Roseovarius crassostreae sp. nov., a member of the *Roseobacter* clade and the apparent cause of juvenile oyster disease (JOD) in cultured Eastern oysters

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An α -proteobacterium has been identified which is believed to be the causative agent of juvenile oyster disease (JOD). Since its first isolation in 1997, the bacterium has been recovered as the numerically dominant species from JOD-affected animals throughout the north-eastern United States (Maine, New York and Massachusetts). Colonies are usually beige to pinkish-beige, although the majority of isolates recovered in 2003 from an epizootic in Martha's Vineyard, Massachusetts, produce colonies with a greenish-yellow appearance. The cells are Gram-negative, aerobic, strictly marine and rod or ovoid in appearance. They are actively motile by one or two flagella, but cells are also observed to produce tufts of polar fimbriae. The principal fatty acid in whole cells is C_{18:1} ω 7c and other characteristic fatty acids are C_{16:0}, C_{10:0} 3-OH, 11-methyl C_{18:1} ω 7c and C_{18:0}. Almost without exception, isolates have 16S rRNA gene sequences that are 100 % identical to each other. Phylogenetic analyses place the organism within the *Roseobacter* clade of the α -Proteobacteria, with moderate bootstrap support for inclusion in the genus *Roseovarius*. DNA–DNA relatedness values from pairwise comparisons of this organism with the type species of the genus (*Roseovarius tolerans*) and the only other described species in this genus, *Roseovarius nubinhibens*, were 11 and 47 %, respectively. Phenotypic and biochemical dissimilarities also support the assignment of this bacterium to a novel species. The name *Roseovarius crassostreae* sp. nov. is proposed, with the type strain CV919-312^T (=ATCC BAA-1102^T=DSM 16950^T).

Juvenile oyster disease (JOD) causes seasonal mortalities among hatchery-produced juvenile *Crassostrea virginica* raised in the north-eastern United States. Single-season crop losses may exceed 90 % of total production at enzootic sites in Maine, Massachusetts and New York (Bricelj *et al.*,

1992; Davis & Barber, 1994; Ford & Borrero, 2001), although management strategies utilizing selected lines and early deployment of hatchery-produced seed have helped to minimize the impact (Barber *et al.*, 1996; Barber *et al.*, 1998; Davis & Barber, 1999).

In 1997, a study to elucidate the aetiology of JOD led to the isolation of a marine α -proteobacterium in the *Roseobacter* clade that was numerically dominant in affected animals (Boettcher *et al.*, 1999). Additional studies revealed the consistent association of this species with JOD-epizootics in Maine and mortalities were reproduced by laboratory challenge with the representative isolate CV919-312^T (Boettcher *et al.*, 2000). Animals experiencing JOD-mortality in New York and Massachusetts were heavily colonized by isolates sharing 99.8–100 % 16S rRNA gene sequence identity with the Maine isolates. In 2004, colonization was confirmed in a population of oysters during the week immediately preceding JOD-onset (A. P. Maloy and K. J. Boettcher, unpublished).

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Abbreviations: Bchl *a*, bacteriochlorophyll *a*; JOD, juvenile oyster disease.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Roseovarius crassostreae* sp. nov. CV919-312^T is AF114484.

A table detailing the fatty acid profiles for isolates of *Roseovarius crassostreae* sp. nov. and *Roseovarius tolerans* EL-172^T and a figure showing the relationships between isolates derived from fatty acid analysis are available as supplementary material in IJSEM Online.

Typically, mortality episodes last only a few weeks and occur coincident with, or shortly after, the onset of external signs (e.g. uneven valve margins, emaciation and conchiolin deposition on the inner shell surfaces) (Barber *et al.*, 1996; Boettcher *et al.*, 1999). The exact cause of death is unknown, but the feeding impairment observed in experimentally infected animals (Boettcher *et al.*, 2000) is consistent with the 'starved' appearance of naturally affected animals. The role of toxins and/or other virulence factors has yet to be elucidated.

All known strains were obtained from cultured *Crassostrea virginica*. Following aseptic dissection, moistened swabs were used to collect material from the soft tissue, mantle and inner shell surfaces. The swabs were then vortexed in 1 ml filter-sterilized sea water (FSSW). Serial dilutions of the suspensions were then plated onto sea water-tryptone (SWT) agar (containing 70 % sea water, 0.5 % tryptone, 0.3 % yeast extract and 0.3 % glycerol solidified with 1.5 % agar; Boettcher & Ruby, 1990) and incubated at 22–24 °C. The isolates used in this study are listed in Table 1 and include representatives from JOD epizootics in Maine during 1997, 1998 and 2000–2003. Also included are isolates

from JOD-affected animals obtained from New York in 2002 and 2003 and from Massachusetts in 2003. Type strains of *Roseobacter denitrificans*, *Roseobacter litoralis*, *Roseovarius tolerans* and *Roseovarius nubinhibens* served as controls in some experiments.

As described previously (Boettcher *et al.*, 1999), young colonies of the JOD-associated α -proteobacterium appear uniformly round, non-mucoid and semi-translucent. Colonies also have a characteristic umbonate shape, a somewhat chalky consistency and leave a noticeable impression when removed from the surface of an agar plate. Isolates from JOD epizootics in Martha's Vineyard during 2003 and 2004 were found to share 100 % 16S rRNA gene sequence identity with the other isolates, but were unusual in that the resulting colonies had a greenish-yellow appearance and were slightly smaller than normal (only about 0.5 mm after 5 days). Typically, colonies are approximately 1.0 mm in diameter and have a cell-associated pinkish-beige pigmentation after approximately 5 days of growth on SWT.

The pink pigment is not, however, attributable to

Table 1. Source and growth characteristics of *Roseovarius crassostreae* sp. nov. used in this study

Isolate	Year	State*	Growth at NaCl (%)†			Growth at temperature (°C)			Reference
			Opt.	Min.	Max.	Opt.	Min.	Max.	
CV919-312 ^T	1997	ME	1.5	1.0	8.0	34	19	40	Boettcher <i>et al.</i> (1999)
CV1010-352	1997	ME	2.0	1.0	7.5	34	19	40	Boettcher <i>et al.</i> (1999)
CV923-115	1998	ME	1.5	1.0	7.0	28	19	40	Boettcher <i>et al.</i> (2000)
CV910-103	1998	ME	1.0	1.0	6.0	34	19	40	Boettcher <i>et al.</i> (2000)
CV1123-045	1998	ME	2.0	1.0	7.0	34	19	40	Boettcher <i>et al.</i> (2000)
CV930-004	1998	ME	2.0	1.0	6.5	34	19	40	This study
DC008	2000	ME	1.0	0.5	6.0	34	10	40	This study
DC010	2000	ME	2.0	1.0	4.5	31	19	40	This study
MB003	2000	ME	1.5	1.0	7.0	31	10	40	This study
MB004	2000	ME	1.5	1.0	4.0	31	19	40	This study
NM001	2000	ME	1.5	1.0	4.0	31	19	40	This study
NM002	2000	ME	1.5	0.5	4.5	34	19	40	This study
SP101	2001	ME	1.0	0.5	7.0	34	13	40	This study
SP102	2001	ME	1.0	0.5	7.0	34	13	40	This study
OB702-1	2002	NY	1.0	1.0	5.0	34	13	40	This study
OB802-1	2002	NY	1.0	0.5	5.0	34	13	40	This study
OB802-2	2002	NY	1.0	0.5	6.0	34	22	40	This study
MSF1002-11	2002	ME	2.0	0.5	4.5	34	10	40	This study
DR1003-23	2003	ME	2.0	1.0	4.5	34	16	40	This study
OB803-6	2003	NY	1.5	0.5	7.0	34	13	40	This study
MTN1003-19	2003	NY	1.5	0.5	6.5	34	13	40	This study
KB903-1	2003	MA	2.0	1.0	5.0	34	19	43	This study
EGP903-25	2003	MA	1.5	1.0	5.0	34	13	40	This study
CCB1003-40	2003	MA	1.5	0.5	6.5	34	22	40	This study
CCBE1103-8	2003	MA	2.0	1.0	8.0	34	22	40	This study
BS1103-19	2003	MA	2.0	1.0	5.0	34	19	43	This study

*Abbreviations: MA, Massachusetts; ME, Maine; NY, New York.

†Medium also contained 0.5 % tryptone, 0.3 % yeast extract and 0.3 % glycerol.

bacteriochlorophyll *a* (Bchl *a*) production. Cells grown in the dark did not produce an absorbance peak in the range of 790–805 nm while such absorbance peaks were observed with preparations of *Roseobacter denitrificans* and *Roseobacter litoralis* (known producers of Bchl *a*; Shiba, 1991). Still, because Bchl *a* is variably produced in the type species of this genus (*Roseovarius tolerans*), we used PCR to try to detect a *pufM* gene (which encodes the M subunit of the bacterial photosynthetic reaction centre). On the basis of *pufM* sequence data in GenBank, we modified the primer sets (designed for the purple phototrophs) by Achenbach *et al.* (2001), so that they would specifically target members of the *Roseobacter* clade. Each 50 µl PCR contained 750 nM each of primer *pufM* 568F, 5'-CGCACCTTGACTGGAC-3' (modified from *pufM* primer 557F of Achenbach *et al.*, 2001) and primer *pufM* 698R, 5'-RTRGCGGGTCACCGC-CAGAA-3' (modified from *pufM* primer 750R of Achenbach *et al.*, 2001), 200 µM each deoxynucleoside triphosphate, 2 mM MgCl₂, 100–200 ng genomic DNA and 1.25 U *Taq* DNA polymerase (Invitrogen) in the manufacturer-supplied buffer diluted to 1× concentration. The thermocycler was set to the following conditions: 3 min of denaturing (95 °C), followed by 35 cycles of 30 s denaturing (95 °C), 30 s of annealing (53 °C) and 45 s of elongation (72 °C), followed by an additional 5 min of elongation at the end of the program. The products were purified using a Centricon-100 microconcentrator (Amicon) and analysed by electrophoresis on a 1.5 % agarose gel. A specific 146 bp product (verified by sequencing) was amplified from DNAs prepared from *Roseobacter denitrificans* and *Roseovarius tolerans* (both known Bchl *a* producers). Conversely, this product was not amplified from CV919-312^T or from *Roseovarius nubinhibens* (which does not produce detectable Bchl *a*; González *et al.*, 2003).

In wet mount preparations, cells of the species appear rod to ovoid shaped and are actively motile. Bacterial suspensions were also settled onto specimen grids, stained with 1 % phosphotungstic acid and viewed with a Phillips CM-10 transmission electron microscope. Cells were photographed and determined to be 1.1–4.8 µm in length (mean was 2.8 ± 1.2 µm) and 0.6–1.2 µm in width (mean was 0.9 ± 0.2 µm). In addition, the majority of cells were observed to possess one or two flagella at either lateral, polar or subpolar positions (Fig. 1a–c). However, occasionally cells were observed with a single polar fimbriae tuft (Fig. 1c, d). In one instance it appeared that both a fimbriae tuft and a flagellum were present at the same pole of a single cell (Fig. 1a). It is reasonable to suspect that these structures are important for colonization of and/or persistence with the oyster host. Indeed, when the inner shell surfaces of some JOD-affected animals were viewed by scanning electron microscopy, numerous bacterial cells were present and many were clearly attached at their poles (C. L. Boardman and K. J. Boettcher, unpublished).

As previously reported, the cells are aerobic, non-fermentative, oxidase and catalase-positive (Boettcher

et al., 1999). Semi-quantitative analysis of some additional enzymes from the strains in Table 1 was accomplished using API ZYM and API-20 NE tests (bioMérieux). Biolog GN2 Microplates (Biolog) were used to screen for carbon source utilization. All other biochemical tests were performed according to standard methods (Smibert & Krieg, 1994). Although there was minor qualitative variation observed among the isolates using the API tests, all produced high levels of esterase (C4), esterase lipase (C8), acid and alkaline phosphatases and leucine arylamidase. Weak lipase and valine arylamidase activities were also uniformly produced. No cysteine arylamidase, trypsin, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, arginine dihydrolase, urease, gelatinase or aesculinase activity was detected from any isolate. All isolates exhibited denitrifying activity in nitrate broths containing Durham tubes and, of the 95 substrates present in a Biolog GN2 Microplate, utilization of only glycerol and β-hydroxybutyrate was detected.

The effect of salinity on growth was tested in media containing 0.5 % tryptone, 0.3 % yeast extract, 0.3 % glycerol and NaCl at final concentrations of 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 %. Cultures were prepared by diluting an overnight SWT-grown culture to an optical density (OD) of 0.02 (at 600 nm) in 5 ml of each growth medium. The cultures were then grown with shaking (200 r.p.m.) and the OD was measured at defined intervals (every 60–90 min). Optimal growth of CV919-312^T was observed at salinities of 1.0–1.5 % and identical concentrations of potassium salts could not be substituted. The effects of temperature and pH were similarly determined, but in SWT broth at temperatures ranging from 4–40 °C and in SWT broth adjusted with HCl or NaOH to pH values between 5.5 and 9.0. Inocula were prepared from overnight cultures as described above and growth was measured by monitoring the OD at 600 nm. Optimal growth of CV919-312^T occurred at 34–37 °C and at pH values between 6.5 and 8.0. No growth was observed at either 4 or 42 °C or at pH values below 6.0. While the requirement for sodium ions suggests that this is a strictly marine organism, the relatively low NaCl and high temperature optima are indicative of adaptation to coastal regions. Interestingly, significant variation was observed among the isolates with respect to their temperature and salinity optima, but there were no observable trends with respect to the year or location of isolation (Table 1).

The dominant fatty acids in whole cells of 12 representative isolates (CV919-312^T, CV1010-352, CV923-115, CV910-115, CV1123-045, CV930-004, DC008, DC010, MB003, MB004, NM001 and NM002) were determined by fatty acid methyl ester (FAME) analysis at Microbial ID. The principal fatty acid in whole cells was determined to be C_{18:1ω7c} (mean was 85.2 ± 1.7 %) and other characteristic fatty acids were C_{16:0} (3.6 ± 0.9 %), C_{10:0} 3-OH

Fig. 1. Electron micrographs of *Roseovarius crassostreae* sp. nov. isolates. (a) Cell which appears to have a single polar flagellum and the remains of a polar 'tuft' at the same pole. (b) Cell with two subpolar flagella. (c) Two cells; one with a lateral flagellum (insertion point indicated by 'f') and one displaying a polar fimbriae 'tuft' (indicated by 't'). (d) Partial cell showing detail of polar fimbriae. Bars, 1 μ m.

(2.1 ± 0.3 %), 11-methyl $C_{18:1}\omega 7c$ (0.8 ± 0.2 %) and $C_{18:0}$ (0.7 ± 0.3 %). As no species with a similar profile was identified in the database, the fatty acid profile of the type species of the genus (*Roseovarius tolerans*, similarly grown on SWT agar) was obtained for comparison. A supplementary table with the complete fatty acid profiles of all analysed strains is available in IJSEM Online. The resulting dendrogram based on cluster analysis suggests that the 12 isolates form two (or three) strain groups. All are clearly distinct from *Roseovarius tolerans*, yet among themselves would be considered members of the same species (Euclidean distance <4). A supplementary figure is available in IJSEM Online. This assumption was verified by pairwise comparisons using DNA–DNA hybridization conducted at the Deutsche Sammlung von Mikroorganismen (DSMZ), Braunschweig, Germany. The methods of De Ley *et al.* (1970) and Huß *et al.* (1983) were employed

with the following results: CV919-312^T and CV1123-045, 102 %; CV919-312^T and NM002, 99 % and CV1123-045 and NM002, 93 %.

The DNA base composition of CV919-312^T was determined (by HPLC at DSMZ) to be 59.0 mol% G + C. Comparisons of the G + C content and other phenotypic traits of this bacterium with closely related α -proteobacteria (as determined by 16S rRNA gene sequence analysis, see below) are given in Table 2.

Regardless of the location or year of the epizootics, all isolates share 99.8–100 % 16S rRNA gene sequence identity with the type strain CV919-312^T (Boettcher *et al.*, 2000 and this study) (GenBank accession no. AF114484). Of described species, the highest nucleotide similarity was to *Roseovarius nubinihibens* (96.1 % identity). To more precisely define the relationship of the JOD-associated

Table 2. Comparison of *Roseovarius crassostreae* CV919-312^T with the type species of the genus and related α -proteobacteria

Strains: 1, *Roseovarius crassostreae* CV919-312^T (data from this study); 2, *Roseovarius tolerans* EL-172^T (Labrenz *et al.*, 1999); 3, *Antarctobacter heliothermus* EL-219^T (Labrenz *et al.*, 1998); 4, *Leisingera methylohalidivorans* MB2^T (Schaefer *et al.*, 2002); 5, *Roseobacter gallaeciensis* BS107^T (Ruiz-Ponte *et al.*, 1998); 6, *Roseobacter litoralis* ATCC 49566^T (Shiba, 1991); 7, *Ruegeria algicola* ATCC 51440^T (Lafay *et al.*, 1995); 8, *Ruegeria atlantica* IAM 14463^T (Rüger & Höfle, 1992); 9, *Ruegeria gelatinovorans* IAM 12617^T (Rüger & Höfle, 1992); 10, *Sagittula stellata* IAM12621^T (González *et al.*, 1997). *Ruegeria algicola*, *Ruegeria atlantica* and *Ruegeria gelatinovorans* were assigned to the genus *Ruegeria* according to Uchino *et al.* (1998). All strains are catalase-positive with a strict requirement for sodium ions. +, Positive reaction; –, negative reaction; ND, not determined or not reported.

Characteristic	1	2	3	4	5	6	7	8	9	10
Cell shape	Rod to ovoid	Rod	Rod	Rod	Ovoid rod	Rod to ovoid	Ovoid	Rod to ovoid	Rod to ovoid	Rod
Rosettes formed	–	–	+	ND	ND	–	–	+	+	+
Pigment	Pink-beige*	Brown-yellow	None	Brown	Pink to red	Red	Beige	Beige	Beige	Cream
Motility	+	+/–	+/–	+	+	+	+	–	+	–
Flagella	Subpolar to lateral	Not found	Subpolar	Subpolar	Polar	Subpolar	Subpolar	Not found	Peritrichous	Loose†
Fimbriae	Polar tuft	ND	ND	ND	ND	ND	ND	ND	ND	+
Bchl <i>a</i>	–	+	–	–	–	+	–	ND	ND	–
NO ₃ [–] reduced to NO ₂ [–]	+	–	+	–	–	–	–	+	+	–
NO ₂ [–] reduced to gas	+	–	–	–	–	–	–	+	–	ND
Growth at 4 °C	–	+	+	ND	–	+	–	–	ND	–
Growth at 37 °C	+	+	+	ND	+	–	+	–	–	+
Salinity optimum (%)	1.0–1.5	1.0–8.0	2.0–6.0	1.5–6.5	1.2	1.5	0.6–12	ND	ND	ND
pH optimum	6.5–8.0	6.2–9.0	6.0–7.8	7.7	7.0	7.0–8.0	ND	ND	ND	5.5–8.5
Aesculinase	–	ND	ND	ND	ND	ND	+	ND	ND	ND
β -Galactosidase	–	ND	ND	ND	ND	ND	+	ND	ND	ND
Gelatinase	–	–	+	ND	–	+	+	–	+	–
G + C content (mol%)	59.0	63.3–63.4	62.6–62.8	60.5	57.6–58.0	56.3–58.1	64‡	54–57	59	65

*Some strains of this species will produce colonies with a greenish-yellow appearance.

†Flagella observed in preparations, but not attached to cells.

‡Data from Labrenz *et al.* (1998).

bacterium to this and other members of the α -proteobacteria, the sequence was manually aligned with those of 23 representative taxa using the Genetic Data Environment 2.2 Macintosh Edition. These data were imported into PAUP*4.0b10 (Sinauer Associates) and phylogenetic analyses were based on a total of 1359 characters. Distance analyses consistently placed the organism into the genus *Roseovarius* as shown in Fig. 2. Trees with overall similar topology were also achieved using distance and parsimony analyses; however, these analyses did not provide strong support for inclusion of this organism in any of the described genera. DNA–DNA hybridization (conducted by DSMZ) with that of the genus type species (*Roseovarius tolerans*) was 11% and was 47% with *Roseovarius nubinihibens* DNA. Thus, despite the low relatedness to *Roseovarius tolerans*, the organism appears sufficiently similar to *Roseovarius nubinihibens* such that it may be placed in the same genus. In conclusion, the phenotypic and phylogenetic analyses support placement of this bacterium as a novel species in the genus *Roseovarius*. The proposed name is *Roseovarius crassostreae* sp. nov.

Description of *Roseovarius crassostreae* sp. nov.

Roseovarius crassostreae (crass.o.stre'ae. N.L. n. *Crassostrea* systematic name of an oyster genus; N.L. gen. n. *crassostreae* of *Crassostrea*).

Aerobic, Gram-negative, ovoid to rod-shaped cells. Young colonies are small, uniformly round and have a characteristic umbonate shape. Older colonies exhibit a pinkish-beige pigmentation or, rarely, have a greenish-yellow appearance. Oxidase- and catalase-positive. Utilizes a limited range of substrates for growth, but grows well on sea water-based complex medium. Strict requirement for sodium ions. Actively motile and one to several polar, subpolar or lateral flagella may be present. Non-flagellated cells have been observed to have a single polar fimbriae tuft. Esterase (C4), esterase lipase (C8), acid and alkaline phosphatases and leucine arylamidase are produced. Weak lipase and valine arylamidase activities are also detected. Nitrate is reduced to gas. Does not produce Bchl *a*. The principal fatty acid in

Fig. 2. Phylogenetic position of *Roseovarius crassostreae* within the *Roseobacter* clade of the α -Proteobacteria. Two *Rhodobacter* species served as the outgroup and the position of a benign oyster symbiont (*Stappia* sp. CV812-530) is also shown. The unrooted tree was derived from distance analysis of 1359 characters in the 16S rRNA gene sequences (*Escherichia coli* positions 46–1448) and excluded positions with missing data and regions of uncertain alignment. The neighbour-joining method was used with 100 bootstrap replicates and a 50% majority consensus rule. Numbers indicate bootstrap values. Bar, 0.01 Jukes–Cantor substitutions per nucleotide.

whole cells is $C_{18:1}\omega 7c$. Other characteristic fatty acids are $C_{10:0}$ 3-OH, $C_{16:0}$, $C_{18:0}$ and 11-methyl $C_{18:1}\omega 7c$. Optimal growth is observed at 34 °C, at salinities of 1.0–1.5 ‰ and at pH values between 6.5 and 8.0. The G+C content is 59.0 mol%.

The type strain is CV919-312^T (=ATCC BAA-1102^T=DSM 16950^T). This and all other strains of the species were isolated from *Crassostrea virginica*, in which it is believed to be the sole aetiological agent of JOD.

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