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Artificial antifreeze proteins can improve NaCl tolerance when expressed in *E. coli*

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Abstract

A chemically synthesized DNA fragment encoding an artificial antifreeze protein was expressed in *E. coli* as a translational fusion with a truncated protein A. Two constructions were made, with two and four antifreeze domains, respectively. The fusion proteins stimulated the growth of their bacterial host cells at inhibitory NaCl concentrations. The fusion protein carrying four antifreeze domains also conferred improved tolerance towards freezing.

Key words: Antifreeze proteins; Protein A; Osmotolerance; *E. coli*

1. Introduction

Living organisms experience environmental water stress when subjected to high or fluctuating salinity, desiccation or freezing. These environmental conditions tend to reduce the amount of active water in the cell, thus altering the ratio of water to macromolecules [1]. Nature has evolved several strategies to combat the increase in osmotic pressure posed on the cell by environmental water stress. The bacterial osmoprotective systems which have received most attention are the regulated gene expression of major cold shock proteins or transport systems for potassium, porins and quarternary ammonium compounds [2]. The induced intracellular formation of small organic molecules also plays a major osmoprotective role for many organisms [3,4]. For instance, glycine betaine, proline betaine and proline often prevent damage in plants from cellular dehydration by balancing the osmotic strength of the cytoplasm with that of the environment [3].

The antifreeze proteins (AFPs) and glycoproteins from polar fish [5] and the thermal hysteresis proteins from arctic insects [6] also assist these organisms to adapt to increased osmotic pressure. These proteins prevent cellular damage caused by ice crystal formation. The AFPs of winter flounder (*Pseudopleuronectes americanus*) are low molecular weight proteins which are induced at sub-zero temperatures in order to prevent the serum from freezing [7]. These alanine-rich α-helical proteins are composed of 11-amino acid imperfect repeats, of which there are usually three [8], though AFPs with four or five have also been reported [9]. These proteins inhibit the ice nucleation in undercooled body fluids [10], causing a non-colligative depression of the freezing point.

AFPs have previously been expressed in yeast in a biologically active form and can act as an inhibitor of ice crystallization in microorganisms [11]. However, small proteins such as the AFPs are vulnerable to proteolysis in a foreign biological environment. To overcome this problem the AFP genes have previously been fused in frame with a portion of the *Staphylococcal* protein A gene (*spa*) without affecting the freezing point depression properties of the protein as demonstrated by in vitro experiments [12].

In this study, chimeric protein A/winter flounder AFPs were produced in *E. coli*. The object was to evaluate the osmoprotecting properties of AFP analogues in bacteria. Increased osmotic pressure was induced by salinity in order to determine whether the osmoprotecting properties of AFPs were only due to its ability to inhibit the formation of ice crystals, or if other mechanisms also were involved. The AFP analogues were of various lengths; fusion proteins with two or four 11-amino acid repeats were produced.

2. Material and methods

2.1. Bacterial strains and plasmids

E. coli F'11, recA((lac, pro) Δthi, rifA, strA, recA/F'lacI^{q}Z$^-$, pro$^+$) was used in standard cloning procedures and growth rate tests [13]. *E. coli* TG1, (lac-pro), supE, thi1, hsdD5/F'traD36, proA$^+$B$^+$, lac^i, lacZ M15 (ung$^+$, dut$^+$), was used in the freezing tolerance experiments. The plasmid vector pLVC106 was kindly supplied by Dr. Gareth J. Warren (DNA Plant Technology Cooperation, Oakland, CA) [12].

2.2. DNA constructions

Restriction enzymes, T4 DNA ligase and polynucleotide kinase were commercially available and used according to the suppliers' recommendations. All other cloning procedures were performed as described by Sambrook et al. [14]. The oligonucleotides used were synthesized at the Biomolecular Unit at Lund University.

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2.3. Expression and analysis of NaCl tolerance

E. coli F'11 cells harbouring plasmids expressing protein A/AFP fusion proteins (pLAF1 or pLAF2) or a control composed of truncated protein A (pLVC 106) were grown overnight at 37°C in LB medium (tryptone 10 g/l, yeast extract 5 g/l, and NaCl 10 g/l) supplemented with various NaCl concentrations up to 0.75 M. To follow bacterial growth the optical density was monitored spectrophotometrically at 550 nm. Generation times and lag phases were estimated from the resultant growth curves in order to assess the salt tolerance.

2.4. Determination of freezing/thawing tolerance

E. coli TG1 harbouring pLVC106, pLAF1 and pLAF2, respectively, were grown in 100 ml LB medium to late exponential phase (OD_{550} = 5.0). The cells were harvested by centrifugation for 5 min at 6000 rpm and resuspended in 10 ml dH_2O. Three 1 ml samples of each cell suspension were transferred to Eppendorf tubes. The samples were frozen at ×3°C for 1 h and then slowly thawed for 1 h. This process was repeated 6 times over a 12 h period. The degree of survival, i.e. the fraction of non-lysed cells, was estimated by determining the conductivity of the cell suspensions [15,16]. The conductivity of completely disintegrated cells was obtained by boiling a sample for 15 min. The measurements were carried out at 4°C using a conductivity meter from Radiometer (Copenhagen) and Gene Pulsar cuvettes from BioRad (0.2 cm electrode distance). The degree of survival was estimated from the ratio: (conductivity of boiled cells−frozen and thawed cells)/(conductivity of boiled cells−cells kept on ice) [15].

3. Results

3.1. Design and construction of antifreeze gene analogues

A double-stranded artificial AFP gene encoding two 11-amino acid repeats was formed by annealing four complementary oligonucleotides. The sequence is shown in Fig. 1. The chemically synthesized AFP genes were designed to be analogous to the most abundant AFP in winter flounder, HPLC-8 [17], using codons preferred by *E. coli* [18]. An *Sph*I site was introduced to simplify screening of the obtained tranformants. HPLC-8 consists of three alanine-rich imperfect repeats of 11-amino acids long units as depicted in Fig. 2. In this study, protein A/AFP fusion proteins were constructed with two and four 11-amino acid long repeats respectively (Fig. 2). The AFP gene was constructed with sticky ends, where the 5'-end has *Nco*I overlapping ends and the 3'-end a *Pst*I site.

Plasmid pLVC106 carries a 371-nucleotide *spa* fragment encoding a single functional IgG binding domain of protein A followed by a polylinker. The AFP gene was inserted between the *Nco*I and *Pst*I sites in pLVC106 thus placing the AFP gene in frame downstreams of the *spa* gene, yielding plasmid pLAF1. The insertion of the AFP gene destroys the *Nco*I site downstream of the *spa* gene, but introduces a new site on the 5'-side of the TAA termination codon. Plasmid pLAF1 could therefore be further digested by *Nco*I and *Pst*I; another AFP gene was introduced in this way, to generate pLAF2 (Fig. 3).

3.2. Expression and purification of the protein A/AFP fusion proteins

E. coli F'11 harbouring pLVC106, pLAF1 and pLAF2, respectively, were grown at 37°C to late exponential phase. The cells were harvested, washed and sonicated to give a crude cell homogenate. Samples were analysed on a 15% SDS-PAGE gel and blotted onto a nylon membrane. The single IgG binding domain encoded by pLVC106 was sufficient to permit visualisation of the fusion proteins with swine anti-rabbit IgG antibodies coupled to horseradish peroxidase. Each sample gave rise to a major band with only minor proteolytic degradation (data not shown). The expression of pLVC106, pLAF1 and pLAF2 resulted in gene products of 14.5 kDa, 17 kDa and 19 kDa, respectively. The fusion proteins in crude cell homogenates were also sufficiently stable to be affinity purified on IgG Sepharose [19]. The chimeric proteins purified in this way were estimated to be at least 90% pure by SDS-PAGE electrophoresis. Furthermore, we could estimate that approximately 0.5% of the total cell protein consisted of protein A/AFP fusion proteins.

3.3. NaCl tolerance

E. coli F'11 harbouring pLAF1 or pLAF2 were grown in LB broth supplemented with NaCl as described in section 2. pLVC106 was used as a control in all experiments. The optical density at 550 nm was monitored during the experiments starting at 0.2 and continuing until the stationary phase was reached. Distinct differences in generation time, lag phase and maximal cell density were observed between cells expressing the chimeric proteins and the truncated protein A. Typical growth curves of *E. coli* cells harbouring pLAF2 and pLVC106 grown in LB medium supplemented with 0.75 M NaCl are depicted in Fig. 4. Bacteria expressing the chimeric AFP exhibited only slightly longer generation times at 0.5 M NaCl than at 0.17 M NaCl. This was in contrast to pLVC106 where the generation time increased substantially between 0.17 and 0.5 M NaCl. The data from three independent growth rate experiments are shown in Table 1. The lag phase times for pLAF2 were also routinely approximately 30% shorter in LB medium containing 0.75 M NaCl than for pLVC 106. An interest-

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                M  H  T  A  S  D  A  A  A  A  A  A  A  L  T  A  A  N  A  A  A  A
5'-C ATG CAC ACT GCT AGC GAT GCC GCC GCG GCC GCT GCT TTG ACA GCT GCT AAC GCC GCC GCG GCA
  3'- GTG TGA CGA TCG CTA CGG CGG CGC CGG CGA CGA AAC TGT CGA CGA TTG CGG CGG CGC CGT

A  A  A  S  M  A  *
GCA GCA GCA TCC ATG GCA TAA GCA ACT GCA-3'
CGT CGT CGT AGG TAC CTG ATT CGT TG-5'
        Nco I
```

Fig. 1. The nucleotide sequence of the AFP analogue constructed by annealing four complementary oligonucleotides. The fragment corresponds to two antifreeze domains. The ends are compatible with *Nco*I and *Pst*I, respectively.

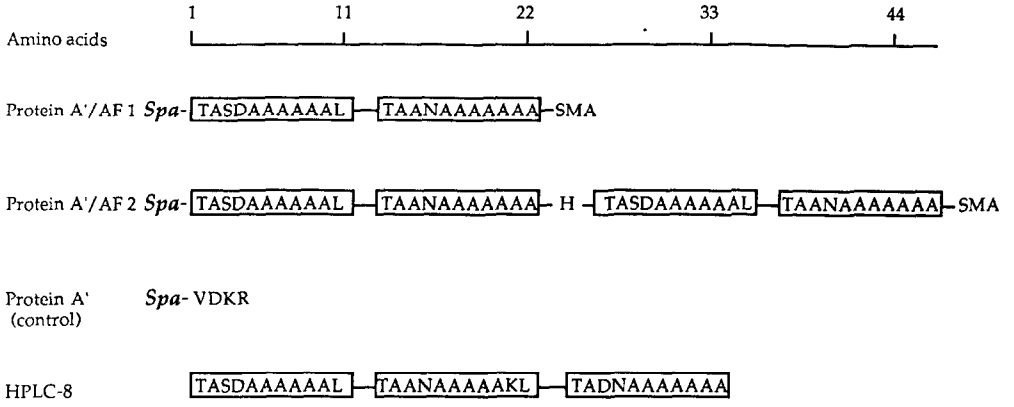

Fig. 2. Primary structures of the most abundant AFP in winter flounder, HPLC-8 [16], AFP analogues and the control protein, truncated protein A.

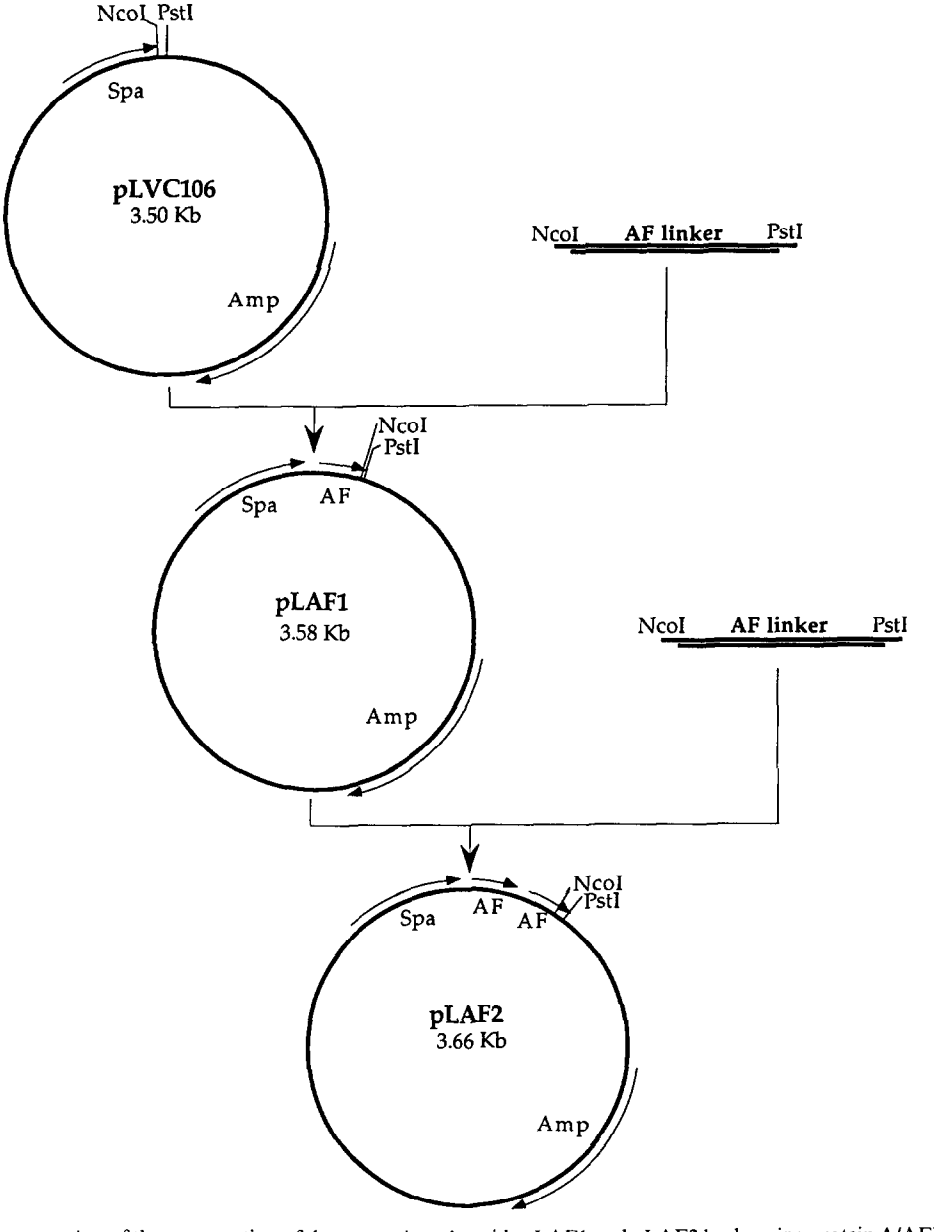

Fig. 3. A schematic representation of the construction of the expression plasmids pLAF1 and pLAF2 harbouring protein A/AFP translational fusions.

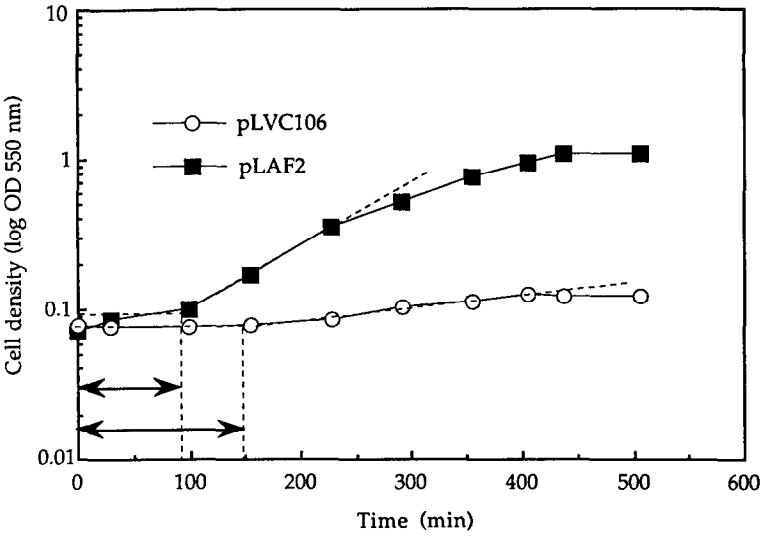

Fig. 4. Typical growth curves of *E. coli* F'11 carrying pLVC 106 (open circle) and pLAF2 (filled square) in LB medium supplemented with NaCl to a final concentration of 0.75 M. The growth lag phases are indicated as double arrows.

ing observation was that the size of the antifreeze domain did not seem to influence the NaCl tolerance, since *E. coli* carrying pLAF1 and pLAF2 exhibited very similar growth behaviour at high salt concentrations.

3.4. Freezing/thawing tolerance

In order to correlate the improved NaCl tolerance with any ability of the AFP analogues to protect the bacteria from damage by freezing, the transformed cells were also subjected to a freezing/thawing procedure. As shown in Fig. 5, antifreeze proteins with four 11 amino acid repeats exhibit improved tolerance toward freezing. These in vivo experiments agree with the results of McKown et al. [11] who have demonstrated that AFPs with fewer than three 11 amino acids repeats have a very marginal ability to inhibit ice crystal formation in vitro.

4. Discussion

Several studies have shown that AFPs inhibit ice crystallization and lower the freezing point [5,6,11,12]. Our results demonstrate that AFP analogues not only protect against cellular damage by freezing but also have a positive influence on osmotic pressure caused by salinity. The mechanisms behind ice crystal inhibition have been thoroughly investigated [6], but an explanation of the salt tolerance conferred by our AFP analogues requires other mechanisms to be considered. High extracellular levels of NaCl will affect the bacterial internal ion composition since the efficiency of various ATP ion pumps will not match the flow through passive ion channels. This leads to a damaging shift of the intracellular ion composition. Brusinsky et al. have demonstrated that AFPs can block these passive ion channels in the cell membranes and effectively suppress calcium and potassium currents in pig granulosa and rabbit parental cells [20,21]. A plausible explanation of the observed improved NaCl tolerance, demonstrated by bacteria expressing AFP analogues, may be an ability of the analogues to inhibit ionic

Table 1
Comparison of growth rates of *Escherichia* F'11 harbouring the listed plasmid

Plasmid[a]	Growth rates, generation time (min), in LB medium supplemented with NaCl		
	0.17 M	0.5 M	0.5 M NaCl
pLVC106	63	136	325
pLAF1	58	87	110
pLAF2	58	89	113

[a] See Fig. 3.

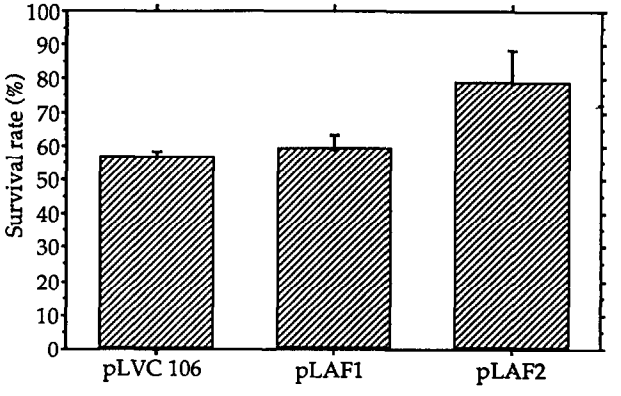

Fig. 5. The degree of survival of *E. coli* TG1 carrying pLVC106, pLAF1 and pLAF2 respectively, when exposed to a freezing/thawing procedure (see section 2). The data presented represent the mean of three independent measurements and the bars represent the standard deviation of the measurements.

currents. However, the size of the AFP moiety of the fusion proteins did not influence the bacterial salt tolerance, in contrast to the effects observed here and elsewhere on ice crystal inhibition [11]. Therefore, in order to further assess the ability of our AFP analogues to depress the passive transport of NaCl to the cell, we are currently coexpressing our plasmids with a luciferase expression vector carrying an osmosensitive promoter to facilitate the determination of internal salt concentrations. Furthermore, we are also evaluating the importance of the primary structures of the AFP analogues by studying the influence of random mutagenesis of the AFP structural gene on the growth rate of bacterial hosts in salt containing media.

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References

[1] Yancey, P.H., Clark, M.E., Hand, S.C., Bowlus, R.D. and Somero, G.N. (1982) Science 217, 1214–1222.
[2] Csonka, L.C. and Hanson, A.D. (1991) Annu. Rev. Microbiol. 45, 569–606.
[3] Le Rudlier, D., Strøm, A.R., Dandekar, A.M., Smith, L.T. and Valentine, R.C. (1984) Science 224, 1064–1068.
[4] Bülow, L. and Mosbach, K. (1991) Gene 109, 125–129.
[5] Devries, A.L. (1983) Annu. Rev. Physiol. 45, 245–260.
[6] Duman, J. and Horwath, K. (1983) Annu. Rev. Physiol. 45. 261–270.
[7] Yang, D.S.C., Sax, M., Chakrabarty, A. and Hew, C.L. (1983) Nature 333, 232–237.
[8] Hew, C.L., Joshi, S.B. and Wang, N.C. (1984) J. Chromatogr. 296, 213–219.
[9] Pickett, M., Scott, G., Davies, P., Wang, N., Joshi, S. and Hew, C.L. (1984) Eur. J. Biochem. 143, 35–38.
[10] Franks, F. (1985) Biophysics and Biochemistry at Low Temperatures, Cambridge University Press, Cambridge.
[11] McKown, R.L. and Warren, J.G. (1991) Cryobiology 28, 474–482.
[12] Mueller, G.M., McKown, R.L., Corotto, L.V., Hague, C. and Warren, G.J. (1991) J. Biol. Chem. 266, 7339–7344.
[13] Rüther, U., Koenen, M., Otto, K. and Müller-Hill, B. (1981) Nucleic Acids Res. 9, 4087–4097.
[14] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning, A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[15] Hömmö, L.M. (1992) Norw. J. Agric. Sci. 7, 39–50.
[16] Teutonico, R.A., Palta, J.P. and Osborne, T.C. (1993) Crop. Sci. 33, 103–107.
[17] Knigth, C.A., Devries, A.L. and Oolman, L.D. (1984) Nature 308, 295–296.
[18] Ernst, J.F. (1988) TIBTECH 8, 196–199.
[19] Nilsson, B., Abrahmsén, L. and Uhlén, M. (1985) EMBO J. 4, 1075–1080.
[20] Brubinsky, B., Mattiolo, M., Arav, A., Barboni, B. and Fletcher, G.L. (1992) Am. J. Physiol. 262, R542–R545.
[21] Negulescu, P.A., Rubinsky, B., Fletcher, G.L. and Machen T.E. (1992) Am. J. Physiol. 263, C1310-C1313.