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(54) Title: PROCESSES AND ORGANISMS FOR THE PRODUCTION OF ANTI-FREEZE PROTEINS

(57) Abstract: The invention relates to a process for preparing a novel anti freezepeptide and to the peptides obtained from bacteria from an aqueous low-temperature environment, such as Marinomonas protea and a novel Pseudomonas species. These anti-freeze peptides can suitably be incorporated in frozen food products such as frozen vegetables and frozen confectionery such as ice-cream.

PROCESSES AND ORGANISMS FOR THE PRODUCTION OF ANTI-FREEZE PROTEINS

Field of the invention

5

The present invention relates to a process for the production of anti-freeze proteins. It further relates to novel organisms useful in the process, to novel proteins obtained thereby and to compositions and uses of such novel

10 proteins. In one aspect, it relates to cultures of the novel organism *Marinomonas protea*; to novel anti-freeze proteins derived therefrom; to the use of such proteins in controlling freezing processes, especially in the production of frozen food; and the foods thereby obtained.

15

Background of the invention

So-called 'anti-freeze proteins' (AFPs) have the property of modifying the growth of ice crystals. They differ in

20 their action from simpler ionic anti-freeze agents such as common salt. For example, aqueous AFP solutions typically have a freezing point that is lower than their melting point (hysteresis). They stabilise ice crystals over a range of temperatures, and inhibit recrystallisation. They

25 seem to assist organisms to survive in temperatures around the freezing point of water, and are accordingly found in several different types of organism. Their properties give them a range of potential uses: in particular in foods that are eaten while frozen, by inhibiting recrystallisation and

30 maintaining a smooth texture. In foods that are frozen only for preservation, AFPs may inhibit recrystallisation during freezing, storage, transport, and thawing, thus

preserving food texture by reducing cellular damage and
also minimising the loss of nutrients by reducing drip (see
Griffith, M. and Vanya Ewart, K. *Biotechnology Advances*,
13, pp 375-402).

5

Various sources of AFPs are known: the commonest are fish
and plants. Some bacteria exhibit anti-freeze properties
(see Griffith et al, supra, p 382). In a few cases, the
isolation of anti-freeze proteins from bacteria has been
10 reported (see for example: Xu H, Griffith M, Patten CL, et
al., Can J Microbiol 44: (1) 64-73 Jan 1998).
Given the advantages to an organism of resistance to
freezing, it may be considered surprising that anti-freeze
proteins are not more widely distributed in Nature and
15 easier to find. This may be because organisms have other
ways of overcoming such problems.

Whereas, fish AFP that can assist production of strong,
hard structures has advantages for some specialised
20 applications, an AFP that is RI-active but does not produce
hard structures has its own, different advantages. For
example, molecules that actively inhibit the re-
crystallisation of ice upon storage may be used to maintain
smooth textures of frozen products during storage. For some
25 frozen foods, such as dairy ice cream, it would be
preferable if the use of such a molecule did not harden the
product excessively.

Therefore there is a desire for anti-freeze peptides which
30 are active in recrystallisation inhibition during thawing
and freezing cycles, and which can be used for maintaining
smooth textures of food products during storage. Desirably

these anti-freeze peptides are heat stable such that they
can be incorporated in food products which are pasteurized
or sterilised, while they maintain their functionality.

5 The hypothesis on which the present invention is (in part)
based is that bacteria are more likely to evolve AFP
proteins if they inhabit a liquid aqueous environment which
is often below the normal freezing point of water: in such
environments (it is postulated) AFPs may be an efficient
10 way of giving bacteria a competitive advantage.

Statement of invention

Accordingly, in a first aspect, the present invention
15 consists of a process for producing AFPs which comprises
collecting one or more samples of bacteria from an aqueous
low-temperature environment, culturing the bacteria and
extracting proteins from the samples, testing the proteins
for anti-freeze properties, selecting protein having
20 superior anti-freeze properties, and producing the
selected protein in amounts sufficient for use as an AFP
food additive.

The invention further comprises novel anti-freeze proteins
25 obtainable by the process, their use in food processing,
and food compositions containing them.

As now claimed, according to one aspect, the present
invention provides a protein showing anti-freeze activity
30 and having an N-terminal amino acid sequence having at least
75% homology to the amino acid sequence shown in SEQ ID NO:
3.

As now claimed, according to another aspect, the present invention provides a protein showing anti-freeze activity isolated from a *Pseudomonas* species.

5 As now claimed, according to a further aspect, the present invention provides a pure bacterial culture of *Marinomonas* species that generates anti-freeze proteins, said bacterial culture showing at least 90% homology in the 16S rRNA gene sequence with the corresponding sequence in the organism

10 *Marinomonas protea* shown as SEQ ID NO: 1.

As now claimed, according to a further aspect, the present invention provides a pure bacterial culture of *Pseudomonas* species that generates anti-freeze proteins, said bacterial

15 culture comprising a 16S rRNA sequence with the nucleotide sequence shown in SEQ ID NO: 2.

Detailed description of the invention

20 The invention will be further described with reference to the accompanying drawings and sequence identifications, in which:

Sequence ID 1 is a DNA sequence from the 16S rRNA gene of
Marinomonas protea;
Sequence ID 2 is a DNA sequence from the 16S rRNA gene of
Pseudomonas species (isolated as "isolate 20").
5 Sequence ID 3 is the N-terminal amino acid sequence of the
anti-freeze peptide isolated from *Marinomonas protea.*

Figure 1 shows the sequence alignment of the 16S rRNA
sequence of *Marinomonas protea* (Figure 3) to the
10 corresponding sequence of *Marinomonas communis;*
Figure 2 is a phylogenetic tree comparing *Marinomonas
protea* with its closest phylogenetic relatives.
Figure 3 shows the 16S rRNA sequence alignment of isolate
20 (seq ID 2, upper case) to Marinononas protea 16S rRNA
15 (Seq ID 1; lower case) showing 89.4% similarity.
Figure 4 shows the 16S rRNA sequence alignment of isolate
20 (seq ID 2) to its closest phylogenetic relative
Pseudomonas synxantha showing 99.4% similarity.
Figure 5 is a phylogenetic tree based on 16S rRNA sequences
20 comparing Isolate 20 (SEQ ID 2) with its closest relatives.
Multiple sequence alignments were created using the Clustal
method with a gap penalty of 10.
Figure 6 shows the result of an RI experiment at time= 0
(6A) and at time = 60 minutes (6B).
25 Figure 7, 8, 9 relate to the Vickers hardness test as
described in the example.

The terms anti-freeze peptide and anti-freeze protein are
used interchangeably in the context of this invention.
30 These terms are abbreviated as AFP.

5

By an aqueous low-temperature environment, we mean an
environment comprising predominantly water that is at a
temperature below 0°C for at least part of the year. We
prefer saline environments, for example having a salt
5 content (salinity) sufficient to depress the freezing point
of ice significantly (for example by 0.2°C or more). The
salinity may be more or less than that of sea water
(35ppt). Preferred environments from which bacteria can be
collected are saline (including hypersaline) lakes, or loci
10 in such lakes. In particular we prefer to collect suitable
bacteria for use in the invention from Antarctic
environments.

Saline lakes in Antarctica are often meromictic or
15 monomictic. Meromictic lakes have a permanent chemically
and thermally stratified water column. They have colder
less saline waters above and more saline warmer waters in
the depths. In Antarctica such lakes are ice-covered for
most of the year, only breaking out briefly in summer.
20 Monomictic lakes in Antarctica are hypersaline lakes, too
saline to develop a proper ice-cover in winter. Ice-cover
acts like a thermal blanket. Without it lake waters in
contact with the air cool to very low temperatures. In
winter their water columns stratify thermally, colder
25 waters above, warmer waters below. In summer the
stratification breaks down and the water column has a
uniform temperature. In the case of both monomictic and
meromictic Antarctic lakes there are times in winter when
water temperatures are well below 0°C.
30

6

The invention further relates to novel bacterial cultures
isolated from the above Antarctic lakes, preferably from
meromictic or monomictic lakes.

5 We further provide pure bacterial cultures of *Marinomonas*
species that generate anti-freeze proteins, said bacterial
cultures showing at least 90% and preferably 95% homology
in the 16S rRNA gene sequence with the corresponding
sequence in the organism *Marinomonas protea* (Sequence ID no
10 1).

The invention further relates to pure bacterial cultures of
Pseudomonas species that generate anti-freeze proteins,
said bacterial cultures showing at least 90% and preferably
15 at least 95% sequence homology in the 16S rRNA sequence
with the gene sequence according to seq ID no 2. Said
cultures are obtainable in the course of carrying out the
process of the invention.

20 The process of the invention comprises four phases:
collection of samples of one or more bactiera; culturing
the bacteria and extraction of proteins; testing and
selection of proteins; and production of proteins.

25 **1. Collection of samples**
Samples of bacteria for use in the process of the invention
are collected from aqueous low-temperature environments.
As noted, by 'low temperature' environments we mean those
in which ice forms for at least part of the year. Such
30 environments occur at high altitudes and high latitudes, or
both. We prefer to collect bacteria from saline
environments, especially saltwater lakes. Particularly

preferred environments are found in the Antarctic, and may
include meromictic or monomictic lakes. Such lakes
comprise loci having a variety of properties, e.g,
temperatures and salt concentrations, and bacterial samples
5 may be collected from a range of such loci for subsequent
laboratory investigation.

2. Protein extraction

Each bacterial sample is cultured under suitable
10 conditions, such that anti-freeze protein is produced, and
protein is extracted from the culture broth by standard
methods, for example by centrifugation of cells, pelleting
and vortexing with glass beads in the presence of buffer.

15 3. Testing of proteins

Proteins extracted in stage 2 are classified by testing
their anti-freeze properties. A variety of suitable tests
are available. We prefer to use a recrystallisation
inhibition test: as described in more detail below. This
20 measures the tendency of ice crystals to increase in size
when stored under freezing conditions in the presence of
the protein. By use of this test, effective AFP proteins,
in the presence of which recrystallisation and crystal
growth is stopped or reduced, may be selected.
25

Proteins with anti-freeze properties are defined as
proteins which preferably show in a recrystallisation assay
as defined in the examples, an ice crystal growth of 6 μm
or less at a concentration of at most 1 mg/ml.
30

8

4. Production of proteins

Proteins selected according to the process of the invention
are produced in quantity for use as AFP food additives.
This may be done in a variety of ways. For example, the
5 original process used to obtain the protein (bacterial
culture followed by protein extraction) may be scaled up.
By experimenting with culture conditions (temperature,
media, etc.) and bacterial selection, yields may be
increased. It is also possible to isolate from the
10 bacterium the DNA sequence coding for the selected AFP,
introduce it into an expression cassette and generate
recombinant protein in a different host cell.

In a preferred process, the protein-containing isolate is
15 subjected to a heat treatment such as pasteurisation or
sterilisation to inactivate live bacteria and proteins
which may affect the formed anti-freeze peptide such as
proteases.
Thus it is a very important feature of the invention that
20 isolated AFPs are preferably resistant to heating. Without
the ability to inactivate proteases by heat treatment,
chemical protease-inhibitors would in many cases need to be
used and most of these are toxic and therefore not
compatible with use in foods.
25 Surprisingly the proteins isolated from the antarctic
sources as indicated are heat stable, despite their
natural, original environment being at very cold
temperatures.

30 Heat stable proteins in the context of the invention are
defined as proteins which show, after a pasteurisation
treatment, a remaining anti-freeze activity of at least

9

50%, preferably at least 80% of the anti-freeze activity of
the protein before the heat treatment.
Most preferred this amount of activity remains after a
sterilisation treatment.

5

In a further aspect the invention relates to a protein
showing anti-freeze properties, obtained by the process of
the invention or isolated from a culture as specified
above.

10 More specifically, the invention comprises the novel
protein marinomonin having anti-freeze properties. It
further comprises isoforms and derivatives, e.g.
glycosylated forms, of marinomonin possessing anti-freeze
properties.

15

Most preferred these proteins show anti-freeze properties
are heat stable.

As is shown in the examples the anti-freeze peptide
20 isolated from *Marinomonas protea* shows an N-terminal amino
acid sequence of Ala-Glu-Gly-Ser-Thr-Gly/Val-Asp-Val-Tyr-
Gln-Asn-Ile-Gln-Tyr-Ala-Gly- (Seq ID no 3). Preferably the
derivatives show at least 75%, more preferably 85% and in
particular at least 95% homology in the N terminal amino
25 acid sequence, with the 16-member peptide sequence given
above.

Isoforms and derivatives of the amino acid sequence of SEQ
ID no 3 having anti-freeze properties are also encompassed
30 in the scope of the invention.

The invention further comprises nucleic acid sequences,
especially DNA, encoding the novel proteins of the
invention. These may readily be obtained from a knowledge
of the protein sequence shown above.

5

In a further preferred aspect the invention relates to a
nucleic acid sequence encoding the amino acid sequence of
sequence ID no 3.

10 The process of the invention comprises culturing an
organism containing a DNA sequence coding for marinomonin
or another anti-freeze protein according to the invention,
under conditions in which marinomonin or the anti-freeze
protein is produced, and recovering this protein.

15

One such organism suitable for use in the invention is the
newly discovered bacterium *Marinomonas protea*. Suitable
conditions and media for culturing this organism are given
in the examples.

20

Other organisms may be discovered containing DNA encoding
marinomonin, which may prove useful in the process of the
invention. An alternative is to employ a genetically
transformed organism comprising a DNA sequence coding for
25 an anti-freeze protein which is marinomonin or an analogue
thereof, the sequence being under control of a gene
promoter adapted to cause it to produce the anti-freeze
protein in the transformed organism. The DNA sequence
coding for the anti-freeze protein may be inserted into a
30 suitable expression vector containing the necessary
elements for transcription and translation into the desired
protein under appropriate conditions, including proper

orientation of the sequence, correct reading frame, and
suitable targeting and expression sequences. Methods for
making suitable vectors, and for using them to transform
many different types of organism, are well understood in
5 the art. Genetically transformed organisms suitable for
carrying out the process of the invention also form a
further aspect of the invention.

In principle, any organism may be modified to produce the
10 desired protein in this way, for example, bacteria, yeasts,
plants, or plant cells, insect cells or animal cells.
Bacteria, yeasts and plants or plant cell systems are
generally preferred.

15 It is also possible to provide suitable genetically
transformed organisms, adapted to produce the AFPs of the
invention, which are themselves useful on account of their
increased frost resistance. In particular this applies to
plants: which may be cereals such as wheat or maize; or
20 dicotyledons such as soya, tomato or lettuce. Such plants
also form part of our invention.

Anti-freeze peptides according to the invention can
conveniently be used in several products, preferably in
25 food products which are frozen or intended for freezing.
Examples of such food products are: frozen food products
such as vegetables, sauces, soups, snacks, dairy products
and frozen confectionery, under which term we include
sorbet, water-ice, granites, frozen fruit purees and milk-
30 containing frozen confections such as ice-cream, frozen
yoghurt or custards, sherbet and ice-milk. Preferred food
products are frozen vegetables and frozen confectionery,

e.g. ice-cream, water-ice. If dry-mixes or concentrates
are used, the concentration may be higher in order to
ensure that the level in the final frozen product is within
the desired range. Preferred levels of AFP are from
5 0.00005 to 0.3%, particularly from 0.0001 to 0.2% by weight
of final product.

It is not necessary to add the AFP in highly purified form:
it may be added as a composition containing the AFP, for
10 example an extract of the organism that produces the AFP.

Frozen confectionery according to the invention can be
produced by any suitable method known to the art.
Preferably all ingredients of the formulation are fully
15 mixed together at or above ambient temperature before
freezing. The level of solids in the frozen confection
(e.g., sugar, fat, flavoring) is suitably adjusted to be at
least 3% and normally in the range 10 to 70%, particularly
40 to 70%, by weight of the final product.
20
The invention will now be illustrated by the following non-
limiting examples

13

Examples

Example 1

5 19 Isolates were collected from various sites in Antarctic
lakes. Two were found to give rise to anti-freeze proteins
(see Table 1 below), isolate 196 and isolate 20. These
bacteria were isolated from Ace lake and Club lake,
respectively. Ace lake is meromictic: Club lake is
10 monomictic. Both bacteria were isolated from near to the
surface of the lake. Isolate 196 was isolated from the
ice/water interface. Isolate 20 was isolated from the top
50cm of water column away from the edge of the lake. More
details of the two lakes are provided below:
15

Ace lake

Brackish at surface - 18ppt; salinity increasing
with depth to 34 ppt.
Ice cover for 10-11 months of the year
20   Meromictic - chemically and thermally stratified

Club lake

Hypersaline (250 ppt)
No ice cover
25 .   Monomictic - stratified thermally in winter,
water column mixed in summer

Culturing of Isolates
Bacterial isolates were inoculated into 200ml of Tryptic
30 Soya Broth (Oxoid).
Isolate 196 (*Marinomonas protea*) was also cultured in 200ml
Sea Salts Broth and 200ml of ½ Sea Water Broth

14

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Tryptic Soya Broth ["TSB"]
Pancreatic digest of Caesin
(Oxoid):                       11.3g/L
Papaic Digest of Soya Bean     2g/L
5 Dibasic Potassium phosphate  1.7g/L
Glucose                        1.7g/L
NaCl                           3.3g/L
pH                             7.3 +/- 0.2


10
Sea Salts Broth ["SSB"]: Sea salts (Sigma)     40g/L
                         Peptone (BDH)           5g/L
                         Yeast Extract (Merck)   2g/L


15 Sea Water Broth ["SWB"]: 'Instant Ocean'      38g/L
                            (Aquarium Systems, France)

                            Yeast Extract (Merck)  1g/L
                            Peptone (BDH)          1g/L
20
   ½ Sea Water Broth ["½SWB"]:'Instant Ocean'    19g/L
                            (Aquarium Systems, France)
                            Yeast Extract (Merck)  1g/L
                            Peptone (BDH)          1g/L
25
```

All cultures were incubated at 15°C until noticeably turbid
(this took 2-6 days depending on the growth rate of each
culture). All cultures were then cold shocked at 5°C for 4
30 days. 200mls of an *E. coli* culture was also produced as a
negative control. This was strain W3110. It was cultured in

Lauria-Bertani ["LB"] media in a shaking vessel at 37°C
overnight.

LB media   Bacto-tryptone   10g/L
5   Bacto-yeast extract 5g/L
                                NaCl   10g/L
                                Adjusted to pH 7.0

Protein extraction
10 The following protocol gives the method used for total
cellular protein extraction.

1.   The cells were collected from the cultures by
centrifugation. A Beckman cooling centrifuge, with a fixed
15 angle JA-14 rotor was used. Samples were spun at a
constant temperature of 4 °C and at a speed of 10,000 rpm
(15,300g) for 10mins. The supernatant was carefully
removed leaving the pellet.
2.   The pellet was resuspended in 1ml of 10mM Tris/HCL
20 buffer (pH 7.0). This was repelleted using the same
centrifuge conditions as step 1. The supernatant was
discarded carefully leaving just the pellet.
3.   The pellet was then resuspended in 1ml of protein
extraction buffer (see below) and then kept on ice for
25 5mins to allow cell lysis. The resuspended pellet was then
sonicated in a thick walled bottle using a Sanyo Soniprep
150 at 15 A for 30 seconds.
4.   The sonicated mixture was then transferred back to a
centrifuge tube and centrifuged as outlined in step 1. The
30 supernatant was removed and frozen at -20°C until required.

16

Protein Extraction Buffer:
25mM Tris/HCL (pH 7.0)
1mM EDTA
1mM PMSF (Sigma)
5 2ug/ml Pepstatin A (Sigma)

Splat Assay
The following protocol is the method used to view the level
of ice re-crystallisation inhibition achieved by each of
10 the protein extractions. It is a modification of the assay
described in Byass et al. [Unilever WO-A-98/04148].

1.   A 60% sucrose solution was prepared. A 20µl sample of
each protein extract was mixed with 20µl of the sucrose
15 solution. These mixtures were made up in 1.5ml Eppendorf
tubes. The 30% sucrose/protein extract solutions were spun
in a MSE Micro Centaur Desk top micro-centrifuge for 10
seconds to make sure all the liquid was mixed at the bottom
of the tube.
20 2.   5µl of the resulting solution was placed between two
circular 16mm diameter coverslips upon which the number of
the extract was written in waterproof maker. These were
then blotted dry.
3.   The coverslips were then dropped into 2,2,4 -
25 Trimethyl pentane that had been pre-chilled to -70°C (using
dry ice). They were super-cooled in this for 2 minutes.
4.   A chilled bath was filled ¾ full of 2,2,4 - Trimethyl
pentane, which was pre-chilled to -6° C using a Haake C
water bath circulator and two Haake temperature control
30 units (PG40 and F4).

5.   The coverslips were removed from the super-cooling
process and placed directly into the -6° C bath for 30mins
to allow re-crystallisation.

6.   The coverslips were then viewed whilst in the -6° C
5 bath using a EF L 20/0.30 160/0-2 objective on a Leitz
Dialux 20 EB stage.

7.   The observed crystal shape, size and density were
related to the level of AFP activity using the Splat
Scoring System outlined below.

10

Splat Score	Observed crystal morphologies
+++++	Very small, very dense crystals.
++++	Small, not dense; or small, quite dense with some medium-sized crystals.
+++	Small, not dense with medium-large crystals; or small-medium, not dense.
++	Medium or large crystals with some small crystals
+	Large, round discrete crystals (e.g. 30% sucrose control).

[A score of +++ or above is required for an isolate to have
any practical utility].

25

Results from the splat assay are shown in Table 1

18

TABLE 1

Bacterial isolate activity	AFP	Bacterial isolate activity	AFP
196 [1/2 SWB]	++++	196 [TSB]	+++
196 [SSB]	++++	18	++
20	+++/++++	26	+
29	+/++	39	+
43	++	44	+
98	++	101	+
104	+	118	++
120	+	125	+
184	+	226	+
288	+	121	+
289	+	E. coli (neg. control)	+
Sucrose only (neg. control)	+		

The results in Table 1 show that out of the 19 isolates of
Antarctic bacteria that were investigated, only isolate 196
and isolate 20 produce proteins with significant amounts of
AFP. Isolate 196 *is Marinomonas protea*, as further
described below.

Example 2

Collection of Isolate 196

Bacteria were isolated from the ice/water interface of Ace
5 Lake, Antarctica (68°28'S, 78°11'E) on 31/6/96. Samples
were collected by drilling a hole through the ice (1600 mm
thick) at the deepest point of the lake. Sections of the
lower part of the core from the ice/water interface were
melted onto agar. The material was designated Isolate 196.
10

Growth characteristics of Isolate 196

Colonies of isolate 196 that formed on half-strength
seawater agar after 3 to 5 days of incubation at 10°C were
non-pigmented (creamy colour), smooth, convex, circular
15 with entire edges and ranged in size from less than 0.5 mm
to 1.5 mm in diameter. On tryptic soya agar the colonies
were beige in colour, mucoid and spreading, and ranged from
2-5 mm in diameter. The isolate was psychrotrophic, growing
at temperatures between <0 to ~25°C (optimal growth between
20 15-22°C), with no growth at 30°C. The bacterium grew
aerobically and under microaerophilic conditions, but would
not grow anaerobically. It did not require seawater for
growth, but could tolerate up to 70‰ NaCl. Cells growing in
60-70‰ NaCl consisted of straight rods and coccoid cells,
25 which appeared more wrinkled and indented than cells in
lower salinity media. Typical growth curves in different
media types are shown in Figures 1 and 2.

30

20

Organism Classification

Analysis of 16S rRNA Phylogenetic from Isolate 196

Identification of the isolate was based on 16S rRNA
analysis. A near complete (1,485-bp) sequence, of the 16S
5 rRNA gene, stretching from nucleotide positions 18 to 1503
(*Escherichia coli* equivalent numbering), was obtained and
is shown in Figure 3. 16S rRNA analyses against species in
the publicly available databanks showed that it represented
a novel species of the genus *Marinomonas* (in the gamma-3
10 subclass of the phylum Proteobacteria), with 74.7%
identity to *Marinomonas communis* (the type species of the
genus).The match between the two sequences is shown in
Figure 4. The name *Marinomonas protea* sp. nov. (*pro´te.a.*
Gr. n. *Proteos*, name of the Greek mythological god of the
15 sea who could appear in many shapes, M.L. fem. adj. *protea*,
polymorphous) was proposed (Prof. Hans G. Trüper, *pers.
comm.*). The bacterium has been deposited at the British
National Collections of Industrial and Marine Bacteria,
Aberdeen (NCIMB) under the number 41006 on 9 February
20 1999.

The 16S rRNA sequences generated in this study were
deposited in GenBank. Percentage similarities for the
sequences are given in Table I. The phylogenetic tree
25 determined using PHYLIP (version 3.57c) is shown in Figure
5. It uses almost complete 16S rRNA sequences, comparing
Marinomonas protea with its closest phylogenetic relatives
and with three other sea-ice isolates identified as
Marinomonas sp. (M. Brown, *pers. comm*). The tree was
30 constructed using the maximum likelihood method
(Felsenstein, 1981).

16S rRNA Phylogenetic Analysis of Isolate 20

An analysis of Isolate 20 found it to be a species of
Pseudomonas. More precisely, isolate 20 was found to have a
similarity value of 99.4% with *Pseudomonas synxantha*.

5 This strain was deposited under the Budapest Treaty with
international depository authority NCIMB Aberdeen, Scotland
under number NCIMB 41076.

Example 3

10 ***Total Cellular Protein Extraction***

A bacterial culture (2 L) of Isolate 196 was grown in
liquid medium (Tryptic Soy Broth) at 15°C until turbid. The
culture was aseptically pipetted into sterile Nalgene™
universals. The cells were harvested by centrifugation at

15 4,200 x g for 12 minutes at 5°C (Sorvall Dupont Econospin).
The pellets were resuspended in 1 ml of ice-cold 10 mM Tris
buffer (Tris [hydroxymethyl]amino-methane)/HCl (pH 7.0)
The cells were pelleted for 10 minutes. The pellets were
then either stored at -20°C overnight, or protein

20 extractions were carried out immediately. The pellet was
resuspended in 1 ml of ice-cold native extraction buffer
(NEB, which consisted of 25 mM Tris/HCl (pH 7.0), 1 mM
EDTA, 1 mM PMSF and 0.1 mM Pepstatin A). The Tris buffer
acts as a biochemical·buffer, the EDTA is a chelating agent

25 and is active against metalloproteases, PMSF is an
inhibitor of serine proteases and Pepstatin A inhibits
proteases. To each resuspended pellet, two volumes of acid-
(70% HCl) washed glass beads were added (1.5 ml of 425-600
μm diameter beads and 0.5 ml of 3 mm diameter beads, both

30 Sigma), or enough to create a viscous adherent coating of
cell suspension over the beads. The beads were acid washed

22

to prevent alkali being liberated from the glass when
vortexed, as this would potentially damage alkali-sensitive
components of the cells. The mixture was then vortexed for
1 minute sessions, with 1 minute intervals on ice. This was
5 repeated 8 times for each sample, after which a further 1
ml of ice-cold NEB was added to the beads, which were re-
vortexed to enable the cell suspension to go into solution.
The cells were left on ice for 5 minutes to allow the glass
beads to settle and the lysates were transferred to 1 ml
10 sterile, ice-cold Eppendorf tubes and centrifuged (Beckman
MSE, 12,000 x g, 5°C, 5 minutes) in order to separate the
cell walls and non-ruptured cells, as well as any glass
beads which were accidentally transferred. The supernatant
was transferred to sterile, ice-cold Eppendorfs and
15 centrifuged once more to remove any further cell debris
that may interfere with the RI assay. The supernatant was
pipetted into 2 ice-cold, labelled Eppendorfs and stored at
-20°C until the assays were carried out.

20 **Example 4**

***Effect of Bacterial Anti-freeze proteins on Ice Crystal
Morphology***
The effect of the anti-freeze protein from isolate 196
(obtained as in Example 3) on ice crystal morphology was
25 assessed by examining total cellular protein extract at -
6°C under the microscope. Hexagonal ice crystals, some
elongated, were clearly visible.

30

23

Example 5

Protein Purification and Sequence Information
Purification of Active Protein
The bacterial anti-freeze protein, which we term
5 marinomonin, was isolated from the crude extract produced
in Example 3 using reverse phase chromatography (RPC) on an
AKTA™ protein purification system, followed by gel
exclusion chromatography (Sephadex® 75, or S75) on a SMART™
system (both Pharmacia Biotech). The active fractions from
10 each separation method were run on a 10% bis-acrylamide SDS
gel: the band obtained ran at a molecular weight of ~38 kDa

Sequence Information
The 38 kDa protein band was electroblotted onto a PVDF
15 membrane and N-terminal sequenced. The following sequence
of 16 amino acids was obtained, with the 6th amino acid
producing an ambiguous result of either Glycine or Valine
(shaded region indicates region of sequence that the
oligonucleotide probe was designed from):
20

Ala-Glu-Gly-Ser-Thr-Gly/Val-**Asp-Val-Tyr-Gln-Asn-Ile-Gln-Tyr-Ala-Gly** (Seq ID 3)

Database Homology Searches
25 Sequence homology searches were carried out using the N-
terminal sequence of the novel AFP protein using FASTA
searches against the SWISS-PROT database, which included
all published AFP sequences. The sequence matches were
random, in that similarities were matched to a wide range
30 of organisms, both bacterial and eukaryotic, and the
similarities were not particularly high. Also, the regions

24

of homology between the protein sequences were not located
at the N-terminus of the proteins on the database, but mid-
sequence: this reduces the likelihood of any real homology
between the protein sequences and the N-terminal sequence
5 of this novel protein.

Example 6

Two important requirements for an AFP-producing micro-
10 organism to be of practical utility is that its culturing
can be readily scaled-up and that the AFP-activity is
stable. For example, susceptibility to denaturation by heat
makes it more difficult to use the AFP in foodstuffs such
as ice cream that are pasteurized during manufacture.
15

**Culturing _Marinomonas protea_ in "shake flasks" (9 litres
scale)**

Isolate 196 (_Marinomonas protea_) was cultured in 18
individual 1 L Erlenmeyer flasks containing 500 ml of
20 sterile Sea Salts Broth (sea salts (Sigma) 40g/L; peptone
(Fisher) 5g/L; yeast extract (Difco) 2g/L). Flasks were
inoculated aseptically with 100 µl of an existing _M. protea_
culture. Cultures were grown statically at 15°C for 7 days
in a Sanyo incubator. The cultures showed signs of
25 turbidity following 2 days at 15°C. On day 7 the incubator
temperature was adjusted to 4°C and maintained at this
temperature for a further 7 days to induce anti-freeze
protein (AFP) production.

30 Following 7 days cold induction at 4 °C, cells from the 9 L
of culture were collected in 6 sterile 300 ml containers by
repeated centrifugation. Centrifugation was carried out at

25

7000 rpm for 20 minutes and at 4 °C using a Sorvall F-16/250 fixed angle rotor in a Sorvall Instruments RC5C centrifuge. Following each centrifugation step the supernatant was discarded and further culture added and
5 centrifugation repeated. In this way, pellets were accumulated in the 6 containers until the 9 L of culture was harvested. The six containers and associated pellets were stored at -80°C until required for protein extraction and SPLAT testing.

26

**Protein extraction from 9 litre culture and splat testing
for AFP activity**

Pellets were washed by gentle resuspension in 10 ml of ice
5 cold 10 mM Tris/HCl (pH 7.0). A 1 ml aliquot of the
resuspended cells was centrifuged in an Eppendorf tube for
5 minutes at 13,000 rpm in a microcentrifuge (MSE Micro
Centaur). The 'wash supernatant' was retained for testing
for AFP activity by the SPLAT assay. The pelleted cells (~
10 0.2 g) was resuspended in 800 µl of ice cold extraction
buffer (50 mM Tris/HCl, pH 7.0) and 200 µl of Bacterial
protease inhibitor cocktail supplied by Sigma. [This
cocktail contains a mixture of protease inhibitors with
broad specificity for the inhibition of serine, cysteine,
15 aspartic and metallo-proteases, and aminopeptidases. It
comprises 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF),
pepstatin-A, trans-epoxysuccinyl-L-leucyl-amido(4-
guanidino)butane (E-64), bestatin, and sodium EDTA]. The
samples were vortexed and then placed in ice, and this
20 process was repeated a total of 8 times. The samples were
then centrifuged and the 'extraction supernatant' retained
for AFP testing by the SPLAT assay.

SPLAT activity

25

Material	SPLAT score
'Wash supernatant'	++++
'Extraction supernatant'	+++

27

Example 7

Induction of AFP by cold-shocking a culture at 8°C

For the AFP production to be readily scaled-up, it is
5 important that cold-shocking can be effective at a range of
different temperatures. For example, it is difficult to
operate fermenters at temperatures below 8°C. Therefore, to
be able to scale-up production in fermenters, it must be
possible to induce expression of AFP by cold-shocking at
10 temperatures of 8°C or above. This example shows that
Marinomonas protea cultures can be induced to express AFP
at 8°C.

Isolate 196 (*Marinomonas protea*) was cultured in a 1 L
15 Erlenmeyer flask containing 500 ml of sterile Sea Salts
Broth (sea salts (Sigma) 40g/L; peptone (Fisher) 5g/L;
yeast extract (Difco) 2g/L). The flask was inoculated
aseptically with 100 µl of an existing *M.protea* culture.
The culture was grown statically at 15°C for 7 days in a
20 Sanyo incubator. The culture showed signs of turbidity
following 2 days at 15°C. On day 7, the culture was
transferred to a Conviron incubator set to 8°C and this
temperature was maintained for a further 7 days to induce
anti-freeze protein (AFP) production.
25
Following 7 days cold induction at 8 °C, the *M. protea*
culture was harvested and tested for AFP activity by the
SPLAT assay. The 500ml of culture was collected in a 50ml
container by repeated centrifugation. Centrifugation was
30 carried out at 9000 rpm for 5 minutes at 4°C using a
Sorvall SS34 rotor in a Sorvall Instrument RC5C centrifuge.
Following each centrifugation step, the supernatant was

discarded and further culture added and centrifugation repeated. In this way a pellet accumulated in the container until the 500ml of culture was harvested. The resulting cell pellet weighed 0.9g.

5

The 0.9g of pelleted cells were gently resuspended in 1ml of ice cold extraction buffer (25 mM Tris/HCl, pH 7.0) and 0.5ml of Bacterial protease inhibitor cocktail supplied by Sigma (see Example 6). The sample was vortexed and then
10 placed on ice, and this process was repeated a total of 8 times. The sample was then centrifuged for 10 minutes at 18000rpm using a Sorvall SS34 rotor in a Sorvall Instrument RC5C centrifuge and the 'extraction supernatant' was filtered using a 0.2 μm filter and retained for SPLAT
15 activity determination (see table).

Material	SPLAT score
'Extraction supernatant'	++++ (+)

20 **Example 8**

Heat stability of AFP extracted from *Marinomonas protea*
An AFP-containing extract was prepared from a culture of *Marinomonas protea*, essentially as described in Example 6.
25   25μl aliquots (5 in total) were placed in 500μl Eppendorf tubes and tested for heat stability. Four of the tubes were placed in a bath of boiling water. Each tube was exposed to the heat treatment for a different length of time: either 1, 2, 5, or 15 minutes. The fifth tube was

29

used as a control and was not exposed to the heat
treatment. After the heat treatment, all tubes were
immediately placed on ice and then assayed for AFP activity
using the splat assay.

5

SPLAT activity following boiling

Treatment	SPLAT score
Marinomonas protea extract, not boiled	+++++
Marinomonas protea extract, *boiled 1* min	+++++
Marinomonas protea extract, boiled 2 min	+++++
Marinomonas protea extract, boiled 5 min	+++++
Marinomonas protea extract, boiled 15 min	++++

As the AFP extracted from *Marinomonas protea* can survive
10 boiling it is suitable for use in food-stuffs that are
pasteurised during their manufacture. For example, an ice-
cream "mix" would typically be pasteurised by holding at a
temperature of 82°C for 25 seconds before cooling and
freezing. This example shows that the AFP extracted from
15 *Marinomonas protea* could undergo such a pasteurisation
process without loss of AFP activity.

30

Example 9

A preferred process is described below for isolation of AFP
from *Marinomonas protea*.

5

a) Culturing cells

10 litres of culture were produced as previously described
in example 6 (growth at 15°C and induction at 4°C). Cells
were harvested by centrifugation and kept at -80°C until
10 required.

b) Protein extraction

Cells were resuspended in extraction buffer (20mM tris,
10mM EDTA, pH 7.1) and then sonicated 10 times for 15
15 seconds at 45 second intervals using a Sanyo Soniprep150
probe at Amplitude 12μ. The extracts were placed in an ice/
ethanol bath during the sonication procedure to avoid
overheating the extracts.

20 *c) Heat treatment to inactivate proteases*

The extract was placed in a boiling water bath for
4.5minutes. The temperature of the samples was measured and
found to reach 73°C. We have found that this is a very
effective way of inactivating the proteases in the sample,
25 and at the same time killing most or all of the bacteria
(*Marinomonas protea*) in the sample.

d) Separation of AFP from cell debris

Extracts were centrifuged for 1 hour at 4°C at 16,000 rpm
30 in a Sorval Centrifuge (SS-34 rotor)   to remove cell
debris. AFP was found to be present in the supernatant.

31

e) Removal of remaining viable bacteria

For many frozen foods, such as ice cream, it is important
to ensure the removal of all viable bacteria. This ensures
that products and equipment can be maintained to high

5 standards of hygiene. For AFP preparations in the
invention, we have found that this can be achieved by
microfiltration. In this example, the supernatant described
above in (d) was filtered with a Nalgene 0.2μm
sterilisation unit. The filtrate was collected in sterile

10 tubes and stored at -80°C until required.

f) Quality assurance – measurement of AFP activity and
viable cells throughout the process

At each step of the process, the extracts were assayed with
15 the splat assay. The extracts were also tested for the
presence of bacterial cells. Petri dishes containing
Trypton Soya agar from Sigma were used (40g diluted in 1L).
Samples were streaked with a loop on a Petri dish or plated
with a spreader (50μl) and incubated for 7 days at 15°C.

20

The samples were

N1: Cells resuspended in Extraction buffer (Splat score
++++).

N2: Cells in Extraction buffer and after sonication (Splat
25 score +++++).

N3: Cells in Extraction Buffer, after sonication and
boiling for 4min30sec (Splat score ++++).

N4: N3 centrifuged for 1 hour and filtered on 0.2μ
sterilisation unit (Splat score +++++).

30

Sample number	N1	N2	N3	N4
3days incubation Loop	✓	✓	✗	✗
3days incubation 50ul	✓	✓	✗	✗
7days incubation Loop	✓✓	✓✓	✗	✗
7days incubation 50ul	✓✓	✓✓	1 colony contaminant	✗

✓ Presence of colonies in the Petri Dish

✗ Absence of colonies

5 Conclusion

AFP produced by this process is: a) active, b) free from
viable cells, and c) free from toxic chemicals.

33

Example 10

In this example it is demonstrated how to determine how
much AFP-containing solution to add to the food-stuff by
5 using the "Re-crystallisation Inhibition (RI) assay". The
RI assay is quantitative but it is also time-consuming and
requires special equipment. An assay for measuring ice re-
crystallisation inhibition has previously been described by
Jarman et al. (WO 99/37782). In this example, the protocol
10 given below was used.

RI Assay protocol
A solution containing AFP from *Marinomonas protea* was
adjusted to a sucrose level of 30 wt% with sucrose
15 crystals. A 3 µl drop of the sample was placed on a 22 mm
coverslip.
A 16 mm diameter cover-slip was then placed on top and a
200 g weight was placed on the sample to ensure a uniform
slide thickness. The edges of the coverslip were sealed
20 with clear nail varnish. The slide was placed on a Linkam
THM 600 temperature controlled microscope stage. The stage
was cooled rapidly (50°C per minute) to -40°C to produce a
large population of small crystals. The stage temperature
was then raised rapidly (50°C per minute) to -6.0 °C and
25 held at this temperature. The ice-phase was observed at -
6.0 °C using a Leitz Ortholux II microscope. Polarised
light conditions in conjunction with a lambda plate were
used to enhance the contrast of the ice crystals. Images
were recorded at T=0 and T=60 minutes and analysed using
30 image analysis software (AnalySIS, Soft-Imaging Software

34

GmbH, D48153, Munster, Hammerstrasse, 89 Germany). The
crystal growth was calculated as follows.

The mean size of a population of ice crystals at a
5 particular time was determined by capturing an image of the
population (using the equipment mentioned above) and then
selecting a region of the image that contained at least 200
ice crystals. The longest diameter of each crystal was
determined by drawing around them and calculating the
10 longest diameter using the image analysis software.

Each RI experiment was carried out 3 times (therefore the
images of at least 600 ice crystals were analysed). The
data in the table record the mean ice crystal growth (i.e.
15 the difference in the mean ice crystal diameter at time
zero and after 60 minutes). Confidence limits were
calculated for a confidence level in the mean of 95%.

Several dilutions of a preparation of *Marinomonas* AFP were
20 made in order to find out how far the sample could be
diluted and still achieve an effective inhibition of re-
crystallisation of ice. In practical terms we consider an
AFP preparation or a dilution of an AFP preparation to
achieve an effective inhibition of re-crystallisation of
25 ice if a sample of said dilution or preparation results in
ice crystal growth in the RI assay of 3µm or less.

RI assay results
Mean crystal growth was determined by analyzing the images
30 of 1200 crystals.

Figures 6A and 6B illustrate typical ice crystal images
simply to show the type of image that was captured and
analysed.

Dilution of AFP preparation	Crystal growth (mean in μm)	Confidence limits
1 in 10	1.96	+/- 0.64
1 in 20	3.72	+/- 0.79
1 in 100	9.87	+/- 2.04
Negative control (no AFP)	12.78	+/- 1.61

5

The results show that the AFP preparation can be diluted
10-fold and still an effective inhibition of the re-
crystallisation of ice is achieved. Therefore, a 1 in 10
10 dilution (= 10%) of this preparation was used in frozen
food prototypes as shown in example 11.

36

Example 11

Preparation of water ice samples

5 Three liquid pre-mixes were made for the preparation of
water-ice:
A) 20% sucrose in water (negative control; not according to
the invention)
B) 20% sucrose in water containing AFP from *Marinomonas*.
10   The AFP had been diluted in the pre-mix to a
concentration that would give an RI value of
approximately 1.9μm. In this instance this was a
dilution of 1 in 10 or 10% – see example 10 for RI data.

15 Therefore, pre-mix B had the following composition:

Ingredient	% by weight
Sucrose	20.00
AFP preparation (in aqueous buffer)	10.00
20 Water	70.00

C) 20% sucrose in water containing fish AFP type III, HPLC
12 as described in WO-A-97/02343. The AFP had been
25   diluted in the pre-mix to a concentration that would
give an RI value of approximately 2.9μm.

Determination of hardness
Cuboid bars of water ice (10mm x 55mm x 95mm) were prepared
30 for each composition (A, B, C) as follows. Each composition
was frozen to -3.5°C in a Gelato Chef 2000 (Magimix UK Ltd)
Ice Cream maker. The mixture was transferred to silicone

rubber moulds with internal dimensions of 10mm x 55mm x
95mm, previously cooled to -30°C. The preparation was then
cooled at -30°C for 1 hour before de-moulding and storing
at -25°C prior to hardness testing, using the Vickers
5 Hardness test.

The Vickers Hardness test
The Vickers Hardness test is an indentation test that
involves pushing a pyramid shaped indentor into the surface
10 of material and recording the force applied as a function
of tip displacement. Force and displacement are measured
during the indentation loading cycle and the unloading
cycle. The test is described in "Handbook of Plastics Test
materials" Ed. R.P. Brown, Pub. George Godwin Limited, The
15 Builder Group, 1-3 Pemberton Row, Fleet Street, London,
1981. The Vickers pyramid geometry is an engineering
industry standard (BSi 427, 1990). It has an apex angle at
the tip of 136°. Hardness is determined as:

$$20 \quad H_V = \frac{F_{max}}{A}$$

where H_V is the Vickers Hardness, F_{max} is the maximum
applied force (see figure 7) and A is the projected area of
the indentation left in the material's surface. The area A
25 is determined by assuming the indentation has the same
geometry as the indentor that formed it, i.e. a Vickers
pyramid, and therefore the projected area can be determined
from the indent depth given by d_i in figure 8.

$$30 \quad A = 24.5 d_i^2$$

38

Determination of strength and modulus

Several (10 or more) thin strips of water ice (50mm x 10mm
x 2mm) were prepared for each composition (A, B, C) as
follows. A silicone rubber mould was accurately cut to give
5 internal dimensions of 50mm x 10mm x 2mm. The mould was
placed on a sheet of acetate and pressed firmly to give a
water tight seal. Each composition was pipetted into one
such mould using a high precision micropipette, and any air
bubbles were removed. A second sheet of acetate was
10 unrolled over the top of the full mould and again any air
bubbles were removed. The composition was frozen by placing
the whole assembly between two nitrogen-cooled blocks at -
30°C for 2 minutes. After this time, the mould was removed
and equilibrated at -20°C . The strips were demoulded by
15 removing the upper sheet of acetate, followed by the
silicone rubber mould before the basesheet was inverted and
peeled back to reveal the strip of water ice. These strips
were stored at -25°C prior to testing for strength and
modulus, using a three point bend test.
20

Three-point bend test

A bend test can be used to determine a number of mechanical
properties of ice confection materials, such as Young's
modulus (apparent) and flexure strength. In a bend test, a
25 test piece is deformed whilst measuring the applied force
and test piece deflection. A schematic data set for an ice
confection is shown in figure 8. The elastic modulus is
determined by the gradient of the initial linear part of
this curve. The general test applied to all types of solids
30 is described in "Biomechanics Materials. A practical
Approach" Ed. J.F.V. Vincent, Pub. IRL Press, Oxford
University Press, Walton Street, Oxford, 1992 and "Handbook

39

of Plastics Test materials" Ed. R.P. Brown, Pub. George
Godwin Limited, The Builder Group, 1-3 Pemberton Row,
Fleet Street, London, 1981. In this particular application,
a 3-point bend test was adapted to evaluate thin strips of
5 water ice (50mm x 10mm x 2mm; prepared as above) whilst in
a temperature controlled cabinet set at -20°C. Testing
involved placing each strip onto 2 supports separated by
30mm and applying pressure from an upper support centrally
onto the strip's surface (figure 9). The force applied in
10 bending and the displacement of the moving contact were
recorded throughout the test. The speed of descent of the
moving support was 10mm per minute.

The apparent elastic modulus of the material is given by
15 the equation;

$$E = \frac{gradient.S^3}{4BD^3}$$

20 where the gradient is that shown in Figure 8, S is the span
(distance) between the supporting contacts beneath the test
strip, B is the width of the strip and D is the depth of
the strip. For these tests the span (S) was 30mm. With
reference to Figure 8, the strength of a material under
25 three point bend conditions, is given as;

$$\sigma_u = \frac{3.F_{max}S}{2BD^2}$$

30 where σ_u is the flexure strength and F_{max} is the maximum
force recorded.

Results of mechanical property testing

Results for the 3 different water ice compositions are
given below. Data were recorded as the mean, as derived
from making 10 measurements. In the case of hardness
5 testing, 10 measurements were made on one water ice bar
(10mm x 55mm x 95mm). In the case of strength and modulus,
one measurement was made on each of 10 individual water ice
strips (50mm x 10mm x 2mm).

Sample	Hardness (MPa)	Strength (MPa)	Modulus (MPa)
A - Negative control (no AFP)	13.64	1.12	413
B - Contains *Marinomonas* AFP	9.00	0.61	248
C - Contains fish AFP type III hplc12	19.34	2.20	1033

10

Surprisingly, the results show that the samples containing
Marinomonas AFP were not hard or brittle, despite the fact
that the fish AFP and *Marinomonas* AFP were each added in
15 similar levels in terms of their RI-activity.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or

5   step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of

10   suggestion that the prior art forms part of the common general knowledge in Australia.
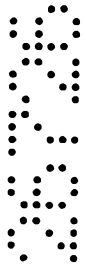
EDITORIAL NOTE

APPLICATION NUMBER - 26726/01

The following Sequence Listing pages 1 to 2 are part of the description. The claims pages follow on pages 41 to 43.

SEQUENCE LISTING

SEQ 1D No.1

5'GTTAGCTCAGATTGAACGCTGGCGGCAGGCTTAAACACATGCAAGTCGAGC
GGTAACAGGGGAGCTTGCTCCTGCTGACGAGCGGCGGACGGGTGAGTAACGC
GTAGGAATCTGCCTAGTAGAGGGGGACAACATGTGGAAACGCATGCTAATAC
CGCATACGCCCTGAGGGGGAAAGGAGGGGACTCTTCGGAGCCTTCCGCTATT
AGATGAGCCTGCGTGAGATTAGCTAGTTGGTAGGGTAAAGGCCTACCAAGGC
GACGATCTCTAACTGGTCTGAGAGGATGACCAGTCACACTGGGACTGAGACA
CGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGC
AAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTAGGGTTGTAAAG
CACTTTCAGGGGTGAGGAAGGGTGATAGGTTAATACGTTATCATCTTGACGTT
AGCCCCAGAAGAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAG
AGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTT
TGTTAAGTCGGATGTGAAATCCCAGGGCTCAACCTTGGAATGGCACCCGATA
CTGGCTAGCTAGAGTATGGTAGAGGGGTGTGGAATTTCCTGTGTAGCGGTGA
AATGCGTAGATATAGGAAGGAACATCAGTGGCGAAGGCGACACCCTGGACT
AATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACC
CTGGTAGTCCACGCCGTAAACGATGTCTACTAGCCGTTGGGTTGTAATGACTT
AGTGGCGCAGCTAACGCAATAAGTAGACCGCCTGGGGAGTACGGCCGCAAG
GTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGG
TTTAATTCGAAGCAACGCGAAGAACCTTACCTACTCTTGACATCCACAGAAC
ATTTGAGAGATCAGATGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGG
CTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGTAACGAGCGCAA
CCCTTGTCCTTATTTGCCAGCACGTAATGGTGGGAACTTTAAGGAGACTGCCG
GTGACAAACCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGGCCCTTACG
AGTAGGGCTACACACGTGCTACAATGGCGTATACAGAGGGCTGCAAGCTAGC
GATAGTGAGCGAATCCCACAAAGTACGTCGTAGTCCGGATTGGAGTCTGCAA
CTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCACGG
TGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGAT
TGCTCCAGAAGTAGCTAGCTTAACCCTTCGGGATGGCGGTTACCACGGAGTG
GTCAATGACTGGGGTTGAAGTCTACGCG-'3

SEQ ID No.2

```
   1 5'GCCCTTGCTC AGATTGAACG CTGGCGGCAG GCCTAACACA TGCAAGTCGA

  51   GCGGTAGAGA GAAGCTTGCT TCTCTTGAGA GCGGCGGACG GGTGAGTAAT

 101   GCCTAGGAAT CTGCCTGGTA GTGGGGGATA ACGTTCGGAA ACGGACGCTA

 151   ATACCGCATA CGTCCTACGG GAGAAAGCAG GGGACCTTCG GGCCTTGCGC

 201   TATCAGATGA GCCTAGGTCG GATTAGCTAG TTGGTGAGGT AATGGCTCAC

 251   CAAGGCGACG ATCCGTAACT GGTCTGAGAG GATGATCAGT CACACTGGAA

 301   CTGAGACACG GTCCAGACTC CTACGGGAGG CAGCAGTGGG GAATATTGGA

 351   CAATGGGCGA AAGCCTGATC CAGCCATGCC GCGTGTGTGA AGAAGGTCTT

 401   CGGATTGTAA AGCACTTTAA GTTGGGAGGA AGGGTTGTAG ATTAATACTC

 451   TGCAATTTTG ACGTTACCGA CAGAATAAGC ACCGGCTAAC TCTGTGCCAG

 501   CAGCCGCGGT AATACAGAGG GTGCAAGCGT TAATCGGAAT TACTGGGCGT

 551   AAAGCGCGCG TAGGTGGTTT GTTAAGTTGG ATGTGAAATC CCCGGGCTCA

 601   ACCTGGGAAC TGCATTCAAA ACTGACTGAC TAGAGTATGG TAGAGGGTGG

 651   TGGAATTTCC TGTGTAGCGG TGAAATGCGT AGATATAGGA AGGAACACCA

 701   GTGGCGAAGG CGACCACCTG GACTAATACT GACACTGAGG TGCGAAAGCG

 751   TGGGGAGCAA ACAGGATTAG ATACCCTGGT AGTCCACGCC GTAAACGATG

 801   TCAACTAGCC GTTGGAAGCC TTGAGCTTTT AGTGGCGCAG CTAACGCATT

 851   AAGTTGACCG CCTGGGGAGT ACGGCCGCAA GGTTAAAACT CAAATGAATT

 901   GACGGGGGCC CGCACAAGCG GTGGAGCATG TGGTTTAATT CGAAGCAACG

 951   CGAAGAACCT TACCAGGCCT TGACATCCAA TGAACTTTCT AGAGATAGAT

1001   TGGTGCCTTC GGGAACATTG AGACAGGTGC TGCATGGCTG TCGTCAGCTC

1051   GTGTTGTGAA ATGTAAGGGC-3'
```

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A protein showing anti-freeze activity and having an N-terminal amino acid sequence having at least 75%
5   homology to the amino acid sequence shown in SEQ ID NO: 3.

2. A protein according to claim 1 having at least 85% homology to the amino acid sequence shown in SEQ ID NO:
10   3.

3. A protein according to claim 2 having an N-terminal amino acid sequence as shown in SEQ ID NO: 3.

15 4. A protein according to any one of claims 1 to 3 having a molecular weight of about 38 kDa.

5. A protein according to any one of claims 1 to 4 which is isolated from a *Marinomonas* species.
20

6. A protein according to claim 5 wherein the *Marinomonas* species is a bacterial strain deposited under Accession No. NCIMB 41006.

25 7. A protein showing anti-freeze activity isolated from a *Pseudomonas* species deposited under Accession No. NCIMB 41076.

8. A nucleic acid sequence encoding a protein according to
30   any one of claims 1 to 6.

9.   A food product comprising a protein according to any of claims 1 to 7.

10. A food product according to claim 9 wherein the food
5   product is selected from the group comprising frozen vegetables and frozen confectionery.

11. A pure bacterial culture of *Marinomonas* species that generates anti-freeze proteins, said bacterial culture
10   showing at least 90% homology in the 16S rRNA gene sequence with the corresponding sequence in the organism *Marinomonas protea* shown as SEQ ID NO: 1.

12. A culture according to claim 11 wherein the *Marinomonas*
15   species is a bacterial strain deposited under Accession No. NCIMB 41006.

13. A pure bacterial culture of *Pseudomonas* species that generates anti-freeze proteins, said bacterial culture
20   comprising a 16S rRNA sequence with the nucleotide sequence shown in SEQ ID NO: 2.

14. A culture according to claim 13 wherein the *Pseudomonas* species is a bacterial strain deposited under Accession
25   No. NCIMB 41076.

15. A protein showing anti-freeze properties isolated *from* a culture according to any one of claims 11 to 14.

16.   A protein according to claim 15 which is heat stable.

DATED THIS 25th day of May, 2004.

5   **UNILEVER PLC**

By Its Patent Attorneys

DAVIES COLLISON CAVE
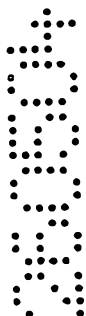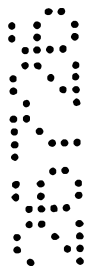
Fig.1.

```
           1                                                          60
196x.seq   GT..................TAGCTCAGATTGAACGCTGGCGGCAGGCTTAAACACATGC
Mcomm.seq  NAAACTGAAGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCTTAA.CACATGC

           61                                                        120
196x.seq   AAGTCGAGCGGTAACAGGGG.AGCTTGCTCCT.GCTGACGAGCGGCGGACGGGTGAGTAA
Mcomm.seq  AAGTCGAGCGGTAACATTGCTAGCTTGCTAGAAGATGACGAGCGGCGGACGGGTGAGTAA

           121                                                       180
196x.seq   CGCGTAGGAATCTGCCTAGTAGAGGGGGGACAACATGTGGAAACGCATGCTAATACCGCAT
Mcomm.seq  CGCGTAGGAATCTGCCTAGTAGTGGGGGGACAACATGTGGAAACGCATGCTAATACCGCAT

           181                                                     . 240
196x.seq   ACGCCCTGAGGGGGAAAGGAGGGGACTCTTCGGAGCCTTCCGCTATTAGATGAGCCTGCG
Mcomm.seq  ACGCCCTACGGGGGAAAGGAGGGNN.TCTTCGGA.CCTTTCGCTATTAGATGAGCCTGCG

           241                                                       300
196x.seq   TGAGATTAGCTAGTTGGTAGGGTAAAGGCCTACCAAGGCGACGATCTCTAACTGGTCTGA
Mcomm.seq  TGAGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTCTAGCTGGTCTGA

           301                                                       360
196x.seq   GAGGATGACCAGTCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGT
Mcomm.seq  GAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGT

           361                                                       420
196x.seq   GGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGC
Mcomm.seq  GGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGC

           421                                                       480
196x.seq   CTTAGGGTTGTAAAGCACTTTCAGGGGTGAGGAAGGGTGATAGGTTAATACGTTATCATC
Mcomm.seq  CTTAGGGTTGTAAAGCACTTTCAGGAGTGAGGAAGGGCGTATAGTTAATACCTGTATGTT

           481                                                       540
196x.seq   TTGACGTTAGCCCCAGAAGAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAG
Mcomm.seq  TTGACGTTAACTCCAGAAGAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAG

           541                                                       600
196x.seq   AGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGT
Mcomm.seq  AGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGCGGTTTGTTAAGT

           601                                                       660
196x.seq   CGGATGTGAAATCCCAGGGCTCAACCTTGGAATGGCACCCGATACTGGCTAGCTAGAGTA
Mcomm.seq  CGGATGTGAAATCCCAGGGCTCAACCTTGGAATGGCACCCGATACTGGCAGGCTAGAGTA

           661                                                       720
196x.seq   TGGTAGAGGGGTGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACA
Mcomm.seq  CGGTAGAGGGGTGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACA

           721                                                       780
196x.seq   TCAGTGGCGAAGGCGACACCCTGGACTAATACTGACACTGAGGTGCGAAAGCGTGGGGAG
Mcomm.seq  TCAGTGGCGAAGGCGACACCCTGGACCGATACTGACGCTGAGGTGCGAAAGCGTGGGGAG

           781                                                       840
196x.seq   CAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTAGCCGTTGGGT
Mcomm.seq  CAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTAGCCGTTGGGG

           841                                                       900
196x.seq   .TGTAATGACTTAGTGGCGCAGCTAACGCAATAAGTAGACCGCCTGGGGAGTACGGCCGC
Mcomm.seq  ATNTATTTCTTTAGTGGCGCAGCTAACGCGATAAGTAGACCGCCTGGGGAGTACGGCCGC

           901                                                       960
196x.seq   AAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAA
Mcomm.seq  AAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAA

           961                                                      1020
196x.seq   TTCGAAGCAACGCGAAGAACCTTACCTACTCTTGACATCCACAGAACATTTGAGAGATCA
Mcomm.seq  TTCGAANNAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTTYCAGAGATGA
```

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Fig.1(cont.)

```
         1021                                                          1080
196x.seq GATGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTG
Mcomm.seq ATTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTG

         1081                                                          1140
196x.seq AAATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTATTTGCCAGCACGTAAT
Mcomm.seq AAATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTATCCTTATTTGCCAGCACTTCG.

         1141                                                          1200
196x.seq GGTGGGAACTTTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGACGACGTCAAG
Mcomm.seq GGTGGNAACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTNGGNNCGACGTCAAG

         1201                                                          1260
196x.seq TCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGTATACAGAGGGCTG
Mcomm.seq TCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGTATACAGAGGGCAG

         1261                                                          1320
196x.seq CAAGCTAGCGATAGTGAGCGAATCCCACAAAGTACGTCGTAGTCCGGATTGGAGTCTGCA
Mcomm.seq CGAACTCGCGAGGGTAAGCAAATCCCAAAAAGTACGTCGTAGTCCGGATTGGAGTCTGCA

         1321                                                          1380
196x.seq ACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCACGGTGAATAC
Mcomm.seq ACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCACGGTGAATAC

         1381                                                          1440
196x.seq GTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGATTGCTCCAGAAGTAG
Mcomm.seq GTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGATTGCTCCAGAAGTAG

         1441                                                          1500
196x.seq CTAGCTTAACCCTTCGGGGATGGCGGTTACCACGGAGTGGTCAATGACTGGGGTTGAAGT
Mcomm.seq CTAGCTTAACCTNC..GGGATGGCGGTTACCACGGAGTGGTCAATGA.............

         1501
196x.seq CTACGCG
Mcomm.seq .......
```

Fig.2.

Spirillum volutans
Oceanospirillum jannaschii
Marinomonas protea
Marinomonas communis
Marinomonas vaga
MMB-1
Oceanospirillum linum
Oceanospirillum maris 2
Oceanospirillum maris
Oceanospirillum multiglobiferum
Oceanospirillum beijerinckii
Oceanospirillum beijerinckii 2
Marinobacter aquaeolei
Marinobacter hydrocarbonoclasticus
Marinobacter sp. CAB
Pseudomonas rhodesiae
Pseudomonas gessardii
Pseudomonas libaniensis
Pseudomonas sp. IC038
Pseudomonas cedrella
Pseudomonas jessenii
Pseudomonas flavescens
Pseudomonas stutzeri
Pseudomonas mandelii
Pseudomonas syringae
Pseudomonas ficuserectae
Gamma proteobacterium 3
Gamma proteobacterium 2
Pseudomonas sp.NKB1
Pseudomonas fluorescens

0.1

Fig.3.

```
  1 GCCCTTGCTCAGATTGAACGCTGGCGGCAGGCCT.AACACATGCAAGTCG 49
     | |||||||||||||||||||||||||||| | |||||||||||||
  1 ..gttagctcagattgaacgctggcggcaggcttaaacacatgcaagtcg 48

 50 AGCGGT.AGAGAGAAGCTTGCTTCTCTTGA.GAGCGGCGGACGGGTGAGT 97
     |||||| | || | ||||||| || ||| ||||||||||||||||||||
 49 agcggtaacaggggagcttgctcctgctgacgagcggcggacgggtgagt 98

 98 AATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTTCGGAAACGGACG 147
     || || ||||||||||| |||| ||||||| ||| | ||||||| | |
 99 aacgcgtaggaatctgcctagtagaggggggacaacatgtggaaacgcatg 148

148 CTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGA..CCTTCGGGCCT 195
    ||||||||||||||| ||| ||| |||||| |||||| | | | ||||
149 ctaataccgcatacgccctgaggggggaaaggagggggactcttcggagcct 198

196 TGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGG 245
    | |||||| ||||||||||| || ||||||||||||||| ||||| ||
199 tccgctattagatgagcctgcgtgagattagctagttggtagggtaaagg 248

246 CTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACAC 295
    | ||||||||||||||||| |||||||||||||||||||| ||||||||
249 cctaccaaggcgacgatctctaactggtctgagaggatgaccagtcacac 298

296 TGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA 345
    ||| |||||||||||| ||||||||||||||||||||||||||||||||
299 tgggactgagacacggcccagactcctacgggaggcagcagtggggaata 348

346 TTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAG 395
    |||||||||||||| |||||||||||||||||||||||||||||||||||
349 ttggacaatgggcgcaagcctgatccagccatgccgcgtgtgtgaagaag 398

396 GTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGTTGTAGATTAA 445
    | ||| || |||||||||||||| || | |||||||||| ||| ||||
399 gccttagggttgtaaagcactttcaggggtgaggaagggtgataggttaa 448

446 TACTCTGCAATTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGT 495
    ||| | || ||||||||| | ||||| |||||||||||||||||||||
449 tacgttatcatcttgacgttagccccagaagaagcaccggctaactctgt 498
```

Fig.3(cont.)i

```
496 GCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTG 545
    |||||||||||||||||||||||||||I·||||||||||||||||||||||
499 gccagcagccgcggtaatacagagggtgcaagcgttaatcggaattactg 548

546 GGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAATCCCCGG 595
    ||||||||||||||||||||||||||||||||| ||||||||||||| ||
549 ggcgtaaagcgcgcgtaggtggtttgttaagtcggatgtgaaatcccagg 598

596 GCTCAACCTGGGAACTGCATTCAAAACTGACTGACTAGAGTATGGTAGAG 645
    |||||||||| ||||   |||   | | |||| ||   ||||||||||||||
599 gctcaaccttggaatggcacccgatactggctagctagagtatggtagag 648

646 GGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAA 695
    ||    ||||||||||||||||||||||||||||||||||||||||||||
649 gggtgtggaatttcctgtgtagcggtgaaatgcgtagatataggaaggaa 698

696 CACCAGTGGCGAAGGCGACCACCTGGACTAATACTGACACTGAGGTGCGA 745
    ||  ||||||||||||||   |||||||||||||||||||||||||||||
699 catcagtggcgaaggcgacaccctggactaatactgacactgaggtgcga 748

746 AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAA 795
    ||||||||||||||||||||||||||||||||||||||||||||||||||
749 aagcgtggggagcaaacaggattagataccctggtagtccacgccgtaaa 798
```

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Fig.3(cont.)ii

```
 796 CGATGTCAACTAGCCGTTGGAAGCCTTGAGCTTTTAGTGGCGCAGCTAAC 845
     |||||||  |||||||||||   |    |  |     ||||||||||||||||||
 799 cgatgtctactagccgttgg..gttgtaatgacttagtggcgcagctaac 846

 846 GCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAAT 895
     |||  ||||| |||||||||||||||||||||||||||||||||||||||||||
 847 gcaataagtagaccgcctggggagtacggccgcaaggttaaaactcaaat 896

 896 GAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAG 945
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 897 gaattgacgggggcccgcacaagcggtggagcatgtggtttaattcgaag 946

 946 CAACGCGAAGAACCTTACCAGGCCTTGACATCCAATGAACTTTCTAGAGA 995
     ||||||||||||||||||||    |||||||||||  ||||  ||  |||||
 947 caacgcgaagaaccttacctactcttgacatccacagaacatttgagaga 996

 996 TAGATTGGTGCCTTCGGGAACATTGAGACAGGTGCTGCATGGCTGTCGTC 1045
     |       ||||||||||||||||  ||||||||||||||||||||||||||
 997 tcagatggtgccttcgggaactgtgagacaggtgctgcatggctgtcgtc 1046

1046 AGCTCGTGTTGTGAAATGTAAGGGC......................... 1070
     ||||||||||||||||||||||   ||
1047 agctcgtgttgtgaaatgttgggttaagtcccgtaacgagcgcaaccctt 1096
```

Fig.4.

```
                    10        20        30        40        50
Isolate 20
           GCCCTTGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGC
           :  :  :::::::::::::::::::::::::::::::::::::::::::::::::
P.synx  AGAGTTTGATCTTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGC
            10        20        30        40        50        60


                    60        70        80        90       100       110
           GGTAGAGAGAAGCTTGCTTCTCTTGAGAGCGGCGGACGGGTGAGTAATGCCTAGGAATCT
           ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
           GGTAGAGAGAAGCTTGCTTCTCTTGAGAGCGGCGGACGGGTGAGTAATGCCTAGGAATCT
              70        80        90       100       110       120


                   120       130       140       150       160       170
           GCCTGGTAGTGGGGGATAACGTTCGGAAACGGACGCTAATACCGCATACGTCCTACGGGA
           ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
           GCCTGGTAGTGGGGGATAACGTTCGGAAACGGACGCTAATACCGCATACGTCCTACGGGA
             130       140       150       160       170       180


                   180       190       200       210       220       230
           GAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTT
           ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
           GAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTT
             190       200       210       220       230       240


                   240       250       260       270       280       290
           GGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCA
           ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
           GGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCA
             250       260       270       280       290       300


                   300       310       320       330       340       350
           CACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACA
           ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
           CACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACA
             310       320       330       340       350       360


                   360       370       380       390       400       410
           ATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAG
           ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
           ATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAG
             370       380       390       400       410       420


                   420       430       440       450       460       470
           CACTTTAAGTTGGGAGGAAGGGTTGTAGATTAATACTCTGCAATTTTGACGTTACCGACA
           ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
           CACTTTAAGTTGGGAGGAAGGGTTGTAGATTAATACTCTGCAATTTTGACGTTACCGACA
             430       440       450       460       470       480
```

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Fig.4(cont.)i

```
     480       490       500       510       520       530
GAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTA
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTA
     490       500       510       520       530       540


     540       550       560       570       580       590
ATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAATCCC
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
ATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAATCCC
     550       560       570       580       590       600


     600       610       620       630       640       650
CGGGCTCAACCTGGGAACTGCATTCAAAACTGACTGACTAGAGTATGGTAGAGGGTGGTG
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
CGGGCTCAACCTGGGAACTGCATTCAAAACTGACTGACTAGAGTATGGTAGAGGGTGGTG
     610       620       630       640       650       660


     660       670       680       690       700       710
GAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCG
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCG
     670       680       690       700       710       720


     720       730       740       750       760       770
ACCACCTGGACTAATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGAT
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
ACCACCTGGACTAATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGAT
     730       740       750       760       770       780


     780       790       800       810       820       830
ACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGAAGCCTTGAGCTTTTAG
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
ACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGAAGCCTTGAGCTTTTAG
     790       800       810       820       830       840


     840       850       860       870       880       890
TGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCA
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
TGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCA
     850       860       870       880       890       900


     900       910       920       930       940       950
AATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCG
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCG
     910       920       930       940       950       960


     960       970       980       990      1000      1010
AAGAACCTTACCAGGCCTTGACATCCAATGAACTTTCTAGAGATAGATTGGTGCCTTCGG
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AAGAACCTTACCAGGCCTTGACATCCAATGAACTTTCTAGAGATAGATTGGTGCCTTCGG
     970       980       990      1000      1010      1020
```
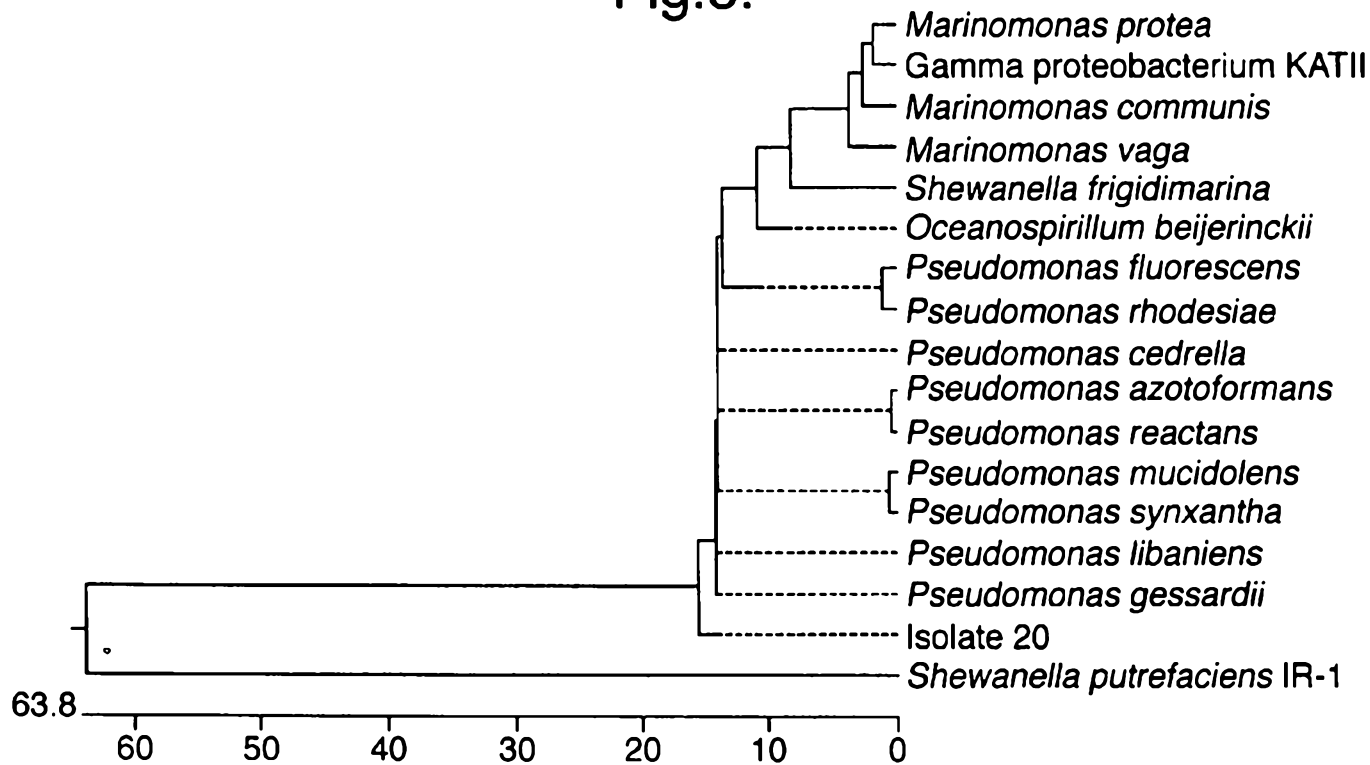
Fig.4(cont.)ii

```
        1020       1030       1040       1050       1060       1070
GAACATTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTAAGGGC
::::::::::::::::::::::::::::::::::::::::::::::: :::: :::: ::
GAACATTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA
        1030       1040       1050       1060       1070       1080


GTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTAATGGTGGGCACTCTAA
        1090       1100       1110       1120       1130       1140
```

Fig.5.

Fig.6A.

Fig.6B.

Fig.7.

Fig.8.

Fig.9.

Force

B

S

D
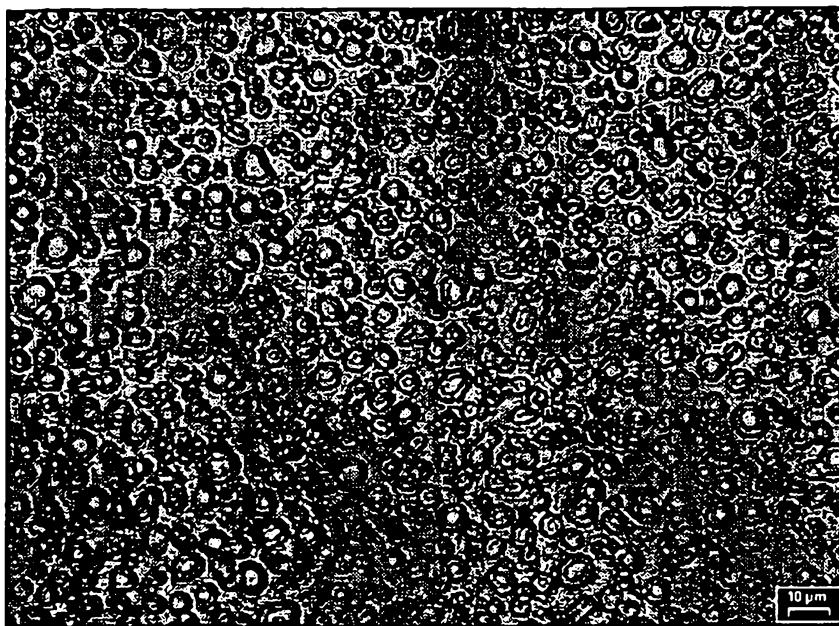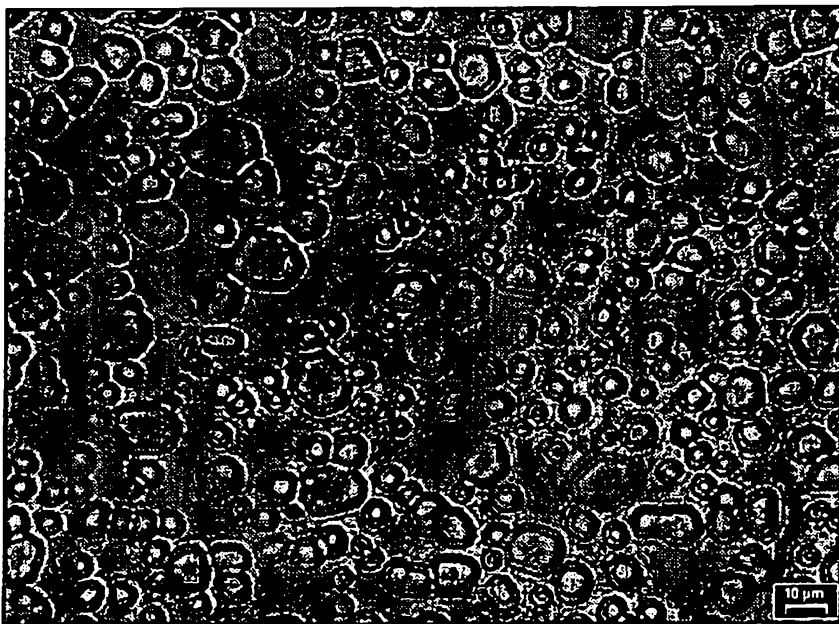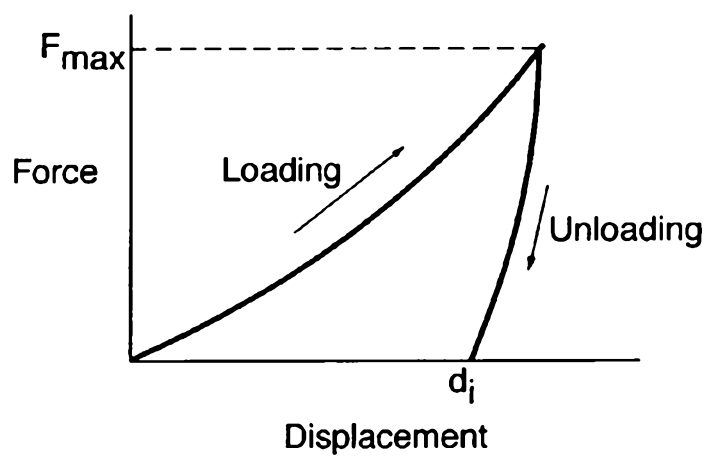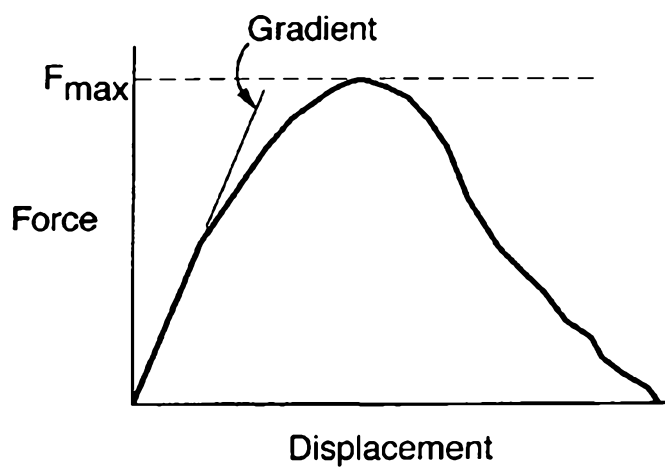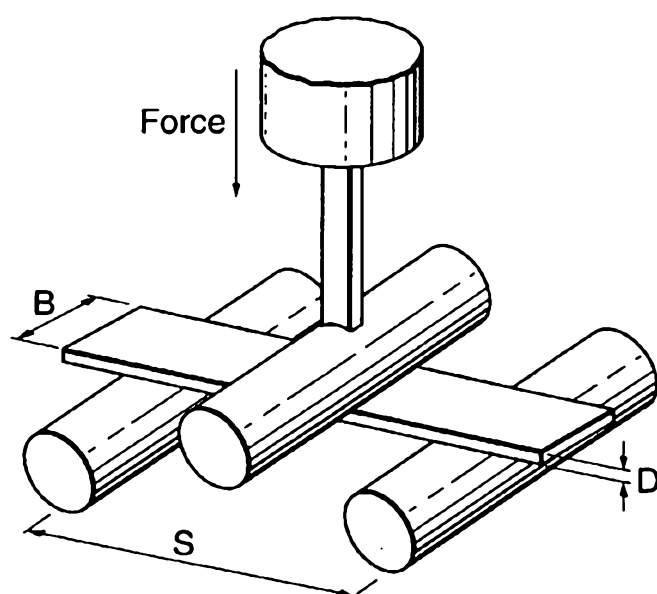
Span (S)   30mm
Depth (D)   ~2mm
Width (B)   10mm