2006-1-94

Hydrobiologia (2006)
DOI 10.1007/s10750-006-0220-2

Primary Research Paper

The study of antagonistic interactions among pelagic bacteria: a promising way to coin environmental friendly antifouling compounds

Hari Datta Bhattarai[1], Yoo Kyung Lee[2], Kyeung Hee Cho[2], Hong Kum Lee[2] & Hyun Woung Shin[1,*]

[1]*Department of Marine Biotechnology, Soonchunhyang University, 646, Eupnae-ri, shinchang-myun, Asan-Si, 336-745 Choongchungnam-do, South Korea*

[2]*Polar BioCenter, Korea Polar Research Institute, KORDI, Ansan, 29 Seoul 425-600, Korea*

*(*Author for correspondence: Tel.: +82-41530-1284; Fax: +82-41530-1493; E-mail: hwshin@sch.ac.kr)*

Received 13 June 2005; in revised form 24 March 2006; accepted 1 April 2006

Key words: *Alteromonas marina*, bacterial repellent, biofilm, secondary metabolites, *Shewanella oneidensis*, 16S rDNA

Abstract

Ten strains of marine bacteria (SCH0401–SCH0410) were isolated from Ayajin, the east coast of South Korea. In spectrophotometer based chemotaxis assay the ethyl acetate extract (300 μg) of SCH0402 decreased the optical density (OD) of the motile target strains SCH0401, SCH0402, SCH0407 and SCH0408 by two to six times when compared to control. Tributyltin oxide (TBTO) decreased the OD of all target strains by only two times. The most active strain SCH0402 was identified as *Shewanella oneidensis* by using 16S rDNA gene sequence analysis. Similarly, the target motile strains SCH0401, SCH0402, SCH0407 and SCH0408 were identified as *Alteromonas marina*, *Shewanella oneidensis*, *Roseobacter gallaeciensis* and *Bacillus atrophaeus*, respectively. The growth inhibition zone produced by the test bacterial extracts against the target strains were three to eight times smaller when compared to that of TBTO. Even though, SCH0402 showed six times weaker antibacterial activity, the repellent activity was three times stronger than TBTO. Therefore, the higher negative chemotactic activity would be better to select eco-friendly antifouling compounds than the other antibacterial activities.

Introduction

Tin and copper based antifouling compounds were proved to be cost effective significant agents (Lewis, 1998), but caused unacceptable environmental hazards in their own merits (Alzieu, 1998). Therefore, the need to develop new environmental benign antifouling compounds has increased in the recent. Natural products from various groups of marine organisms including bacteria (James et al., 1996; Burgess et al., 1999) have been continuously screened.

Bacteria are the primary colonizer of marine biofilm (Armstrong et al., 2001). Colonization of bacteria on the surface of substratum is affected by different parameters, temperature, motility behavior (Kiφrboe & Jackson, 2001), surface chemistry and energy (Ista et al., 2004). Competition for space and nutrition among microorganisms determines the microbial composition of biofilm. Chemical substances secreted by one species of microorganisms greatly influence the colonization of the other species (Holmström et al., 2002).

Motile bacteria move towards favorable environments and away from areas where harmful substances are accumulated (Chet & Mitchel, 1976). This behavior of bacteria can be used to screen nontoxic antifouling compounds. The

bacterial repellent based screening method allowed the identification of bioactive metabolites that were missed during traditional antibacterial screening strategies (Boyd et al., 1999). In this study, negative chemotactic behaviors of bacteria were used to screen antifouling compounds producing marine bacterial strains.

Materials and methods

Site and date of sampling

The sea water samples were collected from Aayajin (38° 12′ 17″ North and 128° 28′ 29″ East), the east coast of South Korea on October 2003.

Bacterial isolation

Unfiltered sea water samples that contained no visible particles were used to culture free living bacteria. Marine agar (Difco) was used to culture the bacterial colonies at room temperature for 3 to 5 days.

Preparation of bacterial extracts

One liter culture grown in marine broth (Difco) with 250 rpm at 28 °C for 5 days was subjected to centrifugation at 13,000 rpm at 4 °C for 20 min. The supernatant was extracted with an equal volume of ethyl acetate. The extraction was performed for three times to ensure the complete extraction. All the extracts were pooled and the solvent was removed by vacuum evaporator at 45 °C.

Antibacterial activity test

Antibacterial assay disks (Advantec, Japan, size 8 mm in diameter) were loaded with 300 μg crude extract dissolved in 10 μl of dimethyl sulfoxide (DMSO). The extracts from all isolated strains were tested against the four motile strains SCH0401, SCH0402, SCH0407 and SCH0408. The disks were placed on the surface of marine agar plates that had been freshly swabbed with an overnight grown liquid culture of target strains. The plates were incubated at 28 °C for 48 h. Disks loaded with only DMSO were served as negative control and the disks loaded with the commercial antifouling agent, TBTO were taken as a positive control. The diameter of the inhibition zone represented the strength of antibacterial activity of the test extracts.

Chemotaxis assay

Spectrophotometer based chemotaxis assay (Boyd et al., 1999) was followed with minor modifications. A final volume of 150 μl of 2% agar gel homogenously mixed with 300 μg of ethyl acetate extract dissolved in 10 μl of DMSO was maintained at 50 °C. The agar mixture was rapidly transferred to the bottom of a cuvette and allowed to cool for 1 h. The target bacterial suspension of marine motile strains, SCH0401, SCH0402, SCH0407 and SCH0408 were made in semisolid medium (SSM) with absorbance of 0.25 at 610 nm. A semisolid medium was prepared by mixing 1.87 g of marine broth (Difco), 1.34 g of synthetic sea salt (Reef Crystal) and 0.2 g of agar per 100 ml of distilled water. DMSO and TBTO served as negative and positive controls, respectively.

Absorbance at 610 nm was measured at an interval of 30 min for 5 h using spectrophotometer (Pharmacia Biotech). The increase and decrease of absorbance indicated the attraction and repulsion activity of the test extract, respectively. The obtained data were tabulated as change in optical density, $\Delta OD_{(t)}$, corresponding with the difference between initial optical density (OD_0) and OD at given time (OD_t).

Bacterial identification

Isolated bacteria were identified by using gram staining and other physiological tests including starch test, hydrolysis of gelatin and casein, urase test, H_2S production test, fermentation of glucose, sucrose and lactose, indole test, citrate test and catalase test. Obtained data were compared with already published literatures and used to support the identification of bacteria by using 16S rDNA gene sequence data.

DNA analysis

All marine strains isolated were identified based on 16S ribosomal DNA (rDNA) sequence analysis.

The isolated genomic DNA of each strain was purified with a Wizard Genomic DNA Purification Kit (Promega). The primer sets 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1518R (5′-AAG GAG GTG ATC CAC CCR CA-3′) were used (Giovannoni, 1991). About 1.5 kb partial sequences of 16S rDNA were determined by ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystem) and an automatic sequence analyzer system (Model 377, Applied Biosystem). The primer set used for sequence determination consisted of 518R (5′-GTA TTA CCG CGG CTG CTG-3′) and 337F (5′-ACT CCT ACG GGA GGC AGC-3′). The nucleotide sequences were deposited in the GenBank database of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) under accession numbers AY881234–AY881243. Sequences of the 16S rDNA were applied to the advanced BLAST search program to identify sequences of closely related organisms. The related sequences were preliminarily aligned with CLUSTALW (Thompson et al., 1994), and complete sequence alignments were performed using PHYDIT (Chun, 1995) and manual comparison. Phylogenetic analyses were performed with PHYLIP (Felsenstein, 1993), and phylogenetic tree was constructed by using neighbor-joining method (Saitou & Nei, 1987).

Results

Isolation of bacteria

More than 50 strains were isolated. Among them four strains were motile. The motile strains were selected as target strains for antibacterial and chemotaxis assays. Including four target isolates, ten strains SCH0401–SCH0410 were selected for screening antibacterial and chemotaxis activity. The ethyl acetate extract of each selected strain showed chemotactic activity to at least one target strain.

Antibacterial assay

The antibacterial activities of ethyl acetate extracts from ten bacterial strains were separately tested against four motile target strains. Each 300 μg disk^{-1} of bacterial extract showed its antibacterial activity with various strengths (Table. 1). The extracts of SCH0401 and SCH0410 showed antibacterial activities against all target strains. The extracts of SCH0403, SCH0405 and SCH0406 showed antibacterial effects only against SCH0401. SCH0407 and SCH0408 showed antibacterial effects against SCH0401 and SCH0408. SCH0402 and SCH0409 produced growth inhibition effects against three strains. SCH0404 showed antibacterial activity against two strains. TBTO inhibited the growth of all target bacterial strains. Quantitatively, the growth inhibition zones produced by the extract of SCH0402 against various target strains were in the range of 0–13 mm in diameter. TBTO produced the average growth inhibition zones in the range of 60–75 mm in diameter.

Chemotaxis assay

When the impregnated bacterial extract was diffused into the overlaid suspension of the motile target bacterium, three different kinds of responses – positive chemotaxis, negative chemotaxis and no response were observed. The target strain, SCH0401 was repelled by the extracts of SCH0401–SCH0408 by two to six times when compared to control (Table 1). Among them the extract of SCH0402 showed the strongest repellent activity producing six times decrease in optical density of the target strain. SCH0409 and SCH0410 showed attraction effect to the strain SCH0401 though the strength of attraction was less than two times. The strain SCH0402 itself was repelled by extracts of nine strains. SCH0404 exhibited no chemotactic activity to SCH0402. SCH0404 showed positive chemotactic and SCH0408 induced no effects to SCH0407. The other eight strains showed various strengths of negative chemotactic activities to SCH0407. The strain SCH0408 was repelled by only two strains SCH0402 and SCH0409. The remaining strains induced positive chemotactic activity to SCH0408. SCH0402 was the only one test strain which induced negative chemotaxis activity to all target strains. TBTO showed only two times decrease in optical density of all target strains when compared to control.

Table 1. Chemotactic[a] and antibacterial[b] activities of the bacterial extracts (300 μg) against motile strains

Test strains	Target strains			
	SCH0401	SCH0402	SCH0407	SCH0408
SCH0401	**(11 ± 1.7)	**(10.5 ± 0.8)	**(12 ± 1.1)	+(14 ± 1.5)
SCH0402	******(10 ± 1)	**(11 ± 1.2)	****(13 ± 1)	**(0)
SCH0403	**(9.3 ± 0.6)	***(0)	***(0)	+(0)
SCH0404	**(13 ± 1.7)	−(8.5 ± 0.5)	+(0)	+(0)
SCH0405	**(14.3 ± 1.5)	***(0)	**(0)	+(0)
SCH0406	***(13.7 ± 2.1)	**(0)	***(0)	+(0)
SCH0407	**(8.3 ± 0.6)	**(0)	**(0)	+(10 ± 0.8)
SCH0408	**(14.3 ± 1.5)	***(0)	−(0)	+(13 ± 1.2)
SCH0409	+(10 ± 1)	**(0)	**(10.5 ± 0.8)	**(10 ± 1)
SCH0410	+(11 ± 1)	****(15 ± 1.4)	**(20 ± 2.1)	+(20 ± 1.8)
TBTO	**(64 ± 6.2)	**(62 ± 7)	**(72 ± 6.5)	**(75 ± 8)

[a]Chemotactic activities of the bacterial extracts were indicated by the symbols (*, +, −). * indicated negative chemotaxis, + indicated positive chemotaxis, − indicated no effect. The number of asterisks indicated the times of decrease in optical density of the target strains when compared to control during spectrophotometer based chemotaxis assay.
[b]Antibacterial activities were indicated in term of diameter of the zone of inhibition (unit: mm). ± Indicated the standard deviation. The data were average among three replicas.

Identification of bacteria

All 10 active isolated strains were identified on the basis of sequence result of 16S rDNA followed by morphological and physiological characters. The isolated five species were under the phylogenic group of Proteobacteria and the remaining five were under the group of Actinobacteria (Fig. 1). The most active repellent producing gram negative motile rod SCH0402 was closest to *Shewanella oneidensis* DML 7 (97.91%). It exhibited catalase and gelatinase activities. H_2S was produced. It utilized lactate, succinate and fumarate as sole carbon sources. It was negative in indole production and glucose fermentation tests. The other gram negative motile rod isolates, SCH0401 and SCH0407 were closest to *Alteromonas marina* SW-47 (97.91%), and *Roseobacter gallaeciensis* CIP 105210^T (97.09%), respectively. The gram negative non motile cocci strains, SCH0403 and SCH0409 were closest to *Paracoccus marcusii* MH 1 (100%) and *Acinetobacter junii* DSM 6964^T (100%) respectively. Other gram positive non motile cocci SCH0404, SCH0405 and SCH0406 were closest to *Kocuria palustris* DSM 11925 (100%), *Micorcoccus luteus* DSM 20030^T (99.5%) and *Kytococcus sedentarius* DSM 20547 (99.72%) respectively. The gram positive motile rod, SCH0408 was closest to *Bacillus atrophaeus* JCM 9070 (99.93%) and gram positive non motile short rod, SCH0410 was closest to *Zimmermanella faecalis* IFO 15706 (99.5%).

Discussion

Bacterium–bacterium antagonism is potentially very common in pelagic ocean. More than 50% of isolates showed antagonistic activities against other pelagic bacteria (Long & Azam, 2001). There is not necessary for a chemotactic repellent to be an antibacterial. Some repellents may be involved in bacterial communication and can alter the species composition in bacterial community (Pesci et al., 1999). Since motile bacteria rapidly colonize to form biofilm (Kiφrboe et al., 2002), and subsequent chemical cues promoted further macrofouling (Satuito et al, 1997), the control of early bacterial fouling by using bacterial repellents instead of toxic antifouling agents could be an environmentally safe strategy to stop further macro fouling on the surface of marine developmental structures (Burgess et al., 1999). Therefore, the organic compounds that induced higher negative chemotactic activities than TBTO on early fouling bacteria with weaker antibacterial

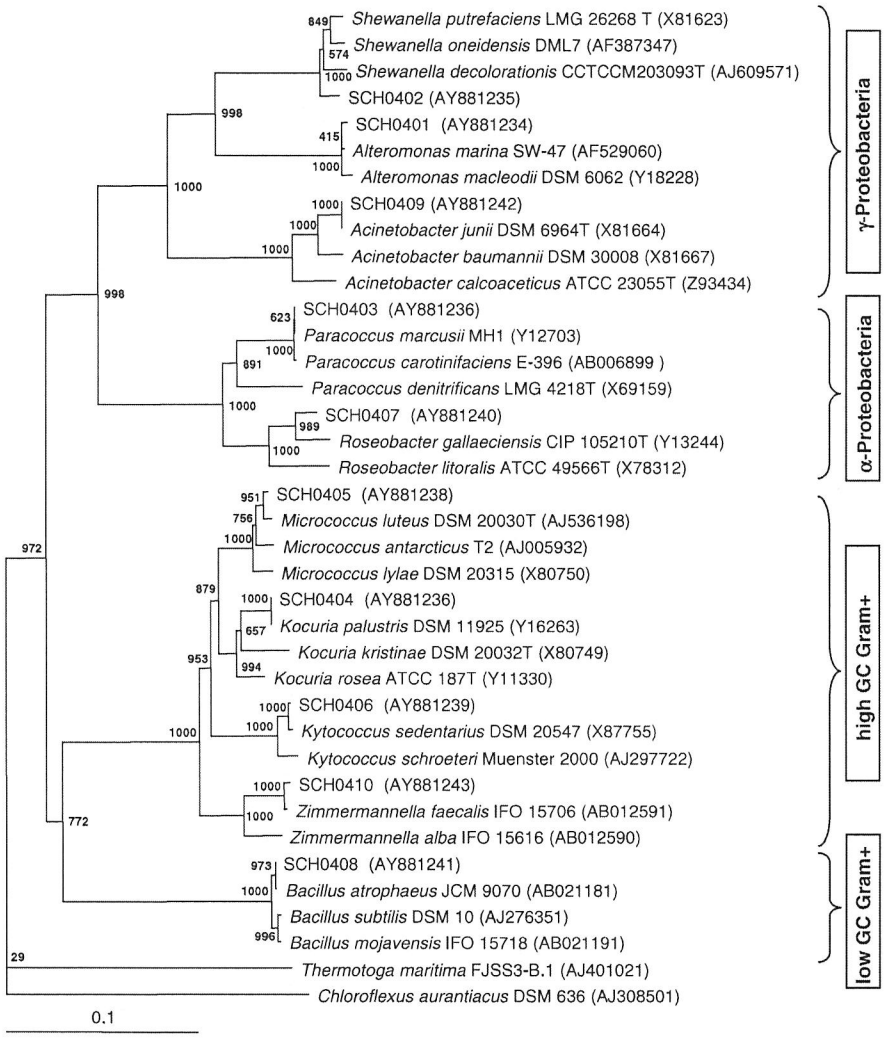

Figure 1. Phylogenetic tree based on partial (approximately 1.5 kb) sequence of 16S rDNA sequence comparison by using neighbor-joining method.

activities can be used as the substitute of the existing toxic antifouling compounds. Several bacterial species including *Pseudoalteromonas tunicata* (James et al., 1996) and *P. ulvae* (Egan et al., 2000) have been reported to produce antifouling compounds and protected their hosts' surfaces against subsequent foulers.

Ten free living marine bacterial strains were isolated and their interactions in term of chemotactic and antibacterial activities against motile bacterial strains were studied. It was observed that each bacterial strain had various antibacterial and chemotactic activities. In addition, there was not any correlation between antibacterial and chemotactic activity induced by the extract of a test strain. The extract of SCH0402 showed repellent activity to all target strains. However, the strengths of repellent activities were various. The antibacterial activity induced by SCH0402 was six times weaker than that of TBTO. But, negative chemotactic activity induced by the same extract was three times stronger than that of TBTO.

The bacterial strains belonging to five orders namely, Alteromonadales, Pseudomonadales,

Rhodobacteriales, Actinomycetales and Bacillales were isolated and identified. These groups of bacteria represented the cultivable marine pelagic bacteria (Giovannoni & Rappé, 2000). The gram negative motile rod SCH0402 was identified as *Shewanella oneidensis*. A number of environmental importance of *S. oneidensis* have been described (Venkateswaran et al., 1999) including bioremediation of organic pollution (Petrovskis et al., 1994), spoilage of proteinous food (Shewan, 1977) and opportunistic pathogens of human and aquatic animals (Aguirre et al., 1994) but the repellent activity shown by *S. oneidensis* is reported here for the first time. Therefore, this particular strain could be an interesting isolate to conduct further research to explore the responsible chemicals. The strain SCH0407 was identified as *Roseobacter gallaeciensis*. The bacterial strain, *Roseobacter litoralis* have been found to induce settlement of larvae of serpulid polychaete *Hydroides* elegans (Lau et al., 2003). Therefore, the repulsion of strain *Roseobacter* might help to deter the larval settlement. SCH0408 was identified as *Bacillus atrophaeus*. The gram positive species was reported as biofilm forming bacteria (Kwon et al., 2002). A number of *Bacillus* species have been described as antibiotic compounds producing bacteria (Bizani & Brandelli, 2002).

The present study of antagonistic interactions among marine pelagic bacterial strains gives a conclusion that strain SCH0402 repelled all target motile strains with less antibacterial activities. The ethyl acetate extract of SCH0402 is expected to contain substances which have less antibacterial activities but higher repellent activities to other fouling bacterial strains. Such characters of the expected chemicals are the prerequisites for eco-friendly antifouling strategy of marine antifouling paints.

Acknowledgements

This work was partially supported by grant to HKL under the National Research Laboratory Program of the Ministry of Science and Technology and partially by grant to HWS under policy research program from the ministry of Maritime Affairs and Fisheries (MOMAF), government of Korea. We are happy to thank Mr. Ki-Cheol Sung for his cooperation and assistance during lab work. We acknowledge the anonymous reviewers for the critical suggestion to improve the quality of the manuscript.

References

Aguirre, A. A., G. H. Balazas, B. Zimmerman & T. R. Spraker, 1994. Evaluation of Hawaiian green turtles (*Chelonia mydas*) for potential pathogens associated with fibropapillomas. Journal of Wildlife Diseases 30: 8–15.

Alzieu, C., 1998. Tributyltin: case study of a chronic contaminant in the coastal environment. Ocean and Coastal Management 40: 23–26.

Armstrong, E., L. Yan, K. G. Boyd, P. C. Wright & J. G. Burgess, 2001. The symbiotic role of marine microbes on living surfaces. Hydrobiologia 461: 37–40.

Bizani, D. & A. Brandelli, 2002. Characterization of a bacteriocin produced by a newly isolated *Bacillus* sp. strain 8 A. Journal of Applied Microbiology 93: 512–519.

Boyd, K. G., A. Mearns-Spragg & J. G. Burgess, 1999. Screening of marine bacteria for the production of microbial repellents using a spectrophotometric chemotaxis assay. Marine Biotechnology 1: 359–363.

Burgess, J. G., E. M. Jordan, M. Bregu, A. Mearns-Spragg & K. G. Boyd, 1999. Microbial antagonism: a neglected avenue of natural product research. Journal of Biotechnology 70: 27–32.

Chet, I. & R. Mitchel, 1976. Ecological aspects of microbial chemotactic behavior. Annual Review of Microbiology 30: 221–239.

Chun J. 1995. Computer-assisted classification and identification of Actinomycetes. Ph.D. Thesis, University of Newcastle, Newcastle upon Tyne, UK.

Egan, S., T. Thomas, C. Holmström & S. Kjelleberg, 2000. Phylogenetic relationship and antifouling activity of bacterial epiphytes from the marine alga *Ulva lactuca*. Environmental Microbiology 2: 343–347.

Felsenstein, J., 1993. PHYLIP (Phylogeny Inference Package), version 3.5c. Department of Genetics, University of Washington, Seattle, WA, USA.

Giovannoni, S. & M. Rappé, 2000. Evolution, diversity, and molecular ecology of marine prokaryotes. In Kirchman, D. L. (ed.), Microbial ecology of the ocean. Wiley-Liss, New York, 47–84.

Giovannoni, S. J., 1991. The polymerase chain reaction. In Stackebrandt, E. & M. Goodfellow (eds.), Nucleic Acid Techniques in Bacterial Systematic. John Wiley & Sons, New York, 177–201.

Holmström, C., S. Egan, A. Franks, S. McCloy & S. Kjelleberg, 2002. Antifouling activities expressed by marine surface associated *Pseudoalteromonas* species. FEMS Microbiology Ecology 41: 47–58.

Ista, L. K., M. E. Callow, J. A. Finlay, S. E. Coleman, A. C. Nolasco, R. H. Simons, J. A. Callow & G. P. Lopez, 2004. Effects of substratum surface chemistry and surface energy

on attachment of marine bacteria and algal spores. Applied and Environmental Microbiology 70: 4151–4157.

James, S. G., C. Hölmstrom & S. Kjelleberg, 1996. Purification and characterization of novel antibacterial protein from the marine bacterium D2. Applied and Environmental Microbiology 62: 2783–2755.

Kiφrboe, T. & G. A. Jackson, 2001. Marine snow, organic solute plumes, and optimal chemosensory behavior of bacteria. Limnology and Oceanography 46: 1309–1318.

Kiφrboe, T., H. P. Grossart, H. Ploug & K. Tang, 2002. Mechanisms and rates of bacterial colonization of sinking aggregates. Applied and Environmental Microbiology 68: 3996–4006.

Kwon, K. K, H. S. Lee, S. Y. Jung, J. H. Yim, J. H. Lee & H. K. Lee, 2002. Isolation and identification of biofilm-forming marine bacteria on glass surfaces in Dae-Ho Dike, Korea. Journal of Microbiology and Biotechnology 40: 260–266.

Lau, S. C., T. Harder & P. Y. Qian, 2003. Induction of larval settlement in the serpulid polychaete *Hydroides elegans* (Haswell): role of bacterial extracellular polymers. Biofouling 19: 197–204.

Lewis, J. A., 1998. Marine biofouling and its prevention on underwater surface. Material Forum 22: 41–46.

Long, R. A. & F. Azam, 2001. Antagonistic interactions among marine pelagic bacteria. Applied and Environmental Microbiology 67: 4975–4983.

Pesci, E. C., J. B. Milbank, J. P. Pearson, S. McKnight, A. S. Kende, E. P. Greenberg & B. H. Iglewski, 1999. Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. Proceedings of the National Academy of Sciences of the United States of America 96: 11229–11234.

Petrovskis, E. A., T. M. Vogel & P. Adriaens, 1994. Effects of electron acceptors and donors on transformation of tetrachloromethane by *Shewanella putrefaciens* MR-1. FEMS Microbiology Letters 121: 357–364.

Saitou, N. & M. Nei, 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4: 406–425.

Satuito, C. G., K. Shimizu & N. Fusetani, 1997. Studies on the factors influencing larval settlement in *Balanus amphitrite* and *Mytilus galoprovinvialis*. Hydrobiologia 358: 275–280.

Shewan, J. M., 1977. The bacteriology of fresh and spoiling fish and the biochemical changes induced by bacterial action. In Proceedings of the Conference of Handling, Processing and Marketing of Tropical Fish, Tropical Product Institute, London, 51–66.

Thompson, J. D., D. G. Higgins & T. J. Gibson, 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22: 4673–4680.

Venkateswaran, K., D. P. Moser, M. E. Dolhopf, D. P. Lies, D. A. Saffarini, B. J. MacGregor, D. B. Ringelberg, D. C. White, M. Nishijima, H. Sano, J. Burghardt, E. Stackebrandt & K. H. Nealson, 1999. Polyphasic taxonomy of the genus *Shewanella* and description of *Shewanella oneidensis* sp. nov. International Journal of Systematic Bacteriology 49: 705–724.