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The water-born protein pheromones of the polar protozoan ciliate, *Euplotes nobilii*: Coding genes and molecular structures

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Abstract

The protozoan ciliate *Euplotes nobilii* found in Antarctic and Arctic coastal waters relies on secretion of water-soluble cell type-specific signal proteins (pheromones) to regulate its vegetative growth and sexual mating. For three of these psychrophilic pheromones we previously determined the three-dimensional structures by nuclear magnetic resonance (NMR) spectroscopy with protein solutions purified from the natural sources, which led to evidence that their adaptation to cold is primarily achieved by increased flexibility through an extension of regions free of regular secondary structures, and by increased exposure of negative charges on the protein surface. Then we cloned the coding genes of these *E. nobilii* pheromones from the transcriptionally active cell somatic nucleus (macronucleus) and characterized the full-length sequences. These sequences all contain an open reading frame of 252–285 nucleotides, which is specific for a cytoplasmic pheromone precursor that requires two proteolytic cleavages to remove a signal peptide and a pro segment before release of the mature protein into the extracellular environment. The 5′ and 3′ non-coding regions are two- to three-fold longer than the coding region and appear to be tightly conserved, probably in relation to the inclusion of intron sequences destined to be alternatively removed to play key regulatory roles in the mechanism of the pheromone gene expression.
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1. Introduction

Among the microorganisms that populate the polar ecosystems, the protozoan ciliates are a major eukaryotic component. Numerous species can be sampled with relative facility from practically everywhere there is availability of liquid water (Petz, 2005; Petz et al., 2007), and used to generate laboratory cultures that provide unique opportunities to study basic aspects of evolution to cold-adaptation in individual eukaryotic cells directly exposed to natural selection.

The ciliate of our research interest is the marine species *Euplotes nobilii*, of which our laboratories host a collection of Antarctic and Arctic wild-type strains

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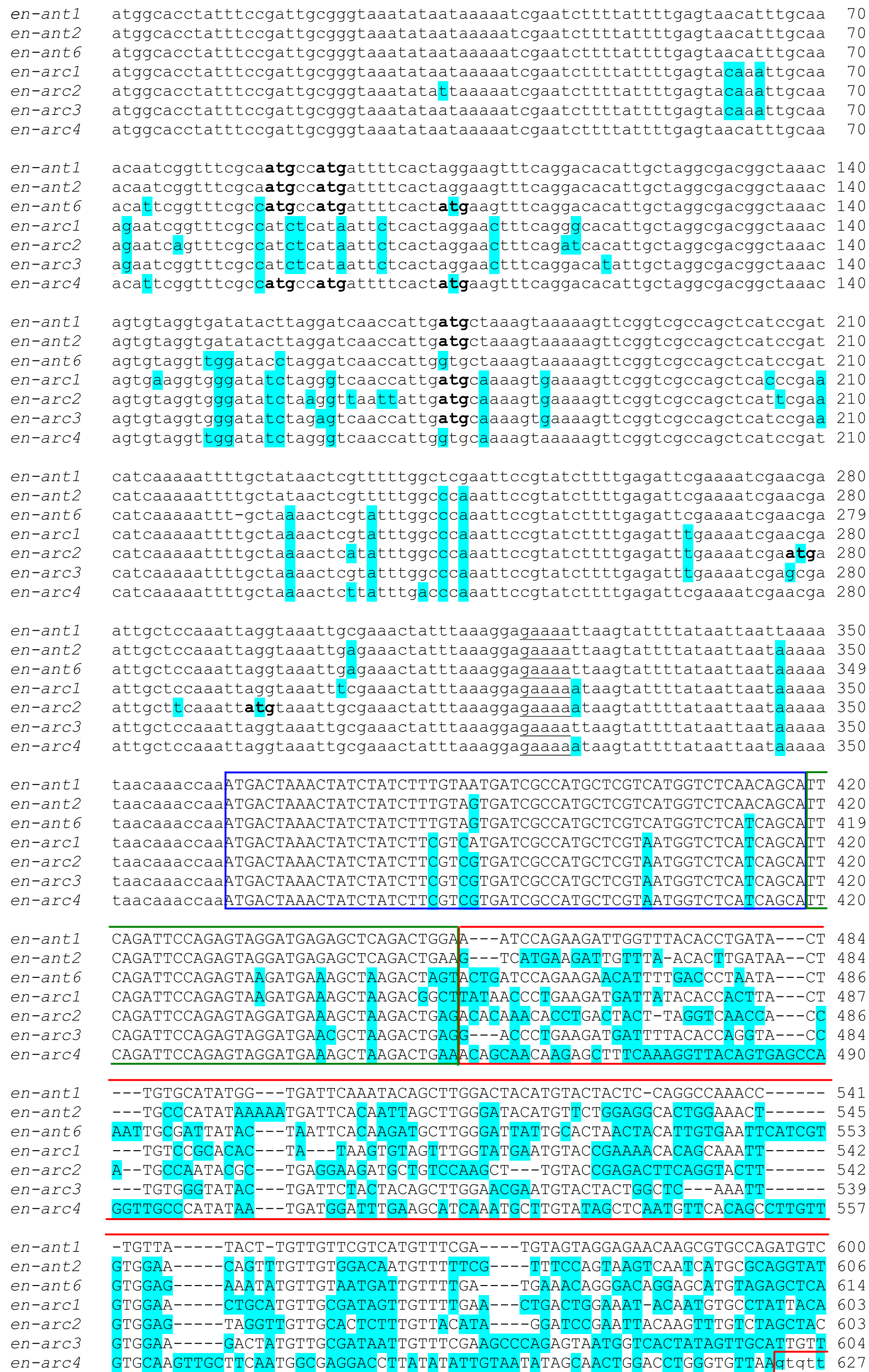

Fig. 1. Multiple nucleotide sequence alignment of the *E. nobilii* pheromone genes generated by Clustal W (Thompson et al., 1994), based on data from La Terza et al. (2009) and Vallesi et al. (2009). The 5′-$(C_4A_4)_3$CCCC and the 3′-$(G_4T_4)_3$GGGG telomeric inverted repeats common to all sequences are not reported. Gaps were inserted to maximize sequence identities. Nucleotide variations with respect to the first gene sequence (*en-ant1*) are highlighted in cyan. Lowercase and capital letters are used for non-coding and coding regions, respectively. In the 5′ non-coding region, supernumerary start codons are evidenced in bold. Blue, green and red boxes contain the regions of the open reading frame that encode the signal peptide, the

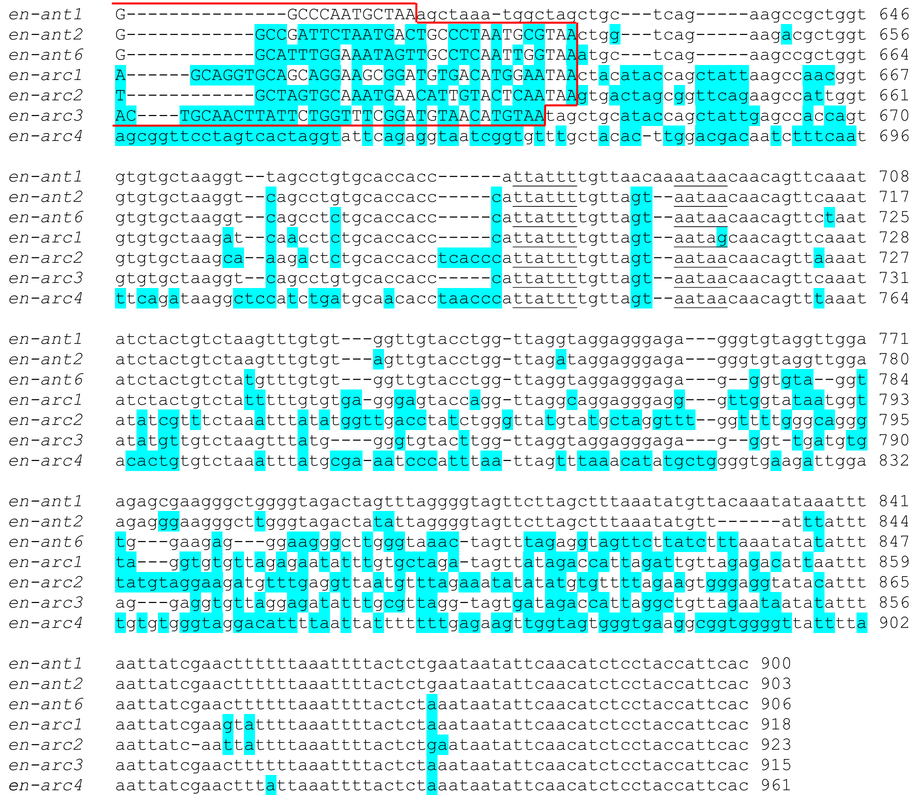

Fig. 1. (*continued*).

that have been isolated from coastal sites of Terra Nova Bay in the Ross Sea, Spitzbergen Island in the Svalbard Archipelago, and Baffin Bay in Western Greenland (Valbonesi and Luporini, 1990; Vallesi et al., 2009). As it occurs in other *Euplotes* species living in temperate waters, such as *Euplotes patella, Euplotes raikovi* and *Euplotes octocarinatus* (reviewed in Dini and Nyberg, 1993; Miyake, 1996; Luporini et al., 2005), *E. nobilii* constitutively secretes a family of structurally homologous cell type-specific signal proteins into the extracellular environment in relation to the mechanisms of self/non-self recognition represented by an apparently "open" system of multiple mating-types (Felici et al., 1999). In each one of these *Euplotes* species, these cell signal proteins (now usually designated as "pheromones", and earlier as "mating-type factors") appear to be specified by series of co-dominant, or serially dominant multiple alleles that segregate in Mendelian fashion at the so-called *mat* (mating-type) locus of the chromosomic genome of the cell transcriptionally silent germinal nucleus (micronucleus) (Dini and Nyberg, 1993). They are then expressed as "gene-sized" DNA molecules in the sub-chromosomic genome of the cell transcriptionally active somatic nucleus (macronucleus) (Jahn and Klobutcher, 2002). The pheromone biological activity varies in a context-dependent manner (Vallesi et al., 1995; Luporini et al., 2005). Cells progress through their vegetative (mitotic) growth in response to the autocrine (autologous) binding of their secreted (self) pheromones, and are induced to shift temporarily from the growth stage of their life cycle to the sexual stage (manifested by mating pair formation) in response to the paracrine (heterologous) binding of a foreign (non-self) pheromone.

Of the *E. nobilii* pheromone family we first isolated some members from culture filtrates of wild-type

pro segment and the secreted (mature) protein, respectively, of the cytoplasmic (immature) pheromone precursor. In the 3′ non-coding region, putative sites for the transcription regulation and polyadenilation are underlined. The sequence positions of the right-most nucleotide in each row are reported on the right. The sequences deposited at the National Centre for Biotechnology Information have the following accession numbers: *en-ant1*, FJ645718; *en-ant2*, FJ645719; *en-ant6*, EF030059; *en-arc1*, FJ645720; *en-arc2*, FJ645721; *en-arc3*, FJ645722; *en-arc4*, FJ645723. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

strains that appeared capable to grow into massive cultures, and analyzed these protein preparations by nuclear magnetic resonance (NMR) spectroscopy to determine the three-dimensional molecular structures in solution (Pedrini et al., 2007; Placzek et al., 2007; Alimenti et al., 2009). More recently, the attention has been directed also to identify and characterize the genes that control the synthesis of these pheromones in the cell macronucleus. Complete nucleotide sequences have been determined for seven allelic pheromone genes (Vallesi et al., 2009). Three sequences are those which encode the pheromones E*n*-1, E*n*-2, and E*n*-6, of which we have already determined the molecular structures (Pedrini et al., 2007; Placzek et al., 2007; Alimenti et al., 2009), and four sequences are specific for pheromones that have not yet been purified and analyzed chemically.

In this study, we concisely review the major structural traits of this array of allelic *E. nobilii* pheromone genes and of the pheromones that they specify.

2. Pheromone gene structures

All seven pheromones genes were amplified by Polymerase Chain Reaction (PCR) of DNA prepared from the transcriptionally active cell somatic macronucleus (La Terza et al., 2009; Vallesi et al., 2009), exclusively represented by sub-chromosomic genes amplified in thousands of copies, each one ending with telomeric inverted C_4A_4 repeats and ranging in size approximately from 500 to 20,000 bp (Jahn and Klobutcher, 2002). Two distinct pheromone-gene sequences were obtained from the Antarctic strain AC-1 (i. e., the *en-ant1* and *en-ant2* sequences) and the Arctic strain 5QAA15 (i. e., the *en-arc2* and *en-arc3* sequences), thus implying that these strains carried heterozygous pheromone-gene combinations in their micronucleus and that their two pheromone genes were represented by similar numbers of copies in their macronucleus. In contrast, single pheromone-gene sequences were obtained from the Arctic strains 4Pyrm4 and 2QAN1 (i. e., the *en-arc1* and *en-arc4* sequences, respectively) and from the Antarctic strain AC-4 (i. e., the *en-ant6* sequence). Thus, these strains either carried their pheromone genes in homozygous combinations or, in case they carried heterozygous combinations, the two pheromone genes were represented in very uneven ratios of macronuclear copies.

The full-length nucleotide sequences of these pheromone genes extend from 900 (*en-ant1*) to 961 bp (*en-arc4*), telomeres excluded (Fig. 1). They contain multiple ATG codons for the initiation of translation, as well as multiple TGA, TAG and TAA codons which are potential stop signals of translation. In *Euplotes*, in fact, TGA is a common codon for cysteine (Meyer et al., 1991; Turanov et al., 2009), and in other ciliates TAA and TAG have been found to specify glutamine, or glutamic acid (Tourancheau et al., 1995; Sánchez-Silva et al., 2003). In any case, of the multiple "open reading frames" (ORFs) that can be identified in each pheromone-gene sequence, the best candidate to be translated is the ORF spanning from the ATG codon occupying the conserved position 362 (361 in *en-ant6*) to a TAA codon at the positions 613 for *en-ant1*, 645 for *en-ant6*, and in-between these positions for the other sequences. This ORF appears to be specific for proteins that vary in length from 83 amino acid residues (in the case of the *en-ant1* sequence) to 94 (in the case of the *en-ant6* sequence), and correspond to the pheromone cytoplasmic precursors in which the three canonical functional domains, i.e., the signal peptide, the pro segment, and the mature protein, are clearly recognized.

When compared to the more canonical organization of the macronuclear genes of the hypotrichous ciliates such as *Euplotes*, in which the coding regions are much longer than their flanking non-coding regions (usually less than 200 bp) (McEwan et al., 2000; Cavalcanti et al., 2004a,b), the *E. nobilii* pheromone genes show an odd organization. Their 5′ and 3′ non-coding regions are nearly three-fold longer than those of the respective coding regions. These regions, which apparently replace the more conventional TATA or CAAT boxes for the regulation of transcription with the motif GAAAA, and the AATAAA polyadenylation signal with the motifs TTATTT or AATAA/G (Ghosh et al., 1994), also show high sequence conservation throughout the entire pheromone-gene family (88–97% identity for the 5′ region, and 43–92% identity for the 3′ region).

The high conservation of the non-coding regions implies that they are responsible for some regulatory activity of the pheromone-gene expression, probably in correlation with the inclusion of intron sequences destined to be alternatively removed to generate other species of mRNAs, in addition to the mRNA specific for the secreted pheromone, as reported in *E. raikovi* (Miceli et al., 1992; Di Giuseppe et al., 2002). This hypothesis is strongly supported by the fact that the 5′ region of all the seven pheromone genes includes multiple ATG start codons in conserved positions, as well as multiple potential consensus splice-site junctions represented by the canonical 5′-GT/AG-3′ intron boundary (Cavalcanti et al., 2004b; Chang et al., 2007; Ricard et al., 2008).

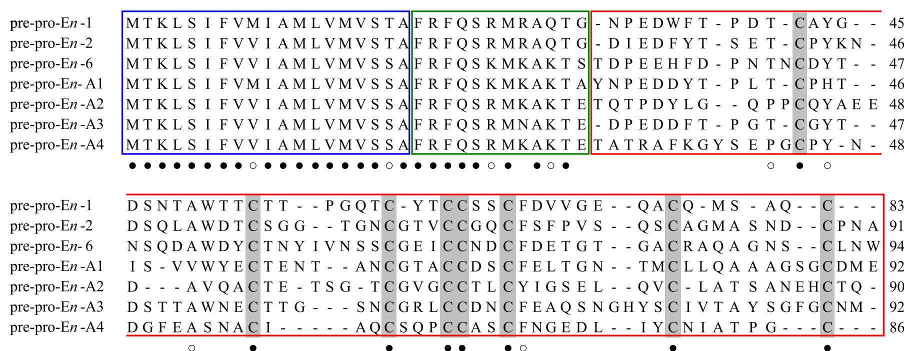

Fig. 2. Multiple amino acid sequence alignment of *E. nobilii* pheromone precursors generated by Clustal W and optimized by gap insertions. Blue, green and red boxes delimit the signal peptide, the pro segment and the secreted pheromone, respectively. Filled and open circles mark positions occupied by one amino acid type, or by two amino acid types, respectively. Shading highlights the eight conserved cysteine residues in the secreted region. The sequence positions of the right-most residue in each row are reported on the right. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Highly conserved intron sequences have also been detected in the pheromone gene structure of another *Euplotes* species, *E. octocarinatus* (Brünen-Nieveler et al., 1998; Möllenbeck and Heckmann, 1999).

3. Cytoplasmic pheromone precursors

As it typically occurs in the secretion of protein hormones, the *E. nobilii* pheromones appear to be synthesized as cytoplasmic, immature precursors (pre-pro-pheromones) requiring two proteolytic cleavages to release the mature and active pheromone forms into the extracellular environment (Fig. 2). The first cleavage is destined to remove the signal peptide of 19 amino acids, which appears to be highly conserved in all the pheromone precursors. The presumed cleavage site Ala-Phe preceded by the combinations Ser-Ser or Ser-Thr would agree well with consensus sequences of known signal peptidases (von Heijne, 1987; Nielsen et al., 1997). The second cleavage would remove the pro segment, which is in all species formed by a highly conserved dodecapeptide segment containing three or four basic residues. The putative site of this cleavage appears to be unconventional for the enzymatic activities of known endopeptidases, with the combinations Gly-Asn/Asp, Ala-Tyr, Ser-Thr and Glu-Thr/Asp that show low conservation among the different species. Thus, while the signal peptide for each of the pheromone precursors could be processed by a single endopeptidase, the removal of the pro segment would require either multiple endopeptidases, or enzymes with multiple specificities.

4. Pheromone amino acid sequences and three-dimensional structures

In contrast to the high conservation of the pre and pro segments of the pheromone precursors, the secreted regions show only 22–52% identity of the amino acid sequences, with full positional conservation limited to a common set of eight Cys residues. However, in spite of this variability in amino acid sequence, the *E. nobilii* pheromones show an overall similarity in their amino acid compositions regardless of whether they are specific of the Antarctic or the Arctic strains (Table 1). In both cases, their compositions appear to be characterized by similar contents in polar amino acids (44.6% on average in the Antarctic

Table 1
Comparison in amino acid composition between Antarctic (E*n*-1, E*n*-2 and E*n*-6) and Arctic (E*n*-A1, E*n*-A2, E*n*-A3 and E*n*-A4) *E. nobilii* pheromones.

Residues (%)	E*n*-1	E*n*-2	E*n*-6	Mean	E*n*-A1	E*n*-A2	E*n*-A3	E*n*-A4	Mean
Polar[a]	46.1	45.0	42.8	44.6	39.3	45.9	49.2	40.1	43.6
Hydrophobic[b]	46.0	43.3	43.0	44.1	45.8	49.3	40.7	52.8	47.1
Charged[c]	11.5	15.0	20.6	15.7	16.4	13.6	16.3	12.7	14.7
Aromatic[d]	11.4	10.0	14.4	11.9	9.7	6.8	13.0	11.0	10.1

[a] Asn, Gly, Gln, Ser, Thr, Tyr.
[b] Ala, Cys, His, Ile, Leu, Met, Phe, Pro, Tyr, Trp, Val.
[c] Arg, Asp, Glu, His, Lys.
[d] His, Phe, Tyr, Trp.

strains *vs.* 43.6% in the Arctic ones), and in the hydrophobic (44.1% *vs.* 47.1%), charged (15.7% *vs.* 14.7%) and aromatic ones (11.9% *vs.* 10.1%).

The evidence that the amino acid composition of the *E. nobilii* pheromones reflects a strong adaptation of these molecules to cold is strongly supported by a comparison with the family of mesophilic pheromones that have been structurally characterized in *E. raikovi* (Luginbühl et al., 1994, 1996; Liu et al., 2001; Zahn et al., 2001), a species of temperate sea waters that is phylogenetically closely related to *E. nobilii* (Vallesi et al., 2008). As reported in other comparisons between psychrophilic, mesophilic and thermophilic proteins (Aahandideh et al., 2007; Adekoya et al., 2006; Bae and Phillips, 2004), the *E. nobilii* pheromones show to be much richer (44% *vs.* 30%) in polar amino acids and poorer (46% *vs.* 57%) in hydrophobic ones. Marked differences exist in particular in the Thr, Asn and Ser contents (11.7% *vs.* 5.7%, 6.8% *vs.* 4.2%, and 7.5% *vs.* 5.7%, respectively) and in the Leu, Pro and Ile contents (2.6% *vs.* 7.3%, 4.9% *vs.* 8.9%, and 2.1% *vs.* 5.7%, respectively). Additional divergences of the *E. nobilii* pheromones from the *E. raikovi* pheromones include the global hydrophilicity and hydrophobicity of these molecules, as revealed by measuring the indexes "GRAVY" (Schiffer and Dötsch, 1996) and "Aliphatic" (Arnórsdóttir et al., 2002). The GRAVY index (indicative of improved interactions with the solvent) showed an average value of −0.35 in the *E. nobilii* pheromones as compared to a value 0.01 in the *E. raikovi* pheromones, while the Aliphatic index (indicative of lower stabilizing hydrophobic forces in the protein core) showed average values of 34.3 for the former and 65.5 for the latter ones.

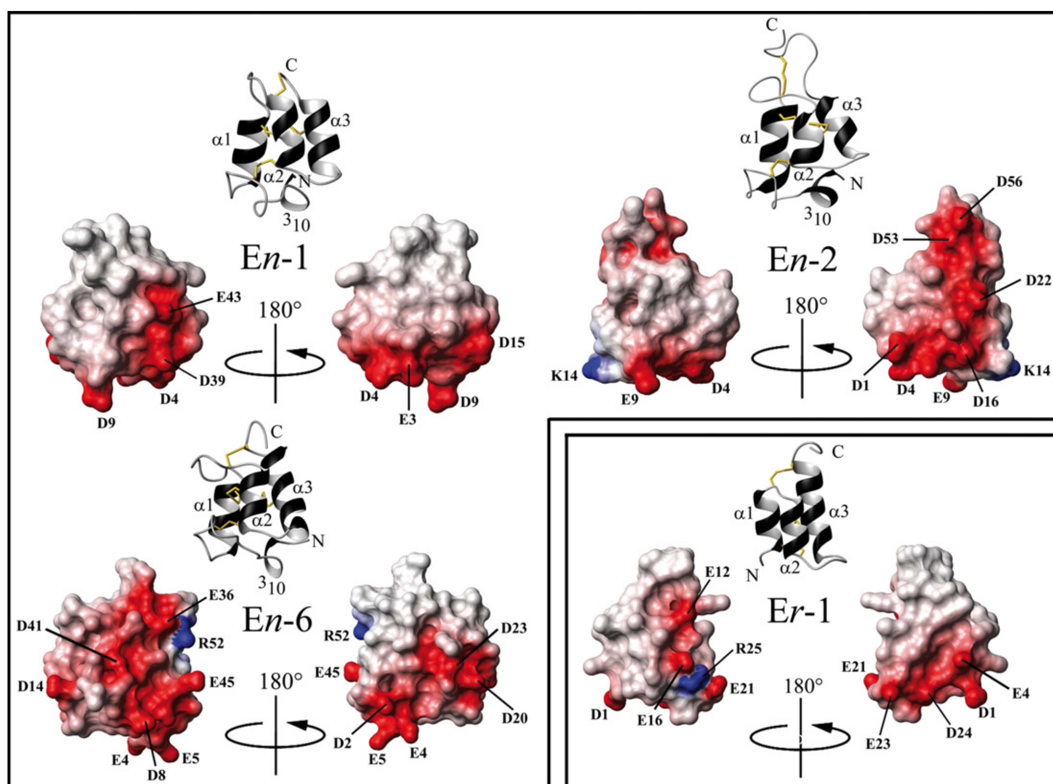

Fig. 3. Ribbon presentations and space-filling models showing the surface electrostatic potential distribution of the psychrophilic *E. nobilii* pheromones E*n*-1, E*n*-2 and E*n*-6, and E*r*-1 as representative of the mesophilic *E. raikovi* pheromone family. The three helices common to all four molecules are indicated as α1, α2 and α3. "3_{10}" identifies a 3_{10}-helical turn that is part of a long segment with otherwise non-regular secondary structure in the *E. nobilii* pheromones, which is not present in the mesophilic *E. raikovi* pheromones. N and C identify the molecule amino and carboxyl termini, respectively, and the disulfide bonds (four in the *E. nobilii* pheromones and three in the *E. raikovi* pheromones) are depicted as yellow stick diagrams. The surface view on the left has the same orientation as the ribbon figure, while in the view on the right the molecule has been rotated by 180° about a vertical axis. Red and blue colours indicate negatively and positively charged surface areas, respectively. The amino acid residues contributing to the electrostatic surface charges are indicated. The *E. nobilii* pheromone structures are from Pedrini et al. (2007) and Placzek et al. (2007), while the *E. raikovi* pheromone structure is from Mronga et al. (1994). The accession numbers of the E*n*-1, E*n*-2, E*n*-6 and E*r*-1 NMR structures in the protein databank (PDB) are 2nsv, 2nsw, 2jms and 1erc, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The peculiarities in the amino acid compositions of the *E. nobilii* pheromones generate unique structural traits in the architecture of these molecules, that can be visually appreciated in the E*n*-1, E*n*-2 and E*n*-6 pheromones of Antarctic origin, for which three-dimensional structures were determined by homonuclear ^{1}H NMR analysis of purified protein preparations in solution (Pedrini et al., 2007; Placzek et al., 2007) (Fig. 3). Two major cold-adaptive strategies appear to have evolved in these *E. nobilii* pheromones. The protein core is substantially in common with the mesophilic *E. raikovi* pheromones and contains an anti-parallel bundle of three α-helices fastened together by three disulfide bonds. One cold-adaptation strategy is represented by insertion of regions devoid of regular secondary structures, leading to enhanced overall backbone flexibility. This involves in particular an N-terminal segment of 15–17 residues, of which only four form an element of regular secondary structure, i. e., a 3_{10}-helix turn, and by a loop of 5–10 residues that connects helices α1 and α2 (Fig. 3). The second strategy is to increase surface exposure of negatively charged side chains that favours the *E. nobilii* pheromone solubility in the thermodynamically adverse conditions of the polar waters.

5. Conclusions

Microorganisms thrive in marine polar habitats, which implies that they were highly successful in adapting their molecular mechanisms to face the drastically reduced metabolic rates imposed by the low thermal energy and high viscosity of their extracellular environment. Proteins hold a central role in these mechanisms; being the most versatile macromolecules in every living system, they serve basic functions in practically all biological processes. Research on biophysical peculiarities and principles associated with cold-adaptation of proteins has so far been primarily based on psychrophilic enzymes extracted from cultivable bacteria and archea, and regarded to be of keen interest also for their powerful biotechnological potential. Further insights into protein strategies of adaptation to cold may be derived from extending this research to other microbial organisms of eukaryotic origin, focusing on non-catalytic and structural proteins. In this respect, the *E. nobilii* protein pheromone system appears to be rather promising. The relative facility by which these small globular proteins can be purified in abundance from the supernatant of stable laboratory cultures has previously enabled us to determine unique features of their cold-adapted molecular structures (Alimenti et al., 2009). The identification and cloning of their coding genes (Vallesi et al., 2009) can now pave the way to further direct experimental analysis of the functional significance of these structural properties.

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References

Aahandideh, S., Abdolmaleki, P., Jahandideh, M., Asadabadi, E.B., 2007. Sequence and structural parameters enhancing adaptation of protein to low temperatures. J. Theor. Biol. 246, 159–166.

Adekoya, O.A., Helland, R., Willassen, N.P., Sylte, I., 2006. Comparative sequence and structure analysis reveal features of cold adaptation of an enzyme in the thermolysin family. Proteins 62, 435–449.

Alimenti, C., Vallesi, A., Pedrini, B., Wüthrich, K., Luporini, P., 2009. Molecular cold-adaptation: comparative analysis of two homologous families of psychrophilic and mesophilic signal proteins of the protozoan ciliate, *Euplotes*. IUBMB Life 61, 838–845.

Arnórsdóttir, J., Smaradóttir, R.B., Magnússon, O., Thorbjarnardóttir, S., Eggertsson, G., Kristjánsson, M., 2002. Characterization of a cloned subtilisin-like serine proteinase from a psychrotrophic *Vibrio* species. Eur. J. Biochem. 269, 5536–5546.

Bae, E., Phillips, G.N., 2004. Structures and analysis of highly homologous psychrophilic, mesophilic, and thermophilic adenylate kinases. J. Biol. Chem. 279, 28202–28208.

Brünen-Nieveler, C., Weiligmann, J.C., Hansen, B., Kuhlmann, H.W., Möllenbeck, M., Heckmann, K., 1998. The pheromones and pheromone genes of new stock of *E. octocarinatus* species complex. Eur. J. Protistol. 34, 124–132.

Cavalcanti, A.R.O., Dunn, D.M., Weiss, R., Herrick, G., Landweber, L.F., Doak, T.G., 2004a. Sequence features of *Oxytricha trifallax* (class Spirotrichea) macronuclear telomeric and subtelomeric sequences. Protist 155, 311–322.

Cavalcanti, A.R.O., Stover, N.A., Orecchia, L., Doak, T.G., Landweber, L.F., 2004b. Coding properties of *Oxytricha trifallax* (*Sterkiella histriomuscorum*) macronuclear chromosomes: analysis of a pilot genome project. Chromosoma 113, 69–76.

Chang, W.J., Addis, V.M., Li, A.J., Axelsson, E., Ardell, D.H., Landweber, L.F., 2007. Intron evolution and information processing in the DNA polymerase α gene in spirotrichous ciliates: a hypothesis for interconversion between DNA and RNA deletion. Biol. Direct 2, 6. doi:10.1186/1745-6150-2-6.

Di Giuseppe, G., Miceli, C., Zahn, R., Damberger, F., Wüthrich, K., Luporini, P., 2002. A structurally deviant member of the *Euplotes raikovi* pheromone family: E *r*-23. J. Eukaryot. Microbiol. 49, 86–92.

Dini, F., Nyberg, D., 1993. Sex in ciliates. In: Jones, J.G. (Ed.), Advances in Microbial Ecology, 13. Plenum press, New York, pp. 85–153.

Felici, A., Alimenti, C., Ortenzi, C., Luporini, P., 1999. Purification and initial characterization of two pheromones from the marine Antarctic ciliate, *Euplotes nobilii*. Ital. J. Zool. 66, 355–360.

Ghosh, S., Jarakzewsky, J.W., Klobutcher, L.A., Jahn, C.L., 1994. Characterization of transcription initiation, translation initiation, and poly(A) addition sites in the gene-sized macronuclear DNA molecules of *Euplotes*. Nucleic Acids Res. 22, 214–221.

Jahn, C.L., Klobutcher, L.A., 2002. Genome remodelling in ciliated protozoa. Ann. Rev. Microbiol. 56, 489–520.

La Terza, A., Dobri, N., Alimenti, C., Vallesi, A., Luporini, P., 2009. The water-borne protein signals (pheromones) of the Antarctic ciliated protozoan, *Euplotes nobilii*: structure of the gene coding for the E *n*-6 pheromone. Can. J. Microbiol. 55, 57–62.

Liu, A., Luginbühl, P., Zerbe, O., Ortenzi, C., Luporini, P., Wüthrich, K., 2001. NMR structure of the pheromone E *r*-22 from *Euplotes raikovi*. J. Biomol. NMR 19, 75–78.

Luginbühl, P., Ottiger, M., Mronga, S., Wüthrich, K., 1994. Comparison of the NMR structures of the pheromones E *r*-1, E *r*-2 and E *r*-10 from *Euplotes raikovi*. Protein Sci. 3, 1537–1546.

Luginbühl, P., Wu, J., Zerbe, O., Ortenzi, C., Luporini, P., Wüthrich, K., 1996. The NMR solution structure of the pheromone E *r*-11 from the ciliated protozoan *Euplotes raikovi*. Protein Sci. 5, 1512–1522.

Luporini, P., Alimenti, C., Ortenzi, C., Vallesi, A., 2005. Ciliate mating types and their specific protein pheromones. Acta Protozool. 44, 89–101.

McEwan, N.R., Eschenlauer, S.C.P., Calza, R.E.R., Wallace, J., Newbold, C.J., 2000. The 3′untranslated region of messages in the rumen protozoan *Entodinium caudatum.* Protist 151, 139–146.

Meyer, F., Schmidt, H.J., Plumber, E., Hasilik, A., Mersmann, G., Meyer, H., Engstrom, A., Heckmann, K., 1991. UGA is translated as cysteine in pheromone 3 of *Euplotes octocarinatus*. Proc. Natl. Acad. Sci. U.S.A. 88, 3758–3761.

Miceli, C., La Terza, A., Bradshaw, R.A., Luporini, P., 1992. Identification and structural characterization of a cDNA clone encoding a membrane-bound form of the polypeptide pheromone E *r*-1 in the ciliate protozoan *Euplotes raikovi*. Proc. Natl. Acad. Sci. U.S.A. 89, 1988–1992.

Miyake, A., 1996. Fertilization and sexuality in ciliates. In: Hausmann, K., Bradbury, P.C. (Eds.), Ciliates, Cells as Organisms. Gustav Fischer, Stuttgart, pp. 243–290.

Möllenbeck, M., Heckmann, K., 1999. Characterization of two genes encoding a fifth so far unknown pheromone of *Euplotes octocarinatus*. Europ. J. Protistol. 35, 225–230.

Mronga, S., Luginbühl, P., Brown, L.R., Ortenzi, C., Luporini, P., Bradshaw, R.A., Wüthrich, K., 1994. The NMR solution structure of the pheromone E *r*-1 from the ciliated protozoan *Euplotes raikovi*. Protein Sci. 3, 1527–1536.

Nielsen, H., Engelbrechtl, J., Brunak, S., von Heijne, G., 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. 10, 1–6.

Pedrini, B., Placzek, W.J., Koculi, E., Alimenti, C., La Terza, A., Luporini, P., Wüthrich, K., 2007. Cold-adaptation in sea-water-borne signal proteins: sequence and NMR structure of the pheromone E *n*-6 from the Antarctic ciliate *Euplotes nobilii*. J. Mol. Biol. 372, 277–286.

Petz, W., 2005. Ciliates. In: Scott, F.J., Marchant, H.J. (Eds.), Antarctic Marine Protists. Australian Biological Resources Study, Canberra, pp. 347–448.

Petz, W., Valbonesi, A., Schiftner, U., Quesada, A., Cynan Ellis-Evans, J., 2007. Ciliate biogeography in Antarctic and Arctic freshwater ecosystems: endemism or global distribution of species? FEMS Microbiol. Ecol. 59, 396–408.

Placzek, W.J., Etezady-Esfarjani, T., Herrmann, T., Pedrini, B., Peti, W., Alimenti, C., Luporini, P., Wüthrich, K., 2007. Cold-adapted signal proteins: NMR structures of pheromones from the Antarctic ciliate *Euplotes nobilii*. IUBMB Life 59, 578–585.

Ricard, G., de Graaf, R.M., Dutilh, B.S., Duarte, I., van Alen, T.A., et al., 2008. Macronuclear genome structure of the ciliate *Nyctotherus ovalis*: single-gene chromosomes and tiny introns. BMC Genomics 9, 587–601.

Sánchez-Silva, R., Villalobo, E., Morin, L., Torres, A., 2003. A new noncanonical nuclear genetic code: translation of UAA into glutamate. Curr. Biol. 13, 442–447.

Schiffer, C.A., Dötsch, V., 1996. The role of protein-solvent interactions in protein unfolding. Curr. Opin. Biotechnol. 7, 428–432.

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

Tourancheau, A.B., Tsao, N., Klobutcher, L.A., Pearlman, R.E., Adoutte, A., 1995. Genetic code deviations in the ciliates: evidence for multiple and independent events. EMBO J. 14, 3262–3267.

Turanov, A.A., Lobanov, A.V., Fomenko, D.E., Morrison, H.G., Sogin, M.L., et al., 2009. Genetic code supports targeted insertion of two amino acids by one codon. Science 323, 259–261.

Valbonesi, A., Luporini, P., 1990. Description of two new species of *Euplotes* and *Euplotes rariseta* from Antarctica. Polar Biol. 11, 47–53.

Vallesi, A., Giuli, G., Bradshaw, R.A., Luporini, P., 1995. Autocrine mitogenic activity of pheromones produced by the protozoan ciliate *Euplotes raikovi*. Nature 4, 1221–1227.

Vallesi, A., Di Giuseppe, G., Dini, F., Luporini, P., 2008. Pheromone evolution in the protozoan ciliate *Euplotes*: the ability to synthesize diffusible forms is ancestral and secondarily lost. Mol. Phylogenet. Evol. 47, 439–442.

Vallesi, A., Alimenti, C., La Terza, A., Di Giuseppe, G., Dini, F., Luporini, P., 2009. Ccharacterization of the pheromone gene family of an Antarctic and Arctic protozoan ciliate, *Euplotes nobilii*. Mar. Genomics 2, 27–32.

von Heijne, G., 1987. SIGPEP: a sequence database for secretory signal peptides. Protein Seq. Data Anal. 1, 41–42.

Zahn, R., Damberger, F., Ortenzi, C., Luporini, P., Wüthrich, K., 2001. NMR structure of the *Euplotes raikovi* pheromone E *r*-23 and identification of its disulfide bonds. J. Mol. Biol. 313, 923–931.

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