

Genetic characterization of the lobster pathogen *Aerococcus viridans* var. *homari* by 16S rRNA gene sequence and RAPD

Spencer J. Greenwood*, Ian R. Keith, Béatrice M. Després, Richard J. Cawthorn

AVC Lobster Science Centre, Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, Prince Edward Island C1A 4P3, Canada

ABSTRACT: A combination of 16S rRNA sequencing and random amplified polymorphic DNA (RAPD) analysis was used to evaluate the genetic diversity within *Aerococcus viridans* var. *homari*, the causative agent of gaffkemia in lobsters. A collection of 7 *A. viridans* var. *homari* strains and 2 avirulent *A. viridans*-like cocci isolated from homarid lobsters harvested from different regions on the Atlantic Coast of North America were analyzed. The isolates are separated geographically and temporally between the years 1947 and 2000. Sequencing of 16S rRNA genes confirmed the inclusion of all 9 isolates in the monophyletic *A. viridans* clade (99.8 to 100 % similarity). RAPD analysis revealed that the 9 *A. viridans* var. *homari* isolates could be separated into 2 distinct subtypes. Subtype 1 included the 7 pathogenic lobster isolates and constituted a homogeneous group regardless of their geographical, temporal or virulence differences. Subtype 2 contained the 2 avirulent *A. viridans*-like cocci that had distinct RAPD patterns and clustered separately with the non-marine *A. viridans*. RAPD analysis represented a useful method for determining molecular subtyping for the intraspecific classification and epidemiological investigations of *A. viridans* var. *homari*.

KEY WORDS: Lobster · *Homarus americanus* · Gaffkemia · *Aerococcus viridans* var. *homari* · RAPD · 16S rRNA

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INTRODUCTION

Aerococcus viridans var. *homari* is the causative agent of gaffkemia, a systemic disease of homarid lobsters (*Homarus americanus* and *H. gammarus*) found in the coastal waters of both North America and Europe (Wood 1965a, Stewart et al. 1966, Audouin & Légli 1971). However, it is within the commercial lobster industry where the major economic impact of gaffkemia occurs, because the disease contributes significantly to post-harvest losses (Stewart 1980). Gaffkemia was first described in dying American lobsters from a holding unit in Maine in 1947, and subsequently the bacterium *A. viridans* var. *homari* (originally named *Gaffkya homari*) was isolated and Koch's postulates fulfilled (Hitchner & Snieszko 1947, Snieszko & Taylor 1947). Subsequently, considerable

research effort has documented the biology of *A. viridans* var. *homari* and the impact of gaffkemia on the commercial lobster industry (reviewed by Stewart 1993).

Aerococcus viridans var. *homari* enters the lobster through breaks in the integument caused by fighting, overcrowding and poor handling practices after capture and during transport (Stewart 1980). Typically, after the lobster's exoskeleton is breached, a fatal septicemia ensues as virulent strains of *A. viridans* var. *homari* appear resistant to the lobster's innate immune response (Stewart 1980). Nevertheless, virulent strains of *A. viridans* var. *homari* maintained *in vitro* lose the capacity to cause disease in lobsters upon re-infection (Stewart 1984, Stewart et al. 2004). Although the exact mechanisms of virulence and its subsequent loss through continuous passage *in vitro* are unknown, it

*Email: sgreenwood@upepei.ca

appears to be correlated with the presence and thickness of the polysaccharide capsule (Stewart 1984, Stewart et al. 2004).

On rare occasions, avirulent *Aerococcus viridans*-like cocci have been isolated from lobsters (Wood 1965b, Stewart et al. 1966, Rabin & Hughes 1968, Stewart et al. 2004). These cocci share many phenotypic characteristics with virulent *A. viridans* var. *homari* but were unable to cause disease in lobsters (Stewart et al. 1966). Previous genotypic characterizations using DNA-DNA hybridization indicated that *A. viridans* var. *homari* was sufficiently similar to be placed in the same taxon as environmental and human clinical isolates of *A. viridans* (Kelly & Evans 1974, Wiik et al. 1986, Bosley et al. 1990). However, the genetic relationship of the avirulent *A. viridans*-like cocci to the lobster pathogen *A. viridans* var. *homari* has remained unresolved (Wiik et al. 1986).

The application of molecular subtyping methods that allow for the characterization of genetic diversity within bacterial species and strains has shown considerable value for the study of intraspecific classification and for epidemiological investigations. The ability to differentiate subtypes is important for the recognition of disease outbreaks, the determination of sources of infection, the detection of particularly virulent strains and host distribution and geographical origin of possible variants of a specific pathogen (Olive & Bean 1999, Soll 2000). The available molecular methods used for subtyping differ widely in their ability to differentiate among strains (Soll 2000). The comparison of 16S rRNA gene sequences has been recognized as an invaluable tool for confirming bacterial species identity but not for differentiating among strains, since the sequence shows limited intraspecific variation (Bottger 1989, Olsen & Woese 1993, Drancourt et al. 2000). In contrast, randomly amplified polymorphic DNA (RAPD) analysis is a sensitive PCR-based method that uses arbitrary primers to generate whole genome DNA fingerprints for discriminating between strains (Welsh & McClelland 1990, Williams et al. 1990). The recent advent of standardized and optimized reagents has greatly improved the accessibility and reproducibility of the RAPD method (Hyytiä et al. 1999). These improvements led to an increase in the number of successful applications of the RAPD method for differentiating strains of marine bacterial pathogens (Romalde et al. 1999, 2002, Magariños et al. 2000, Ravelo et al. 2003).

In this study, a combination of 16S rRNA sequencing and RAPD analysis was used to characterize the genetic diversity within *Aerococcus viridans* var. *homari*. A collection of 7 *A. viridans* var. *homari* strains and 2 avirulent *A. viridans*-like cocci isolated from homarid lobsters harvested from different regions on

the Atlantic Coast of North America were analyzed. The isolates are separated geographically and temporally between the years 1947 and 2000. The usefulness of RAPD analysis for intraspecific classification and epidemiological studies of *Aerococcus viridans* var. *homari* was evaluated.

MATERIALS AND METHODS

Bacterial strains. *Aerococcus viridans* var. *homari* strains included in this study (Rabin, NVI 1030 and NVI 1032) and 2 *A. viridans*-like cocci (37R and 88B) were kindly provided by Dr. J. E. Stewart, (Department of Fisheries and Ocean, Bedford Institute of Oceanography, Dartmouth, NS, Canada). In addition, *A. viridans* var. *homari* strains (ATCC 10400 and 29838) and a non-marine *A. viridans* isolate (ATCC 700406) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Two purified *A. viridans* var. *homari* isolates (Newfoundland and Maine) derived from lobster pathology cases submitted to the Atlantic Veterinary College from commercial holding pounds were also included (Table 1).

The strains were routinely cultured on tryptic soy agar (TSA, Oxoid) at 28°C for 24 h. Stock cultures were stored at –80°C in trypticase soy broth (TSB, Oxoid) supplemented with 15% (v/v) glycerol.

Phenotypic characterization. Standard identification of aerococci includes Gram-reaction, cell morphology and arrangement, motility, catalase, hemolysis, hydrolysis of hippurate and esculin, deamination of arginine, utilization of pyruvate and sensitivity to 30 µg vancomycin disc (Facklam & Elliott 1995). Growth was assessed at 10°C, 45°C, and at pH 9.6 (Facklam & Elliott 1995). Other physiological tests recommended are salt (6.5 and 10% NaCl), bile (40%) and tellurite (0.04%) tolerance (Facklam & Elliot 1995). Glucose oxidation/fermentation followed (Lemos et al. 1985), while additional carbohydrate fermentations (fructose, galactose, maltose, melibiose, sucrose, glycerol, sorbitol, and inulin) were tested by conventional tube test (Facklam & Elliott 1995). L-arabinose, D-mannitol, D-lactose, D-raffinose, and glycogen were tested by the Analytical Profile Index (API) 20 Strep product (Fertally & Facklam 1987). The isolates were further tested for production of 1-pyrrolidonyl-β-arylamidase (PYR), leucine aminopeptidase (LAP) (Bosley et al. 1990) and other enzymes by the API 20 Strep and API ZYM products (BioMérieux). Additionally, the production and detection of acetoin (MacFaddin 1980, Fertally & Facklam 1987), nitrate reduction (MacFaddin 1980) and gelatin liquefaction (Facklam 1972) were evaluated.

Each isolate in the study was passed on TSA (Oxoid) at 28°C at least 3 times before serving as seed inocu-

lum for broth or agar plate growth for phenotypic tests. Twenty-four hour growth on TSA (Oxoid) or Columbia blood agar (BA) (Oxoid), supplemented with 5 % defibrinated sheep's blood, were the inocula for the API and heart infusion broth-based tests, respectively. Appropriate positive and negative control isolates used were as described by Cowan et al. (1993).

Genomic DNA extraction. Bacterial colonies were picked from TSB plates for inoculation of liquid media. Bacteria were grown in TSB supplemented with 3 % (w/v) NaCl at 28°C for 24 h. DNA was extracted using the GenElute™ Bacterial Genomic DNA kit (Sigma-Aldrich) supplemented with mutanolysin (250 U l⁻¹, Sigma-Aldrich). DNA concentration was determined both spectrophotometrically and by an ethidium bromide-spot assay (Sambrook et al. 1989). Quality of the genomic DNA was assessed by electrophoretic separation in a 0.8 % agarose gel containing 0.5 µg ml⁻¹ ethidium bromide.

PCR amplification and sequencing of 16S rRNA genes. The 16S rRNA gene of all 10 bacterial isolates was amplified using modified versions of the universal bacterial primers BSF-8/20 (5'-AGAGTTTGATCCTGGCTCAG-3'), positions 8 to 27 in the *Escherichia coli* 16S rRNA gene and BSR-1541/20 (5'-AAGGAGGTGATCCAGCCGCA-3'), *E. coli* positions 1541 to 1522 (for both primers the polylinker regions were removed) (Weisburg et al. 1991, Daly et al. 1993, Wuyts et al. 2002). Approximately 10 ng of bacterial genomic DNA was amplified in a 50 µl reaction containing 10 pmol of each primer BSF-8/20 and BSR-1541/20 in the presence of the following reagents (Fermentas International), 200 µM of each dNTP (A,G,C and T), 1.5 mM MgCl₂, 1 × PCR buffer (10 mM Tris, pH 8.8, 50 mM KCl and 0.08 % Nonidet P40) and 1.25 U of Taq DNA polymerase. A negative control, consisting of the same

reaction mixture but with molecular biology grade water (Sigma-Aldrich) instead of template DNA, was included in each run. The amplification protocol was carried out in a MJ Research PTC-200 thermal cycler (MJ Research) under the following conditions: an initial denaturation at 94°C for 2.5 min, followed by 25 cycles consisting of denaturation at 94°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for 1.5 min. Final extension was at 72°C for 5 min. The amplified DNA was purified using a GenElute™ PCR clean-up kit (Sigma-Aldrich) and separated by electrophoresis in a 0.8 % agarose gel containing 0.5 µg ml⁻¹ of ethidium bromide. Direct sequencing of purified PCR products was performed on an ABI Prism 377 sequencer using Big-Dye™ terminators (Applied Biosystems) at the Guelph Molecular Supercentre (Laboratory Services Division, University of Guelph, ON, Canada). Sequencing primers were derived from a 16S rRNA primer collection at the European Ribosomal RNA Database (<http://oberon.fvms.ugent.be:8080/rRNA/index.html>, Wuyts et al. 2002). Forward sequencing primers used were BSF-8/20 and BSF-784/16 (5'-AGGATTAGATACCCTG-3') *E. coli* positions 784 to 799 and reverse sequencing primers BSR-1541/20 and BSR-799/16 (5'-CAGGGTATCTAATCCT-3') *E. coli* positions 799 to 784.

The 16S rRNA sequences were assembled by manual alignment using Bio-Edit (Hall 1999) incorporating the published *Aerococcus viridans* 16S rRNA sequence (ATCC 1156, GenBank accession number M58797) as a guide. To ensure homogeneous single-species sequence information prior to phylogenetic reconstruction, all 16S rRNA sequence electropherograms were carefully examined to ensure that only single peaks and therefore single species DNA was amplified and sequenced (Drancourt et al. 2000). Sequences were further screened for

Table 1. *Aerococcus viridans* isolates used in the present study

Isolate	Identification	Origin	Location, Year of isolation	Virulence status ^a
ATCC 10400 ^b	<i>A. viridans</i> var. <i>homari</i>	<i>Homarus americanus</i>	Maine, USA, 1947	Avirulent
ATCC 29838 ^c	<i>A. viridans</i> var. <i>homari</i>	<i>Homarus americanus</i>	England	Virulent
Rabin	<i>A. viridans</i> var. <i>homari</i>	<i>Homarus americanus</i>	Massachusetts, USA, 1963	Virulent
Newfoundland	<i>A. viridans</i> var. <i>homari</i>	<i>Homarus americanus</i>	Newfoundland, Canada, 1991	Virulent
Maine	<i>A. viridans</i> var. <i>homari</i>	<i>Homarus americanus</i>	Maine, USA, 2000	Virulent
NVI 1030 ^d	<i>A. viridans</i> var. <i>homari</i>	<i>Homarus americanus</i>	NVI, Norway, 1977	Virulent
NVI 1032 ^d	<i>A. viridans</i> var. <i>homari</i>	<i>Homarus americanus</i>	NVI, Norway, 1977	Virulent
37R	<i>A. viridans</i> -like coccus	<i>Homarus americanus</i>	Prince Edward Island, Canada, 1964	Avirulent
88B	<i>A. viridans</i> -like coccus	<i>Homarus americanus</i>	Nova Scotia, Canada, 1962	Avirulent
ATCC 700406 ^e	<i>A. viridans</i>	Environment	Not reported	Avirulent

^aVirulence status at time of the present study
^bOriginal isolate of Sniesko & Taylor (1947), virulent at time of isolation
^cOrigin likely North America (J. E. Stewart pers. comm.)
^dNational Veterinary Institute, Norway—infected lobsters imported from St. Lawrence area, Canada, 1977
^eBioMerieux Vitek, Inc. (API 78-12-095)—quality control strain for API products

the presence of chimeric sequence using the programs Chimera Check (Maidak et al. 1999, Cole et al. 2003) and Bellerophon (Hugenholtz & Huber 2003).

16S rRNA phylogenetic analysis. The following sequences were obtained from GenBank: *Aerococcus viridans* (accession no. M58797) (Woese et al. unpubl.), uncultured feedlot manure bacterium B87 (AF317386) (Ouwerkerk & Klieve unpubl.), *Aerococcus* sp. LV 65.5:W1 (AF076639) (Bratina et al. 1998), *Pediococcus urinaeequi* (D87677) (Nageshima unpubl.), bacterium B16S (AF300974) (Allen et al. 2001), *Aerococcus urinae-hominis* (AJ278341) (Lawson et al. 2001a), *Aerococcus christensenii* (Y17005) (Collins et al. 1999), *Aerococcus urinae* (M77819) (Aguirre & Collins 1992), *Aerococcus sanguicola* (AJ276512) (Lawson et al. 2001b), *Facklamia hominis* (Y10772) (Collins et al. 1997).

Sequences were analyzed with programs in the Ribosomal Database Project-II (RDP-II) (Maidak et al. 1999, Maidak et al. 2001, Cole et al. 2003). Sequences were aligned using the conserved 16S rRNA secondary structure. Phylogenies were reconstructed using PHYLIP (Felsenstein 1993) with a similarity matrix computed by the method described by Kimura (1980) and a dendrogram via neighbor-joining (Saitou & Nei 1987). The constructed *Aerococcus viridans* RDP-II alignment was then imported into MEGA-2 (Molecular Evolutionary Genetics Analysis-2) (Kumar et al. 2001) software for phylogenetic analysis using neighbor-joining (Kimura 2-parameter with gaps and missing data handled by complete deletion) and maximum parsimony. Statistical evaluation of phylogenetic tree branching order was bootstrap resampled 1000 times (Felsenstein 1985). Phylogenetic trees were visualized using TreeExplorer (Kumar et al. 2001) and TreeView (Page 1996).

RAPD analysis. RAPD analysis was performed using Ready-To-Go RAPD Analysis Beads™ (Amersham Biosciences). These commercial beads have been optimized for PCR amplifications and contain thermostable polymerase (Ampli-Taq™ DNA polymerase and Stoffel fragment), dNTPs (0.4 mM each), BSA (2.5 µg), and buffer (3 mM MgCl₂, 30 mM KCl and 10 mM Tris, pH 8.3, in a 25 µl reaction volume). Twenty-five pmol of the selected arbitrary primer, 50 ng of template DNA and molecular biology grade water was added to achieve the 25 µl reaction volume.

The amplification was performed in a MJ Research PTC-200 thermal cycler (MJ Research) under the following conditions; an initial denaturation at 95°C for 5 min, followed by 45 cycles consisting of denaturation at 95°C for 1 min, annealing at 36°C for 1 min, and extension at 72°C for 2 min, with a final

extension at 72°C for 5 min. The RAPD products were electrophoresed in a 1.5% agarose gel containing 0.5 µg ml⁻¹ of ethidium bromide and a DNA molecular weight marker, GeneRuler 100 bp DNA Ladder Plus (Fermentas International) was used to determine RAPD fragment sizes. All agarose gels were visualized under UV light and images captured (.TIFF files) by a gel documentation system, GeneSnap 4.0 (Syngene). Images of agarose gels were analyzed by manually transforming all DNA fragments into a binary data matrix. Individual DNA fragments obtained using a given primer were scored as present (1) or absent (0) for each isolate. All reproducibly visible DNA fragments (n = 3, for each isolate and primer combination) were included in the RAPD analysis. The data obtained from all 3 RAPD primers (1, 5 and 6) were combined into a single data matrix. All computations were performed with the program FreeTree (Pavalíček et al. 1999, Hampl et al. 2001). Genetic distances (*d*) for each isolate were calculated from Nei-Li's coefficient of similarity (*s*), where $d = (1 - s)$ (Nei & Li 1979). Phylogenetic trees were constructed from the similarity matrix by neighbor-joining (Saitou & Nei 1987) incorporating 1000 bootstrap resamplings (Felsenstein 1985). Phylogenetic trees were visualized using TreeView (Page 1996).

RESULTS

Phenotypic characterization

All isolates of lobster origin and the single non-marine isolate ATCC 700406 satisfied the criteria set out by Stewart et al. (1966) for *Aerococcus viridans* var. *homari*: pinpoint colonies of Gram-positive cocci, arranged singly, paired, or in tetrads or other small

Table 2. Phenotypic differences among *Aerococcus viridans* var. *homari* isolates and a non-marine *A. viridans* (ATCC 700406). βGLU: β-glucosidase; βGUR: β-glucuronidase; HIPP: hydrolysis of hippurate; GAL: fermentation of galactose; Inulin: fermentation of inulin; Starch: hydrolysis of starch; TEL: tolerance to 0.04% tellurite

Isolate	βGLU	βGUR	HIPP	GAL	Inulin	Starch	TEL
ATCC 10400	-	+	+	+	+	-	+
ATCC 29838	-	+	+	+	+	-	+
Rabin	-	+	+	+	+	-	+
Newfoundland	-	+	+	+	+	-	+
Maine	-	+	+	+	+	-	+
NVI 1030	-	+	+	+	+	-	+
NVI 1032	-	+	+	+	+	-	+
37R	+	-	-	+	-	-	+
88B	-	+	+	+	+	-	+
ATCC 700406	-	-	+	-	-	+	-

groups but not in chains or grape-like clusters; hemolytic on BA (sheep); catalase-negative. All isolates also satisfied the criteria for *A. viridans* given by Bergey's manual of determinative bacteriology: non-motile; growth at 10°C but not at 45°C; growth at pH 9.6, in 10% NaCl, and in 40% bile; gelatin was not liquified; nitrate was not reduced; and production of acid from oxidative and fermentative metabolism of glucose (Holt et al. 1994, p. 527). Production of PYR, no production of LAP and sensitivity to vancomycin were consistent with *A. viridans* (Ruoff 2003).

There was phenotypic clustering of the virulent *Aerococcus viridans* var. *homari* isolates based on β -glucosidase, β -glucuronidase, hippurate, galactose, inulin, tellurite, and starch hydrolysis (Table 2). However, there was no clustering of the avirulent and non-marine *A. viridans* group, but the variability within this group clearly differentiated them from the virulent group. No phenotypic tests were specific for the virulent strains. These results are in agreement with Wiik et al. (1986).

16S rRNA gene analysis

A total of 1419 nucleotides (nt) of unambiguous sequence information was generated from the universal primers (BSF-8/20 and BSR-1541/20) for all 7 *Aerococcus viridans* var. *homari* isolates, the 2 avirulent *A. viridans*-like cocci and the non-marine *A. viridans* (ATCC 700406). Therefore, approximately 26 nt and 59 nt of sequence are missing from the 5' and 3' ends of the gene (between the primers), respectively, in relation to the *Escherichia coli* nucleotide positions. The GenBank accession numbers are as follows: *A. viridans* var. *homari* NVI 1030 (AY707775), NVI 1032 (AY707776), ATCC 10400 (AY707777), ATCC 29838 (AY707778), Maine (AY707780), Newfoundland (AY707781), Rabin (AY707782), *A. viridans*-like cocci 37R (AY707773) and 88B (AY707774) and *A. viridans* ATCC 700406 (AY707779).

The 7 *Aerococcus viridans* var. *homari* isolates, the 2 avirulent *A. viridans*-like cocci and the non-marine *A. viridans* (ATCC 700406) isolates from the present study exhibited 99.9 to 100% similarity over 1419 nucleotides (Table 3). The 16S rRNA sequences of *A. viridans* var. *homari* isolates ATCC 10400, ATCC 29838, Rabin, Newfoundland, Maine, 1030, 1032 are identical to that of *Pediococcus urinaeequi* (Table 3). The *A. viridans*-like cocci 37R and 88B isolates share the same 16S rRNA sequence with the non-marine strain ATCC 700406 and the Lake Vanda (Antarctica) isolate *Aerococcus* sp. LV 65.5:W1 (Table 3).

The *Aerococcus viridans* var. *homari* isolates only differ from each other at 1 nucleotide position; ATCC

Table 3. Levels of similarity (%) of *Aerococcus viridans* var. *homari* isolates and reference strains derived from 16S rRNA gene sequences. Kimura 2-parameter genetic distances are shown below the diagonal

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	
<i>A. viridans</i> var. <i>homari</i> ATCC 10400	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
<i>A. viridans</i> var. <i>homari</i> Rabin	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
<i>A. viridans</i> var. <i>homari</i> Newfoundland	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
<i>A. viridans</i> var. <i>homari</i> Maine	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
<i>A. viridans</i> var. <i>homari</i> ATCC 29838	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
<i>A. viridans</i> var. <i>homari</i> NVI 1030	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
<i>A. viridans</i> var. <i>homari</i> NVI 1032	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
<i>Pediococcus urinaeequi</i> D87677	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9
<i>A. viridans</i> -like cocci 37R	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
<i>A. viridans</i> -like cocci 88B	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
<i>A. viridans</i> ATCC 700406	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
<i>Aerococcus</i> sp. LV65.5:W1 AF076639	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
<i>A. viridans</i> M58797	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9
Uncultured feedlot manure bacterium AF317386	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9
Bacterium B16S AF300974	98.6	98.6	98.6	98.6	98.6	98.6	98.6	98.6	98.7	98.7	98.7	98.7	98.7	98.7	98.7	98.7	98.7	98.7	98.7	98.7
<i>A. urinae</i> M77819	93.5	93.5	93.5	93.5	93.5	93.5	93.5	93.5	93.5	93.5	93.5	93.5	93.5	93.3	92.4	92.4	92.4	92.4	92.4	92.4
<i>A. sanguicola</i> AJ276512	94.8	94.8	94.8	94.8	94.8	94.8	94.8	94.8	94.8	94.8	94.9	94.9	94.9	94.8	94.8	94.1	95.4	94.1	95.4	94.1
<i>A. christensenii</i> Y17005	92.6	92.6	92.6	92.6	92.6	92.6	92.6	92.6	92.7	92.7	92.7	92.7	92.7	92.6	92.5	91.6	95.4	94.6	94.6	94.6
<i>A. urinaehominis</i> AJ278341	94.7	94.7	94.7	94.7	94.7	94.7	94.7	94.7	94.6	94.6	94.6	94.6	94.6	94.5	94.4	93.6	94.0	94.3	93.1	93.1
<i>Facklamia hominis</i> Y10772	88.1	88.1	88.1	88.1	88.1	88.1	88.1	88.1	88.2	88.2	88.2	88.2	88.2	88.1	88.0	86.8	88.9	87.8	87.6	89.0

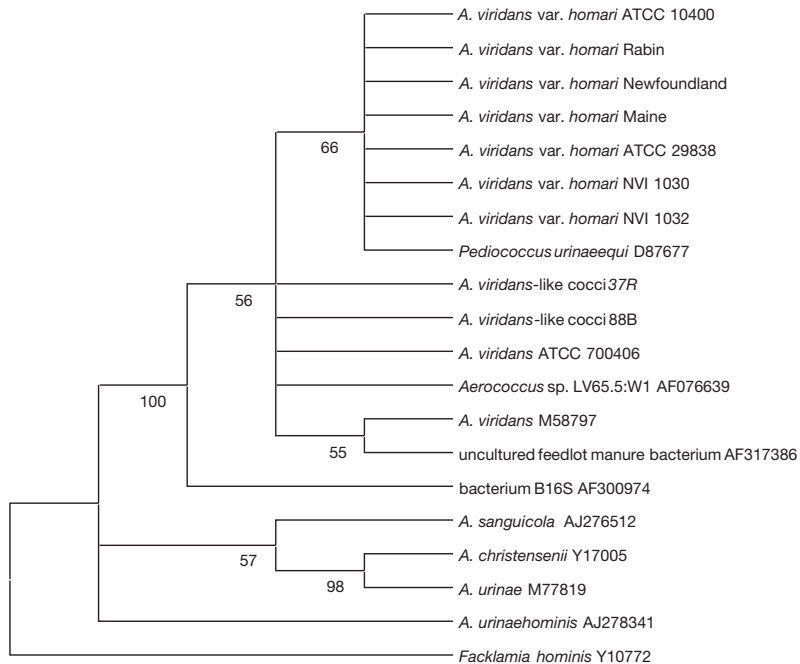


Fig. 1. *Aerococcus viridans* var. *homari*. Neighbor-joining tree based on the alignment of 1238 nucleotides of the 16S rRNA gene sequences indicating the phylogenetic position of the *A. viridans* var. *homari* isolates. Numbers at nodes are the percentages of bootstrap replications (only values greater than 50% are shown). GenBank accession numbers are given beside species names

10400, ATCC 29838, Rabin, Newfoundland, Maine, NVI 1030, NVI 1032 have a G at nucleotide position 150 (of the 1419) while the *A. viridans*-like cocci 37R and 88B have an A at the same position.

A phylogenetic analysis based on 1238 nucleotides from the 16S rRNA gene of the *Aerococcus viridans* isolates from this study was performed by comparison with 10 reference sequences obtained from GenBank. A neighbor-joining bootstrap-consensus tree (1000 iterations and reported bootstrap values >50%) was constructed (Fig. 1). The *A. viridans* isolates are supported (100% bootstrap value) as a monophyletic clade within the genus *Aerococcus* (Fig. 1). The association of the *A. viridans* var. *homari* isolates with *Pediococcus urinaeequi* was supported by a bootstrap value of 66% (Fig. 1). The *A. viridans*-like cocci 37R and 88B isolated from lobsters are more closely associated with the non-marine *A. viridans* isolate ATCC 700406 and the Lake Vanda (Antarctica) isolate *Aerococcus* sp. LV 65.5:W1 (Fig. 1). A phylogenetic tree constructed using maximum parsimony gave a similar result (not shown).

RAPD analysis

Initially, RAPD analysis on *Aerococcus viridans* var. *homari* strains ATCC 10400 and the *A. viridans*-like

cocci 37R was performed using each of the 6 arbitrary primers provided in the commercial kit. Primers (1, 5 and 6) were selected for further analysis with all strains, based on producing the most discriminatory DNA fingerprint profile (number of DNA fragments and the best differential pattern). The other primers (2, 3 and 4) gave a small number of DNA fragments or very poor amplification and were not considered further in the present study (data not shown). The reproducibility of the results was verified by repeating all RAPD reactions for each strain at least 3 times. No significant differences were observed in the patterns, although some bands varied in intensity.

The DNA fingerprint profiles from all 3 primers revealed that the 7 recognized *Aerococcus viridans* var. *homari* lobster isolates were identical (Fig. 2). Two other isolates previously documented as avirulent *A. viridans*-like cocci (37R and 88B) isolated from lobsters had DNA fragment patterns that were more similar to each other than to either the recognized *A. viridans* var. *homari* lobster isolates or the non-marine *A. viridans* isolate (Fig. 2).

A total of 71 reproducible DNA fragments from all 3 primers, ranging in size from 350 to 2100 bp were used to build the similarity matrix for the 10 *Aerococcus viridans* isolates and 1 *Escherichia coli* strain (Fig. 3). The *Aerococcus* isolates formed 2 assemblages with the 7 *A. viridans* var. *homari* lobster isolates being supported with bootstrap values of 100% and a weaker association of the non-marine isolate with the *A. viridans*-like cocci, 37R and 88B (mean bootstrap value, 43%) (Fig. 3).

DISCUSSION

This study describes the genetic characterization of 7 isolates of *Aerococcus viridans* var. *homari* and 2 avirulent *A. viridans*-like cocci isolated from American lobsters originating on the Atlantic Coast of North America. To resolve the phylogenetic affinities of the *A. viridans*-like cocci and to confirm the identity of the *A. viridans* var. *homari* strains, all isolates were initially characterized by sequencing their 16S rRNA genes. This confirmed unequivocally the identity of all 9 isolates as members of the monophyletic clade *A. viri-*

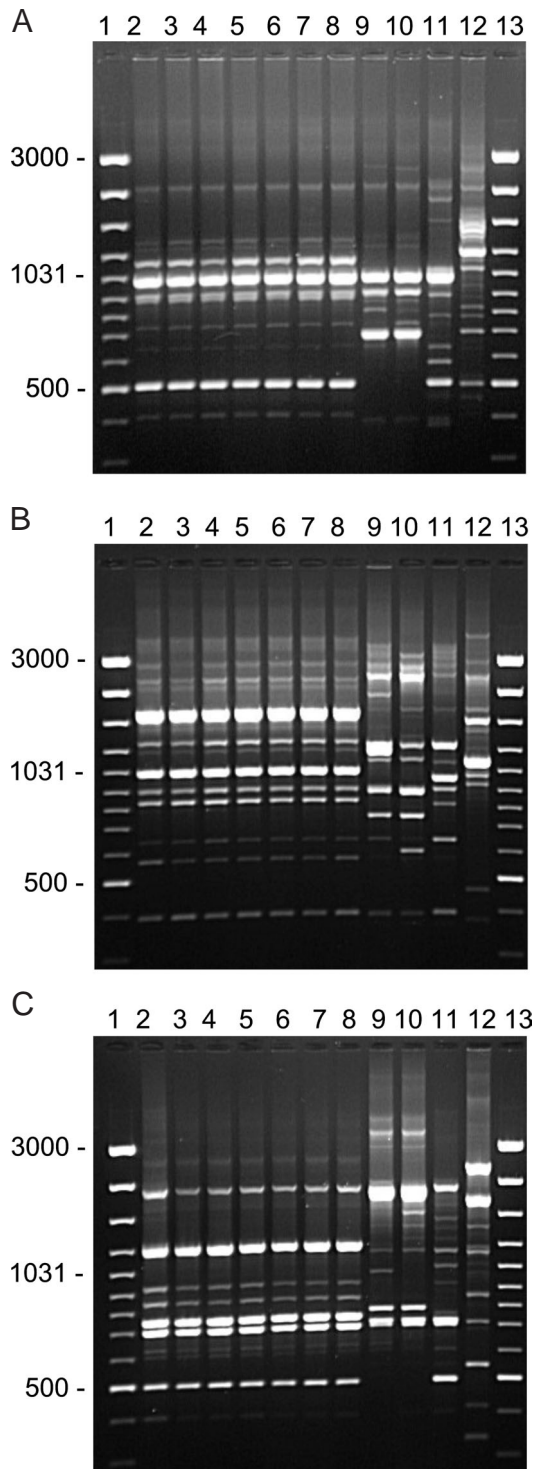


Fig. 2. *Aerococcus viridans* var. *homari* isolates RAPD profiles. (A) RAPD primer 1; (B) RAPD primer 5; (C) RAPD primer 6. Lanes: 1, GeneRuler 100 bp DNA Ladder Plus (sizes of specific bands in base pairs noted on left); 2, ATCC 10400; 3, Rabin; 4, Newfoundland; 5, Maine; 6, ATCC 29838; 7, NVI 1030; 8, NVI 1032; 9, 37R; 10, 88B; 11, ATCC 700406; 12, *Escherichia coli* (strain BL21); 13, GeneRuler 100 bp DNA Ladder Plus

dans. The placement of *Pediococcus urinaeequi* within the *A. viridans* clade was noted earlier (Collins et al. 1990, Christensen et al. 1997). However, the present analysis suggests that *P. urinaeequi* may be an *A. viridans* var. *homari* strain based on having an identical 16S rRNA sequence. Currently, there is no accepted cut-off value for 16S rRNA sequence similarity that defines a species, but the majority of related species within a genus differ by at least 1% or more of the nucleotide positions (Fox et al. 1992, Stackebrandt & Goebel 1994, Drancourt et al. 2000, Song et al. 2003). Using this criterion, all members of the *A. viridans* clade belong to the same species since they differ at only 1 nucleotide position (sequence similarity values of 99.8 to 100%).

A previous genotypic comparison of *Aerococcus viridans* var. *homari* based on DNA hybridization indicated a close relationship of all isolates from gaffkemic lobsters (80 to 100% hybridized DNA) but questioned the inclusion of the *A. viridans*-like cocci in the same group as the lobster pathogen, because of low hybridization values of 48 to 56% (Wiik et al. 1986). Strains of the human urinary tract pathogen, *Aerococcus urinae*, can be separated into 2 biotypes based on phenotypic differences in esculin hydrolysis. Yet, the *A. urinae* biotypes are 98.2 to 99.3% similar based on their 16S rRNA sequences and show considerable variation in DNA reassociation (62 to 81% hybridized DNA) (Christensen et al. 1997). The genotypic heterogeneity demonstrated by DNA hybridization differences between *A. urinae* led Christensen et al. (1997) to suggest that the species may comprise 2 or more subspecies. Therefore, although the *A. viridans* var. *homari* 16S rRNA sequences are very similar, the DNA hybridization data (Wiik et al. 1986) indicates that there is considerable genetic variation between the different strains. Thus, a significant genetic variation at the strain level may be a common feature of the genus *Aerococcus*.

The RAPD analysis indicated the presence of 2 distinct *Aerococcus viridans* var. *homari* subtypes. Subtype 1 included the 7 previously recognized *A. viridans* var. *homari* isolates which have the same DNA fingerprint profile irrespective of the primer employed, and therefore, constitute a genetically homogeneous group regardless of their geographical, temporal or virulence differences. Subtype 2 contained only the 2 naturally occurring avirulent *A. viridans*-like cocci. These *A. viridans*-like cocci exhibited distinct RAPD patterns from the other *A. viridans* var. *homari* isolates (subtype 1) but clustered separately with the non-marine *A. viridans* strain.

The homogeneity of the *Aerococcus viridans* var. *homari* subtype 1 was surprising, given they are temporally quite disparate (1947 to 2000) and found over

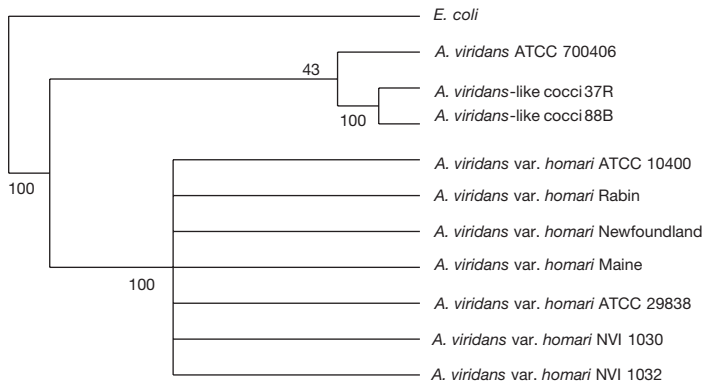


Fig. 3. *Aerococcus viridans* var. *homari*. Neighbor-joining tree constructed using 71 RAPD characters indicating the phylogenetic position of the *A. viridans* var. *homari* isolates. Numbers at nodes are percentages of bootstrap replications

the geographic range of the present study (Massachusetts to Newfoundland). Historically, subtype 1 contains the 2 strains that have been the focus of the greatest amount of research, ATCC 10400 (Snieszko & Taylor 1947) and Rabin strain (Rabin & Hughes 1968, Stewart et al. 1966). The identical DNA fingerprint profile of subtype 1 may be explained by one or more of the following: (1) the persistence of the bacteria in a stable marine environment; (2) the dispersal of the bacteria by water currents within the Atlantic region; (3) the natural history of an evolutionarily long-standing host–pathogen relationship; (4) the importation of lobsters from different regions for processing, which may lead to the movement of infected lobsters or asymptomatic carriers. The pathogens could potentially spread to susceptible populations through waste water run-off from processing plants. Similar scenarios have been implicated in the spread of fish diseases and for the appearance of pathogens with the same DNA fingerprint pattern in different countries (Hänninen & Hirvelä-Koski 1999, Ravelo et al. 2003).

Stewart et al. (2004) have recently shown that virulence in *Aerococcus viridans* var. *homari* can be induced by culturing avirulent strains in the presence of sterile lobster hemolymph serum. The induction of virulence coincides with the restoration of the polysaccharide capsule, which in turn prevents agglutination and killing by lobster phagocytes during infection. The variation in capsule thickness under different culture conditions had been noted earlier (Hitchner & Snieszko 1947, Stewart 1984). The explanation of induced virulence is consistent with the current RAPD analysis of *A. viridans* var. *homari* strains in which no genetic variation was observed between the avirulent and virulent members of subtype 1. However, in the context of the study of Stewart et al. (2004), our RAPD results also

indicate that there are at least 2 distinctly different genetic strains of *A. viridans* var. *homari* on the Atlantic Coast of North America that have the potential to cause disease in lobsters. Since the *A. viridans*-like cocci (which are *A. viridans* var. *homari*) share an identical 16S rRNA sequence with the non-marine strain ATCC 700406 and the Lake Vanda (Antarctica) isolate *Aerococcus* sp. LV 65.5:W1, this implies that there potentially are more strains of *A. viridans* var. *homari* than have been previously recognized.

Previous work on the development of an indirect fluorescent antibody technique (IFAT) for detection of *A. viridans* var. *homari* documented differences in IFAT staining between virulent (Rabin, NVI 1030, NVI 1032) and avirulent strains (ATCC 10400, 37R and 88B) that were correlated with variation in capsule thickness (Marks et al. 1992). The results of our genetic characterization of *Aerococcus viridans* var. *homari*, combined with the studies of Stewart et al. (2004) and Marks et al. (1992), suggest that the variation observed in virulence among the different strains of *A. viridans* var. *homari* is probably a function of changes in gene expression that result in antigenic variation (protein or carbohydrate) in the context of the environment. It would be interesting to extend the present study to include more isolates from similar locations to assess both the local, regional and global genetic variation that may exist within *A. viridans* var. *homari* and to explore the potential variation in pathogenesis that may exist within the 2 *A. viridans* var. *homari* subtypes.

In summary, the combination of 16S rRNA gene sequencing and RAPD analysis has revealed that all 9 *Aerococcus viridans* var. *homari* isolates are members of the monophyletic *A. viridans* clade and that the lobster pathogen can be separated into at least 2 subtypes on the Atlantic coast of North America. On this basis, RAPD analysis represents a useful method for discriminating molecular subtypes for both intraspecific classification and epidemiological investigations of *A. viridans* var. *homari*.

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