The Oyster Bacterial Biome: Who Else Is Inside the Shell?

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Introduction

A recent approach to the analysis of bacterial communities inhabiting various environments uses ribosomal RNA gene sequences (rDNA) to identify the various species present.[1] DNA is extracted, the rDNA genes are amplified by PCR, and the ribosomal amplicons are sequenced.[2] We applied this technique to a specialty food, the oyster, which contains heterogeneous populations of bacteria, some of which can be harmful to humans. *Crassostrea virginica*, the Eastern oyster, is the species most consumed in the United States (www.agmrc.org/aquaculture/profiles/oysterprofiles.pdf). The ecology of its microbial symbionts is not well studied, and published reports differ widely in the bacterial species found in oysters.[3-5]

EPICENTRE's MasterPure™ Plant Leaf DNA Purification Kit was used to extract DNA from *C. virginica*. The isolated DNA contains both oyster genomic DNA (shown by amplification of metallothionein genes) and DNA from a number of bacterial species in the oyster. Bacterial identification was made by amplifying 16S rDNA, cloning the PCR products using EPICENTRE's CopyControl™ PCR Cloning Kit, and sequencing the cloned genes. Eight species of gram-negative bacteria, some unidentified, were found. No known human pathogens were represented by the amplified rDNAs. However, an ampicillin-resistant *Pseudomonas* species was found.

Materials and Methods

DNA isolation

Mucopolysaccharides and glycogen in shellfish often make the isolation of nucleic acids difficult. The MasterPure Plant Leaf DNA Purification Kit effectively removes polysaccharides and isolates DNA from glycogen and carbohydrate-rich organisms, such as mollusks. *C. virginica* was harvested in New England and purchased in Madison, WI. Samples of 120 to 180 mg of freshly shucked oyster were macerated in 1.5-ml tubes using a plastic pestle and 300 µl of the kit's DNA Extraction Reagent. The remainder of the isolation was done as described in the kit literature, without organic solvents or enzymatic treatments. Yields were approximately 1 to 1.5 µg of DNA for each of four oyster tissue samples. Figure 1 shows DNA purified from the four samples. The lower part of the lanes shows eukaryotic, nucleosomal DNA bands.

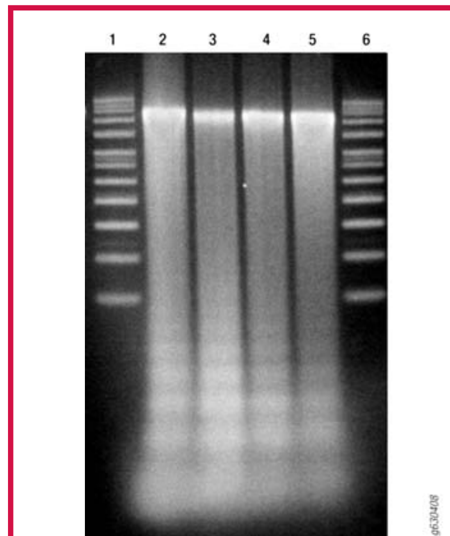

Figure 1. Oyster DNA was purified using the MasterPure™ Plant Leaf DNA Purification Kit. Aliquots from four preparations of oyster DNA were electrophoresed in a 1% agarose gel and stained with SYBR® Gold. Lanes 1 and 6, DNA ladder; Lanes 2 to 5, oyster DNA.

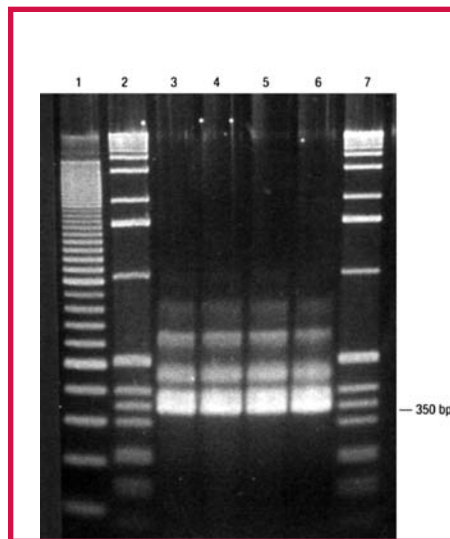

Figure 2. The 16S ribosomal DNA sequences from four oyster samples were amplified using the FailSafe™ PCR System and universal eubacterial primers. After amplification, 5 µl of each 50-µl reaction was assayed in a 2% agarose gel and stained with SYBR® Gold. Lane 1, 100 bp ladder; Lanes 2 and 7, kb ladder; Lanes 3 to 6, oyster DNA PCR products. The bands above the approximately 350-bp rDNA amplicons are probably heteroduplexes, as described in the text.

PCR amplification

16S rDNA sequences were amplified using 1 µl of oyster DNA, universal eubacterial primers[6] and the FailSafe™ PCR 2X PreMix B, which previously had been determined to be optimal with these primers and similar templates. Primers used were EB-L, 5′-CTG CTG CCT CCC GTA GGA GT-3′ and EB-R, 5′-AGA GTT TGA TCC TGG CTC AG-3′. Cycling conditions were: 96°C (2 minutes), followed by 28 cycles of 96°C (30 seconds), 55°C (30 seconds), and 72°C (45 seconds).

Figure 2 shows the 16S rDNA PCR products (approximately 350 bp) from the four DNA samples. Each lane contains additional, slower migrating amplicons, which are probably heteroduplexes arising from the annealing of rDNAs from different species. These heteroduplexes can be removed by a procedure called "PCR reconditioning".[7] If cloned, heteroduplexes can be repaired in *E. coli* after transformation, producing mosaic rDNA sequences.[7]

For oyster-specific genes, PCR primers were designed to amplify oyster metallothionein genes, which code for a family of detoxifying proteins.[8] Primers used were 5′-ATG TCT GAT CCA TGT AAC TGC ACT-3′ and 5′-TCA TTC AAG AAT TAG ATA TCG AGC T-3′. To quickly determine the optimum PCR reaction conditions for oyster genomic DNA and the metallothionein primers, reactions were set up with PreMixes from the FailSafe™ PCR PreMix Selection Kit, as directed. Cycling conditions were: 95°C (3 minutes), followed by 28 cycles of 95°C (30 seconds), 55°C (30 seconds), and 72°C (30 seconds), then 72°C (2 minutes).

Figure 3 shows PCR results from reactions set up with Premixes A through I from the FailSafe PCR PreMix Selection Kit. These primers amplified the oyster metallothionein genes from the purified genomic

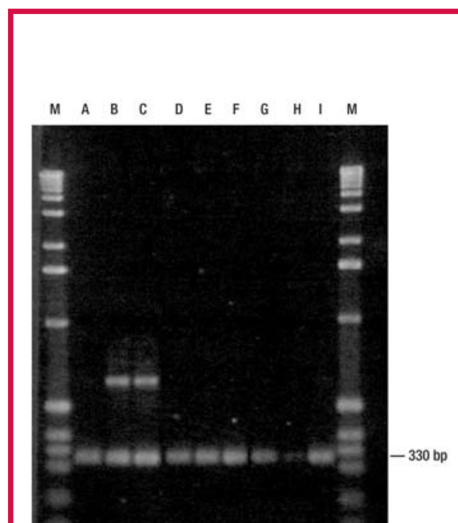

Figure 3. Oyster metallothionein genes were amplified using the FailSafe™ PCR PreMix Selection Kit. After amplification, PCR products were electrophoresed in a 2% agarose gel and stained with SYBR® Gold. Lanes A to I correspond to the FailSafe PreMixes used; Lanes M, kb marker.

DNA under all of the reaction conditions. PreMixes B and C gave additional products and PreMix H did not give optimal results, but any of the other PreMixes could be used with this template and these primers in future reactions.

PCR cloning

Amplified rDNAs were end-repaired and cloned into the CopyControl™ pCC1™ (Blunt Cloning-Ready) Vector as directed in the CopyControl PCR Cloning Kit. Ligations were electroporated into the kit's TransforMAX™ EPI300™ Electrocompetent *E. coli* and plated on LB plates containing 12.5 µg/ml of chloramphenicol and 1X CopyControl™ Induction Solution. Clones were grown under inducing conditions to increase the plasmid copy number and facilitate screening and plasmid purification. (For more information on the advantages of using the inducible CopyControl™ System to stably maintain clones at single copy, please see www.epicentre.com/ccpcr.asp.) Clones containing one to three copies each of the rDNA amplicons were selected using the Colony Fast-Screen™ (Size Screen) reagents included in the kit.

Sequencing and analysis of rDNAs

Selected clones were grown as described in the CopyControl PCR Cloning Kit. DNA was isolated, using either EPICENTRE's new FosmidMAX™ DNA Purification Kit or a spin-column plasmid isolation kit from another supplier, and sequenced. Sequencing results were consistently better with plasmids isolated using the FosmidMAX DNA Purification Kit than with the spin-column plasmid isolation kit (data not shown). Results were compared by BLAST analysis to known bacterial 16S rDNA sequences.

Results

Table 1 shows that of nine sequenced clones, eight are unique. Three clones represent *Pseudomonas sp.*, which are principal agents in the spoilage of fresh fish and shellfish, where they may inhibit the growth of other spoilage organisms.[9] The abundance of *Pseudomonas sp.* in oysters purchased in Wisconsin may reflect the length of time between their harvest and molecular analysis. *Shewanella sp.* have the interesting ability to reduce Fe III in the absence of oxygen, and have been found in many environments around the world. *S. baltica*, previously named *S. putrifaciens*, produces odorous compounds, such as trimethylamine, during fish spoilage.

Notably, 16S sequences of several clones (clones OB7, 14, 19, and 21) match the closest GenBank species by 94 to 98%, and may represent previously unidentified bacteria. Also, only gram-negative bacteria were found. Because the bacterial DNA was isolated without lysozyme treatment, gram-positive bacteria may have been present in the oyster, but not detected here. EPICENTRE offers the MasterPure™ Gram Positive DNA Purification Kit specifically for isolating DNA from hard-to-lyse gram-positive bacteria.

There are no pathogenic bacterial sequences in this set of rDNA genes. However, plating the surface bacteria from an oyster on antibiotic media revealed an ampicillin-resistant, culturable strain. No organisms resistant to 10 µg/ml tetracycline were found. The rDNA sequences were amplified from single colonies of the ampicillin-resistant strain and sequenced directly. The rDNA sequence is a 100% match with *Pseudomonas sp.* NZ101, isolated in New Zealand, which causes a disease in cultured mushrooms (Godfrey, S.A.C., Harrow, S.A., Marshall, J.W. and Klena, J.D., unpublished). Ampicillin-resistant bacteria are common in many rivers in the United States[10] and by inference might be found in river estuaries that support oysters.

Conclusion

EPICENTRE offers a wide variety of molecular tools for environmental DNA analysis, including the MasterPure Plant Leaf DNA Purification Kit, the

Table 1. Bacteria identified by rDNA sequences.

Clone	Closest database match*	% similarity	Putative phylum
OB1	*Flavobacterium sp.* A43	100	gram-negative
OB4	*Pseudomonas sp.* MSB2046	100	γ-proteobacteria, gram-negative
OB7	trout intestinal bacterium B76	97	gram-negative
OB12	*Shewanella baltica* OS155	100	γ-proteobacteria, gram-negative
OB14	*Pseudomonas Sp.* AU4899	94	γ-proteobacteria, gram-negative
OB17	*Shewanella baltica* CSQ1	100	γ-proteobacteria, gram-negative
OB19	trout intestinal bacterium B76	98	gram-negative
OB21	*Pseudomonas tolaasii* NZ032	98	γ-proteobacteria, gram-negative
OB26	eubacterium dtb25	99	gram-negative

*Gene assignment by BLAST searching the NCBI GenBank collection of bacterial 16S rDNA sequences.

CopyControl PCR Cloning Kit, FailSafe PCR PreMix Selection Kit, FosmidMAX DNA Purification Kit, and the MasterPure Gram Positive DNA Purification Kit.

References

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