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Identification and analysis of two sequences encoding ice-binding proteins obtained from a putative bacterial symbiont of the psychrophilic Antarctic ciliate *Euplotes focardii*

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Abstract: We identified two ice-binding protein (IBP) sequences, named EFsymbAFP and EFsymbIBP, from a putative bacterial symbiont of the Antarctic psychrophilic ciliate *Euplotes focardii*. EFsymbAFP is 57.43% identical to the antifreeze protein (AFP) from the *Stigmatella aurantiaca* strain DW4/3-1, which was isolated from the Victoria Valley lower glacier. EFsymbIBP is 53.38% identical to the IBP from the *Flavobacteriaceae bacterium* strain 3519-10, isolated from the glacial ice of Lake Vostok. EFsymbAFP and EFsymbIBP are 31.73% identical at the amino acid level and are organized in tandem on the bacterial chromosome. The relatively low sequence identity and the tandem organization, which appears unique to this symbiont, suggest an occurrence of horizontal gene transfer (HGT). Structurally, EFsymbAFP and EFsymbIBP are similar to the AFPs from the snow mould fungus *Typhula ishikariensis* and from the Arctic yeast *Leucosporidium* sp. AY30. A phylogenetic analysis showed that EFsymbAFP and EFsymbIBP cluster principally with the IBP sequences from other Antarctic bacteria, supporting the view that these sequences belong to an Antarctic symbiontic bacterium of *E. focardii*. These results confirm that IBPs have a complex evolutionary history, which includes HGT events, most probably due to the demands of the environment and the need for rapid adaptation.

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Key words: antifreeze, biology, cold-adaptation, genome, horizontal gene transfer, Protozoa

Introduction

The Southern Ocean began to cool approximately 40 million years ago (m.y.a.), reaching the current temperature range of +2°C to -1.8°C approximately 25 m.y.a. (Clark *et al.* 2004). The formation of ice crystals in the water would normally be fatal for living organisms, due to the physical destruction of membranes and the resulting chemical damage from processes such as osmotic shock. Antarctic organisms have developed a variety of molecular strategies to thrive in these low-temperature environments, such as increasing molecular flexibility to modulate the kinetics of key enzymes and developing biological membranes that are more fluid through the accumulation of polyunsaturated fatty acyl chains (Morgan-Kiss *et al.* 2006). Another adaptive strategy involves the use of ice-binding proteins (IBPs), which include antifreeze proteins (AFPs), to combat the destructive effects of ice crystals. IBPs can inhibit ice growth by binding to specific ice planes and lowering the freezing point to below the melting point of the ice. The difference between the freezing and melting temperatures is called thermal hysteresis (TH) and is used as a measure of antifreeze activity (Scotter *et al.* 2006).

Initially identified in Antarctic marine fishes, AFPs protect their hosts from freezing by binding to, and preventing the growth of, seed ice crystals (DeVries & Wohlschl 1969). In fishes, four types of AFPs have been characterized: types I, II and III, and antifreeze glycoproteins (Fletcher *et al.* 2001); interestingly, these AFPs are apparently unrelated. Type II AFPs, which are derived from C-type lectin genes, are the only type that have been found in three phylogenetically disparate fish species: the Atlantic herring (Clupeiformes), the smelt (Salmoniformes) and the sea raven (Scorpaenoformes). This finding suggests that type II AFPs have evolved through the duplication and divergence of the C-type lectin genes at least three times (Ewart *et al.* 1992). Subsequently, AFPs were found in other freeze-tolerant organisms, such as plants (Sidebottom *et al.* 2000), fungi (Kondo *et al.* 2012) and bacteria (Raymond *et al.* 2008, Do *et al.* 2012). The bacterial AFPs stop the growth of ice crystals through ice re-crystallization inhibition (IRI) in frozen tissues, rather than preventing the freezing of

tissues (Gilbert *et al.* 2004). Gilbert *et al.* (2004) isolated 11 bacterial strains from Antarctica with IRI activity. Among these, *Marinomonas primoryensis* Romanenko *et al.* produces a hyperactive, Ca^{2+}-dependent AFP, which lowers the freezing point of water by 2°C (Gilbert *et al.* 2004). This mode of action resembles that of insect AFPs (Duman 2001); ice crystals formed in the presence of this AFP do not have distinct facets and are typically round in shape.

Over the past decade, IBPs/AFPs have proven to be potent agents in the cryopreservation of food and pharmaceutical materials. For example, the AFP from carrots (*Daucus carota* L.) has been shown to improve the leavening of frozen dough, and to help maintain loaf volume and dough softness during frozen storage (Yeh *et al.* 2009). Additionally, the type I AFP produced in *Lactococcus lactis* (Lister) improves the fermentation capacity of frozen dough and the conservation of frozen meat (Yeh *et al.* 2009).

The mechanisms of molecular adaptation to low-temperature environments remain poorly understood, largely due to the limited amount of available molecular data. To obtain sufficient sample data necessary to identifying the molecular adaptation in psychrophiles, the entire macronuclear genome from the Antarctic ciliate *Euplotes focardii* Valbonesi & Luporini was sequenced recently. Using this approach, we expected to discover new genes that may be responsible for cold-adaptation, including genes encoding IBPs.

Euplotes focardii was isolated from the coastal seawater near Terra Nova Bay and is endemic to the Antarctic continent (Valbonesi & Luporini 1993). It has been maintained in laboratory culture since its first isolation. Its psychrophilic phenotypes include optimal survival and multiplication rates at temperatures between 4–5°C (Valbonesi & Luporini 1993), lack of a transcriptional response of the Hsp70 genes to thermal stress (La Terza *et al.* 2001), and modifications in the primary structure of the tubulin (Marziale *et al.* 2008, Pucciarelli *et al.* 2009, Chiappori *et al.* 2012) and ribosomal stalk proteins (Pucciarelli *et al.* 2005). As in all ciliates, *E. focardii* possesses two nuclei: a diploid micronucleus involved only in sexual exchange and a polyploid macronucleus responsible for all gene expression, which is renewed after each phenomenon of sexuality (Hoffman *et al.* 1995). In the latter, the genome is organized in small linear chromosomes (nanochromosomes). Each nanochromosome contains a single genetic unit that is flanked by regulatory regions and capped by telomeres (Hoffman *et al.* 1995). Among the assembled nanochromosomes characterized by regular *Euplotes* telomeres, DNA sequences that lack telomeres and thus do not belong to the *E. focardii* nanochromosomes have been identified. We report the identification and sequence analysis of two IBP genes obtained from the *E. focardii* genome.

Materials and methods

Materials

Taq polymerase, DNA modifying enzymes and restriction enzymes were purchased from Fermentas (Milan, Italy). Oligonucleotides were synthesized by Sigma/Genosys (Milan, Italy). All routine chemicals were of analytical grade and supplied by Sigma Aldrich (Milan, Italy).

Cell strains and growth conditions

Cell cultures of *E. focardii* strain TN1 (Valbonesi & Luporini 1993) were used for this study. They were isolated from coastal sediment and seawater samples collected in Terra Nova Bay, Ross Sea (temperature -1.8°C, salinity 35‰, pH 8.1–8.2). The cultures were grown at 4°C and fed on the green algae *Dunaliella tertiolecta* Butcher.

Isolation of Euplotes focardii *macronuclear DNA and rapid amplification of telomeric ends*

The macronuclear DNA of *E. focardii* was purified as described by Miceli *et al.* (1989). Organization in small nanochromosomes facilitates the characterization of the 5'- and 3'-untranslated region sequences using the rapid amplification of telomeric ends-PCR (RATE-PCR) technique (Pucciarelli *et al.* 2005, Marziale *et al.* 2008). To determine whether the genes encoding the AFPs were present in the *E. focardii* genome, we used degenerate forward and reverse primers designed against the conserved amino acid sequences (Table I).

The PCR reactions were started with only one of the listed primers. After ten cycles, the telomeric oligonucleotide

Table I. Degenerate forward and reverse primers designed against the conserved amino acid (AA) sequences, used to determine whether the genes encoding the antifreeze proteins were present in the *E. focardii* genome.

Forward primers	Corresponding AA sequence	Reverse primers	Corresponding AA sequence
EFsymbAFP_FW: 5'-ACTTTAACGCCNGGNCTATACAAA-3'	TLTPGLYK	EFsymbIBP_FW: 5'-TTGTATAGNCCNGGCGTTAAAGT-3'	TLTPGLYK
EFsymbAFP_RV: 5'-TATTCAAAAGTACTATTRATAAAC-3'	AVFVIQIN	EFsymbIBP_RV 5'-NAANCCTTCNACTTTCCANAA-3'	FWKVEGLV

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5'-$(C_4A_4)_4$-3' was added to complete the strategy: 1 minute at 94°C, 1 minute at 55°C and 1 minute at 72°C for 35 cycles. Amplified products were cloned into the pTZ57R/T vector following the procedure recommended by the manufacturer (Fermentas, Milan, Italy). The recombinant vectors were sequenced by BMR Genomics (Padua, Italy).

Colony blotting

Colony blotting was performed as previously described by Marziale *et al*. (2008). Probes corresponding to the sequence of the AFP genes from the *E. focardii* symbiont were used, following the procedure described by Yu *et al*. (2012).

Sequence analysis, alignment and phylogenetic tree construction

Sequences were obtained from the analysed reads and assembled contigs of the *E. focardii* macronuclear genome sequencing performed in collaboration with Dr Vadim Gladishev's research group (Harvard Medical School, USA).

For the tBLASTn analysis, we used the *Fragilariopsis cylindrus* (Grunow) Krieger AFP sequences (GenBank: ACT21449.1, ACT21447.1, ACT21445.1, ACT21443.1, ACT21448.1, ACT21446.1, ACT21444.1, ABT17158.1, ACX36856.1, ACX36854.1, ACX36852.1, ACX36855.1 and ACX36853.1), a dominant species within the polar sea ice assemblages (Bayer-Giraldi *et al*. 2010), to query the genome of *E. focardii*.

To verify the tandem organization of the two IBP gene sequences on the bacterial chromosome, we performed PCR using the forward primer 5'-ACAACA ACACCAGGGCGAAAAG-3' and the reverse primer 5'-TTCGTTGTAAGCGATTAATAAATC-3'. We used macronuclear DNA isolated from unperturbed *E. focardii* cells or from cells treated with antibiotics to remove the symbiotic bacteria (Vannini *et al*. 2004). DNA from the cells treated with antibiotics was used as a control. The expected band was of *c*. 750 bp. Furthermore, to

```
>contig03294  length=3221   numreads=477
ACGAAATGGGCTTTTTTTGTGGTTGCTTGGTTTGTCAATTATTACTCGTTTTTCATTCTAAGTTACAAGC
AATACTCCATTCAAATTCCAAACACGCATACCAGAAATGACGCTGTCAAAATAGTATCTCAGTCTAAAAC
TCCTAACCGTTATTTAGTATTAGACAGATAATTAGAATCCCAATAAGTGTGAACTATGTAAGTCGTAGTA
ATAGTTGTGTAACACATTCTGAAACAATAGTTCGCAACTTTGATTCGTGAAAACTATTTCACAATTGTTG
CGCCAAAAGCGCAAATAAAAAAAAGGGAAATGAAAAAAATAAAAATAACAATGTTAACCGCAACTGTTCT
ATTCGGACTTTTAACTGTGGTTGGGTGCAAAAAAGAAAAAAATGATCCCACAACACCTGGAACAACTACA
ACTGTAATCCCTCTGCAAACAACTGTACAGACGCCGATAACTTTGGGTTCAGCCAATAATTTTGCAGTAA
TCGCTGGGTCATCCGTTACGAACACTGGCGCAACAAACATTACAGGAGATCTTGGTTTAAGTCCGGGAAC
ATCAATTGGTGGATTTCCTCCTGGAATTTTAAATGGTACCCTTCACATAAACGACGCCATTGCCAATCAG
GCAAAACTTGATATTACAACGGCCTACAATGATGCTGCGGCAAGAGTTGCAAGTGATATGGTAACAATTT
CGGGGAACATTGGTGGTTTGACTTTAACGCCCGGGCTATACAAATCGACATCATCGCTTGCCGTTTCTTC
GGGCGATGTTACGTTCGATGCGTTGGGAGATCCAAGTGCTATTTTTGTAATACAAATCGCATCAACGCTT
ACAACAACACCAGGGCGAAAAGTACTATTAAGTGGTGGAGCACTTGCCTCTAATATTTACTGGCAAGTGA
GCAGTTCAGCATCTTTTGGAACGACTACGTCTTTTAAAGGAACCGTAATTGCTTTGGAATCCATCACTTT
CGATACCGGAGCTACACTGGAAGGAAGGGCACTTGCTAGAAATGGAGCTGTCACAATGGAAGGAAATACA
TTTGTACTTCCTTAAATAGCATCTTACGTTTTAATCCTATTAGGCCTTACTCAACATTATGAGTAAGGTC
TTTTTGTTTGTCGATTCTACCGTGAACCAAAACACAAAAATGTGAGAACTATGTAACTATGGAGAGAGAT
TGTATAACATAAGATGAAGTATAAAACCTCATCTTGAAGTGTGAAAATGATTTCACACAACTAAATAAAA
CAAAATGAAAACACAATTACTTCACCTACTACTCATTGTCTTTCCGCTATGTGCGCCACAATTAGTATTT
GGCCAAGCACCAAATTTAGGCACAGCTTCAAATTTTGTACTCTTTTCAACAAATGGAGCCGTTAGTAATT
CAGGTATTTCACATATAACAGGTGAAGTTGGAACAAATAACGGATCAAGTACTTCATTCGGAAACGTTGA
TGGTTCTATGCACGACGGAGACGCCGCAAGTATCCAATGTGCAGCAGATTTATTAATCGCTTACAACGAA
CTAAATGCTGTTATTCCTGGATTCTTTCCTGCCCCTTTACTTGGAAATGGTCAAACACTAAACGCGGGTA
CCTATTCTATACCGGGTGCCTCAACGTTAAACCTAAATCTAAATTTGGATGCCCAAGGAGATCCAAACGC
TGTGTTTATTTTTCAAATTCAAGGCCCCTTATCAACCAATGCTGATTCCAAAGTTAAACTGTTAAATGGA
GCTTTGGCTTGCAATGTTTTCTGGAAAGTGGAAGGATTGGTAAGTATGGCTTCTGGCAGCACAATGAGAG
GAACTATCATTGCGAATAATGCCGCAATTGAAATGAATACGGGTGACACTCTTGAAGGCAGAGCACTTTC
TACAGCGGGAGCAATTACAGTTGATGGAATATTGGCATACACACCAATCGGTTGTGGAAGTCCTGTACTT
GACGGCCCAATAGCTCCTACTCTTGGAGCGGCTGCCTGTTATGCAATATTCTCTACTGACGGTGCCGTAA
CAAATACGGGCACGACAACAATTACTGGAGATGTAGGGTCTAATAGTGGTTCTCCAACAGGCTTCGATCC
TTTGCTTGTCACAGGTGAAATTCACTTGATTCCAGATGGCTCAACTGCACAATGTGCGAATGATTTACTC
GTTGCCTATAATTATATAAATACGTTGCCCTATGATATCGAACTGTTGTATCCTGCACAATTTGGAAAAA
ATCTAGTTCTTACCCCTCACACCTATTTAATGGGAGGTGCGGCAACATTTACTGATTCTCTCTACTTGAA
TGCTCAAGGAAATCCTGATGCCGTTTTTGTTATTCAGATTAATGGAGCTCTTTCTACGAGTACTTATTCA
AAAGTACTATTGATAAACGGAGCGCAATCGAAAAATGTGTATTGGAAAGTTGAAGGAGCTGTAAGCATTA
ACGATTATTCAGTATTCTGCGGAACTATTATCTGTAATAATGGTGCCTTGGGCGCAATAAATACTGGAGT
AACTCTAAATGGACGAGCACTTACAACTGCAGGCGCACTAAATACTTTTTCAATTGACGCAATTGCTCCA
AATTTACCCTTGAACTGCGAGTCTGTAGGAGTCTCAACTATTGAGATTACAGATGAAGTAATGGCTATTT
ATCCGAATCCCTTCAATCAAATGACGACCATTTCGATTCATGATGCTTCTGAGAGTAATAGTTATGTTTT
AGAAATCTACAATGCGATGGGAGAGCAAATGATCAATACAATCATTACTAATCCGTCAACGCCTCTTGAT
TTTACAGACTTTAATTCAGGAATGTTTTTCTACAAGGTATTCAGTAATCAACAGGTCATTCAAACAGGTA
AATTGATTGCCCAATAAATTAATGTCTAGTCCTAAATAACCGTTACAGGTTTTAGACTAAGAAACTTTAT
TTATTAAATAGCGAAGAGAACGTTCTCTTCGCTGTTTTTTCTTTTCTGGAATCCTTTAATTCGCACACAA
AATAAAATTGGCAGACATGAAAAAGAGAATTCCTATCTATCATTTTATCGCTTTTGGGCTTCTCGTAAAA
TCTTTATGATTAATCTGGCGAATAGCCCAAAGTGTGAATCATGTAAGTAGTAGGAATAGTAGTGTAACAG
ATAGCTAACTAGGTATTCTCACCTTTGTTGTGTATTACATAAAACACTAAAAATGAAAAAAGTAAATCTA
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Fig. 1. Partial nucleotide sequence of the bacterial symbiont contig (3211 bp; contig 03294) identified in the *E. focardii* genome. The two open reading frames encoding for the ice-binding protein and antifreeze protein, respectively, are shaded in grey.

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Table II. Percentage identities of ice-binding protein sequences from the *E. focardii* symbiont and from other cold-adapted bacteria.

	EFsymbAFP	*S. aurantiaca* AFP	Uncultured bacterium	EFsymbIBP	*F. bacterium* IBP	*P. torquis* IBP
EFsymbAFP	100%	57.43%	39.44%	31.73%	16%	32.89%
S. aurantiaca AFP		100%	39.32%	22.30%	15%	17.55%
Uncultured bacterium			100%	16.20%	19.81%	13.40%
EFsymbIBP				100%	53.38 %	23.82%
F. bacterium IBP					100%	27.53%
P. torquis IBP						100%

Percentage identities were obtained from the ClustalW2 tool (http://www.ebi.ac.uk/Tools/msa/clustalw2/). AFP = antifreeze protein, IBP = ice-binding protein.

exclude the possibility that the IBP sequences were derived from associated bacteria or endosymbionts of the algae in the culture medium rather than from the *Euplotes*, we performed a PCR reaction using DNA purified from *E. focardii* or *Dunaliella* cells.

Sequence similarity and conserved domain analysis were performed using the Basic Local Alignment Search Tool (BLAST) program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the default parameters. Multiple sequence alignments were performed using

```
EfSymbAFP                  ----------------------------------------------------
S.aurantiaca AFP           ---MRSLKTGVLFLLFSLMTACG---------QQLVEFPDDTNDPGDDAG 38
Uncultured bacterium AFP   MKITKNLLPALVLAVFIFGCNCHSCGDGGGNTTPTTTNLVSATDPADGAD 50

EfSymbAFP                  ----------------------------------------------------
S.aurantiaca AFP           ----------------------------SGGDAGSGGDAGSGGDAGIS- 58
Uncultured bacterium AFP   GVFINRKIAATFSGEMDSATITDTTFTIMQGTTSVSGAVAYSGVTAVFTP 100

EfSymbAFP                  ----------------------------------------------------
S.aurantiaca AFP           ------------------------------------------PTVVST 64
Uncultured bacterium AFP   ASNLVVNTEYTATITTGVADLEGRALANEYTWSFTTGVTADTTAPTVAST 150

EfSymbAFP                  ----------------------------------------------------
S.aurantiaca AFP           RPANAATSVAVNAPITATFSTEMNPATLSTAFTLR--QDATFVAG--DVA 110
Uncultured bacterium AFP   DPVNENVDVLLNRKIAATFSEAMDALTITTDNFIVSGPDATLVTGTVDYV 200

EfSymbAFP                  ----------------------------------------------------
S.aurantiaca AFP           YLGTTATLTPKGNLAPGSVFTATISTASKDLEGHALATDYTWSFTT--DA 158
Uncultured bacterium AFP   LVGNVVTFTPENDLEVSTTYTATMMTAAADLAGNELASDYVWSFTTGADV 250

EfSymbAFP                  --------------------------------MKKIKITMLTATVLFGLL 18
S.aurantiaca AFP           HIISPTVIASSPVNAATNVSTNKRIMATFNKGMNPATITTSTFTVYQGAT 208
Uncultured bacterium AFP   DVNAPTLVSTNPSDADTSVALNKTITATFNEAMDALTITTENFLVTGPGT 300
                                                   *.  .**  .  *

EfSymbAFP                  TVVGCKKEKNDPTTPGTTTTVIPLQT------TVQT-------------- 48
S.aurantiaca AFP           AVAGAVT-WSAATNEATFTPTLPLELSQTYTATISTGAQDAAGSSLATNH 257
Uncultured bacterium AFP   TPVIGTVDYDLINNIASFNPGSDLSANTEYTATVTTGSRDLADNALANDN 350
                           : .      .  .. .: ..   *.       *: *

EfSymbAFP                  -----------------PITLGSANNFAVIAGSSVTNTGATNITGDLGLS 81
S.aurantiaca AFP           DWSFTT-----GACSQLPVELGSAGNFAVMAGSTVTSTGQTSVTGDLGVS 302
Uncultured bacterium AFP   VWNFTTSDQVAQTVIQESVSLGVSSNFAILASAAITNIATSAITGDVGLT 400
                                     .: ** :.***::*.:::*. . : :***:*::

EfSymbAFP                  P--GTSIGGFPP----GILNGTLHINDAIA-----------NQAKLDITT 114
S.aurantiaca AFP           S--GTAITGFPP----GKLIGAKHAGDPTA-----------AQGIADLTA 335
Uncultured bacterium AFP   PDSGSNISGFSVPETCPEITGTMYAVDAAGPACALIDPTLLANAKTAAET 450
                           .  *: * **.       : *: :  *. .             :.     :

EfSymbAFP                  AYNDAAARVASDMVTISGNIGGLTLTPGLYKSTSSLAVSSG-DVTFDALG 163
S.aurantiaca AFP           AYNNAAGRSLCP-VTVAGNLGGQTLTPGLYKSTSSLAISEG-DLTLDAKG 383
Uncultured bacterium AFP   AFIDARDAVRGTPQEISGNLNGLTLYPGLYESGSSLEISPGGFLYLDAQG 500
                           *: :*          ::**:.* ** ****:* *** :* *  : :** *

EfSymbAFP                  DPSAIFVIQIASTLTTTPGRKVLLSGGALASNIYWQVSSSASFGTTTSFK 213
S.aurantiaca AFP           DGDAVFIFQMASTLTTTAGRQVVLTGGARSTNIFWQVGTSATFGTTSSFQ 433
Uncultured bacterium AFP   DTNAVFVIRSATSITTEAASEVVLTKGAKANNVYWTAGSTVTLGTTSIMK 550
                           * .*:*::: *:::** .. :*:*: ** :.*::* ..::.::***: ::

EfSymbAFP                  GTVIALESITFDTGATLEGRALAR---NGAVTMEGNTFVLP-- 251
S.aurantiaca AFP           GTVMADQAITLNTGATLNGRALAR---IGAVALDDNTIVKPAP 473
Uncultured bacterium AFP   GTMIAGTSLSLLTGANLEGRALNQGAAAEAVTLDSCIITVPSP 593
                           **::*  :::: ***.*:**** :     **:::.  :. *
```

Fig. 2. Sequence comparison of EFsymbAFP with the antifreeze proteins (AFPs) from *S. aurantiaca* (ZP_01462925.1) and an uncultured bacterium (EKD52074.1), using the ClustalW2 multiple sequence alignment tool. The predicted secretory signal at the N-terminus is shaded.

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```
EfSymbIBP           MKTQLLHLLLIVFPLCAPQLVFGQAP----NLGTASNFVLFSTNGAVSNSGISHITGEVG 56
F.bacterium IBP     MNKFLLLAASVAF-MSFSGKAHAQAP----TLGAAANFALFTTAGAVTNTGLSHITGDVG 55
P.torquis IBP       MKTIILSIAIIAY--SFSSIAQDEQPPIDIYLGTAANFILFTGAGAVANTGVSEITGDVG 58
                    *:. :*    :.:  . .  .  : *     **:*:** **:  ***:*:*:*.***:**

EfSymbIBP           TNNGSSTSFGN---VDGSMHDGDAASIQCAADLLIAYNELNAVIPGFFPAP-LLG--NGQ 110
F.bacterium IBP     TNNAASTNFGN---VDGVMQDSNGATSAAAADLLIAYNLLNAAIPTATLAP-LLG--NGT 109
P.torquis IBP       SHAGAIAGFGLPTVLNGTIQNTNSITAQALLDLAAACVQLQNIPATITDHSGIFGSLEGE 118
                    :: .: :.**    ::* ::: :. :  .  **  *   *:   .     . ::*  :*

EfSymbIBP           TLNAGTYSIPGASTLNLNLNLDAQGDPNAVFIFQIQGPLSTNADSKVKLLNGALACNVFW 170
F.bacterium IBP     TLTAGNYFIGQGASLSGTLTLDGGGNSNSVFIFKIQGALSSAANTQVLLTNGALACNVFW 169
P.torquis IBP       TIYPGVYSNAAAVSLTGTLTLDAQGDPDAMFIFKITGALNSVAGATVLLANGASSENVYW 178
                    *: .* *    . :*. .*.**. *:.:::***:* *.*.: *.: * * *** : **:*

EfSymbIBP           KVEGLVSMASGSTMRGTIIANNAAIEMNTGDTLEGRALSTAGAITVDGILAYTPIGCGSP 230
F.bacterium IBP     KVEGLVDLATNTVMKGNVVANNAAIVLQSGVSLEGRALSTTGAITVTGVTVRKPILCGSA 229
P.torquis IBP       IAVGALALGANTTMKGTAIAYPGAVSLGAGASIDGSLYSTVGAIAINSTVGTKPTYN-TP 237
                     . * : :.:.:.*:*. :*  .*: : :* :::*   **.***:: .    .*    :.

EfSymbIBP           VLDGPIAPTLGAAACYAIFSTDGAVTNTGTTTITGDVGSNSGSPTGFDPLL--VTGEIHL 288
F.bacterium IBP     VLTGPVAPNLGTVVCYTIFSGNGALTNAGITYVTGDVGTNVGLTTGFQADN--VNGTIHS 287
P.torquis IBP       FGCDINAYLFQDNDVYTIDLASGSSYEIATDITTGDIN-----ATGYNPVDGYIWGSLSS 292
                    .  .  *  :     *:*   .*:  : .    ***:.     .**::.    : * :

EfSymbIBP           IPDGSTAQCANDLLVAYNYINTLP-YDIELLYPAQFGKNLVLTPHTYLMGGAATFTDSLY 347
F.bacterium IBP     NPDTSTAQAALDLNNAYTYLNTLP-TDIELLYPAAFGQNLVLTPHTYLLNAATVLNGKVT 346
P.torquis IBP       -PEKTIVRVGKNFNTTSYYIDELPSSDTKIGDVSADGIYYLKGEDTTYYKIDLNPSSADF 351
                     *: : .: . ::  :  *:: **  * ::   :  *   :   .*         ..

EfSymbIBP           LNAQGNPDAVFVIQINGALSTSTYSKVLLINGAQSKNVYWKVEG----AVSINDYSVFCG 403
F.bacterium IBP     LDAQGNENAVFVIKINGALSTTVNASVELINGAIAKNVFWKVDG----AVDLNDYTKFKG 402
P.torquis IBP       AQHQSTESLSQNISIDDWAFNAVDGNLYAIEKIS--NILYRIDPSDGNVQTMGEVPILSG 409
                     : *.. .    *.*:.   .:. ..:  *:     *: ::::     .  :.: . : *

EfSymbIBP           TIICNNGALGAINTGVTLNGRALTTAGALN-------TFSIDAIAPNLPLNCESVGVSTI 456
F.bacterium IBP     SVIGNNGAV-IINTGVEIEGRVLSTSGGIS-------TFGINAQMT---PGCELLGTGSN 451
P.torquis IBP       STYTYDAVYFDVDGRFYISASEIGTIFVVQDVQDLDGSNAIDSNLFAFGPSSSNSDGARC 469
                    :    :..   ::  . :..  : *   :.       : .*::       ...  . .

EfSymbIBP           EITDEVMAIYPNPFN-----------------QMTTISIHDASESNSYVLEIYNAMGEQM 499
F.bacterium IBP     TVAIQAAKFYPNPFS-----------------SVLNVTMEDLNGGS--TLTIYNAAGSQV 492
P.torquis IBP       PTALVAQEICDNGIDDDGDGLIDCEDPSCSGYGSCPIIESNTSGGNDGGLESNNRLSDKI 529
                      :  .  :  * :.                  :   : . ..   *   *  ..::

EfSymbIBP           INTIITNPSTPLDFTD---------------FNSGMFFYKVFSNQQVIQTGKLIAQ---- 540
F.bacterium IBP     FSKVLSTKTTSLSMK----------------LPAGVYFYQMIGKNGAKQAGKLIAKP--- 533
P.torquis IBP       SQRNYNRAKINYRFDRGVARRVSKSSNYAKRSPNSNFQLQNFIPLTVIDEDYVIDSTPID 589
                     .   .  .    :                    . :  : :    . : . :* .

EfSymbIBP           --------------
F.bacterium IBP     --------------
P.torquis IBP       LLGITNAVDVYSVD 603
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Fig. 3. Sequence comparison of EFsymbIBP with the ice-binding proteins (IBPs) from *F. bacterium* (gi_255534643) and *P. torquis* (strain ATCC700755, YP_006867144), using the ClustalW2 multiple sequence alignment tool. The predicted secretory signal at the N-terminus is shaded.

the ClustalW2 tool (http://www.ebi.ac.uk/Tools/msa/clustalw2/) or the PRALINE tool (http://www.ibi.vu.nl/programs/pralinewww/) using the default parameters. Percentage similarities were estimated using the ClustalW2 tool. The evolutionary history was inferred using the maximum parsimony (MP) method. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 5 in which the initial trees were obtained by the random addition of sequences (ten replicates). The analysis involved 22 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 118 positions in the final dataset. Evolutionary analyses were conducted in MEGA 5 (Tamura *et al.* 2011).

The sequences were obtained from the National Center for Biotechnology Information (NCBI) database: *Sphaerochaeta globus* Abt *et al.* (strain Buddy) YP_004248731, *Navicula glaciei* Van Heurck AAZ76251, *Fragilariopsis cylindrus* Grunow, Willi Krieg. CN212299, *Fragilariopsis curta* (Van Heurck) Hustedt ACT99634, *Chlamydomonas* sp. EU190445, *Typhula ishikariensis* S.Imai BAD02897, *Flammulina populicola* Redhead & Petersen ACL27144, *Lentinulaedodes edodes* (Berk.) Pegler ACL27145, *Glaciozyma antarctica* Thomas-Hall & Boekhout ACX31168, *Psychromonas ingrahamii* Auman *et al.* ZP_01349469, *Colwellia* sp. DQ788793, *Deschampsia antarctica* E.Desv. ACN38296, *Lolium perenne* L. ACN38303, *Marivirga tractuosa* (Lewin) (strain DSM4126) YP_004052221, *Cytophaga hutchinsonii* Winogradsky (strain ATCC33406) YP_676864, *Chaetoceros neogracile* VanLandingham ACU09498, *Nitzschia stellata* Manguin AEY75833, *Psychroflexus torquis* Bowman *et al.* (strain ATCC700755) YP_006867144, *Stigmatella aurantiaca* Berkeley & Curtis (strain DW4/3-1) ZP_01462925.1, an uncultured bacterium (EKD52074.1), and *Flavobacteriaceae bacterium* Bernardet *et al.* (strain 3519-10) gi_255534643.

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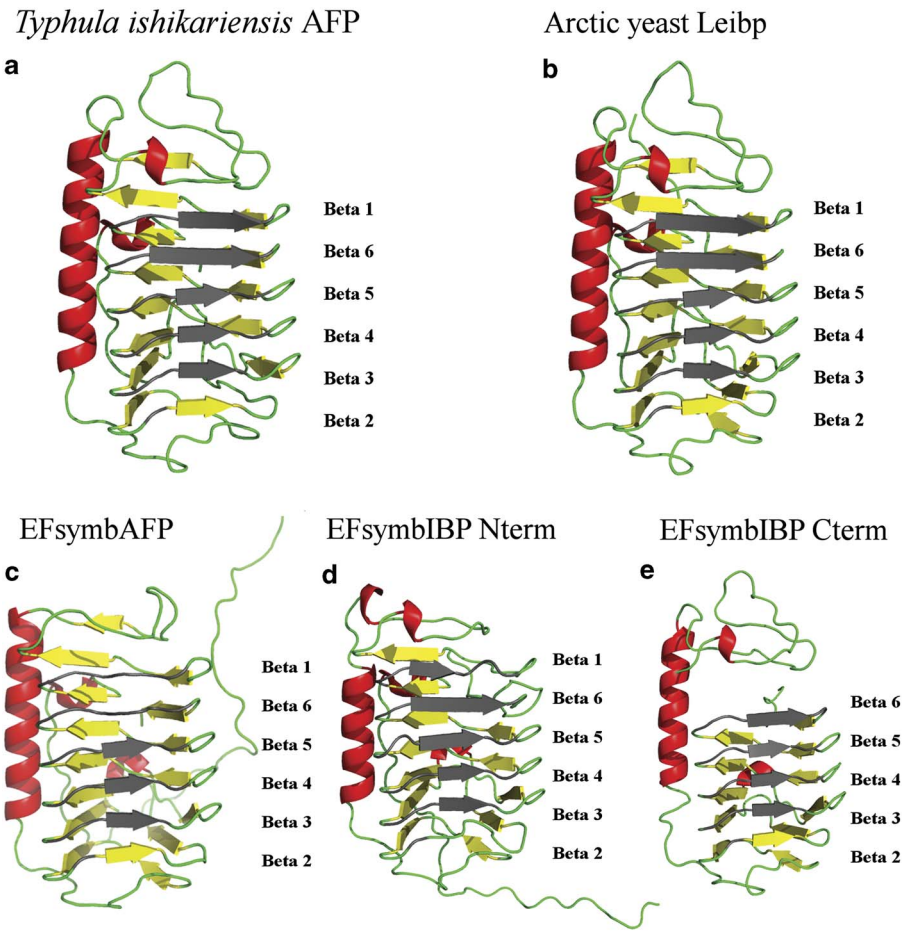

f

Beta 1	18	ASTGVS-TVPQ	27	Beta 4	173	VAGAVNIEVG	182
EFsymbAFP	62	AGSSVT-NTGA	71	EFsymbAFP	200	VSSSASFGTT	209
EFsymbIBP_N	38	STNGAVSNSGI	48	EFsymbIBP_N	172	VEGLVSMASG	181
EFsymbIBP_C		----------		EFsymbIBP_C	458	ITDEVMAIYP	469
Beta 6	209	AQTSVA-LQS	217	Beta 3	146	IDGTLDVATG	155
EFsymbAFP	236	ARNGAVTMEG	245	EFsymbAFP	173	IASTLTTTPG	182
EFsymbIBP_N	209	STAGAI-TVD	217	EFsymbIBP_N	145	IQGPLSTNAD	154
EFsymbIBP_C	494	AMGEQM-INT	502	EFsymbIBP_C	431	ALNTFSIDAI	440
Beta 5	192	KT-AVTFKTG	200	Beta 2	121	KWTSSVG-A-	128
EFsymbAFP	219	LE-SITFDTG	227	EFsymbAFP	145	KSTSSLA-VS	153
EFsymbIBP_N	191	NNAAIEMNTG	200	EFsymbIBP_N	118	SIPGASTLNL	127
EFsymbIBP_C	477	SI-HDASESN	185	EFsymbIBP_C	406	ICNNGAL-G-	413

Fig. 4. Molecular models of EFsymbAFP and EFsymbIBP. The 3D structures were modelled using the routine multiple template model from MODELLER 9v11 (Eswar *et al.* 2006). **a.** Antifreeze protein (AFP) structure from the snow mould fungus *T. ishikariensis* (PDB: 3VN3_A) (Kondo *et al.* 2012). **b.** AFP structure from the Arctic yeast *Leucosporidium* sp. AY30 (PDB: 3UYU_A) (Lee *et al.* 2012). **c.** EFsymbAFP. **d.** EFsymbIBP N-terminal domain. **e.** EFsymbIBP C-terminal domain. **f.** Sequence alignment of the ice-binding sites (IBSs) from the *T. ishikariensis* AFP, EFsymbAFP and EFsymbIBP (N- and C-terminal domains). Hydrophobic residues are in yellow and residues putatively involved in hydrogen bonds are in red. The residues shaded in grey represent the IBS of the *T. ishikariensis* AFP.

Modelling of tertiary structures and putative cleavage site prediction

The 3D structures of EFsymbAFP and EFsymbIBP were modelled, with a homology modelling method, on the structure of the snow mould fungus *T. ishikariensis* AFP (PDB: 3VN3_A) (Kondo *et al.* 2012) and the IBP from Arctic yeast *Leucosporidium* (sp. AY30, PDB: 3UYU_A; LeIBP) (Lee *et al.* 2012) using the routine multiple template model from MODELLER 9v11 (Eswar *et al.* 2006). EFsymbAFP displays a sequence identity of 35% and 34% compared to the fungal AFP and the Arctic yeast LeIBP, respectively; whereas EFsymbIBP displays 33% and 28% of sequence identity, respectively. Models were obtained from the alignment with Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo)

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using default parameters. The resulting models were visualized using the PyMol software. The putative cleavage site of the signal peptide was predicted using the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/).

Results

Identification and analysis of the ice-binding protein sequences

As a first approach to characterizing the IBPs in *E. focardii*, the entire macronuclear genome was explored using the AFP sequences from the diatom *Fragilariopsis cylindrus* as query sequences. A 3221 bp contig (KC691985) differed from the typical *Euplotes* nanochromosomes, i.e. it lacked the telomeric sequences at the ends of the nanochromosomes. Computer-assisted analysis revealed two open-reading frames (ORFs; shaded in grey in Fig. 1). The first ORF encodes a polypeptide of 251 amino acids, similar to the AFP from the DW4/3-1 strain of *S. aurantiaca* (NC_014623.1, max. identity: 54%, coverage: 91%, e-value: 1e-179), a bacterium isolated from the lower glacier of Victoria Valley. The second ORF encodes a polypeptide of 540 amino acids that is similar to the IBP from the 3519-10 strain of *F. bacterium* (EU694412, max. identity: 62%, coverage: 81%, e-value: 4e-75), a bacterium isolated from the glacial ice of Lake Vostok at a depth of more than 3000 m. To exclude the possibility that these sequences were derived from associated bacteria or endosymbionts of the algae in the culture medium, a PCR reaction using DNA purified from *E. focardii* or *Dunaliella* cells was performed. Only the sample containing *E. focardii* DNA gave the expected band of 750 bp (Supplemental Fig. S1 found at http://dx.doi.org/10.1017/S0954102014000017). Considering the possibility that these sequences were derived from a symbiotic bacterium of ciliate *E. focardii*, we named the two sequences EFsymbAFP and EFsymbIBP.

EFsymbAFP is 57.43% identical to the AFP from *S. aurantiaca*, and 39.44% identical to the AFP from an uncultured bacterium obtained from a metagenomic environmental sample situated at 1645 m above sea level (Rifle, CO, USA) (Table II). The low percentages are due to the different lengths of the two sequences (Fig. 2). All these proteins contain a predicted signal peptide (shaded in grey in Fig. 2). The N-terminal half of the *S. aurantiaca* and uncultured bacterium AFPs is completely absent in EFsymbAFP. However, EFsymbAFP overlaps fairly well with the N-terminus of a number of IBPs, such as that from *Acanthamoeba castellanii* strain Neff sh90004976 (XP_004344590).

EFsymbIBP is 53.38% identical to the IBP from *F. bacterium*, and 23.82% identical to that from *P. torquis* (YP_006867144) a psychrophilic species from Antarctic sea ice (Bowman *et al*. 1998) (Table II). The less conserved region is the predicted signal peptide (shaded in grey in Fig. 3).

Both EFsymbAFP and EFsymbIBP, as well as many other AFPs, possess the DUF3494 family domain of unknown function, which is duplicated in EFsymbIBP. This characteristic suggests that the DUF3494 family domain may be a fingerprint for IBPs. The multiple sequence alignments of EFsymbAFP and EFsymbIBP indicate that these sequences maintain similar physical-chemical properties with respect to those from other polar and non-polar organisms, although the amino acid composition is not identical (see Supplemental Figs S2 and S3 found at http://dx.doi.org/10.1017/S0954102014000017).

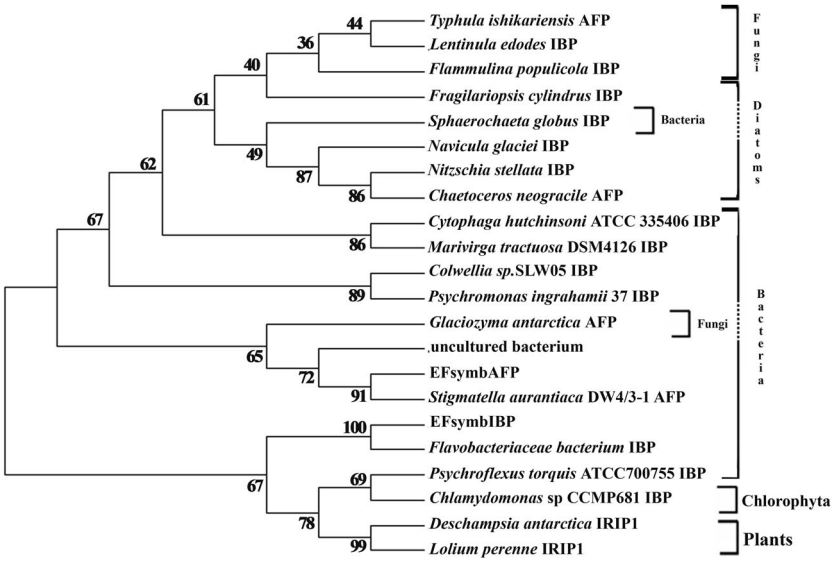

Fig. 5. Maximum parsimony phylogenetic tree based on the amino acid sequences of antifreeze (AFP) and ice-binding proteins (IBP) from bacteria, diatoms, fungi, plants and protists. The most parsimonious tree with length = 1016 is shown. For the parsimony-informative sites, the consistency index is 0.681000, the retention index is 0.495253 and the composite index is 0.337267 (the composite index for all sites is 0.339755). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches.

Further analysis of EFsymbIBP revealed that the N-terminal and C-terminal halves of the protein are nearly 42% similar, suggesting that the protein is the result of gene duplication. This hypothesis is supported by the model of the tertiary structure. Both the N- and C-terminal domains of EFsymbIBP displayed predicted 3D structures that were similar to the *T. ishikariensis* and *Leucosporidium* AFPs (Fig. 4). The main structural element is an irregular β-helix, composed of six, non-sequentially arranged loops that lie alongside an α-helix. The ice-binding site (IBS) lies on the flattest β-helical surface of the protein, defined as the b-face (indicated in grey in Fig. 4). The b-face of the Arctic and Antarctic yeast AFPs are composed of six β-sheets (Fig. 4), whereas those from the bacterial symbiont appear to be composed of four (EFsymbAFP) or five (EFsymbIBP, both N- and C-terminal domains) β-sheets (Fig. 4).

Through sequence and structural homology, we identified the IBSs in EFsymbAFP and EFsymbIBP. As reported for the homologues from bacteria, diatoms and fungi, the IBSs are poorly conserved at the sequence level (Fig. 4f). However, they maintain a high content of hydrophobic residues and repetitions of residues that may form hydrogen bonds, including serine, threonine and asparagine, suggesting a mechanism of ice crystal binding by the 'anchored clathrate' mode of action.

Genomic organization of the antifreeze proteins

To determine whether the AFP genes of the *S. aurantiaca* strain DW4/3-1 and the *F. bacterium* strain 3519-10 were organized in tandem in their respective chromosomes, as with those found in the *E. focardii* symbiont, the entire genome from both species was analysed (GenBank database: NC_014623 and NC_013062, respectively). Both bacterial chromosomes contained only one copy of the genes encoding the AFPs, suggesting that either a gene duplication or a horizontal gene transfer (HGT) event occurred in the *E. focardii* symbiont. If the former hypothesis were true, the two bacterial sequences must share an amino acid sequence identity higher than that shared with either the *S. aurantiaca* or *F. bacterium* AFPs. The two symbiont sequences are only 31.73% identical (Table II), a percentage that is lower than the shared sequence identities with either the *S. aurantiaca* or *F. bacterium* AFP. Therefore, the low identity of the two sequences excludes the gene duplication hypothesis. The construction of a phylogenetic tree (Fig. 5) revealed that the two sequences are more closely related to their orthologs from *S. aurantiaca* and *F. bacterium* than to each other. Therefore, the tandem organization of the two AFP gene sequences in the *E. focardii* symbiont was most probably derived from an HGT event between bacterial strains.

To exclude the possibility that the tandem organization of the two genes was the consequence of a misassembly of

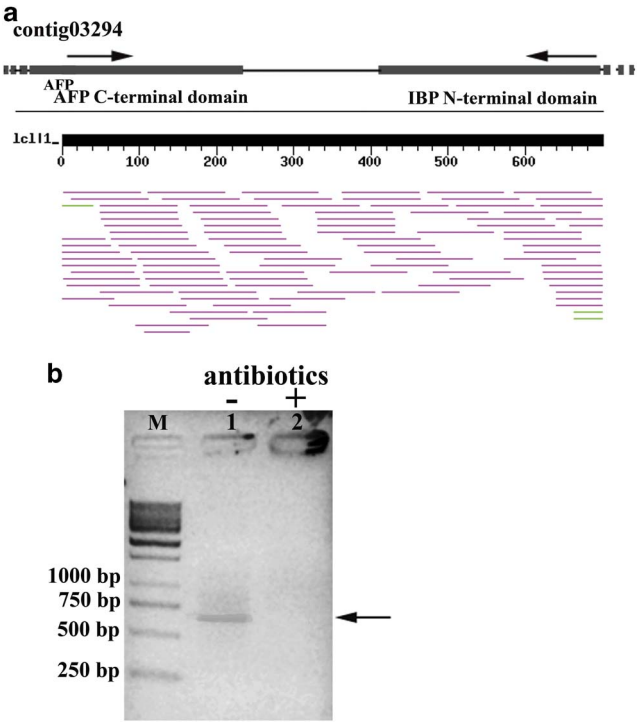

Fig. 6. Tandem organization of the two ice-binding protein gene sequences. **a.** Schematic representation of the 700 bp fragment of the *E. focardii* genomic contig (contig 03294) containing the C-terminal domain of EFsymbAFP and the N-terminal domain of EFsymbIBP; this contig was used to search for the corresponding genomic reads (indicated in pink). **b.** Agarose gel of the PCR reactions performed using DNA extracted from untreated cells (lane 2) or cells treated with antibiotics (lane 3). The arrow indicates the PCR product obtained in the reaction from the untreated DNA sample.

the genomic reads, the assembly of the reads that overlap on the two genes were checked manually. By BLASTing a fragment of 700 bp from the *E. focardii* genomic contigs, which included the C-terminal domain of the AFP and the N-terminal domain of the IBP, nearly 100 reads that covered the entire fragment of 700 bp were found (Fig. 6a). A PCR reaction using DNA extracted from untreated cells or from cells treated with antibiotics, only produced a reaction with the untreated cells (Fig. 6b), confirming that the two IBP sequences are of bacterial origin and are organized in tandem on the bacterial chromosome.

The HGT may have occurred between the bacteria and *E. focardii*. If this had occurred, the sequences would be found in the *E. focardii* genome within the typical nanochromosome organization, i.e. short molecules containing a single ORF protected at the extremities by telomeres composed of the nucleotide repetition C_4A_4. Therefore, a RATE-PCR was performed using degenerated primers (according to the codon usage of

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E. focardii) that corresponded to conserved motives of the coding region of the AFPs and primers that bind the telomeric sequences. The PCR products were analysed on an agarose gel (data not shown). In the reaction performed with forward primers (FW and C_4A_4), no PCR products were detected. However, in the reaction performed with reverse primers (RV and C_4A_4), a smear was obtained which was subsequently subcloned. The resulting colonies were screened using a probe that corresponded to the AFP coding region. However, this screening method did not identify any positive clones. Sequencing randomly chosen clones revealed that the PCR products were derived from non-specific binding of the primers. These results confirm that these bacterial sequences are not represented in the *E. focardii* genome, unless they are contained in exceptionally long nanochromosomes. This finding, together with the inability to amplify the genes following antibiotic treatment, corroborates the hypothesis that the AFP and IBP genes are of bacterial origin and excludes the possibility of HGT moving into the *E. focardii* genome.

Phylogenetic analysis

To assess the phylogenetic relationships between the IBPs encoded by the putative *E. focardii* symbiont and similar proteins from other organisms, a phylogenetic tree was constructed using the coding sequences of both EFsymbAFP and EFsymbIBP (Fig. 5), as well as the AFPs/IBPs from different species of bacteria, diatoms, fungi and plants.

EFsymbAFP, the AFPs from *S. aurantiaca* and an uncultured bacterium clustered with the AFP from the Antarctic yeast *G. antarctica* (Fig. 5). This cluster appears to be the sister group of the clade containing the AFPs/IBPs from all other bacteria, including the Arctic *Psychromonas* and the Antarctic *Colwella*, and the clade that includes other Basidiomycota species and diatoms. In contrast, EFsymbIBP and the IBP from the Antarctic bacteria *F. bacterium* and *P. torquis* clustered with those from *Chlamydomonas* and plants. These latter sequences appear to derive from the *P. torquis* IBP, most probably as a consequence of HGT.

The lineage of AFPs/IBPs forms two separate clades, those from bacteria, fungi and diatoms, and those from *Chlamydomonas* and plants, suggesting that these proteins followed different evolutionary histories.

Discussion

We report the identification and sequence analysis of two bacterial IBPs, EFsymbAFP and EFsymbIBP. The corresponding bacterial contig was found among the genomic sequences of the Antarctic ciliate *E. focardii*, suggesting that these sequences belong to an unidentified bacterial symbiont of the ciliate. We excluded the possibility that these sequences are derived from associated bacteria or endosymbionts of the algae in the culture medium (Supplemental Fig. S1 http://dx.doi.org/10.1017/S0954102014000017). The two genes are 31.73% identical at the amino acid level and are organized in tandem on the chromosome. Tandem organization of genes encoding AFPs has been reported in cold-adapted fishes (Scott *et al.* 1988, Wang *et al.* 1995), but amongst bacteria it appears to be unique to this symbiont.

The relatively low sequence identity and the tandem organization suggest the occurrence of HGT between two bacterial species. The phenomenon of HGT between bacteria, and from bacteria to eukaryotes, is well documented; HGT frequently occurs in cases of selective pressure from harsh environmental conditions and is favoured by endosymbiosis (Sorhannus 2011). Sorhannus (2011) reported four examples of HGT of AFP genes: one from *Polaribacter irgensii* to a group of four proteobacterium species, one from the diatom *Chaetocerus neogracile* to the copepod *Stephos Longipes*, one from a basidiomycete to the diatoms *Fragilariopsis curta* and *Fragilariopsis cylindrus*, and one from an ascomycete lineage to the Antarctic bacterium *S. aurantiaca*. The latter constitutes a rare example of HGT from eukaryotes to prokaryotes (Keeling & Palmer 2008); however, this gene transfer event is disputed because neither of the ascomycete species is known to occur in polar regions (Sorhannus 2011). In our phylogenetic tree, the *S. aurantiaca* AFP appears to branch from the Antarctic basydiomycete *G. antarctica*, suggesting that *S. aurantiaca* acquired the AFP gene from basydiomycetes. Furthermore, our phylogenetic analysis showed that both EFsymbAFP and EFsymbIBP cluster principally with homologues from other Antarctic bacteria. The high bootstrap values strongly suggest that the EFsymbAFP and EFsymbIBP genes came from *S. aurantiaca* and *F. bacterium* lineages. However, it is possible that these sequences derived from close relatives of *S. aurantiaca* and *F. bacterium* that were not included in the current sample. Thus, the presence of the two genes in the *E. focardii* genome can be explained by two separate symbiotic events, after which the two genes were retained by *E. focardii* due to their high adaptive significance in cold environments.

From the modelling analysis, EFsymbAFP and EFsymbIBP appear similar to the AFPs from the fungus *T. ishikariensis* and from the Arctic yeast *Leucosporidium* sp. AY30. Both N- and C-terminal domains of EFsymbIBP are similar to the structure of the AFP from the fungi (Fig. 4); this supports the hypothesis that EFsymbIBP resulted from a gene duplication event. This gene organization is also present in *F. bacterium* (Raymond *et al.* 2008), therefore the gene duplication

most probably happened prior to the symbiotic relationship between the bacterium and *E. focardii*.

The EFsymbAFP and EFsymbIBP IBSs appear to maintain a relatively high content of hydrophobic and hydrogen-bond forming residues; these features are shared with other AFPs and help bind ice crystals through the 'anchored clathrate' mode of action, i.e. through the contributions of both hydrophobic effects and hydrogen bonds (Garnham *et al.* 2011).

Genes coding for proteins with antifreeze properties probably evolved independently in a number of lineages, while other organisms acquired the genes through HGT (Kelley *et al.* 2010). The evolution of the prokaryotic genome is thought to have been profoundly influenced by HGT (Keeling & Palmer 2008). Most of the AFPs are derived from gene duplication events and from diversification of pre-existing genes that perform other functions. For example, the type II AFPs come from the duplication and diversification of the C-type lectin genes, and the Antarctic Notothenoid AFP is derived from a trypsinogen-like serine protease. This raises an interesting question: where did the bacterial AFP come from? Most of these bacterial sequences share a conserved domain (DUF3494), the function of which is unknown, but that may be considered as a fingerprint for IBPs. However, EFsymbAFP, EFsymbIBP and their closest homologues share a sequence similarity that ranges from 42% to 52% with the alpha 5 type IV collagen isoform 3 from *Rhodococcus* sp. JVH1. It has been reported that collagen peptides derived from the alcalase hydrolysis of bovine gelatine are able to inhibit the re-crystallization of ice in ice cream (Wang & Damodaran 2009), suggesting that a bacterial collagen-like protein may represent the ancestor of the bacterial AFPs.

To conclude, our results confirm that IBPs and AFPs have complex evolutionary histories, which includes gene/domain duplication and HGT events, most probably due to the demands of the environment and the need for rapid adaptation.

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Supplemental material

Three supplemental figures will be found at http://dx.doi.org/10.1017/S0954102014000017.

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