arcsine transformation (49) prior to a standard one-way analysis of variance and Dunnett's post-hoc test (α = 0.05) using the Statmost 3.5 statistical package (Dataxiom Software, Inc., Los Angeles, Calif.).

Phenotypic analyses of bacteria. Determinations of Gram reaction, motility (in a wet mount), catalase activity, oxidase activity, nitrate reductase, and reaction in oxidative-fermentative media were performed according to standard methods (45). The capacity for anaerobic growth was tested using an anaerobic-bag system (Marion Scientific, Kansas City, Mo.). The API 20 NE system (bioMérieux Vitex, Inc., Hazelwood, Mont.) was used to test for arginine dihydrolyase, urease, esculin hydrolysis, gelatinase, β-galactosidase, and assimilation of 12 different substrates. The test strips were prepared according to the instructions of the manufacturer and were incubated at 23°C for 5 days before interpretation. Biolog GN microplates (Biolog Inc., Hayward, Calif.) were also used to test for the ability of strains to utilize 95 different carbon sources. The 96-well plates were inoculated with cells suspended in sterile 1.5% saline at an OD₆₀₀ of 0.2. After incubation for 48 h at 23°C, the plates were read with the Biolog microplate reader.

The ability of selected isolates to inhibit the growth of CVSP strain CV919-312 was tested in a disk-diffusion assay. Cultures of CV919-312 and each test strain were grown in SWT broth at 23°C until the OD₆₀₀ reached 1.0 (approximately 5 h). A sterile cotton swab moistened with the CV919-312 culture was used to inoculate the surface of each 150-mm-diameter SWT agar plate. A sterile filter disk (6-mm diameter) was then placed into the center of each plate and moistened with 15 µl of each test culture. The plates were incubated inverted for 3 days at 23°C before inspection.

Molecular characterization of isolates. Genomic DNA was prepared from bacterial cultures using the Qiagen tissue kit (Qiagen Inc., Valencia, Calif.) and was diluted to 10 µg ml⁻¹ in 10 mM Tris-HCl-1 mM EDTA (pH 8.0). Primers used for amplification of 16S rDNA were previously described (30) and correspond to *Escherichia coli* positions 8 to 27 (primer 27F) and 1492 to 1510 (primer 1492R). Each 50-µl PCR mixture contained 50 ng of genomic DNA, 2 mM MgCl₂, amplification primers (100 nM each), and 200 µM each deoxynucleoside triphosphate in 1× thermophilic DNA polymerase buffer. After an initial 4-min denaturing step (94°C), 1.25 U of *Taq* polymerase (Life Technologies, Inc., Rockville, Md.) was added to each reaction tube, and the tubes were held at 94°C for an additional 2 min. The thermocycler was then set to run 35 cycles under the following conditions: 40 s denaturing (94°C), 30 s annealing (62°C), and 60 s elongation (72°C), followed by an additional 7-min elongation period at the end of the program. PCR products were purified using the QIAquick gel extraction kit (Qiagen), and the purity and concentration of DNA were analyzed by electrophoresis on a 1% agarose gel. Purified products were sequenced at the University of Maine's DNA sequencing facility and were analyzed using Sequence Navigator software (Applied Biosystems, Inc., Foster City, Calif.) and BLAST analysis (1).

Nucleotide sequence accession numbers. The nucleotide sequences of the strains CV812-530 and CV902-700 rDNAs have been submitted to GenBank and assigned accession no. AF246614 and AF246615, respectively.

RESULTS

JOD-associated mortalities at field deployment sites. No animals from either group developed signs of JOD when maintained at the Maquoit Bay site, nor did any animals from the first group develop signs of JOD at the Damariscotta River site. However, JOD signs and mortalities were observed in animals from the second group deployed on the Damariscotta River beginning on 23 September in one of the trays (no. 3). Mortalities were not observed in the other two trays until over a month later. The last sampling date was 29 October, at which time cumulative mortalities were 0.7, 0.1, and 7.1% for trays no. 1, 2, and 3, respectively.

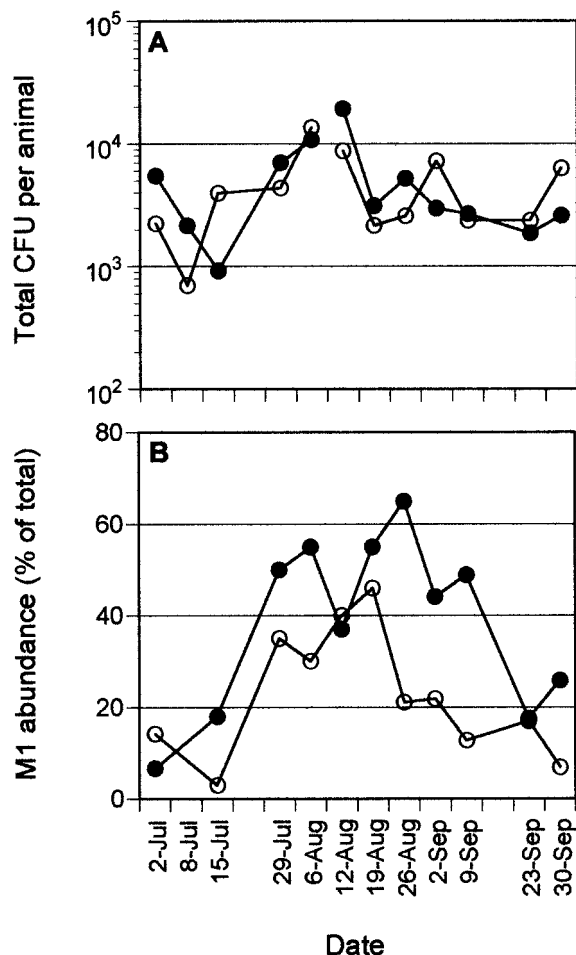


FIG. 1. Results of bacteriological analysis of oysters from the first deployment. (A) Total heterotrophic bacteria recovered from animals (data from 2 July to 8 August acquired from meat samples; data from 12 August to 30 September acquired from swab samples). (B) Estimated abundance of M1 bacteria expressed as a percentage of total CFU. Symbols represent the means of results from three bags. ●, Damariscotta River samples; ○, Maquoit Bay samples.

Bacteriological analysis of oyster groups. Between 10^3 and 10^4 CFU per oyster were recovered from the meats of animals originating from the first group (Fig. 1A). We have noticed that CVSP bacteria are more efficiently recovered from swab samples of animal surfaces, but this is an impractical method for sampling animals with a shell height of less than 15 mm. After 12 August, the animals were sufficiently large (15 to 20 mm) that swab sampling replaced the procedure of sampling of entire meats. The total CFU recovered from these swab suspensions typically ranged from 10^3 to 10^4 CFU per ml (Fig. 1A). Regardless of the sampling protocol, no CVSP-like bacteria were isolated at any time from animals in this group. Instead, another specific type of bacterium was consistently observed to be present at levels of between 10 and 50% of total CFU (Fig. 1B). These colonies (designated M1) had a very mucoid consistency and a distinctive light-brown pigmentation after 3 days of incubation on SWT agar. Seventeen M1 isolates recovered from this first group were stored for subsequent phenotypic characterization. Two of these M1 strains were also subjected to 16S rDNA analysis. These were strain CV729-100, which was isolated on 29 July from an animal held at Maquoit Bay, and strain CV812-530, which was isolated on 12 August

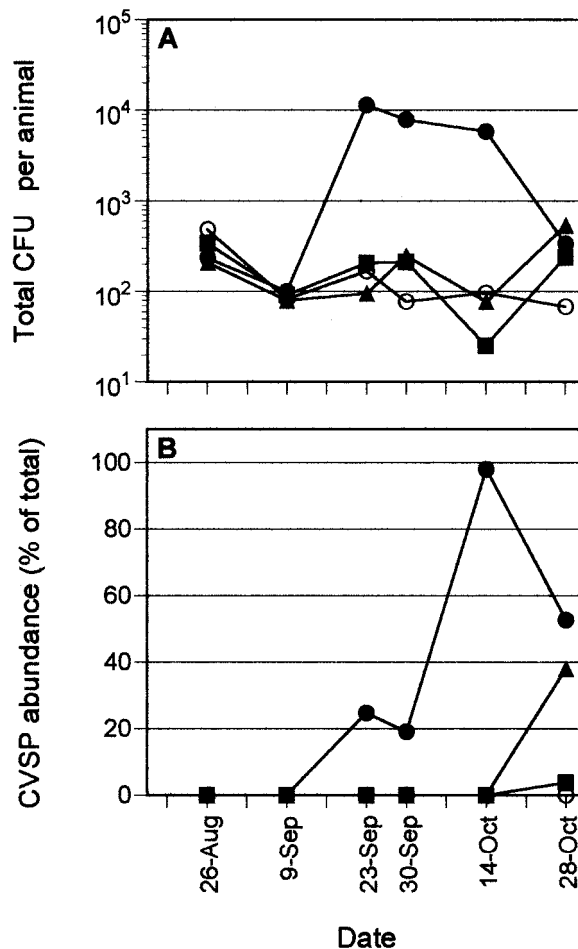


FIG. 2. Results of bacteriological analysis of oysters from the second deployment. (A) Total heterotrophic bacteria recovered from animals. (B) Estimated abundance of CVSP bacteria expressed as a percentage of total CFU recovered. Symbols: Damariscotta River samples, Tray 1 (▲), Tray 2 (■), Bag 3 (●); Maquoit Bay samples, ○.

from an animal deployed on the Damariscotta River. No other specific types of colonies besides the M1 type were consistently isolated from these animals over the course of the study.

Total CFU from animals originating from the second group typically ranged from 10^2 to 10^3 CFU mg (wet weight) of meat⁻¹ (Fig. 2A). However, total CFU recovered from animals removed from tray no. 3 at the Damariscotta River site increased to approximately 10^4 CFU mg⁻¹ during the weeks in which JOD mortalities were observed. Similarly, total CFU in animals from the remaining two trays were elevated on the date when mortalities in those animals were observed. Bacterial colonies morphologically identical to those of CVSP bacteria were recovered from animals exhibiting signs of JOD and coincided with the timing of the mortalities (Fig. 2B). Additionally, when present, the CVSP-type colony was abundant. Typically, CVSP-like CFU comprised at least 25% of total CFU recovered and reached a maximum of 99% of total CFU on one occasion (tray no. 3, on 14 October). Two isolates from JOD-affected animals, CV923-115, isolated on 23 September from an animal in tray no. 3, and CV1028-008, isolated on 28 October from an animal in tray no. 1, were subjected to molecular analysis.

On three dates (2 September, 23 September, and 30 Sep-

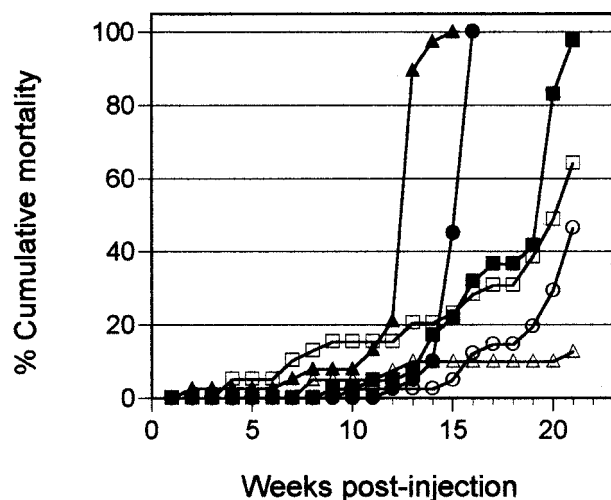


FIG. 3. Cumulative weekly mortalities observed in laboratory-maintained animals that had been injected either with a culture of CVSP bacteria (solid symbols) or with an equivalent amount of medium alone (open symbols). Triangles, circles, and squares indicate aquaria no. 1, 2, and 3 in each group, respectively.

tember), low levels (<5%) of CFU with the M1 colony morphology were recovered from animals in tray no. 1 of the second deployment group. To reflect that these isolates were recovered from the second group, they were designated "M2." Similar low levels of M2-type colonies were isolated from animals in tray no. 2 on 23 September and 30 September, and from animals in tray no. 3 on 23 September only. Two M2 isolates, recovered from the animals in tray no. 1 on 2 September and 23 September and designated CV902-700 and CV923-700, respectively, were subjected to further molecular and phenotypic analysis.

Challenge experiments. When animals in laboratory aquaria were exposed to CVSP bacteria via the addition of a culture of CVSP strain CV919-312 to aquaria water, there was no difference ($P \geq 0.05$) in the mortality rate of exposed oysters compared to that of controls. After 5 months, cumulative mortalities did not exceed 0.5% in any of the treatment or control tanks, nor were any CFU resembling CVSP bacteria recovered from animals sampled 1 month after repeated exposure to the strain. In contrast, all four animals sampled (two each from a treatment and a control aquarium) were extensively colonized by M1 bacteria. The M1 colonies comprised between 10 and 30% of the total CFU recovered from each of these animals. One M1 isolate (CV910-004) was stored and subjected to further phenotypic and molecular characterization.

Juvenile *C. virginica* isolates obtained in 1999 were exposed to CVSP strain CV919-312 via injection of a culture into the mantle cavities of the animals. No effect of the challenge was observed in any of the treatment tanks until 6 weeks postinjection, when these animals exhibited a reduced capacity to clear the daily algal additions. This effect was noted almost simultaneously in all three treatment aquaria. Significant mortalities were not observed until 12 weeks postinjection, and they initially occurred in only one of the treatment aquaria (Fig. 3). While abnormal conchiolin deposition was not observed in the dead oysters, CVSP bacteria (at levels approximating 20% of total CFU) were recovered from animals that were sampled during the mortality events (aquaria no. 1 and 3). The 16S rDNAs from two of these isolates (CV102-1001 and CV211-3001 from aquaria no. 1 and 3, respectively) were

amplified and sequenced (see below). By 15 weeks, all animals in two of the three treatment aquaria had died. Cumulative mortality in the third treatment aquaria was 97% after 21 weeks. Cumulative mortalities at this time in each of the control aquaria were 64.1, 46.3, and 12.5%, respectively. A one-way analysis of variance of the mortality data (following arcsine transformation) demonstrated that these differences were statistically significant ($P \leq 0.05$).

Molecular characterization of isolates. A total of six CVSP strains isolated from JOD-affected animals were analyzed. Two of these were from animals in the second group (CV923-115 and CV1028-008), two strains were isolated from JOD-affected oysters given to us by commercial growers (CV910-103 and CV1123-045), and two were isolated from laboratory-maintained animals that had been exposed to CV919-312 by injection (CV102-1001 and CV211-3001). The 16S rRNA genes of each isolate were amplified and sequenced, and all sequences were shown to be identical across the resulting 1,390-bp region. BLAST analysis revealed that these sequences were also 100% identical to the CVSP bacteria (CV919-312 and CV1010-352) isolated from the 1997 JOD episode (7). This sequence (GenBank accession no. AF114484 and AF114485) also shares extensive similarity with *Roseobacter* sp. strain ISM, marine α -proteobacterium MBIC3951, and *Roseovarius tolerans* (accession no. AF098485, AB018689, and Y11551, respectively). The percent sequence identities (excluding gaps) of the CVSP sequence with these strains are 96.1, 95.5, and 94.6%, respectively.

The 16S rRNA genes from three M1 isolates (CV729-100, CV812-530, and CV910-004) and two M2 isolates (CV902-700 and CV923-700) were similarly amplified and sequenced. The sequences of the M1 isolates were identical to each other across the resulting 1,394 bp, and BLAST analysis revealed a 100% identity (and no gaps) of this sequence to the marine α -proteobacteria MBIC1535 and MBIC3993. The sequence was also identical (excluding gaps) to that of *Agrobacterium stellulatum* (the new designation for this species is *Stappia stellulata*) (25, 47). The 16S rRNA sequences from the two M2 isolates were identical to each other and were almost identical to the M1 strain, with the exception of an unambiguous difference between the M1 and M2 sequence at nucleotide position 1043. The 16S rDNA nucleotide sequences of M1 isolate CV812-530 and M2 isolate CV902-700, have been submitted to GenBank (see "Nucleotide sequence accession numbers" above).

Phenotypic characterization of M1 and M2 isolates. Eighteen M1 isolates (CV729-100, CV812-530, CV910-004, and 15 others) and two M2 isolates (CV902-700 and CV923-700) were phenotypically characterized. Like *S. stellulata*, all were gram-negative motile rods, were oxidase positive, grew (albeit poorly) under anaerobic conditions, and formed star-shaped-like aggregates when grown in liquid media. Further, all isolates tested produced urease and β -galactosidase, reduced nitrate to gas, and hydrolyzed esculin. No strains were positive for arginine dihydrolase or gelatinase. Interestingly, all M1 isolates exhibited a very strong catalase activity, while this activity was barely detectable in the M2 isolates. Other differences were observed between the M1 and M2 isolates with respect to utilization of some carbon sources, and only M1 strains inhibited the growth of CVSP strain CV919-352 in a disk-diffusion assay (Table 1).

All M1 and M2 isolates assimilated the glucose, arabinose, mannose, mannitol, *N*-acetyl-glucosamine, maltose, gluconate, adipate, malate, and citrate present in the API 20 NE strips, while caprate and phenyl acetate were not utilized. In the GN microplate assay, all isolates strongly oxidized dextrin, glyco-

TABLE 1. Phenotypic differences between the M1 and M2 isolates and comparison with *S. stellulata*

Characteristic	Test result ^a		
	M1 isolates ^b	M2 isolates ^c	<i>S. stellulata</i> ^d
Catalase	+	W	+
Inhibition of CVSP bacteria ^e	+	–	ND
Utilization of:			
D-Alanine	–	W	ND
Adipate	+	+	–
Quinate	+	+	–
DL- α -Glycerol phosphate	–	+	ND
Glucose-1-phosphate	–	+	ND
Glucose-6-phosphate	–	+	ND

^a Symbols: +, positive; W, weakly positive; –, negative; ND, not determined.

^b Eighteen isolates were tested; symbol denotes the reaction of $\geq 94\%$ of isolates.

^c Two isolates were tested; symbol denotes the reaction of both isolates.

^d Data from reference 41. The previous designation for this strain was *A. stellulatum*.

^e Any visible zone of inhibition was scored as a positive.

gen, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, cellobiose, *i*-erythritol, D-fructose, L-fucose, D-galactose, gentiobiose, α -D-glucose, *m*-inositol, α -D-lactose, maltose, D-mannitol, D-mannose, D-melibiose, β -methyl D-glucoside, psicose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, turanose, methyl pyruvate, monomethyl succinate, *cis*-aconitic acid, citric acid, formic acid, D-gluconic acid, D-glucuronic acid, α -hydroxybutyric acid, β -hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α -ketobutyric acid, α -ketoglutaric acid, α -ketovaleric acid, DL-lactic acid, malonic acid, propionic acid, D-saccharic acid, succinic acid, alaninamide, L-alanine, L-alanyl-glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-glutamic acid, L-histidine, hydroxy L-proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyrroglutamic acid, L-serine, L-threonine, DL-carnitine, γ -aminobutyric acid, urocanic acid, inosine, thymidine, and glycerol. Substrates that were weakly oxidized included Tween 40, α -lactose, acetic acid, D-galactonic acid lactone, γ -hydroxybutyric acid, quinic acid, succinamic acid, glucuronamide, uridine, phenylethylamine, and 2-aminoethanol. Variable reactions were observed with bromosuccinic acid and glycyl-L-aspartic acid. The isolates did not utilize α -cyclodextrin, Tween 80, D-raffinose, xylitol, D-glucosaminic acid, sebamic acid, D-serine, putrescine, or 2,3-butanediol.

DISCUSSION

While the severity of the 1998 annual JOD epizootic was low compared with those of previous years, it impacted at least three separate culture sites on Maine's Damariscotta River. Bacteriological analysis of JOD-affected animals from each of these epizootics revealed the presence of CFU morphologically indistinguishable from those of the CVSP marine α -proteobacterium that was found to be associated with the 1997 epizootic (7). The 16S rRNA genes from isolates in the present study were amplified, and the sequence was determined to be identical to that of the previously described CVSP strains. CVSP-type CFU were not isolated from nonaffected animals raised at the JOD enzootic site or in cohorts raised at a JOD-free site. Thus, these results demonstrate that the presence of CVSP bacteria is a reliable indicator of JOD and constitutes strong correlative evidence for the role of these marine α -proteobacteria in JOD mortality.

An attempt to reproduce JOD signs and mortalities in laboratory-maintained *C. virginica* via the addition of CVSP bac-

teria to aquaria water was unsuccessful. However, when cells of CVSP strain CV919-312 were injected directly into the mantle cavities of oysters, the inoculated animals exhibited a reduced capacity to filter the algal food suspension (after 6 weeks) and suffered earlier and significantly elevated mortalities compared to that of controls (Fig. 3). CVSP-type CFU were recovered from animals during these mortality events, and their identity was confirmed by amplification and sequencing of the 16S rDNAs.

While these results support the hypothesis that CVSP bacteria are the etiological agent of JOD, the case would be more convincing if the characteristic conchiolin deposits had also been observed. However, if the oysters are not in good health and actively growing prior to the onset of JOD, mortalities can occur in the absence of this host response (32, 38). Because no net growth was observed during our experiment (data not shown), it is possible that the animals similarly may not have possessed the metabolic resources to produce the characteristic conchiolin deposits. Prolonged lack of a natural diet may also partially account for the unexpected mortalities in the control aquaria after 20 weeks.

In a previous study where JOD signs were reproduced by injection of animals with homogenates from affected animals, JOD signs developed within 1 month and researchers noted a higher prevalence of JOD signs in animals receiving more than one inoculation (38). Thus, the timing and extent of laboratory-induced JOD signs may depend upon the aggressiveness of the exposure regimen, as well as the infectivity of the source material. We suspect that the effect of CVSP bacteria will be observed sooner in animals exposed to homogenates from CVSP-inoculated animals than in those injected with cultured cells. This hypothesis will be addressed in subsequent experiments that will also include additional controls (e.g., heat-killed CVSP bacteria and a known nonpathogenic bacterium) and an increased and/or supplemented algal ration to improve the likelihood of a vigorous metabolic host response.

The apparent avirulence of CVSP when provided to juvenile *C. virginica* as a suspension in aquaria water should also be addressed. One possibility is that the cells experienced a rapid loss of viability upon addition to the artificial seawater. However, in preliminary experiments, we observed that CVSP bacteria survive and multiply in this water at rates approaching those observed in SWT medium (data not shown). Another possibility is that the animals did not concentrate the CVSP bacteria in the course of their normal filter-feeding activity. Given that oysters are very efficient at clearing bacterial cells from suspension (26, 35), this is considered unlikely. An alternative explanation may center on the production of factors important for bacterial colonization of the juvenile oysters. Like *Sagittula stellata* (another marine α -proteobacterium) (22), CVSP bacteria produce a polar holdfast structure in addition to polar flagella (data not shown). The method of exposure (immersion versus injection) may be significant if these potential colonization factors are not produced or are lost before the oysters encounter CVSP cells when present in suspension.

It is also important to consider the source of the animals used in each challenge experiment. Animals exposed to the CVSP bacteria by immersion were members of the first group that was deployed in the 1998 field study. Both groups used that year were progeny of oysters that had been bred for fast growth and had increased resistance to JOD mortalities (4, 12), but only animals in the second group became colonized by CVSP bacteria and experienced any JOD-associated mortalities. In addition, animals from the first group were stably colonized by the M1 genotype of an *S. stellulata*-like bacterium,

while the related M2 genotype was only isolated from the animals in the second group. The ability of the M1 strains (but not the M2 strains) to inhibit the growth of CVSP strain CV919-312 in a disk-diffusion assay raises the possibility that prior colonization of *C. virginica* by this species may prevent colonization by CVSP bacteria. A probiotic effect of the M1 strains could explain the apparent immunity of the first group to JOD both in the field and when challenged with CVSP bacteria in the immersion experiment. Animals used in the injection experiment had not been bred for JOD resistance, and no M1-like CFU were detected upon bacteriological analyses of the animals.

The body surfaces of oysters, as filter feeders, are in near-continuous contact with bacteria present in the ambient water. However, bacterial diseases of postmetamorphic oysters are relatively rare (13), and when examined by transmission electron microscopy, the external surfaces of the Pacific oyster (*Crassostrea gigas*) have been shown to be essentially free of any bacterial colonization (19). It is also generally accepted that if oysters do have a "normal microbiota," these organisms are predominantly associated with the gut and digestive gland (24, 27). In this study, both the CVSP and *S. stellulata*-type α -proteobacteria were consistently isolated from samples of the external tissue and the inner shell surfaces of *C. virginica*. These bacteria are not normal microbiota in the sense that they were not isolated from all animals, yet there is no question that they can initiate and persist in the association. This raises the interesting question of how oysters (and perhaps other bivalves) prevent colonization of tissues by most bacteria, and what factors allow these α -proteobacteria to overcome such defenses.

The marine α -proteobacteria are common and abundant in coastal ecosystems (20). Often referred to as the *Roseobacter* group (20, 21), these organisms also inhabit "extreme" environments such as hypersaline lakes (28), Antarctic ice (23), and hydrothermal vents (48). Several species of marine α -proteobacteria are known to be associated with a variety of marine plant (2, 29, 43) and animal species (5, 10, 42), although, to our knowledge, none of the animal symbionts have been successfully cultivated and characterized. *S. stellulata* (originally described as a marine *Agrobacterium*) has been isolated from marine sediments and seawater (41, 46), but this study provides the first evidence that this bacterium can also associate with a defined animal host. Conversely, CVSP bacteria have been isolated only from JOD-affected *C. virginica* (7 and this study), but they too are likely to be a normal member of marine bacterioplankton and sediment communities.

In sum, our results are not consistent with a multifactorial etiology for JOD and, instead, support the hypothesis that the CVSP α -proteobacterium is the sole etiological agent of JOD. This system represents a unique opportunity to study the bacterium-host interactions of both CVSP bacteria and the *S. stellulata*-like strains with *C. virginica*. As members of the marine α -proteobacteria, these bacteria are unrelated taxonomically to other common fish and shellfish pathogens. In addition, there is little known about the environmental role of this entire group of bacteria, and this study demonstrates that it will be important to consider host associations as factors influencing the abundance and distribution of planktonic marine α -proteobacteria. We are currently developing a PCR-based molecular beacon assay for the detection of CVSP bacteria in the environment and for use as a diagnostic tool.

ACKNOWLEDGMENTS

This study was funded by a Maine/New Hampshire Sea Grant development award.

We are grateful to Chance Along Farm, Dodge Cove Marine Farms, Mook Sea Farms, and Pemaquid Oyster Company for their very generous assistance with this study. We also thank P. B. Singer and K. Williams for sequencing services, L. Costello and R. B. Carnegie for assistance in the field, K. Mitchell for help with the challenge experiments, and M. A. Killam for assistance with the phenotypic characterizations.

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