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FEMS Microbiology Letters 160 (1998) 257–262

FEMS MICROBIOLOGY LETTERS

Putative environmental transmission of sulfur-oxidizing bacterial symbionts in tropical lucinid bivalves inhabiting various environments

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Received 22 September 1997; revised 23 January 1998; accepted 23 January 1998

Abstract

Four tropical lucinids, *Codakia orbiculata*, *C. pectinella*, *Linga pensylvanica*, which inhabit sea-grass beds, and *Lucina pectinata*, which inhabits mangrove swamps, harbor sulfur-oxidizing endosymbiotic bacteria within bacteriocytes of their gill filaments. To elucidate the symbiont transmission mode in these bivalves, symbiont-specific oligonucleotides were designed and used in polymerase chain reaction amplifications (PCR). For all species investigated, each primer set was unsuccessful in amplifying symbiont DNA targets from ovaries and testis, whereas successful amplifications were obtained from symbiont-containing gill tissue. These data suggest that the transmission mode is environmental, independently of the lucinid habitat, as it is in the other tropical lucinid *Codakia orbicularis*.

Keywords: Bivalvia; Sulfur-oxidizing bacterium; Endosymbiont; Transmission mode; PCR; 16S rRNA gene

1. Introduction

Symbioses between sulfur-oxidizing bacteria and marine invertebrates, first discovered in deep-sea hydrothermal vent fauna [1,2], are known to be distributed across at least seven host taxa in the marine environment [3–9]. The greatest diversity of intracellular thioautotrophic symbioses is found among Bivalvia [6,7]. In all the species studied so far, these associations appear obligatory, specific, and stable geographically and over the time [10,11]. All symbiotic associations must maintain their symbiont host relationships over successive generations by using selective mechanisms that ensure successful symbiont inheritance by the new host generation. This could be achieved by an individual host through one of three ways: (i) by vertical transmission – from parents to offsprings – which may include incorporation of symbionts in or on the gametes of the host parent [12], (ii) by horizontal transmission which involves infection of aposymbiotic individual from symbiotic individual [13], and (iii) by environmental transmission which involves the infection of the new host generation from an environmental stock of free-living symbiont form [14].

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0378-1097/98/$19.00
PII S0378-1097(98)00041-X

To date, the transmission mode of sulfur-oxidizing endosymbionts in marine invertebrates was elucidated for only few species, due to the difficulty to cultivate both symbionts and hosts. Environmental transmission mode is strongly suggested in two oligochaetes [15] and in two vestimentiferans [16]. In bivalves, the eight species studied harbor gill endosymbionts which are vertically transmitted [16–19], except the tropical lucinid *Codakia orbicularis* in which symbionts are environmentally transmitted to the new host generation [20].

For a better understanding of the transmission mode in lucinid-bacterial associations, four tropical lucinid species were analyzed. Three of them, *Codakia orbiculata*, *C. pectinella*, and *Linga pensylvanica* inhabit the same type of shallow water sea-grass beds (*Thalassia testudinum* environment) as *C. orbicularis*. Phylogenetic studies based on 16S rDNA analysis have shown that these lucinid species are colonized by the same symbiotic bacterium [10,21]. On the other hand, *Lucina pectinata*, which lives in black reducing mud in mangrove swamps, harbors a distinct bacterial symbiont appearing as the most divergent microorganism of the Lucinacea cluster [10].

These four bivalves remain uncultivable as induced spawning and fertilization of stripped oocytes remain unsuccessful. Therefore, unlike for *C. orbicularis* [22], eggs, veligers (D-larvae), and juveniles could not be analyzed. Species-specific oligonucleotide primers, able to detect symbiont-specific target sequences at low-copy number within mixture of symbiont host genomic DNA, were designed using 16S rDNA sequences from lucinid symbionts.

The present study focuses on the bacterial endosymbiont detection in the gonads of four tropical lucinids, whose larval developments are unavailable, by amplifying symbiont target DNA.

2. Materials and methods

2.1. Specimen collection

Adult specimens were collected during their period of gonad repletion, from sea-grass beds in Guadeloupe for *C. orbiculata* and *C. pectinella*, and in Martinique for *L. pensylvanica*. *L. pectinata* was collected from mangrove swamps in Guadeloupe. Specimens were kept alive in 5 μm-filtered and UV-treated sea water until DNA extraction, which was performed few hours after collection.

2.2. DNA preparation

Total DNA was extracted from symbiont-containing gill tissues and *Vibrio alginolyticus* as described elsewhere [10,21]. Following the recent study on the gill endosymbiont transmission mode in *C. orbicularis* [20], DNA was extracted from gonad tissues by using the Isoquick Nucleic Acid Extraction kit (ORCA Research Inc., Bothell, USA) according to the manufacturer's instructions. Samples of DNA were independently obtained from eight specimens (four males and four females) for each lucinid species with the exception of *L. pensylvanica*, of which only two specimens of each sex were available. DNA extraction from ovaries was performed from samples of 50 mm^3 corresponding to $\sim$10 000 oocytes. Before PCR amplification, DNA concentration and purity in each sample were determined spectrophotometrically.

2.3. Oligonucleotide primers

Computer-assisted analysis of available bacterial 16S rDNA sequences identified one region that was specified to the *L. pectinata* symbiont. This sequence was used as PCR reverse primer SymLp (5′-TCGCAGGTTCGCTACCGT-3′; *Escherichia coli* 1252 to 1269 [23]) with the bacterial-specific primer 27f [24]. The primer set Symco1-1492r described elsewhere [20] was used for PCR detection of *C. orbicularis* symbiont and consequently of *C. orbiculata*, *C. pectinella* and *L. pensylvanica* symbiont.

2.4. PCR conditions

PCR amplification was performed in a 25 μl reaction volume using 200 mM of each dNTP, 2.5 μl of 10× reaction buffer (Bioprobe Systems, Montreuil, France), 25 pmoles of each primer, 0.5 unit of Hi Taq™ DNA polymerase (Bioprobe Systems, France), and 25 ng of DNA. Samples were amplified using a DNA Thermal cycler 480 (Perkin Elmer Inc., Norwalk, USA) under the following conditions: initial denaturation at 94°C for 8 min followed by 25 cycles

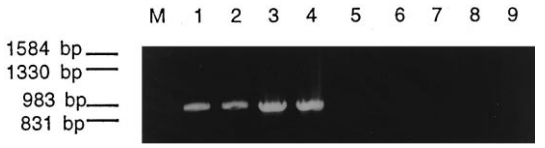

Fig. 1. Specificity of the *C. orbicularis* primer set. The *C. orbicularis* primer set was used in amplifications of genomic DNA extracted from a variety of non-homologous templates. Lanes: M, DNA marker; 1, *C. orbicularis* gill; 2, *C. orbiculata* gill; 3, *C. pectinella* gill; 4, *L. pensylvanica* gill; 5, *C. costata* gill; 6, *L. pectinata* gill; 7, *L. nassula* gill; 8, *V. alginolyticus*; 9, negative control.

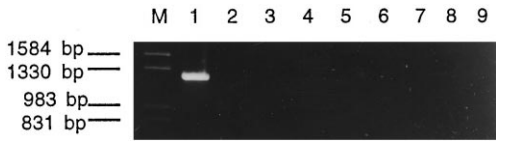

Fig. 2. Specificity of the *L. pectinata* primer set. The *L. pectinata* primer set was used in amplifications of genomic DNA extracted from a variety of non-homologous templates. Lanes: M, DNA marker; 1, *L. pectinata* gill; 2, *C. orbicularis* gill; 3, *C. orbiculata* gill; 4, *C. pectinella* gill; 5, *L. pensylvanica* gill; 6, *C. costata* gill; 7, *L. nassula* gill; 8, *V. alginolyticus*; 9, negative control.

of denaturation at 94°C for 1 min, annealing at 50°C (Symco1) or 60°C (SymLp) for 1 min and extension at 72°C for 1.5 min, followed by a final elongation time of 7 min at 72°C. Five microliters of each PCR sample were used for a second amplification under the same conditions. PCR products were examined by horizontal electrophoresis in 0.8% agarose gel.

The specificity of the primer sets was determined in a series of amplification reactions (25 cycles) with a variety of non-homologous templates extracted from a bacterial strain and from symbiont-containing gill tissue of other tropical lucinids. The sensitivity of the primer sets to detect target sequences in a mixture of prokaryotic and eukaryotic DNA was determined through a series of amplification reactions (2×25 cycles) in which host symbiont template concentrations were titrated from 1 ng to 1 pg at 10-fold dilutions.

3. Results and discussion

PCR amplification (25 cycles) performed with the primer set (Symco1-1492r) produced an expected single band (∼870 bp) from DNA extracted from *C. orbicularis*, *C. orbiculata*, *C. pectinella* and *L. pensylvanica* gills, whereas no amplification products were detected with DNA extracted from *Codakia costata*, *Lucina nassula* and *L. pectinata* gills, from symbiont-free tissues (negative controls) and from *V. alginolyticus* (Fig. 1). On the other hand, the *L. pectinata* primer set (27f-SymLp) produced an expected single band (∼1200 bp) only from DNA extracted from *L. pectinata* gills (Fig. 2). Thus, both primer sets (Symco1-1492r and 27f-SymLp) are specific to the target organism and give the expected results based on the previously obtained 16S rDNA sequences [10,11,21]. The detection limit of both PCR tests (2×25 cycles) was below 10 pg of target DNA in a mixture of eukaryotic and prokaryotic DNA extracted from symbiont-containing host tissues (Fig. 3). The genomic DNA of a bacterium is about 10 fg, and we worked with a mixed genomic DNA for which the ratio prokaryotic DNA/total (eukaryotic and prokaryotic) DNA was unknown, but undoubtedly decreases the detection level under 10^3 cells. For the *C. orbicularis* primer set, this detection limit was not improved even after Southern blotting and hybridization with a specific probe [20].

Repeated attempts to amplify the symbiont DNA target (2×25 cycles) from DNA extracted and purified from ovaries and testis were unsuccessful (Fig. 4). Addition of the target DNA (25 ng of mixed DNA extracted from symbiont-containing gill tissue) produced successful amplification and hence rules

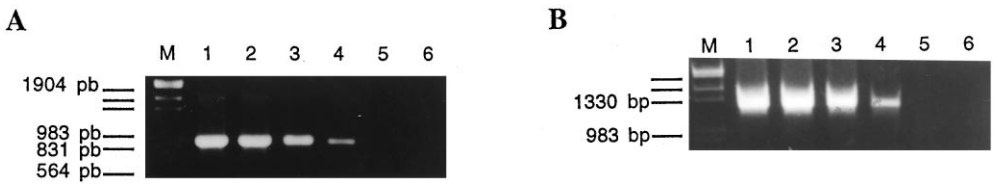

Fig. 3. Detection sensitivity for A: *C. orbicularis* and B: *L. pectinata* primer sets. Template DNA was titrated from 1 ng to 1 pg at 10-fold dilutions. Lanes: M, DNA marker; 1, 10 ng; 2, 1 ng; 3, 100 pg; 4, 10 pg; 5, 1 pg; 6, negative control.

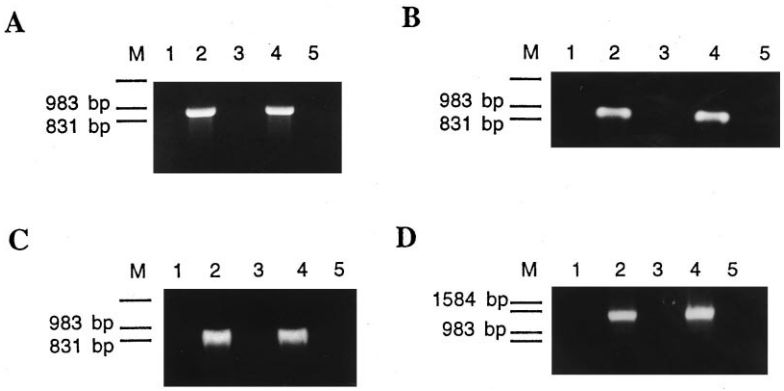

Fig. 4. PCR detection of symbiotic DNA target from male and female gonads of *C. orbiculata* (A), *C. pectinella* (B), *L. pensylvanica* (C), and *L. pectinata* (D). Lanes: M, DNA marker; 1, ovary; 2, ovary+25 ng of gill DNA; 3, testis; 4, testis+25 ng of gill DNA; 5, negative control.

out the hypothesis of PCR inhibition (Fig. 4). Comparable PCR experiments have been performed with vertically transmitted symbionts and no problem of detection occurred in the bivalve gonads with template concentration identical or below those we used [17,19]. Moreover, the detection levels of the primer sets used in this study are of the same order of magnitude as those previously described [17,20]. In the case of *C. orbicularis*, juveniles reared in sterile conditions did not host symbiont although juveniles issued from the same spawn but cultivated in unsterilized sea-grass sand were infected [20]. These facts refute the endospore-like stage hypothesis in this species. The early life stages of the four bivalves investigated in this study are not available for developmental studies, hence did not permit to formally negate the hypothesis of an endospore-like stage which could be present in oocytes or in sperm, and be resistant to nucleic acid extraction and to the PCR conditions. However, like in *C. orbicularis* [20], TEM observation of thin sections from mature ovaries and testis of *L. pectinata* supports these PCR results as no cryptic form of bacteria were observed either in oocytes, in follicle cells [25], and in (or on) sperm (Mouëza, unpublished data). Hence, the fact that no amplification was obtained from ovaries and testis for the four species strongly suggests that gametes, and the different cells constituting gonads as follicle cells in ovaries, do not contain endosymbiotic bacteria.

C. orbiculata, *C. pectinella*, and *L. pensylvanica*, which live in low sulfide sea-grass beds, are colonized by the same sulfur-oxidizing bacterium as *C. orbicularis* [10,21] in which gill endosymbionts are environmentally transmitted to the new host generation [20]. The lack of amplification of symbiotic DNA target from gonads in the three species inhabiting the same environment as *C. orbicularis*, strongly suggests that this symbiotic bacterium is also environmentally transmitted in these three lucinid hosts. The lack of juveniles reared either in sterile or unsterilized sediment, does not permit to initiate symbiosis by environmental symbiosis-competent bacteria. Nevertheless, a set of facts strongly supports the conclusion that the symbiont transmission mode in the four lucinids investigated in this study and inhabiting various environment, is environmental as it is in the other tropical lucinid *C. orbicularis* [20].

Moreover, recently we have PCR amplified specific target DNA of (i) the free-living symbiont form of *C. orbicularis* from sea-grass sediment and (ii) the free-living symbiont form of *L. pectinata* from mangrove mud (Gros et al., unpublished data). These new results strengthen the hypothesis of an environmental symbiont transmission mode for these four lucinids.

The knowledge of the transmission mode of the *L. pectinata* gill endosymbiont provides significant information. This bivalve is an unusual species in comparison to the other lucinids: it is the most abundant species living deeply burrowed in black sulfide mud of mangrove swamps and the most resistant to anaerobic conditions and H_2S accumulation. Moreover, its bacteriocytes have remarkable cytological features like an unusual high concentration of cytoplasmic

hemoglobin [26], and its symbiont appears as the most divergent microorganism from the cluster of Lucinacea symbionts [10]. These features are important enough to suppose metabolic pathways in the symbiont and the host to be quite different in *L. pectinata* than in other lucinid symbiont association. However, its symbiont transmission mode seems to be horizontal or environmental like in lucinids inhabiting low sulfide environment. Thus, we can allow for supposition that, at least in Bivalvia, the transmission mode of endosymbionts could be specific to a family as environmental in Lucinidae ([20], this study), and vertical in Solemyidae [17,18] and Vesicomyidae [16,19] independently of environmental conditions or host metabolic pathways. Nevertheless, more investigations are needed to check this hypothesis, notably studies on the gill endosymbiont transmission mode of other temperate or deep-sea lucinids and of thyasirids, the other family of Lucinacea in which some members harbor sulfur-oxidizing gill endosymbionts.

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