A PCR-based diagnostic assay for the detection of *Roseovarius crassostreae* in *Crassostrea virginica* affected by juvenile oyster disease (JOD)

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ABSTRACT: We have developed a PCR-assay for the diagnosis of juvenile oyster disease (JOD) based on the detection of *Roseovarius crassostreae* directly from affected oysters. Species-specific primers are used to amplify the 16S-23S rDNA internal transcribed spacer (ITS) of *R. crassostreae*, and confirmation of product identity is accomplished by restriction enzyme analysis. No false positives were obtained with either closely related bacterial species or from other DNAs present in oyster samples. The assay has the potential to detect as few as 10 cells of *R. crassostreae* per oyster when samples are taken from the inner valve surfaces of the animal. Inclusion of material from soft body surfaces is not necessary, and may reduce sensitivity approximately 10-fold. In a JOD-affected population, a positive PCR result was obtained from all oysters from which these bacteria were subsequently cultured. The assay also detected the presence of *R. crassostreae* in 2 oysters from which no *R. crassostreae* isolates were recovered. No *R. crassostreae* was detected by either PCR or bacteriology in oysters from a population that was not exhibiting JOD-signs. This assay is expected to advance regional disease management efforts and provide valuable insights into the disease process and epizootiology of JOD.

KEY WORDS: Juvenile oyster disease · JOD · *Roseobacter* · PCR · *Crassostrea virginica* · *Roseovarius crassostreae*

INTRODUCTION

Epizootics of juvenile oyster disease (JOD) are seasonally common throughout the northeastern United States and result in substantial losses of cultured C*rassostrea virginica.* Epizootics vary in severity, but may result in over 90% mortality in first-year crops (Bricelj et al. 1992, Davis & Barber 1994, Ford & Borrero 2001). Despite the success of husbandry-based management techniques (Barber et al. 1996, 1998), JOD is still a significant threat to the industry. In recent years it has spread to previously JOD-free locations in Maine, USA (Barber & Boettcher 2002), Massachusetts, USA (Boettcher & Maloy 2004a), and possibly France (Renault et al. 2001).

The etiological agent of JOD is believed to be a member of the *Roseobacter* clade of the marine α-Proteobacteria. The bacterium has been consistently and exclusively isolated as the numerically dominant bacterium from JOD-affected oysters in Maine, New York, and Massachusetts (Boettcher et al. 1999, 2000, 2005). Mortalities have also been reproduced in laboratory-held *Crassostrea virginica* by exposure to the type strain CV919-312[T] (Boettcher et al. 2000), and colonization by this species has been detected among a commercial population of oysters 1 to 2 wk prior to the development of JOD-signs (A. P. Maloy et al. unpubl. data). Molecular and biochemical characterization revealed that the bacterium is a novel species, and originally the designation *Roseimarina crassostreae* (gen. nov., sp. nov.) was proposed (Boettcher 2002, Maloy & Boettcher 2003). Based on evaluation of additional strains and the description of a closely related species, we have now assigned this bacterium to the

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established genus *Roseovarius*, as *Roseovarius crassostreae* (sp. nov.) (Boettcher et al. 2005).

Generally, epizootics occur late in the summer (after water temperatures have reached 20°C), and predominantly affect oysters less than 25 mm in shell height (Bricelj et al. 1992, Barber et al. 1996, 1998, Ford & Borrero 2001). Recently, however, heavy colonization by *Roseovarius crassostreae*, accompanied by mortality, has been observed among adults in Massachusetts (A.P. Maloy et al. unpubl. data). Once some oysters in a population are affected by JOD, the condition soon after appears in neighboring oysters (Lewis et al. 1996). This situation generally results in a high frequency of disease among oysters held in dense culture, which is the case in most grow-out operations. Because signs can appear as soon as 2 wk after deployment, it has been a high priority to determine the origin of colonizing *R. crassostreae*. In 2003, we analyzed strains of *R. crassostreae* from JOD-affected individuals throughout the northeastern USA and concluded that most epizootics result from site-specific acquisition of the bacterial agent (Boettcher & Maloy 2004b). However, we have also found instances where outbreaks were most probably caused by the movement of previously colonized individuals (Boettcher 2002). Therefore, some means of intervention may help to prevent the spread of JOD.

The overt signs of JOD include stunted growth, uneven valve margins, conchiolin deposition, and pale and watery tissues. Unfortunately, visual inspection is a poor screening method because mortalities occur coincident with or very shortly after (within 1 wk) the manifestation of such signs (Bricelj et al. 1992, Ford & Borrero 2001). Further, it is often difficult to detect the presence of *Roseovarius crassostreae* by culture techniques. For example, extended exposure to temperature extremes prior to sampling greatly reduces the efficiency of *R. crassostreae* recovery (A. P. Maloy & K. J. Boettcher unpubl. data). Further, it typically takes 3 d for *R. crassostreae* to form a visible colony, and 5 to 7 d for those colonies to exhibit morphologically distinguishing features. Thus, their presence is easily masked by interspecific inhibition and/or overgrowth of faster growing species. Such competitive disadvantages limit the effectiveness of culture-based assays, especially when *R. crassostreae* is present in low numbers. Detection using bacteriological techniques is also time consuming, and correct identification of *R. crassostreae* colonies is dependent on the experience of the investigator.

The development of a molecular assay to directly detect *Roseovarius crassostreae* in seed oysters has the potential to greatly improve the accuracy and efficiency of JOD diagnosis. For example, direct amplification of bacterial (Brasher et al. 1998), viral (Renault & Arzul 2001) and protistan parasite (Marsh et al. 1995, Carnegie et al. 2000) nucleic acids have improved the diagnostic capability for many other shellfish diseases. Amplification and analysis of the 16S rRNA gene often serves as the basis for the detection and identification of bacteria, but the internal transcribed spacer (ITS) region between the 16S and 23S rRNA genes can also provide useful information (Scheinert et al. 1996, Söller et al. 2000). Unlike 16S rDNA, appreciable differences exist in the length of this region among eubacterial species, and thus, discrimination can be achieved with little effort. Analyses of sequence variations allow for additional discrimination among species, and in some cases, restriction fragment length polymorphism (RFLP) analyses of this region have provided accurate differentiation between strains of the same species (Gürtler & Stanisich 1996). We have similarly been successful in using amplification and RFLP analysis to identify strain variation among isolates of *R. crassostreae* recovered from diseased animals (Maloy et al. 2002, Boettcher & Maloy 2004b). While these techniques have been useful for preliminary epidemiological investigations, a detection assay for screening and diagnostic applications is still needed. We describe herein a method based on PCR amplification of the *R. crassostreae* ITS for the sensitive, specific, culture-independent detection of the bacterium directly from oyster samples.

MATERIALS AND METHODS

PCR reaction and thermocycler protocol. The primers ROS-109F (5′-GTTGGTTCTACCTGACGG-3′) and ROS+955R (5′-CTCTTTGAATGTCCTACCGC-3′) were used to amplify a 1071 or 1100 bp fragment containing the majority of the *Roseovarius crassostreae* 16S–23S rDNA ITS region. (These primers correspond to positions 109–126 bp before the end of the 16S rRNA gene, and positions 955–974 of the ITS region of *R. crassostreae* CV919-312^T, respectively). Each 25 µl PCR reaction contained either a 1 µl suspension of approximately 10^5 cells (for screening of isolates), 50 ng bacterial DNA (for specificity trials), or 2 µl of DNA extracted from swab samples (for sensitivity and field trials). Each reaction also included $MgCl_2$ at 1.5 mM for reactions using whole cells or 2.0 mM for reactions containing DNA, 0.75 µM of each amplification primer, 200 µM of each deoxynucleoside triphosphate, 1.25 U *Taq* polymerase (Invitrogen), and 2.5 µl 10X PCR buffer. The remaining volume was completed with nuclease free water. Each reaction was held in a programmable thermal Controller PTC-100™ (MJ Research) for 5 min (95°C), followed by 40 cycles of 30 s denaturing (94°C), 30 s annealing (60°C), 1 min elongation (72°C), and 1 final 7 min elongation (72°C).

Amplification products were resolved on a 1.5 % agarose gel and stained with ethidium bromide.

Assay specificity. PCR amplifications using the primers ROS-109F and ROS+955R were performed using 50 ng of genomic DNA template (extracted with the QIAamp® DNA Micro Kit; Qiagen) from pure cultures of *Roseovarius crassostreae* (CV919-312[T]), *Roseovarius nubinhibens* (ISM[T]), *Roseovarius denitrificans* (OCH114[T]), *Roseovarius litoralis* (ATCC 49566[T]), *Roseovarius tolerans* (EL-172[T]) and *Ruegeria algicola* (ATCC 51440[T]). Products were analyzed by agarose gel electrophoresis of a 5 µl aliquot from each PCR reaction.

Preparation of swab samples and DNA extractions. Oysters were individually rinsed in 70 % filter (0.2 µm)-sterilized seawater (FSSW) to remove external debris. Following aseptic dissection, the entire soft tissue was removed, and the inner valve surfaces were rinsed (×3) with FSSW to remove any remaining fluids. A sterile cotton applicator (moistened with FSSW) was then used to collect material from the inner valves, and a suspension was created by vortexing the swab in 1 ml of 70 % FSSW. Suspensions were pelleted at $19\,000 \times g$ for 5 min at 4°C, and a pipette was used to remove the supernatant. Total genomic DNA was extracted from each pellet using the QIAamp® DNA Micro Kit (Qiagen) following the manufacturer's supplied protocol *Isolation of Genomic DNA from Swabs* (starting with Step #2). The DNA was eluted in 25 µl of the supplied buffer and 2 µl of this preparation was used as template for PCR reactions.

Assay sensitivity using simulated samples. To determine the theoretical detection limit of the assay, a parallel set of swab samples was prepared from unaffected *Crassostrea virginica*. The first sample of each set consisted of material collected from the inner valve surfaces only (as described above), and the second contained material from both the inner valve and host tissue surfaces. A 5 µl aliquot of SWT media (70 % seawater, 0.5 % tryptone, 0.3 % yeast extract, and 0.3 % glycerol; Boettcher & Ruby 1990) containing a known number of *Roseovarius crassostreae* cells was then added to each suspension. Final cell concentrations ranged (at 10-fold increments) from 10^6 to 1 cell ml^{-1}. An additional set of control suspensions which contained 5 µl of SWT medium only (no cells) was prepared for use as a negative control. Total genomic DNAs were extracted from each of these simulated samples (as described above) and used as templates in subsequent PCR reactions.

PCR vs. culture-based analyses of field samples. Cultured *Crassostrea virginica* from oyster populations in Maine and Massachusetts were shipped overnight to the laboratory. Upon arrival, oysters were cleaned of external debris, aseptically dissected and visually assessed for JOD signs (conchiolin and uneven valve margins). Suspensions of material from the inner valve surfaces were prepared as described above, and an aliquot from each suspension was 10-fold serially diluted in FSSW to 1:10^4. Twenty microliters of each dilution were spread on SWT agar plates and incubated for 7 d at room temperature before enumeration of colonies. The original (i.e. undiluted) suspensions were then pelleted, and the total genomic DNA was extracted and PCR-amplified as described above.

After incubation, growth and morphological characteristics were used to identify *Roseovarius crassostreae*-like colonies. Such colonies were isolated in pure culture and reexamined after subsequent growth on SWT agar. Presumptive identification as *R. crassostreae* was confirmed via PCR as follows. Isolates with morphological features consistent with *R. crassostreae* were resuspended in SWT, and the cell concentration was estimated by determining the optical density at 600 nm. The density was adjusted (if necessary) to a concentration of 10^8 cells ml^{-1}. A 1 µl aliquot (containing approximately 10^5 cells) was used as template, and reaction conditions were as described above for the PCR assay using whole cells.

Verification of PCR-product identity. The presence of a 1071 or 1100 bp product from amplifications using the primers ROS-109F and ROS+955R is interpreted as evidence for the presence of *Roseovarius crassostreae*. However, for additional confirmation, PCR products from swab samples and bacterial isolates were also subjected to RFLP analysis by digestion with the restriction enzyme *Ava*I. Each 10 µl reaction contained 5 µl of PCR product, 1 µl of NEB Buffer #4, 5 U of *Ava*I (New England Bio Labs), and was brought to volume with nuclease-free water. The digestions were incubated overnight at 37°C, and analyzed by gel electrophoresis.

Products from both a simulated swab sample and a JOD-affected individual from Massachusetts were also sequenced to further validate the PCR approach. Sequencing was carried out at the University of Maine's DNA sequencing facility using an ABI model 3730 DNA Analyzer (Applied Biosystems) with the BigDye Version 3.1 Cycle Sequencing Kit per manufacturer's instructions. Raw ABI files were edited with Chromas (Technelysium), imported into GeneDoc (Pittsburgh Supercomputing Center), and compared to the sequence of the *Roseovarius crassostreae* type strain CV919-312[T].

RESULTS

Specificity

In PCR reactions containing the primers ROS-109F and ROS+955R, a 1100 bp product was amplified from *Roseovarius crassostreae* CV919-312[T] genomic DNA.
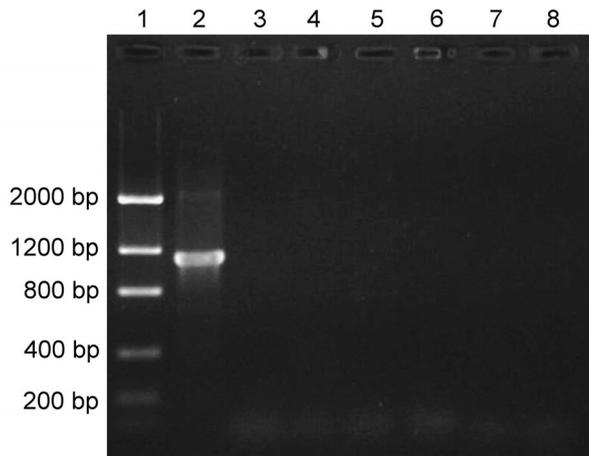
Fig. 1. Agarose gel electrophoresis of PCR products amplified with the primers ROS-109F and ROS+955R. Bacterial genomic DNA (50 ng) were used as template in each respective reaction. Lane 1: Invitrogen low DNA mass ladder (2 μl); Lane 2: *Roseovarius crassostreae* (CV919-312^T); Lane 3: *Roseovarius nubinhibens* (ISM^T); Lane 4: *Roseovarius denitrificans* (OCH114^T); Lane 5: *Roseovarius litoralis* (ATCC49566^T); Lane 6: *Roseovarius tolerans* (EL-172^T); Lane 7: *Ruegeria algicola* (ATCC51440^T); Lane 8: negative PCR control

Fig. 2. *Crassostrea virginica.* Agarose gel electrophoresis of PCR products from simulated swab samples prepared from unaffected specimens of *C. virginica.* Material was taken from either: (A) the inside surface of oyster valves and tissue surfaces, or (B) the inside surface of rinsed valves. Known numbers of *Roseovarius crassostreae* cells were then added to the samples, and the DNA was extracted and used as template for PCR reactions as described in 'Materials and methods'. Lane 1: Invitrogen low DNA mass ladder; Lane 2: sample with no additional cells; Lane 3: addition of 10^6 cells; Lane 4: addition of 10^5 cells; Lane 5: addition of 10^4 cells; Lane 6: addition of 10^3 cells; Lane 7: addition of 100 cells; Lane 8: addition of 10 cells; Lane 9: addition of 1 cell; Lane 10: positive PCR control (*R. crassostreae* DNA); Lane 11: negative PCR control (water)

In contrast, no products were amplified from these primers when genomic DNAs from other *Roseovarius* spp. or other closely related members of the *Roseobacter* clade were used as template (Fig. 1).

Sensitivity

A range in product band intensity was observed that directly reflected the number of *Roseovarius crassostreae* cells in each simulated swab sample (Fig. 2). In samples that contained material from the surfaces of host tissues, products were detectable with as few as 100 added cells per suspension (Fig. 2A). (The actual PCR reactions contain a fraction of the total DNA eluate, representing in this case the DNA from approximately 12.5 cells). However, when swabs contained only material from the inner valve surfaces, as few as 10 added cells per suspension resulted in detectable amplification products (Fig. 2B). In this case, the template would have contained the DNA from only about 1.2 cells.

Detection in naturally affected oysters

Samples from Maine oysters which showed no signs of JOD yielded no amplification products when assayed by PCR, nor were any *Roseovarius crassostreae* colonies recovered from these individuals (Fig. 3, Table 1).

Of 21 oysters from a population in Massachusetts (which was experiencing JOD-mortality) 16 exhibited overt signs of JOD, while the remaining 5 showed no such signs. *Roseovarius crassostreae* colonies were identified in all but 1 of the samples prepared from the 16 apparently affected individuals. The total numbers of colony forming units (CFUs) recovered from these oysters were also 10- to 1000-fold greater than those recovered from oysters without JOD-signs. Much of this increase was attributed to *R. crassostreae* which, when present, averaged 40% of the total recoverable
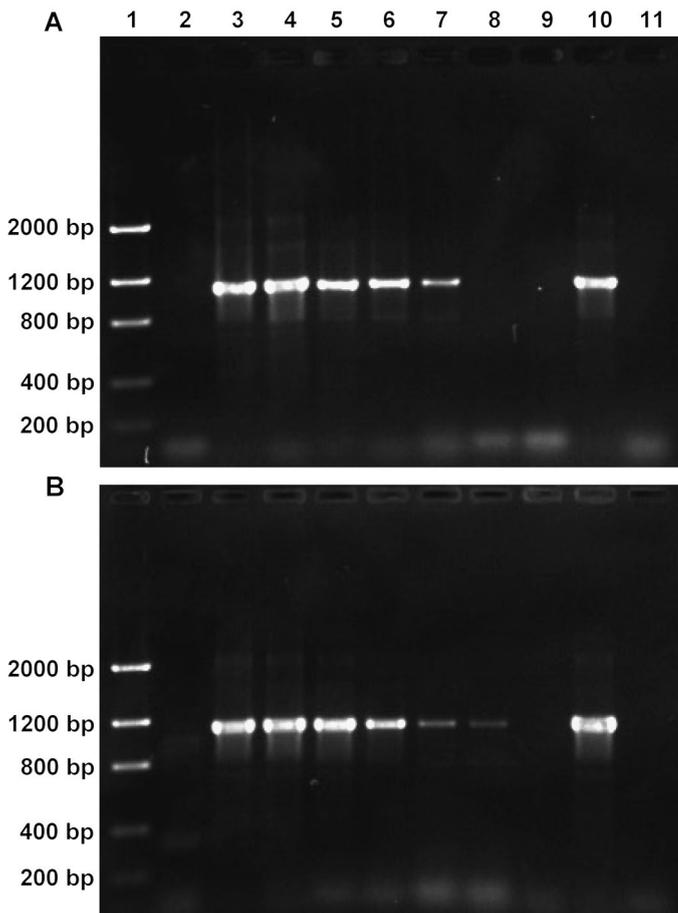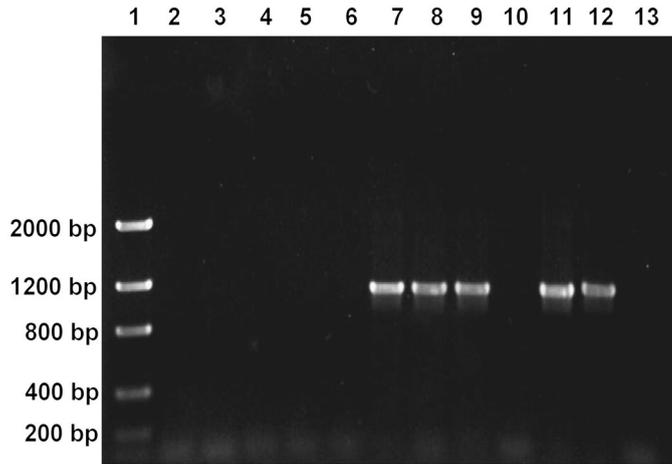
Fig. 3. *Crassostrea virginica.* Agarose gel electrophoresis of PCR products amplified from cultured *C. virginica*. An unaffected population (from Maine), and a group experiencing juvenile oyster disease (JOD)-mortality (from Massachusetts) were sampled. Lane 1: Invitrogen low DNA mass ladder; Lane 2–6: Maine oysters 1–5, respectively; Lanes 7–11: Massachusetts oysters 1–5, respectively; Lane 12: positive PCR control (*Roseovarius crassostreae* DNA); Lane 13: negative PCR control. Oyster 4 from Massachusetts (Lane 10) originated from an affected population, but showed no overt signs of JOD

bacterial community. Individuals with the most pronounced signs of JOD were usually the most heavily colonized (Table 1). When analyzed using the PCR assay, products of a size consistent with *R. crassostreae* were detected in all 16 of those individuals with obvious signs. The assay also indicated the presence of *R. crassostreae* in 1 of the 5 oysters that did not have overt signs of the disease. When analyzed using bacteriology, *R. crassostreae* was not recovered from this individual or from any of the remaining 4 oysters that did not have obvious JOD-signs (Table 1).

RFLP analysis of the ITS amplicons

Depending on the strain, *Ava*I restriction analysis of the PCR-amplified ITS region of *Roseovarius crassostreae*, yields 1 of 2 distinct patterns. The first genetic signature (GSI) is part of an 1100 bp amplicon and contains 2 *Ava*I sites. These result in *Ava*I restriction fragments of 430, 360 and 310 bp (Fig. 4). The other genetic signature (GSII) is identified by a 1071 bp amplicon and contains 1 *Ava*I site. Digestion of this product results in restriction fragments of 641 and

Table 1. *Roseovarius crassostreae* from *Crassostrea virginica.* PCR-based detection and recovery of *R. crassostreae* from JOD-affected (+: uneven value margins and conchiolin) and unaffected (–) field populations of *C. virginica*. ITS PCR: internal transcribed spacer PCR (+: 1071 or 1100 bp PCR product). GS: genetic signature. GS of each isolate determined by *Ava*I digestion. NA: not appliable. CFU: colony-forming units

| Location	Oyster	Signs of JOD	ITS PCR	GS	*R. crassostreae* recovered (CFU)[a]	Total CFU recovered[a]	Percent *R. crassostreae*
Maine	1	–	–	NA	0	5.5E+02	0
	2	–	–	NA	0	2.0E+02	0
	3	–	–	NA	0	3.5E+02	0
	4	–	–	NA	0	1.5E+03	0
	5	–	–	NA	0	5.0E+01	0
Massachusetts	1	+	+	II	1.8E+05	4.6E+05	38.0
	2	+	+	II	1.5E+04	1.9E+05	7.9
	3	+	+	II	2.5E+04	4.3E+05	5.9
	4	–	–	NA	0	4.8E+03	0
	5	+	+	II	1.6E+06	1.9E+06	86.5
	6	+	+	II	2.2E+05	2.3E+05	95.6
	7	–	–	NA	0	1.2E+04	0
	8	+	+	II	1.7E+06	2.7E+06	64.2
	9	+	+	II	2.5E+03	7.5E+03	33.3
	10	–	+	II	0	4.4E+04	0
	11	+	+	II	1.7E+05	2.5E+05	66.0
	12	+	+	II	3.5E+05	1.8E+06	20.0
	13	+	+	II	0	5.0E+02	0
	14	+	+	II	1.3E+06	3.4E+06	38.8
	15	+	+	II	1.0E+06	2.1E+06	47.6
	16	+	+	II	1.0E+02	6.0E+02	16.7
	17	+	+	II	2.0E+05	2.1E+06	9.5
	18	–	–	NA	0	6.4E+03	0
	19	+	+	II	3.5E+05	2.0E+06	17.5
	20	+	+	II	4.0E+05	9.0E+05	44.4
	21	–	–	NA	0	2.9E+03	0
[a]Cultured from swab samples							
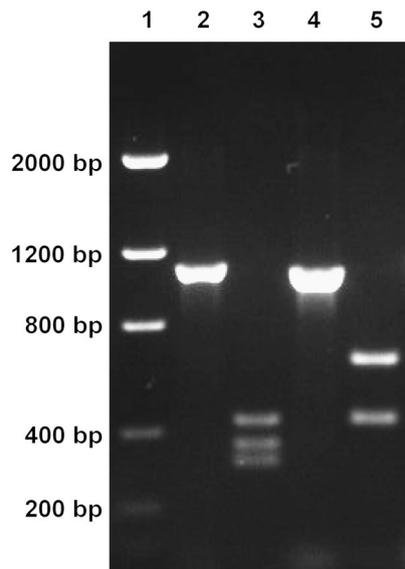
Fig. 4. *Roseovarius crassostreae.* Agarose gel electrophoresis of PCR-amplified *R. crassostreae* DNA (using the ITS primers) and subsequent *Ava*I restriction digests depicting each recognized genetic signature (GS). Lane 1: Invitrogen low DNA mass ladder; Lanes 2 and 3: a GSI PCR amplicon and restriction pattern, respectively (from *R. crassostreae* CV919-312[T]); Lanes 4 and 5: a GSII PCR amplicon and restriction pattern, respectively (from an isolate from Massachusetts oyster 2)

430 bp in size (Fig. 4). All reactions which were determined to be positive for *R. crassostreae* (on the basis of the presence and size of the amplification products) were verified as such, and simultaneously classified as GSI or GSII, by *Ava*I restriction analysis.

ITS sequence analyses

The DNA sequence of the PCR-products amplified from a simulated swab sample was confirmed to be identical to that of the inoculating strain (*Roseovarius crassostreae* CV919-312[T], a GSI isolate). In addition, sequence data was obtained for products that were amplified from swab samples of naturally affected oysters and from *R. crassostreae* isolates that were subsequently cultivated from these individuals. The sequences obtained by each method were identical, and the results confirmed the determination (arrived at by RFLP) that these were GSII strains.

DISCUSSION

The PCR assay described herein targets the 16S-23S rDNA ITS region of *Roseovarius crassostreae* and represents the first culture-independent assay for JOD diagnosis. We have demonstrated that the primer set

ROS-109F and ROS+955R provides the required specificity and excellent sensitivity. The specificity was established in tests using DNA from cultured bacteria closely related to *R. crassostreae*. In these assays, no cross-reactivity (i.e. no amplification) was observed with any tested member of the *Roseobacter* clade (Fig. 1). In addition, no products of a size similar to the *R. crassostreae* ITS were amplified from the heterogeneous DNAs present in material collected from unaffected oysters. Such material would have included any bacteria that remained associated with the internal valve surfaces following gentle rinsing. Thus, the primer set ROS-109F and ROS+955R is effective at directing amplification of only the *R. crassostreae* ITS region, even in samples containing potentially large amounts of non-target DNA.

Based on these results, an amplicon of 1071 or 1100 bp may be considered credible evidence for the presence of *Roseovarius crassostreae*. It is also important to note that non-specific products were never obtained from direct amplifications of material taken from oysters. In contrast, non-specific amplifications were sometimes observed when screening random colonies cultured from oysters, but such products were always of a size different than that expected for *R. crassostreae*. Still, this finding raises the possibility that the primers may amplify a DNA segment from some unknown, unrelated bacterium that would be close enough in size to be mistaken for *R. crassostreae*. Therefore, to test for the occurrence of such 'false positives', we conducted RFLP analyses on all amplicons produced in this study. As mentioned previously, 2 major sequence variations have been observed among *R. crassostreae* isolates, and these can be differentiated by digestion with the restriction enzyme *Ava*I (Fig 4). In every instance, samples identified as positive (based on amplification products of the predicted size) yielded 1 of the 2 expected fragment patterns when digested with *Ava*I. Some of these products were also directly sequenced, and their identity as *R. crassostreae* DNAs was thereby irrefutably established.

For this assay to be of maximum benefit, it must also be able to detect low numbers of the bacterium. Therefore, to determine the theoretical detection limit in oysters, we conducted a series of simulated infections. In these experiments, sensitivity was assessed using 2 parallel sampling approaches. Swab samples were either taken from the internal valve surfaces or from the surfaces of the internal valves and host tissues combined. The material was then resuspended and known numbers of *Roseovarius crassostreae* cells were added prior to DNA extraction and amplification (as described above). When samples contained only host material originating from the internal valve surfaces, a product was consistently amplified from samples con-

taining as few as 10 *R. crassostreae* cells (Fig. 2). Because this represented an approximate 10-fold increase in sensitivity compared to the second set of samples, it would appear that there is an inhibitory effect associated with the soft tissues. This effect was reproducible, and also noted to occur when extrapallial fluid was present in the sample (data not shown). The host tissue and associated mucus may contain enzymatic inhibitors or otherwise compromise the efficiency of the filter columns used in the initial DNA extractions.

Sampling of the internal valve surfaces not only prevents the introduction of potentially inhibitory substances, but also directly targets what is believed to be the site of *Roseovarius crassostreae* colonization. In experiments to culture *R. crassostreae* from the hemolymph, tissues surfaces, and interior valve surfaces of naturally affected oysters, significantly greater numbers of *R. crassostreae* were recovered from the interior valve surfaces (A. P. Maloy et al. unpubl. data). It is also relevant to note that past JOD-studies showed no obvious evidence of a pathogen infecting the tissues, but rather, bacteria were found associated with mantle lesions and trapped between layers of conchiolin (Bricelj et al. 1992, Perkins 1996, Ford & Borrero 2001, C. L. Boardman & K. J. Boettcher unpubl. data)

The method was further validated using oysters from a JOD-epizootic in Massachusetts. Amplification products were detected from all samples which, on the basis of culture results, were shown to contain *Roseovarius crassostreae* (Table 1). While it is impossible to determine the actual limit of sensitivity from these experiments, we know that it is at least as low as 100 per oyster (this was the lowest number recovered from any culture-positive sample) (Table 1). Using the PCR assay, *R. crassostreae* was also detected in 2 oysters (from the JOD-affected population) from which no *R. crassostreae* were recovered in culture. So it is possible that the sensitivity of the assay at least approaches the theoretical sensitivity as determined by the simulated infections.

In addition to diagnostic and screening applications, this assay holds promise for elucidating the pathogenesis of JOD. In earlier studies, mantle lesions and coccoid bodies were observed in oysters approximately 2 to 3 wk prior to the onset of gross JOD signs (Bricelj et al. 1992, Ford & Borrero 2001). Tissues of affected oysters also have a starved appearance, and colonization by *Roseovarius crassostreae* has been shown to result in decreased filter-feeding and subsequent mortality (Boettcher et al. 2000). Conchiolin formation has also been induced by exposure to *R. crassostreae* (Maloy & Boettcher 2003), but aside from these observations, little is known about the pathogenesis of JOD. In fact, a major obstacle in establishing *R. crassostreae* as the etiological agent of JOD was the fact that the bacteria had been isolated exclusively from populations of oysters that already showed signs of JOD. Then in 2004, we were able to document the presence of *R. crassostreae* in a population of Massachusetts oysters 1 to 2 wk before they developed JOD signs (A. P. Maloy & K. J. Boettcher unpubl. data). Access to a population of oysters prior to and throughout a JOD epizootic was unavailable for this study, but we are confident that the application of these techniques will more accurately define the period and conditions associated with the initial colonization process.

This basic methodology may also be adopted to screen environmental samples for the presence of *Roseovarius crassostreae*. Understanding both the origin of *R. crassostreae* in the environment and the timing and progression of colonization, will provide valuable information for the improvement of regional management schemes. Ultimately, selective breeding programs for the development of JOD-resistant lines are believed to offer the best long-term management strategy. Nevertheless, caution will always be warranted when moving seed oysters between locations. It is very likely that transfer of affected seed has been the cause of some JOD-epizootics in the past, and may also play a significant role in the introduction of *R. crassostreae* stains into new environments. The assay described herein for detection of the JOD-agent could be easily adapted for the purpose of 'certifying' seed oysters as *R. crassostreae*-free prior to relocation.

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