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Cryoprotective Properties of Exopolysaccharide (P-21653) Produced by the Antarctic Bacterium, *Pseudoalteromonas arctica* KOPRI 21653

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Twenty-five bacterial strains that secrete mucous materials were isolated from sediment obtained from King George Island, Antarctica. Seven of these strains proved capable of producing cryoprotective exopolysaccharides. The strain KOPRI 21653 was selected for the further study of an anti-ice-nucleating polysaccharide (ANP), which originated from a polar region. KOPRI 21653 was identified as *Pseudoalteromonas arctica* as the result of 16S rRNA analysis. The exopolysaccharide, P-21653, was purified completely from the KOPRI 21653 cell culture via column chromatography and protease treatment. The principal sugar components of P-21653 were determined to be galactose and glucose, at a ratio of 1:1.5, via GC-MS analysis. The cryoprotective activity of P-21653 was characterized via an *E. coli* viability test. In the presence of 0.1% (w/v) P-21653, the survival ratio of *E. coli* cells was as high as 82.6% over three repeated freeze-thaw cycles. The survival ratio decreased drastically to 71.5 and 48.1%, respectively, in five and seven repeated cycle conditions; however, the survival ratios were greater over three (96.6-92.1%) to seven (100.5-91.6%) freeze-thaw cycles in the presence of 0.5 and 1.0% (w/v) P-21653. In addition, at much lower concentrations (0.1-1.0%), P-21653 resulted in survival ratios (83.1-98.4%) similar to those of two commercially available cryoprotectants (V_{EG} plus X-1000, 92.9% and V_{M3}, 95.3%), which were utilized at the recommended concentrations (90%). The biochemical characteristics of exopolysaccharide P-21653 reflect that this compound may be developed as a useful cryoprotectant for use in medical applications and in the food industry.

Keywords: cryoprotective property, *Pseudoalteromonas arctica*, cryoprotectant

Freeze-thaw cycles are quite common in the cold regions of the Arctic and Antarctic. Cold-adapted microorganisms are accustomed to being frozen within their habitats. Such organisms are also expected to have evolved adaptations to survive repeated freezing and thawing, as these processes tend to damage living cells and attenuate cell viability (Mazur, 1970).

Several diatoms, cyanobacteria, and bacteria generate abundant quantities of exopolysaccharide (Palmisano and Sullivan, 1985; Cooksey and Cooksey, 1995; Costerton *et al.*, 1995; Stoderegger and Herndl, 1998), which is stored as a thick gel surrounding the cells. The primary ecologically significant characteristic of exopolysaccharide is that it can form and maintain protective microhabitats around microorganisms in aquatic and cold environments (Decho, 1990). The physical, rheological, and chemical properties of exopolysaccharide are affected by the length of the polymer chain, which is the principal determinant of the molecular weight (Christensen, 1999). As the length of the polymer increases, a greater opportunity for complex entanglement of polymer chains and intramolecular associations occurs, and these interactions contribute to the tertiary structure and physical behavior of the polymer (Sutherland, 1994). *Phomaherbarum*, a fungal strain isolated from Antarctic soil, generates a glucose homosaccharide with a molecular mass of 7.4×10^6 kDa, and this exopolysaccharide was suggested to function as a cryoprotectant within the Antarctic environment (Selbmann *et al.*, 2002).

Researchers have attempted to develop methods to permit 100% preservation of diverse cellular specimens after freezing and thawing (Albrecht *et al.*, 1973; Dumont *et al.*, 2003). In an effort to improve cell preservation, some cryoprotectants, including glycerol or dimethyl sulfoxide, can be utilized (Hubalek, 2003). These molecules improve cell preservation via the minimization of the cell water content (Dumont *et al.*, 2003), thereby bolstering vitrification (Ablett *et al.*, 1992), and protecting the constitutive macromolecules of the cell (Anchordoguy *et al.*, 1987; Adam *et al.*, 1994)

The principal objective of this study was to assess the influence of an exopolysaccharide obtained from the newly isolated Antarctic bacterium, *Pseudoalteromonas arctica,* on the *E. coli* cell survival ratio after repeated freeze-thaw treatments. In addition, we assessed the utility of this new cryoprotective exopolysaccharide from Antarctic bacteria.

Materials and Methods

Culture conditions and isolation

Seaside soil and sediment samples from Barton Peninsula on King George Island were diluted with saline (0.85% NaCl), spread onto ZoBell medium (Zobell, 1946), and incubated for three days at 25°C. Bacterial colonies, which secrete a

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viscous material on the surfaces of cells, were isolated as candidates and subsequently maintained in a 20% (v/v) glycerol suspension at -80°C. For the selection of exopolysaccharide-generating strains, each isolated colony was incubated in 20 ml of ZoBell medium. After 3 days of incubation at 25°C with shaking at 120 rpm, the cells were removed via 20 min of centrifugation at 10,000×g, and 6 ml of cold ethanol was added to 3 ml of the supernatant in a test tube (10 ml). Subsequently, the reaction mixture was incubated for 24 h at 4°C. Exopolysaccharide-generating bacterial strains were selected on the basis of the relative quantity of the resultant polymer complex that was dehydrated and then flocculated by ethanol. All tested strains are listed in Table 1.

Identification

Total genomic DNA was extracted from the isolates using an AccuPrep genomic DNA Extraction kit (Bioneer, Korea). The 16S rRNA genes were amplified from the genomic DNA via PCR using the 27F; 5'-AGAGTTTGATC(C/A)T GGCTCAG-3' and 1492R; 5'-GGTTACCTTGTTACGACTT-3' primer sets (Lane, 1991). The PCR products were then sequenced, the resulting DNA sequences being analyzed using the GenBank database.

Exopolysaccharide purification

Bacterial cells were removed from the culture medium via 30 min of centrifugation at 12,000×g at 4°C. The exopolysaccharides were then separated from the supernatant via the addition of two volumes of ethanol followed by 24 h of precipitation at 4°C. The precipitated exopolysaccharides were then collected via 20 min of centrifugation at 10,000×g at 4°C, dissolved in dH_2O, and lyophilized. For the exclusion of proteins, crude polysaccharides were treated for 30 min with protease (500 units/L) at 37°C, dialyzed against dH_2O using Viva-Flow (Sartorius, Germany), and lyophilized. The crude exopolysaccharides were then dissolved in dH_2O and re-precipitated via the addition of a 10% solution of cetylpyridinium chloride (CPC). The precipitated CPC-exopolysaccharide complex was subsequently collected via 20 min of centrifugation at 10,000×g at 4°C and re-dissolved in 10% NaCl. The precipitated polysaccharide was recovered via the addition of three volumes of ethanol. The extracted exopolysaccharide was dissolved in dH_2O, dialyzed twice against dH_2O, and lyophilized.

Analytical procedures

The sugar composition was determined via GC-MS analysis using sugar standards as the controls. P-21653 (10 μg) was mixed with 0.5 ml of methanolysis reagent in a 10 ml Pyrex tube. The methanolysis reagent was prepared via the dissolution of 100 ml of dry methanol in 4.65 ml of acetyl chloride (Sigma, USA; Chaplin, 1994). The samples were left for 20 h of methanolysis at 80°C. After the methanolysis reaction was complete, the samples were evaporated to dryness under a moderate nitrogen stream. The dry samples were then dissolved in 200 μl of anhydrous acetonitrile, after which 25 μl of heptaflurobutyric acid anhydride (HFBAA) was added to each sample. The mixed samples were heated for 30 min at 150°C in a heating block. After cooling at room temperature, the samples were evaporated with a light nitrogen gas stream in order to remove any excess reagents. The final evaporated samples were dissolved in 100 μl of anhydrous acetonitrile and subjected to GC-MS analysis.

Cryoprotective properties of exopolysaccharide

In order to determine the cryoprotective properties of the exopolysaccharides generated from the bacteria, *E. coli* was subjected to freeze-thaw cycles in the presence or absence of the exopolysaccharide. An equal volume of exopolysaccharide was mixed with a suspension of *E. coli* in 2 ml freezing vials (Nalgene Cryoware cryogenic vials, Nalgene Nunc International, USA), after which the mixture was frozen at -80°C for 30 min. Each tube was thawed for 20 min in a water bath at 25°C, after which it was again frozen at -80°C. For the *E. coli* cell viability test, 20 ml of an *E. coli* culture in late log phase was harvested via 5 min of centrifugation at 10,000×g. The cell pellet was resuspended in 20 ml of saline solution (0.85% NaCl). After three washings in saline, the pellets were resuspended in 10 ml of saline. A bacterial viability kit (LIVE/DEAD® BacLight™ Bacterial Viability Kits; Molecular Probes, L7012, USA) was employed in conjunction with a fluorescence microplate reader (Perkin Elmer, Envision 2103, USA). The *E. coli* suspensions were adjusted to 2×10^8 cells/ml ($O.D._{670}$=0.06). The adjusted 100 μl *E. coli* sample was then mixed with an equal volume of exopolysaccharide solution. In accordance with the manufacturer's instructions, 6 μl of staining dye A and 6 μl of staining dye B were mixed, and then 2 ml of filter-sterilized dH_2O was added and mixed thoroughly. After the freeze- thaw cycle, the mixture of 100 μl of *E. coli* and an equal volume of

Table 1. List of the isolated strains that produce exopolysaccharides

Strain	Sample type	Collection site
KOPRI 21650[a]	Sediment	King George Island, 62° 13' S, 58° 47' W
KOPRI 21653[a]	Sediment	King George Island, 62° 14' S, 58° 44' W
KOPRI 21654[a]	Seaside soil	King George Island, 62° 13' S, 58° 44' W
KOPRI 21663[a]	Sediment	King George Island, 62° 13' S, 58° 46' W
EP 144	Seaside soil	King George Island, 62° 13' S, 58° 47' W
EP 205	Seaside soil	King George Island, 62° 13' S, 58° 47' W
EZ 291	Seaside soil	King George Island, 62° 14' S, 58° 43' W

[a] The strains were given KOPRI (Korea Polar Research Institutes) Collection Numbers.

exopolysaccharide was dispensed into the wells of a 96 well flat-bottom microtiter plate. Prepared staining dye (100 μl) was then mixed with the sample. All mixed samples were incubated at room temperature for 15 min in darkness. The excitation wavelength was 485 nm, and the fluorescence intensity was measured at 530 nm (emission 1, green) and 630 nm (emission 2, red). The cell survival ratio was calculated as follows: Cell survival ratio (%)=(A/B)× 100 in which A=fluorescence ratio $_{G/R}$ after the freeze-thaw cycle and B=initial fluorescence ratio $_{G/R}$. The ratio $_{G/R}$=Emission 1 (485 nm, 530 nm) / Emission 2 (485 nm, 630 nm).

V_{EG}, V_{M3}, and X-1000 (21st Century Medicine, USA) were utilized as commercialized cryoprotectants. The concentration of each commercial cryoprotectant was adjusted in accordance with the protocols recommended by 21st Century Medicine. A 90% (v/v) V_{EG} solution with 1% (v/v) X-1000 and 90% (v/v) V_{M3} solutions were assessed via the *E. coli* cell viability test technique, as described above.

Results and Discussion

Isolation and identification of bacterial strains

Approximately 25 bacterial strains that generated mucous materials were isolated from solid ZoBell medium. Among the isolated strains, seven strains were shown to generate mucous polysaccharide. The exopolysaccharide produced by the strain KOPRI 21653, which is designated P-21653, coated *E. coli* cells during the freeze-thaw cycle (Fig. 1) and evidenced profound cryoprotective properties compared with the exopolysaccharides obtained from other strains (Fig. 2). After three freeze-thaw cycles in the presence of P-21653, the *E. coli* cell survival ratio was 90.53±0.60%, whereas the survival rates of *E. coli* in the presence of other exopolysaccharides ranged from 44.80±0.41% to 33.03±0.53%. The control (saline only) treatment resulted in a survival ratio of only 13.24±0.32%.

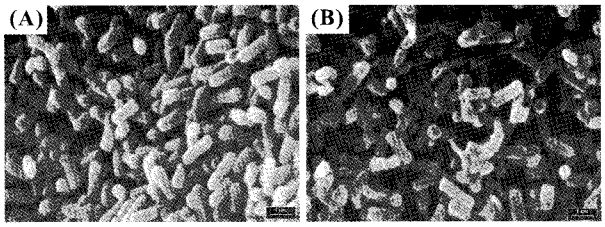

Fig. 1. Scanning electron micrograph of *E. coli* cell. Bar, 1 μm. (A) Control: *E. coli* cell in the presence of saline solution. (B) *E. coli* cell in the presence of 0.1% P-21653.

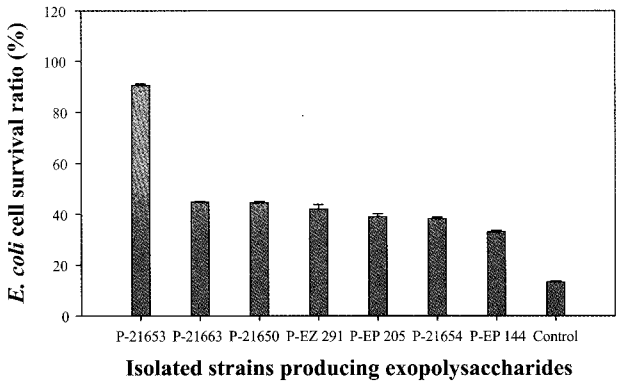

Fig. 2. *E. coli* cell survival ratio following a third freeze-thaw cycle in the presence of 0.2% (w/v) crude exopolysaccharide solution from selected strains. An equal volume of saline solution was added to the control rather than exopolysaccharide.

After 24 h of growth in ZoBell medium at 25°C, the KOPRI 21653 strain was rod-shaped, 3-5 μm in length, and 0.3-0.5 μm in diameter. The 16S rDNA sequence of KOPRI 21653 (1,400 bp) was determined and analyzed with the Advanced BLAST search software. On the basis of our 16S rDNA analysis, KOPRI 21653 was found to evidence the highest similarity to *Pseudoalteromonas arctica* A 37-1-2^T (100% 16S rDNA similarity) and lesser but significant similarities to *Pseudoalteromonas elyakovii* KMM162^T (99.64%), *Pseudoalteromonas distincta* KMM638^T (99.64%), *Pseudoalteromonas nigrifaciens* NCIMB-8614^T (99.57%), and *Pseudoalteromonas paragorgicola* KMM3548^T (99.50%).

Characterization of the exopolysaccharide P-21653

The GC-MS analysis of the hydrolysis products of the exopolysaccharide was conducted (Zanetta *et al.*, 1999). Four peaks were obtained, at 11.2, 11.6, 11.7, and 12.2 min on the GC chromatogram. The peaks evidenced retention times

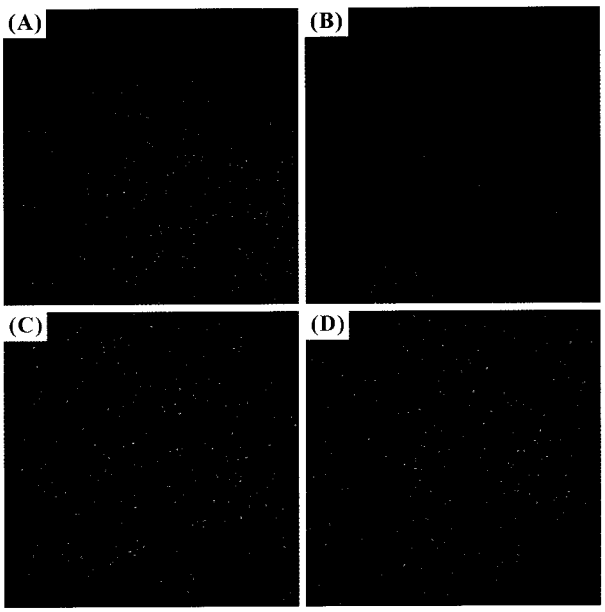

Fig. 3. Confocal scanning microscope image (×400) of *E. coli* cells stained with bacterial viability kits. Bacteria with intact cell membranes appear fluorescent green, whereas bacteria with damaged membranes appear fluorescent red. (A) *E. coli* cells emitted green and red fluorescence in the controls (saline solution) prior to the freeze-thaw cycle. (B) *E. coli* cells emitted red fluorescence in the control experiment (saline only) after five freeze-thaw cycles. (C) *E. coli* cells emitted green and red fluorescence in the presence of 0.5% (w/v) P-21653 solution prior to the freeze-thaw cycle. (D) *E. coli* cells emitted both green and red fluorescence in 0.5% (w/v) P-21653 solution after five freeze-thaw cycles (compare panels B and D).

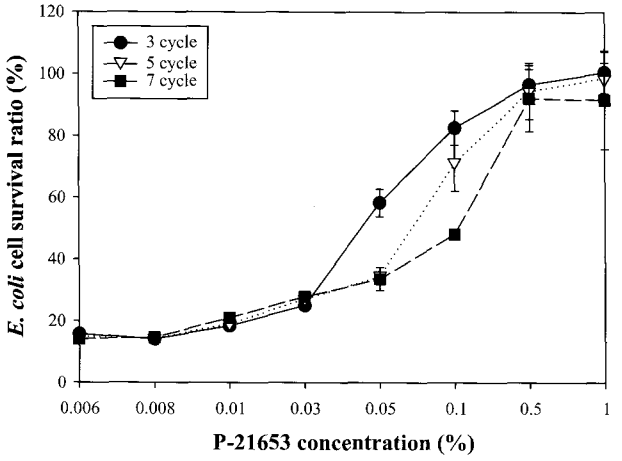

Fig. 4. *E. coli* cell survival ratio at various concentrations of P-21653 after freeze-thaw cycles (as indicated). An equal volume of saline solution was added to the control in the place of the exopolysaccharide.

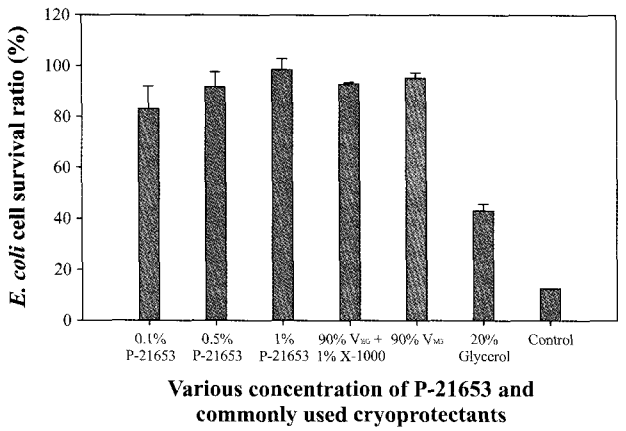

Fig. 5. The *E. coli* cell survival ratio at various concentrations of P-21653, 20% (v/v) glycerol, and commercial cryoprotectant (V_{EG} mixed X-1000 and V_{M3}) after three freeze-thaw cycles. An equal volume of saline solution was added to the control rather than exopolysaccharide.

and fragmentation patterns identical to those of the glucose (11.6 and 11.7 min) and galactose (11.2 and 12.2 min) standards. Thus, the principal sugar constituents of P-21653 are glucose and galactose, at a molar ratio of 1.5:1.

Cryoprotective effect of P-21653 on the survival of E. coli

To assess the cryoprotective properties of P-21653 on *E. coli* during freezing and thawing, the relationship between the concentration of P-21653 and the frequency of freeze-thaw cycles was assessed. *E. coli* cells were mixed with 0.001 to 1% (w/v) P-21653 solution and subjected to freeze-thaw cycles (3-7 times). The effects of the P-12653 concentration on the *E. coli* cell survival ratio according to freeze-thaw cycle frequency are provided in Figs. 3 and 4. The *E. coli* cell survival ratio increased as the concentration of P-21653 increased from 0.01 to 1% (w/v). More specifically, after the third freeze-thaw cycle, the *E. coli* cell survival ratios were 18.41±0.42%, 24.99±1.10%, 58.29±4.46%, 82.63±5.60%, 96.61±4.94%, and 100.57±6.70% in the presence of 0.01%, 0.03%, 0.05%, 0.1%, 0.5%, and 1% of P-21653, respectively. The *E. coli* cell survival ratio, however, was reduced with an increasing frequency of freeze-thaw cycles. After five and seven freeze-thaw cycles, the survival ratio was reduced from 58.29±4.46% to 34.87±0.44% with 0.05% (w/v) P-21653 and from 71.51±9.38% to 48.12±0.35% with 0.1% (w/v) P-21653. By way of contrast, after five and seven freeze-haw cycles, dramatic variations in survival ratio were not observed for *E. coli* exposed to 1% and 0.5% P-21653. These results clearly show that only exopolysaccharide P-21653 treated with protease influences the *E. coli* cell survival ratio. This exopolysaccharide significantly improved the freeze-thaw survival ratio of *E. coli*, which is a non-polar species, thereby indicating that the exopolysaccharide may exert a cryopreservative effect.

Comparison of P-21653 and commercial cryoprotectant on E. coli cell survival ratio

In order to compare the protective effects of P-21653 with other cryoprotectants, commercial V_{EG}, V_{M3} and X-1000 were used. V_{EG} and V_{M3} are cryoprotectants, and X-1000 is an additive which enhances the performance of the vitrification solution. Following a third freeze-thaw cycle, the *E. coli* cell survival ratios were 95.30±2.06%, 92.91±0.61%, 98.48±4.37%, 91.69±5.99%, 83.12±8.93%, and 43.09±2.61% after incubation with 90% (v/v) V_{M3} solution, 90% (v/v) V_{EG} solution with 1% (v/v) X-1000, 1% (w/v) P-21653 solution, 0.5% (w/v) P-21653 solution, 0.1% (w/v) P-21653 solution, and 20% (v/v) glycerol solution, respectively (Fig. 5). P-21653 incubation resulted in a high *E. coli* cell survival ratio of more than 90% at low concentrations, as compared to those of the commercial cryoprotectants. Considering that glycerol is generally employed as a bacterial cryoprotectant, the *E. coli* cell survival ratio of only 43.09% observed in the presence of 20% (v/v) glycerol indicates the cryoprotective power of P-21653. These results support the possibility of using P-21653 as a cryoprotectant material.

The identification and characterization of such cryotolerance-related adaptations would greatly contribute to our understanding of the mechanisms that facilitate the survival of soil and sediment-associated bacteria in cold environments. Furthermore, such studies have the potential to contribute to the development of novel cryoprotectant materials.

Of the 25 isolated bacterial strains that generate polysaccharides, seven strains from Antarctic seaside soil and sediment generated mucous polysaccharide. The results of 16S rDNA sequence analysis showed that KOPRI 21653 evidences a sequence similarity of 100% with that of *Pseudoalteromonas arctica*. KOPRI 21653 generated mucous polysaccharide, designated P-21653, with high cryoprotective properties on the basis of the *E. coli* cell survival ratio. The cryoprotective properties of P-21653 were determined to enable bacteria to withstand repeated freezing and thawing under laboratory conditions. This polysaccharide may prove a useful cryoprotective agent, and may shed light on the mechanisms exploited by bacterial organisms inhabiting extreme environments.

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References

Ablett, S., J.M. Izzard, and P.J. Lillford. 1992. Differential scanning calorimetric study of frozen sucrose and glycerol solutions. *J. Chem. Soc. Faraday Trans.* 88, 789-794.

Adam, M.M., K.J. Rana, and B.J. Mcandrew. 1994. Effect of cryoprotectants on activity of selected enzymes in fish embryos. *Cryobiol.* 32, 92-104.

Albrecht, R.M., G.R. Orndorff, and A.P. MacKenzie. 1973. Survival of certain microorganisms subjected to rapid and very rapid freezing on membrane filters. *Cryobiol.* 10, 233-239.

Anchordoguy, T.J., A.S. Rudolph, J.F. Carpenter, and J.H. Crowe. 1987. Modes of interaction of cryoprotectants with membrane phospholipids during freezing. *Cryobiol.* 24, 324-331.

Chaplin, M.F. 1994. Monosaccharides, p. 1-41. *In* M.F. Chaplin and J.F. Kennedy (eds.), Carbohydrate analysis. A practical approach. Oxford University Press, Oxford, UK.

Christensen, B.E. 1999. Physical and chemical properties of extracellular polysaccharides associated with biofilms and related substances, p. 144-154. *In* J. Wingender, T. Neu, and H.C. Flemming (ed.), Microbial extracellular substances: characterization, structure and function. Springer, New York, USA.

Cooksey, K.E. and B. Cooksey. 1995. Adhesion of bacteria and diatom to surfaces in the sea: a review. *Aquat. Microb. Ecol.* 9, 87-96.

Costerton, J.W., Z. Lewandowski, D.E. Caldwell, D.R. Korber, and H.M. Lappin-Scott. 1995. Microbial biofilms. *Ann. Rev. Microbiol.* 49, 711-745.

Decho, A.W. 1990. Microbial exopolymer secretions in ocean environments: their role(s) in food webs and marine processes. *Oceanogr. Mar. Biol. Ann. Rev.* 28, 73-153.

Dumont, F., P.A. Marechal, and P. Gervais. 2003. Influence of cooling rate on *Saccharomyces cerevisiae* destruction during freezing: unexpected viability at ultra-rapid cooling rates. *Cryobiol.* 46, 33-42.

Hubalek, Z. 2003. Protectants used in the cryopreservation of microorganisms. *Cryobiol.* 46, 205-229.

Lane, D.J. 1991. 16S/23S rRNA sequencing, p. 115-175. *In* E. Stackebrandt and M. Goodfellow (eds.), Nucleic acid techniques in bacterial systematics, Chichester, John Wiley and Sons, New York, N.Y., USA.

Mazur, P. 1970. Cryobiology: the freezing of biological systems. *Science* 168, 939-949.

Palmisano, A.C. and C.W. Sullivan. 1985. Growth, metabolism and dark survival in sea ice microalgae, p. 131-146. *In* R.A. Horner (ed.), Sea Ice Biota. CRC Press, Boca Raton, FL, USA.

Selbmann, L., S. Onofri, M. Fenice, F. Frederico, and M. Petriuccioli. 2002. Production and structural characterization of the exopolysaccharde of the Antarctic fungus *Phoma herbarum* CCFEE 5080. *Res. Microbiol.* 153, 585-592.

Stoderegger, K. and G. Herndl. 1998. Production and release of bacterial capsular material and its subsequent utilization by marine bacterioplankton. *Limnol. Oceanol.* 43, 877-884.

Sutherland, I.W. 1994. Structure function relationships in microbial exopolysaccharides. *Biotechnol. Adv.* 12, 393-448.

Zanetta, J.P., P. Timmerman, and Y. Leroy. 1999. Gas-liquid chromatography of the heptafluorobutyrate derivates of the O-methyl-glycosides on capillary columns: a method for the quantitative determination of the monosaccharide composition of glycoproteins and glycolipids. *Glycobiol.* 9, 255-266

ZoBell, C.E. 1946. Marine Microbiology, XV, p. 240. Chronica Botanica Co., Waltham, Massachusetts, USA.