Detection of *Arcobacter* spp. in the Coastal Environment of the Mediterranean Sea

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The occurrence of *Arcobacter* spp. was studied in seawater and plankton samples collected from the Straits of Messina, Italy, during an annual period of observation by using cultural and molecular techniques. A PCR assay with three pairs of primers targeting the 16S and 23S rRNA genes was used for detection and identification of *Arcobacter butzleri*, *Arcobacter cryaerophilus*, and *Arcobacter skirrowii* in cultures and environmental samples. Only one of the *Arcobacter* species, *A. butzleri*, was isolated from seawater and plankton samples. With some samples the *A. butzleri* PCR assay gave amplified products when cultures were negative. *A. cryaerophilus* and *A. skirrowii* were never detected by culture on selective agar plates; they were detected only by PCR performed directly with environmental samples. Collectively, our data suggest that culturable and nonculturable forms of *Arcobacter* are present in marine environments. The assay was useful for detecting *Arcobacter* spp. both as free forms and intimately associated with plankton. This is the first report showing both direct isolation of *A. butzleri* and the presence of nonculturable *Arcobacter* spp. in the coastal environment of the Mediterranean Sea.

The genus *Arcobacter* was first described by Ellis et al. in 1977 as a taxon that contains gram-negative, spirillum-like bacteria isolated from bovine and porcine fetuses (7). This genus, together with genera *Campylobacter* and *Helicobacter*, forms a phylogenetically distinct group referred to either as rRNA superfamily VI or as the epsilon division of the class *Proteobacteria* (23). These bacteria have been placed in the genus *Arcobacter* and differentiated into five species: *A. butzleri*, *A. cryaerophilus*, *A. skirrowii*, *A. nitrofigilis*, and *A. sulfidicus* (23, 24, 25, 26). Three of these species, *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii*, have been associated with human and animal enteric diseases (25). *A. butzleri* and *A. cryaerophilus* have been isolated mainly from stool specimens from patients with diarrhea (11, 13). Invasive *A. butzleri* infection in humans has been described in a patient with liver cirrhosis and in a patient with acute gangrenous appendicitis (12, 29). *A. cryaerophilus* was isolated from the blood of a uremic patient with hematogenous pneumonia and from a traffic accident victim (9, 28).

The role of *A. skirrowii* in human illness is not clear. *A. nitrofigilis* has been isolated from the roots of *Spartina alterniflora*, a salt marsh plant (15). *A. sulfidicus*, a coastal marine sulfide-oxidizing autotrophic producer of filamentous sulfur, has been isolated from a variety of habitats (27). It has been suggested that water may play an important role in the transmission of *Arcobacter* spp. both to animals and to humans (19, 20). *A. butzleri* and *A. cryaerophilus* have been isolated from a drinking water reservoir in Germany (10), from canal water in

Thailand (6), from river water in Italy (17), from groundwater sources (20), and from sewage (22). Since *Arcobacter* spp. have been isolated from water and mussels from a brackish lake near Messina, Italy (14), the present study was undertaken to analyze the presence of *Arcobacter* spp. in the coastal waters of the Straits of Messina and in plankton by using both classical methods and molecular techniques. The molecular methods were used to evaluate nonviable and viable but noncultivable forms (3, 4, 5, 18). The latter state is a sophisticated strategy developed by many gram-negative bacteria to survive under adverse environmental conditions. Conditions that have been shown to induce nonculturability differ according to the organism and include factors such as starvation, salinity, temperature, visible light, osmotic stress, and desiccation. The loss of culturability may not guarantee a loss of pathogenicity.

Knowledge of the survival strategies of arcobacters in the environment is very important for control of both water quality and transmission of disease. The presence of *Arcobacter* isolates in food animals and water and their association with human and animal diseases mean that there must be reliable methods for correct identification of these organisms, as well as for monitoring the spread of *Arcobacter* isolates in different environments. Culture techniques are often time-consuming and give poor results for species that are difficult to culture, while DNA analysis based on nucleic acid amplification is very rapid and dependable. The presence of *Arcobacter* spp. in the marine environment of the Straits of Messina has special importance, since seawater is known to contain both autochthonous and allochthonous microorganisms, the latter coming from shore areas.

MATERIALS AND METHODS

Sampling. Monthly sampling was carried out from April 2001 to March 2002. Surface seawater samples were collected at a station located in the Straits of

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Messina ca. 50 m from the coast. The surface water temperature was measured in situ with a mercury thermometer. After collection, aliquots of seawater and plankton samples were used for culture and molecular analyses.

Seawater samples. Seawater samples (3 liters) were collected by using presterilized bottles. To collect free-living *Arcobacter* spp., seawater samples were filtered first through a 200- μ m-pore-size net and then through a 64- μ m-pore-size net. The 64- μ m-pore-size net was saved for analysis of small plankton (see below). The filtrate was concentrated by using 0.2- μ m-pore-size membrane filters (Millipore Corp., Bedford, Mass.). The filters (four to eight filters for each 3-liter sample) were washed with sterile seawater to obtain a final volume of 3 ml, which was divided into two aliquots; one of these aliquots was used for cultural analysis, and the other was used for molecular analysis.

Small plankton. To collect small plankton, the 64- μ m-pore-size net was washed with sterile seawater to obtain a final volume corresponding to 1/1,000 of the volume of the original seawater sample. Suspensions were used for plankton, culture, and PCR analyses.

Large plankton. Large plankton (>200 μ m) were collected from a boat by using a horizontal tow at a depth of 1.5 to 2 m with a 200- μ m-pore-size plankton net (WP2). The retained large plankton and adhering bacteria were resuspended in 500 ml of sterile seawater, which was divided into three aliquots; these aliquots were used for plankton, cultural, and molecular analyses.

Culture conditions. Aliquots of seawater and small and large plankton were directly inoculated into the appropriate culture media. Before inoculation, samples of large plankton were homogenized for 1 min in a glass homogenizer at 5,800 rpm.

All samples were inoculated into *Arcobacter* broth CM965 (Oxoid) supplemented with CAT (cefoperazone, amphotericin B, teicoplanin) selective supplement SR 174E, which was selective for growth of *Arcobacter* species, and with CCDA (cefoperazone, amphotericin B) selective supplement SR 155 for selective enrichment of *A. butzleri*. After aerobic incubation at 30°C for 24 h, liquid cultures were streaked onto plates containing the same media containing agar. Colonies were picked and restreaked for purification on the same media. The pure cultures were tested for Gram staining, motility, oxidase and catalase production, oxidative or fermentative glucose metabolism, and gas production. Gram-negative isolates and isolates that did not ferment glucose were maintained and investigated further. Isolates were also studied to determine whether they grew at 15, 25, 37, and 42°C in Bacto Nutrient broth (Difco, Detroit, Mich.) and whether they grew on MacConkey agar (Oxoid) at 37°C for 2 days. Biochemical and antibiotic susceptibility characteristics of the isolates were determined by using the API CAMPY identification system (bioMérieux Italia S.p.A.). Phenotypic characteristics of the isolates were compared with those of *A. butzleri* ATCC 49616, *A. cryaerophilus* ATCC 43157, and *A. skirrowii* ATCC 51132, which were included as reference strains.

Extraction of DNA from seawater and plankton samples. A 1-ml aliquot of each concentrated sample was centrifuged at $8,000 \times g$ for 20 min. The resulting pellet was resuspended in 500 μ l of Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0). Four methods were used for DNA extraction. (i) For method A 50 μ l of a pellet resuspended in TE buffer was boiled for 5 min at 100°C and then was stored at 4°C prior to PCR. (ii) For method B the remaining 450 μ l of a sample was centrifuged at 13,000 rpm for 20 min at 15°C, and the pellet was resuspended in 300 μ l of lysis buffer (20 mM Tris-HCl [pH 8.0], 0.5% Tween 20) with vortexing once. The sample was digested with proteinase K to obtain a final concentration of 0.5 mg/ml. After incubation at 55°C for 1 h, heating at 98°C for 10 min was used to deactivate the proteinase K. Then 50 μ l of the solution was used directly as a template in PCR. (iii) For method C 300 μ l of phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) was added to the remaining solution with vortexing once. Following centrifugation at 13,000 rpm for 10 min, 30 μ l of the aqueous phase was used as a template in PCR. (iv) For method D the remaining DNA was precipitated with 750 μ l of ice-cold 100% ethanol and 30 μ l of 3 M sodium acetate (pH 5.2). Following gentle mixing the sample was kept at -80°C for 30 min and then centrifuged at 13,000 rpm for 15 min at 4°C. The pellet was air dried, resuspended in 30 μ l of sterile water, and used as a template for PCR (16).

In selected experiments, to control for the presence of PCR inhibitors, 1×10^6 heat-killed (100°C for 5 min) *A. butzleri* cells were added to seawater samples. These samples were then extracted by using the four methods described above and subjected to PCR analysis with *A. butzleri*-specific primers.

Extraction of DNA from *Arcobacter* spp. isolates. For extraction of DNA from *Arcobacter* spp. isolates, a Dneasy tissue kit (Qiagen, Milan, Italy) was used according to the manufacturer's recommendations. One colony was harvested from an agar plate, added to a lysis buffer, and homogenized by vortexing. Twenty microliters of a proteinase K solution (20 mg/ml) was then added, and this was followed by incubation at 55°C for 3 h. A second buffer provided in the

kit was added, and the sample was incubated at 70°C for 10 min. Next, 200 μ l of ethanol was added. The mixture was then loaded onto the DNeasy minicolumn and centrifuged at $6,000 \times g$ for 1 min. The Dneasy minicolumn was placed in a 2-ml collection microtube, and the tube containing the mixture was discarded. The column material was washed with 500 μ l of the first washing buffer and with 500 μ l of the second washing buffer provided in the kit. Finally, the DNA was eluted with 200 μ l of a third buffer provided in the kit.

PCR conditions. *Arcobacter* genomic DNA was amplified with a HotStarTaq Master MixKit (Qiagen) and a PCR Sprint thermal cycler (Hybaid, Ashford, United Kingdom). The reaction mixtures used for PCR contained (per 50 μ l) $1 \times$ PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 1.5 mM $MgCl_2$), each deoxynucleoside triphosphate at a concentration of 200 μ M, each primer at a concentration of 0.25 μ M, and 2.5 U of HotStarTaq DNA polymerase, and 5 μ l aliquots of sample were used as templates. The temperature profile for the PCR was as follows: an initial step of 15 min at 95°C, followed by denaturation for 1 min at 94°C, annealing for 1 min at 50°C, and primer extension for 1 min at 72°C. After the 35th cycle, the extension step was prolonged for 10 min to complete synthesis of all strands, and then the samples were kept at 4°C until analysis. Negative and positive controls were included in every experiment. PCR products were detected by gel electrophoresis. Samples (5 μ l) of final PCR products were loaded onto 1.5% agarose gels and subjected to electrophoresis in $1 \times$ TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA) for 60 to 90 min at 100 V. The gels were stained with ethidium bromide and photographed with UV light transillumination. A 100-bp DNA ladder (New England Biolabs) was included in each gel as a molecular size standard.

Oligonucleotide primers. Three pairs of primers targeting the 16S and 23S genes were used in PCR assays for detection and identification of *Arcobacter* spp. These primers amplified a 257-bp fragment from *A. cryaerophilus* (ArcCRY), a 401-bp fragment from *A. butzleri* (ArcBUTZ), and a 641-bp fragment from *A. skirrowii* (ArcSKIR) (8). The sequences of the primers were as follows: ArcBUTZ Top, 5'-CCT GGA CTT GAC ATA GTA AGA ATG A-3'; Bot 16S-rDNA, 5'-CGT ATT CAC CGT AGC ATA GC-3'; ArcCRY Top, 5'-TGC TGG AGC GGA TAG AAG TA-3'; Bot 23S-rDNA, 5'-AAC AAC CTA CGT CCT TCG AC-3'; ArcSKIR Top, 5'-GGC GAT TTA CTG GAA CAC-A3'; and Bot 16S-rDNA, 5'-CGT ATT CAC CGT AGC ATA GC-3'. The primers were synthesized by GENSET SA (Paris, France).

PCR sensitivity. In preliminary experiments we determined the analytical sensitivity of the PCR. To do this, DNA was extracted from serial dilutions of *Arcobacter* spp. broth cultures. Dilution series (10^0 to 10^{-9}) were prepared by using a Dneasy tissue kit (Qiagen). In these dilution series the actual number of CFU was determined by culturing. The following strains were used: *A. butzleri* ATCC 49616, *A. cryaerophilus* ATCC 43157, and *A. skirrowii* ATCC 51132. The detection limits were approximately 5, 7, and 3 CFU for *A. cryaerophilus*, *A. skirrowii*, and *A. butzleri*, respectively.

Sequence analysis. Ten randomly selected PCR products having different molecular weights obtained from environmental samples were purified with a JET quick PCR purification spin kit (Celbio s.r.l., Milan, Italy) used according to the manufacturer's instructions, and these products were used for nucleotide sequencing. Both DNA strands were sequenced with a ThermoSequenase fluorescently labeled primer cycle sequencing kit (Amersham Pharmacia) by using an ALFexpress DNA sequencer (Amersham Pharmacia). A homology analysis was carried out at the BCM Search Launcher server (<http://searchlauncher.bcm.tmc.edu/seq-util/seq-util.html>) (21). Blast software (<http://www.ncbi.nlm.nih.gov/blast/>) was used to conduct homology searches of the GenBank database (1).

Plankton analysis. Aliquots of samples were preserved in formaldehyde at a final concentration of 4%. After sedimentation in an Utermöhl chamber, samples were analyzed by using both inverted and stereoscopic microscopes (Zeiss) to determine the number of individuals per cubic meter and broad taxonomic groups.

RESULTS

Data for the presence of *Arcobacter* spp. associated with seawater and small and large plankton, as determined by traditional culture methods and by the *Arcobacter*-specific PCR test, are shown in Tables 1 and 2. Only some isolates could be clearly identified as *A. butzleri* on the basis of the phenotypic analysis. The other isolates were provisionally identified only to the genus level since the cultural and biochemical test results were not perfectly consistent with those for any of the

TABLE 1. Detection of *Arcobacter* spp. in water and plankton samples collected in the Straits of Messina, Italy, by PCR and cultural methods from April to September 2001

Month	Surface water temp (°C)	Detection method	Organisms detected in:		
			Marine water	Small plankton (64–200 µm)	Large plankton (>200 µm)
April	11.5	PCR	<i>A. butzleri</i> , <i>A. cryaerophilus</i>	ND ^a	<i>A. butzleri</i> , <i>A. cryaerophilus</i>
		CM ^b	<i>A. butzleri</i>	ND	<i>A. butzleri</i>
May	17.0	PCR	<i>A. butzleri</i> , <i>A. cryaerophilus</i>	<i>A. butzleri</i>	<i>A. butzleri</i>
		CM	<i>A. butzleri</i>	ND	<i>A. butzleri</i>
June	20.0	PCR	<i>A. butzleri</i>	<i>A. butzleri</i>	<i>A. butzleri</i> , <i>A. cryaerophilus</i>
		CM	<i>A. butzleri</i>	ND	<i>A. butzleri</i>
July	21.5	PCR	<i>A. butzleri</i> , <i>A. cryaerophilus</i>	<i>A. butzleri</i> , <i>A. cryaerophilus</i>	<i>A. butzleri</i> , <i>A. cryaerophilus</i>
		CM	<i>A. butzleri</i>	<i>A. butzleri</i>	<i>A. butzleri</i>
August	22.0	PCR	ND	ND	ND
		CM	ND	ND	ND
September	22.5	PCR	<i>A. butzleri</i>	ND	<i>A. butzleri</i> , <i>A. cryaerophilus</i> , <i>A. skirrowii</i>
		CM	ND	ND	ND

^a ND, no arcobacters detected.^b CM, cultural method.

American Type Culture Collection type strains. The lack of definite biochemical test methods for species identification prompted our search for a PCR protocol that could specifically identify all *Arcobacter* species. Primers used in this study were tested with our isolates of *Arcobacter* spp.; the specificity of the primers was tested by using the DNA extracted from *A. butzleri* ATCC 49616, *A. cryaerophilus* ATCC 43157, and *A. skirrowii* ATCC 51132 as templates. All of our isolates were definitively identified by PCR as *A. butzleri*. Figure 1 shows the amplified products of four isolates from the samples collected in April and May 2001. No PCR products for *A. cryaerophilus* and *A. skirrowii* were obtained with water and plankton samples. Our data suggested that the isolates were phenotypically heterogeneous. After PCR identification the cultural data showed that *A. butzleri* was readily cultured from seawater samples, as well as from large plankton, from April to July and in November and March and from small plankton in July, November, and March. The results of the temperature studies indicate that *A. butzleri* may be culturable in all months.

Arcobacter spp. were directly detected in water and plankton

samples by PCR analysis by using four different extraction methods, and the results were compared with the results obtained when the standard culture method was used. The direct boiling method (method A) and lysis-protease treatment (method B) yielded larger amounts of DNA from the samples than the other two methods (methods C and D) yielded. Methods A and B always enabled *Arcobacter* detection. Additional steps, including phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation (methods C and D), resulted in a loss of sensitivity (data not shown). A comparison of the sequences of 10 randomly selected PCR products obtained from samples with known 16S rRNA and 23S rRNA gene sequences in the GenBank database showed that there was complete homology between our amplified products and *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii* sequences (4, 4, and 2 sequences, respectively).

The *Arcobacter* detection rate for PCR amplification was much better than the detection rate for the traditional culture method (77.8 versus 41.7% for *A. butzleri*; 55.5 versus 0% for *A. cryaerophilus*; and 8.3 versus 0% for *A. skirrowii*).

TABLE 2. Detection of *Arcobacter* spp. in water and plankton samples collected in the Straits of Messina, Italy, by PCR and cultural method from October 2001 to March 2002

Month	Surface water temp (°C)	Detection method	Organisms detected in:		
			Marine water	Small plankton (64–200 µm)	Large plankton (>200 µm)
October	20.0	PCR	<i>A. butzleri</i>	ND ^a	<i>A. butzleri</i>
		CM ^b	ND	ND	ND
November	20.0	PCR	<i>A. butzleri</i> , <i>A. cryaerophilus</i>	<i>A. butzleri</i>	<i>A. butzleri</i> , <i>A. cryaerophilus</i>
		CM	<i>A. butzleri</i>	<i>A. butzleri</i>	<i>A. butzleri</i>
December	18.0	PCR	<i>A. butzleri</i> , <i>A. cryaerophilus</i>	<i>A. butzleri</i> , <i>A. cryaerophilus</i>	<i>A. butzleri</i> , <i>A. cryaerophilus</i>
		CM	ND	ND	ND
January	11.0	PCR	<i>A. butzleri</i> , <i>A. cryaerophilus</i>	<i>A. butzleri</i> , <i>A. cryaerophilus</i>	<i>A. cryaerophilus</i>
		CM	ND	ND	ND
February	15.5	PCR	<i>A. butzleri</i> , <i>A. cryaerophilus</i>	<i>A. cryaerophilus</i>	<i>A. butzleri</i> , <i>A. cryaerophilus</i> , <i>A. skirrowii</i>
		CM	ND	ND	ND
March	15.5	PCR	<i>A. butzleri</i> , <i>A. cryaerophilus</i> , <i>A. skirrowii</i>	<i>A. butzleri</i>	<i>A. butzleri</i>
		CM	<i>A. butzleri</i>	<i>A. butzleri</i>	<i>A. butzleri</i>

^a ND, no arcobacters detected.^b CM, cultural method.

FIG. 1. Species-specific identification of four strains of *Arcobacter* isolated from samples collected in April and May 2001 by PCR. The gel is an ethidium bromide-stained 1.5% agarose gel containing amplified products generated with primers ArcBUTZ, ArcCRY, and ArcSKIR. Lane 1, 100-bp ladder; lanes 2, 5, 8, and 11, PCR products obtained with *A. butzleri*-specific primers; lanes 3, 6, 9, and 12, PCR products obtained with *A. cryaerophilus*-specific primers; lanes 4, 7, 10, and 13, PCR products obtained with *A. skirrowii*-specific primers; lane 14, PCR product of *A. butzleri* ATCC 49616 DNA amplified with *A. butzleri*-specific primers; lane 15, PCR product of *A. cryaerophilus* ATCC 43157 DNA amplified with *A. cryaerophilus*-specific primers; lane 16, PCR product of *A. skirrowii* ATCC 51132 DNA amplified with *A. skirrowii*-specific primers.

Arcobacter spp. were found more frequently in seawater and in large-plankton samples than in small-plankton samples. No amplification was observed with DNA extracted from any of the environmental samples collected in August (Tables 1 and 2). Amplified products of *A. butzleri* were detected in seawater samples collected in all months except August. *A. cryaerophilus* was detected in all seawater samples except those collected in June, August, September, and October, while *A. skirrowii* was observed only in March. Figure 2 shows the amplified products obtained from the seawater sample collected in March 2002 when the four different DNA extraction methods were used.

The PCR assays used to examine the occurrence of *Arcobacter* spp. associated with small plankton yielded specific products in May, June, July, November, December, January, February, and March. Only *A. butzleri* was detected in samples collected in May, June, November, and March. *A. butzleri* with *A. cryaerophilus* were observed in July, December, and January. Only *A. cryaerophilus* was detected in February. Figure 3 shows the amplified products of *A. butzleri* and *A. cryaerophilus* from the small-plankton sample collected in December 2001. No *A. skirrowii* PCR product was obtained from small plankton.

The PCR assay with the large-plankton samples showed that *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii* were present in the samples collected in September and February. *A. butzleri* and *A. cryaerophilus* were present in April, June, July, November, and December (Fig. 4). Only *A. butzleri* was detected in May, October, and March, and only *A. cryaerophilus* was detected in January.

The level of large plankton varied greatly during the time studied and was higher during the period from July to September. Copepods accounted for 86.13% of all individuals. Phytoplankton was present in the small fraction, and it was mainly diatoms.

FIG. 2. PCR products amplified from a single seawater sample obtained in March 2002 after different extraction methods were used. The gel is an ethidium bromide-stained 1.5% agarose gel containing amplified products generated with primers ArcBUTZ, ArcCRY, and ArcSKIR. Lane 1, 100-bp ladder; lanes 2 to 4, extraction method A; lanes 5 to 7, extraction method B; lanes 8 to 10, extraction method C; lanes 11 to 13, extraction method D; lanes 2, 5, 8, and 11, PCR products obtained with *A. butzleri*-specific primers; lanes 3, 6, 9, and 12, PCR products obtained with *A. cryaerophilus*-specific primers; lanes 4 and 7, PCR products obtained with *A. skirrowii*-specific primers; lanes 10 and 13, PCR products obtained with *A. skirrowii*-specific primers; lane 14, PCR product of *A. butzleri* ATCC 49616 DNA amplified with *A. butzleri*-specific primers; lane 15, PCR product of *A. cryaerophilus* ATCC 43157 DNA amplified with *A. cryaerophilus*-specific primers; lane 16, PCR product of *A. skirrowii* ATCC 51132 DNA amplified with *A. skirrowii*-specific primers.

DISCUSSION

Arcobacter species are common human and animal pathogens. The association of these organisms with enteritis (11, 13)

FIG. 3. PCR products amplified from a single small-plankton sample obtained in December 2001 after different extraction methods were used. The gel is an ethidium bromide-stained 1.5% agarose gel containing amplified products generated with primers ArcBUTZ, ArcCRY, and ArcSKIR. Lane 1, 100-bp ladder; lanes 2 to 4, extraction method A; lanes 5 to 7, extraction method B; lanes 8 to 10, extraction method C; lanes 11 to 13, extraction method D; lanes 2, 5, and 8, PCR products obtained with *A. butzleri*-specific primers; lanes 3, 6, and 9, PCR products obtained with *A. cryaerophilus*-specific primers; lane 11, PCR products obtained with *A. butzleri*-specific primers; lane 12, PCR products obtained with *A. cryaerophilus*-specific primers; lanes 4, 7, 10, and 13, PCR products obtained with *A. skirrowii*-specific primers; lane 14, PCR product of *A. butzleri* ATCC 49616 DNA amplified with *A. butzleri*-specific primers; lane 15, PCR product of *A. cryaerophilus* ATCC 43157 DNA amplified with *A. cryaerophilus*-specific primers; lane 16, PCR product of *A. skirrowii* ATCC 51132 DNA amplified with *A. skirrowii*-specific primers.

FIG. 4. PCR products amplified from a single large-plankton sample obtained in December 2001 after different extraction methods were used. The gel is an ethidium bromide-stained 1.5% agarose gel containing amplified products generated with primers ArcBUTZ, ArcCRY, and ArcSKIR. Lane 1, 100-bp ladder; lanes 2 to 4, extraction method A; lanes 5 to 7, extraction method B; lanes 8 to 10, extraction method C; lanes 11 to 13, extraction method D; lanes 2, 5, 8, and 11, PCR products obtained with *A. butzleri*-specific primers; lanes 3, 6, 9, and 12, PCR products obtained with *A. cryaerophilus*-specific primers; lanes 4, 7, 10, and 13, PCR products obtained with *A. skirrowii*-specific primers; lane 14, PCR product of *A. butzleri* ATCC 49616 DNA amplified with *A. butzleri*-specific primers; lane 15, PCR product of *A. cryaerophilus* ATCC 43157 DNA amplified with *A. cryaerophilus*-specific primers; lane 16, PCR product of *A. skirrowii* ATCC 51132 DNA amplified with *A. skirrowii*-specific primers.

in humans and animals (2) indicates their potential public health importance. In humans, exposure to contaminated water may be a principal risk factor (11). Assessing the prevalence of arcobacters in water is useful for determining the clinical significance and zoonotic potential of these new microorganisms. Since biochemical tests based on the phenotypes of *Arcobacter* species are limited, molecular approaches have been used to improve species level identification. Isolates were saved and retained for verification by genetic probing. Species-specific primer pairs were used and were tested to determine their sensitivity and specificity. *A. butzleri* was the only *Arcobacter* species isolated by cultural methods from environmental samples. The *A. butzleri* PCR assay for direct detection in marine samples gave amplified products when cultures were negative. *A. cryaerophilus* and *A. skirrowii* were never detected by culture on selective agar plates. The high sensitivity of the PCR assays compared with the sensitivity of the cultural method for detecting *A. cryaerophilus* and *A. skirrowii* is interesting. There were 20 PCR-positive samples that were culture negative for *A. cryaerophilus* and 3 PCR-positive samples that were culture negative for *A. skirrowii*. Selective and enrichment media are known to provide decreased sensitivity for detection of natural populations for a variety of reasons, including poor or no growth on selective media, as well as competition with other bacteria comprising the microbial community in the natural environment when enrichment broth is employed. Our extensive cultivation attempts, which included the use of defined artificial medium, enrichment, and direct plating, failed to isolate *A. cryaerophilus* and *A. skirrowii* and sometimes failed to isolate *A. butzleri*.

Data obtained in the present investigation suggest that

DNA-based techniques are more rapid and sensitive than traditional culture techniques for detecting *Arcobacter* species.

PCR assays characterized at the species level isolates that were phenotypically identified only at the genus level and detected *Arcobacter* spp. in a nonculturable state, both free living in marine water and intimately associated with plankton. In this study, the boiling method (method A) and the lysis-protease method (method B) yielded the largest amounts of DNA from the samples, resulting in the highest sensitivity for PCR detection, even if the extracts contained a slightly larger amount of PCR inhibitors than the extracts obtained by the other two methods contained. Indeed, the loss of sample DNA with additional steps (methods C and D) was not compensated for by the removal of PCR inhibitors (data not shown).

This is the first time that *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii*, which are associated with disease in humans and animals, were detected in a coastal marine environment. Molecular methods investigated in this study have distinct advantages over traditional culture methods for detection of arcobacters in environmental samples. PCR offers the advantages of specificity, sensitivity, rapidity, and the capacity to detect small amounts of target nucleic acid in a sample. PCR and DNA extraction have the potential to provide reliable identification of both routine and ambiguous pathogens, as well as mixtures of pathogens. Such accurate information is critical for disease diagnosis and epidemiological studies for both research and essential applications. It is evident from this study that *Arcobacter* spp. are members of the bacterial flora of seawater and are associated with zooplankton in the coastal environment of Messina, Italy.

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