Characterization and recombinant expression of a divergent ice nucleation protein from '*Pseudomonas borealis*'

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Isolates of '*Pseudomonas borealis*' were recovered after ice-affinity selection of summer-collected soils. '*P. borealis*' DL7 was further characterized and shown to have ice nucleation activity (INA), a property that allows the crystallization of ice at temperatures close to the melting point, effectively preventing the supercooling of water. INA was optimally detected after culturing at temperatures consistent with psychrophilic growth. The sequence encoding the '*P. borealis*' ice nucleation protein (INP) was obtained using both PCR and chromosome walking. When expressed in *Escherichia coli*, the resulting *inaPb* recombinants had INA. The '*P. borealis*' sequence, dubbed *inaPb*, is clearly related to previously cloned INP genes, but it shows greater divergence. Sequence analysis suggests that there are two opposite flat surfaces, one relatively hydrophobic that likely serves as an ice template, and the other that could function as a complementary face to facilitate interprotein interaction for ice-step formation.

INTRODUCTION

It is only the presence of ice nucleators that ensures the crystallization of water at temperatures close to 0 °C; without them water would supercool. Apart from ice itself, the most active known ice nucleators are found in bacteria. Several decades ago the plant epiphyte *Pseudomonas syringae* was discovered to have ice nucleation activity (INA) (Maki *et al.*, 1974). It was speculated that INA contributed to its pathogenicity since freezing and subsequent wounding of the leaves and stems would allow these strains ready access to plant nutrients (Lindow *et al.*, 1978). More recently, INA has been reported in *Pseudomonas fluorescens* and *Erwinia herbicola*, showing similar serological properties to *P. syringae* (Deininger *et al.*, 1988). Isolation of ice nucleation protein (INP) sequences from these, as well as from *Xanthomonas campestris*, has shown that they are similar despite the evolutionary divergence of the host bacteria (Abe *et al.*, 1989; Warren & Corotto, 1989). It has been speculated that the sequence was horizontally transferred by transduction events between plant epiphytes (Edwards *et al.* 1994). These INPs have a conserved amino terminus and a highly repeated middle region of 960–1296 amino acids consisting of three 16-residue repeats (yielding a 48 amino acid unit). This internally periodic middle region accounts for 80 % of

the protein (Warren & Corotto, 1989) and is followed by a 41–68-residue carboxyl-terminal sequence.

INA is not necessarily correlated with high freeze–thaw resistance. Indeed, in experiments to select for microbes with this phenotype from soil samples, no microbes with previously known INPs were recovered (Walker *et al.*, 2006). However, when bacteria from several temperate soils were adsorbed to growing multicrystalline ice, several isolates, including two '*Pseudomonas borealis*' strains (DL7 and YIC), identified on the basis of their 16S rRNA sequences, were recovered in the melt water (Wilson *et al.*, 2006). They were subsequently shown to have INA. Since INA had not been previously observed in these bacteria, the phenotype was examined further to determine if this activity could explain ice association and the gene sequence was isolated to compare it to better known species with this activity.

METHODS

Bacterial strains. '*P. borealis*' DL7 originated from soil samples collected from the Tundra Ecosystem Research Station at Daring Lake in the North West Territories, Canada (64°52'N, 111°35'W). This isolate was recovered after ice-affinity selection as previously described (Wilson *et al.*, 2006). *Chryseobacterium* sp. C14 was originally isolated by freeze–thaw selection (Walker *et al.*, 2006) and has no INA. Other strains used were *P. syringae* B728a, *Escherichia coli* TG2 and *E. coli* BL21 (Novagen).

Characterization of '*P. borealis*' DL7. A single colony of '*P. borealis*' DL7 was inoculated in 10 % tryptic soy broth (TSB), cultured

Abbreviations: INA, ice nucleation activity; INP, ice nucleation protein.

The GenBank/EMBL/DBJ accession number for the sequence of the '*P. borealis*' INP gene and protein is EU573998.

at 22 °C for 24 h, stained with Gram dyes according to standard protocols, and examined microscopically at 1000 $\times$. The antibiotic-resistant phenotype was determined on 10% TSB plates supplemented with different antibiotics and agents, and verified by liquid cultures. The different agents and their concentrations were: ampicillin (100 mg ml⁻¹), kanamycin (50 mg ml⁻¹), tetracycline (15 mg ml⁻¹), streptomycin (100 mg ml⁻¹), and CuSO₄ (0.8 mM).

The optimal growth temperature of the isolate was investigated by inoculating a single colony into 10% TSB, shaking, and monitoring growth at 22 °C. Once the cultures reached stationary phase, new cultures were initiated by inoculating 1% of the stationary phase cells into fresh 10% TSB and maintaining the culture temperature at 4 °C, 8 °C, 22 °C or 37 °C. Growth was monitored by both OD₆₀₀ and viable cell counts (c.f.u. ml⁻¹).

Ice nucleation assays and ice affinity. Ice nucleation was assessed using different sample volumes. A thermal analyser was used to determine the freezing point, as assessed by the heat of fusion of first-order freezing (Borchardt & Daniels, 1957). A thermister was placed in 1.4 ml samples that were chilled from 5 °C to -15 °C at 0.2 °C min⁻¹; temperatures were automatically recorded on a spread sheet and subsequently plotted (Walker *et al.*, 2008). Smaller volume samples were assayed essentially as previously described (Vali, 1971; Maki *et al.*, 1974; Kozloff *et al.*, 1983; Wilson *et al.*, 2006). Freezing was monitored visually by a fibreoptic light source directed through crossed polarized filters as the temperature was lowered from -1 °C to -15 °C (at 0.2 °C min⁻¹) and expressed as the logarithm of the number of ice nuclei per cell (Nemecek-Marshall *et al.*, 1993).

Ice affinity was determined as previously described (Wilson *et al.*, 2006). Briefly, the temperature of a hollow brass finger was set at -1 °C to establish a thin ice coat, and then it was lowered into a beaker containing the bacteria of interest (1.0 $\times$ 10⁴ c.f.u. ml⁻¹). By keeping the ice finger at -1 °C, an ice hemisphere could be slowly formed. After 2 days, ~50% of the liquid fraction was frozen, during which time the temperature of the ice finger was lowered incrementally. Subsequently, the hemisphere was rinsed and the ice was melted and poured onto 10% TSB plates. Identification of the bacteria as well as the numbers of viable cells (c.f.u. ml⁻¹) was then determined by using plates without additives or containing the appropriate discriminating antibiotics (tetracycline at 15 mg ml⁻¹, ampicillin at 100 mg ml⁻¹ and streptomycin at 100 mg ml⁻¹).

INP sequence determination. Amino acid sequences from known INPs were aligned and degenerate primers were designed to amplify the conserved, repetitive repeats. After initial trials, two primers, INPup (5'-AACATGGCCGATCAYTGCGG-3') and INPdown (5'-GTAVCKYTTSCCGTCCCAG-3'), were used. DNA from '*P. borealis*' DL7 was isolated using standard phenol/chloroform methods in PCR experiments under the following conditions: 94 °C for 5 min, followed by 94 °C for 0.5 min, 55 °C for 0.5 min, 72 °C for 4 min for 35 cycles, finishing with an extension at 72 °C for 30 min. The products were separated on agarose gels, purified using standard methods (Nucleotide removal kit, Qiagen), and the recovered PCR products were ligated into pGEMT vector (Promega). A plasmid with the appropriately sized insert was sequenced by ACGT Corporation and named pGEMcoreinp.

Based on the known sequence, four more primers were used to extend the 5'-terminal sequence of the putative '*P. borealis*' INP gene. The template for first reverse PCR was '*P. borealis*' genomic DNA, which was cut by *Sall* and self-ligated overnight, then purified as previously indicated. The PCR product (1 μ l) of the first two primers (INPR887: 5'-ATAGAATTCTGCCGTAGCCGCGAGTGAGAT-3' and INPL1549: 5'-CCTCGGGTTATGGCAGTTCCC-3') was used as template for a second PCR with two new primers (INPR188: 5'-TCAGCCTGC-ATATTCAAATGCG-3' and INPL2573: 5'-ACCCAGACTGCCAGG-

AAGA-3'). A similar method was used for 3'-terminal extensions. The product (1 μ l) from the first PCR amplification (using primers INPL3396: 5'-ATAGAATTCCGGGCGACCGAAGCAAACCTATTG-3' and INPR3350: 5'-CGGCCATCTGGATGCTGTGCGG-3') were used in a second amplification (with INPL3581: 5'-CTGACGGGCGGCGGAACCTCG-3' and INPR2768: 5'-TGCCTACTTCCATAACCTG-CGACCACT-3'). Both PCR products were ligated into pGEMT and positive colonies were designated pGEM5extension and pGEM3extension, respectively.

After sequencing, alignments were made using BioEdit for pGEMcoreinp, pGEM5extension and pGEM3extension. Similarities between the putative '*P. borealis*' INP sequence and all 13 known INPs in the database were determined by BLAST (Altschul *et al.*, 1997) and a consensus sequence was produced using the statistical tool hidden Markov models (HMM) according to Schuster-Böckler *et al.* (2004). Alignments with multiple sequences were made with the coding regions of INP genes from the following microbes: '*P. borealis*', '*P. fluorescens* (inaW)', '*P. syringae* B728a', '*P. syringae* (inaV)', '*Pantoea ananatis* (inaA)', '*Pantoea agglomerans*', '*X. campestris* (inaX)' and '*Xanthomonas translucens*'. Separate alignments were assembled for the repetitive region, as well as the amino- and carboxyl-terminal regions using BioEdit. The maximum-likelihood model was employed using ProML (Felsenstein, 2004), which does not assume a molecular clock, and this was used for the phylogenetic trees.

Expression of recombinant '*P. borealis*' INP. PCR was performed to introduce a *NotI* site to pGEM3extension with primers EC32 (5'-GCGAATTCTCATTATTCCTCCCGGATTTATTCACG-3') and EL2351 (5'-GGATCCATGGCAGGCTTCCAAAGCACATTGAT-3'), and the product was ligated to pGEMT and named pGEM2351. Subsequently, pGEM2351 was cut with *Sall* and ligated to pGEMcoreinp that had been digested by the same enzyme, and the recovered plasmid was designated pGEM3core. Another PCR was performed to introduce an *EcoRI* site at the 5'-terminal site of pGEM5extension using primers EN11 (5'-GGGAATTCGGGATGAACGATGACAAAG-3') and EN22 (5'-CGTAGATCTATCGCCAGCGGTTTCGGTA-3'). The resulting amplified product was ligated to pGEMT and designated pGEM1122, which was then digested using *XhoI* and ligated to pGEM3core, which had been digested with same restriction enzyme. The sequence of pGEMeinp was then obtained. Finally, the putative INP gene was introduced to pET24a at the *EcoRI* and *NotI* sites by digesting both the vector and pGEMeinp. This last plasmid was designated pET24aINP.

A single colony of *E. coli* BL21(DE3) harbouring pET24aINP was used to inoculate 10 ml Luria-Bertani (LB) broth containing 30 μ g kanamycin ml⁻¹. After culture at 37 °C, IPTG was added to 1 mM when the OD₆₀₀ reached 0.6. Subsequently the culture was shifted to 4 °C for 3 h. INA was assessed in the transformed cells as well as control cultures treated like the transformed cells and as previously stated.

RESULTS

Growth and characterization of '*P. borealis*' DL7

'*P. borealis*' DL7, which had been previously isolated by ice affinity (Wilson *et al.*, 2006), was microscopically visualized as Gram-negative cells, ~0.5 $\times$ 3 μ m (not shown). Cultures grew well on 10% TSB, with a maximum cell density at 10⁹ c.f.u. ml⁻¹, corresponding to an OD₆₀₀ of ~1.1. Colonies on 10% TSB plates were white with round and smooth margins. '*P. borealis*' DL7 can be broadly classified as psychrophilic since there was growth at 22 °C

(doubling time of 3.3 h), but cultures also grew well at 8 °C and 4 °C (with a doubling time of 12.6 h and 13.6 h, respectively). Even after 1 year of storage at temperatures at 0 °C, with ice floating on the surface of the medium, the cultures remained viable with no diminution of INA (see below).

When characterized in the presence of selected additives (see Methods), '*P. borealis*' DL7 continued to divide in the presence of ampicillin, but growth was inhibited by kanamycin, tetracycline and streptomycin, as well as by CuSO₄, amongst others (not shown). Thus the resistance phenotype was distinct from that of *E. coli* TG2 cells, which were inhibited by ampicillin but not by tetracycline.

INA and ice affinity

INA in '*P. borealis*' DL7 was shown using several different methods. As demonstrated in other studies (Vali 1995), absolute ice nucleation temperatures depend not only on cell number, but also on sample size. Thermal profiles of '*P. borealis*' ($\sim 10^9$ c.f.u. ml⁻¹; 1.4 ml volume) cultured at 22 °C and held at 4 °C for 2 days showed a mean freeze temperature of -2.8 °C, compared to a mean freeze temperature of -2.4 °C for *P. syringae* cultures under the same conditions (Fig. 1 inset). Thermal profiles for the two *Pseudomonas* species were significantly different from those for *E. coli* TG2 and *Chryseobacterium* C14, with mean freeze temperatures of -6.8 °C and -7.1 °C ($P < 0.03$). Ice nucleation spectra showed the same trend, although at temperatures consistent with the smaller volumes used to assess large numbers of samples, and confirmed that '*P. borealis*' had levels of INA (expressed as the logarithm of the number of ice nuclei per cell) that were slightly lower than that of *P. syringae* (Fig. 2).

Although INA appeared to be independent of growth phase (not shown), it was affected by culture temperature. There was little INA (log ice nuclei per cell between -6 and -7) in '*P. borealis*' cultures at 22 °C, but substantially more activity (log ice nuclei per cell between -2 and -4) at 4 °C or 8 °C. Likewise, when cells cultured at 22 °C reached stationary phase and were transferred to 4 °C for 2 or 4 days, a substantial increase in INA was observed (log ice nuclei per cell increased from about -7 to -4 and -2, respectively). There was no INA and no growth at 37 °C.

'*P. borealis*' DL7 was originally isolated after incorporation into growing ice and therefore its relative ice affinity was compared to that of *E. coli*. Identification of the two bacteria was facilitated by their sensitivity to different antibiotics. Although it was not possible to accurately estimate '*P. borealis*' cell numbers in the unfrozen liquid fraction because it is a psychrophile, there was an average fivefold higher recovery of '*P. borealis*' in the ice fraction compared to *E. coli* TG2 (not shown). This higher recovery could only be partially attributed to the threefold higher survival of '*P. borealis*' compared to *E. coli* in the ice fraction ($P < 0.001$).

Fig. 1. INA of '*P. borealis*' DL7. Typical thermal profiles showing the release of the heat of crystallization for '*P. borealis*' (◇; -1.9 °C) and *E. coli* BL21 (□; -7.9 °C); transformed *E. coli* BL21 contained either pET24a (-; -6.0 °C) or pET24aINP (▲; -5 °C) containing the '*P. borealis*' *inaPb* coding region. The inset shows further control experiments with '*P. borealis*' DL7 (A; -2.5 °C) compared to *P. syringae* B728a strain (B; -1.8 °C) cultures as well as control samples of *E. coli* TG2 (C; -7.9 °C) and *Chryseobacterium* sp. C14 (D; -7.7 °C). Typical thermal profiles are shown; experiments were done three to five times using independent cultures.

Isolation, sequence and analysis of the '*P. borealis*' INP gene

Previously reported INP gene sequences (Wolber *et al.*, 1986) allowed the design of 'consensus' DNA primers for the amplification of a DNA fragment from '*P. borealis*'. Eventually, degenerate primers (see Methods) were used to successfully amplify a 3.6 kb product (not shown). After cloning in pGEMT, subsequent sequencing allowed the synthesis of 'exact match' primers, which were used in a reverse PCR strategy to extend the sequence in both directions.

After assembly of the sequenced fragments, the resulting putative '*P. borealis*' INP DNA sequence conceptually encoded 1244 residues. The amino-terminal region extended for about 163 residues and accounted for 13 % of the gene, with the carboxyl-terminal region making up 41 residues. The remainder (~84 %) of the sequence was highly repetitive, with 65 typical 16 amino acid repeats (GenBank accession EU573998). The pattern was generally of two relatively hydrophilic repeats followed by a more hydrophobic 16-residue repeat for each 48 amino acid repeat unit. Comparison of the '*P. borealis*' DNA sequence indicated that there was no homologue amongst previously

Fig. 2. INA expressed as the logarithm of the cumulative number of ice nuclei per microbial cell (10 μ l samples) for decreasing temperatures. Samples tested included '*P. borealis*' DL7 ($\diamond$), *P. syringae* B728a ($\blacksquare$), *E. coli* TG2 ($\blacktriangle$) and pET24aINP ($\times$). Cultures were grown at 22 $^{\circ}$ C and transferred to 4 $^{\circ}$ C for 2–4 days. Typical data are shown with the experiments done in triplicate.

known INP genes in the first 500 bp. Overall there was ~66 % identity with *P. syringae* INP. Phylogenetic analysis (Fig. 3a) showed that '*P. borealis*' INP was divergent from the better-known INPs but overall had more similarity to the *P. fluorescens* protein. Not unexpectedly, the repeat sequences showed the highest identity with other INPs. '*P. borealis*' INP (e.g. position 164–1076) had a consensus sequence of AGYGSTxTAGxxSxLi/t (Fig. 3b), compared to the consensus AGYGSTQTsGseSsLT repeat in *P. syringae* InaZ (Kajava & Lindow, 1993).

Recombinant '*P. borealis*' INP is functional

To confirm that the PCR-amplified sequence encoded the INP, separate DNA fragments were amplified using primers containing judiciously chosen restriction sites. Subsequently, digestion with the appropriate restriction enzymes allowed the fragments to be assembled by ligation such that the entire 3735 bp sequence was introduced into pET24a. After inducing the *E. coli* host and transferring to 4 $^{\circ}$ C, the expressed recombinant INP showed modest but consistent INA: 1.4 ml samples froze at a mean of -5° C (Fig. 1) compared to a mean of -7° C for control cells (non-transformed or *E. coli*-transformed with an 'empty' pET24a plasmid). Ice nucleation spectra confirmed that transformed *E. coli* had modest levels of INA (log ice nuclei

Fig. 3. The INP in '*P. borealis*' DL7. (a) Phylogenetic tree of the INP from '*P. borealis*' with other representative INPs (BioEdit). (b) The 16 amino acid repeat (positions 164–1076) in '*P. borealis*' is depicted showing the frequency of each amino acid as indicated by the relative size of the letters. The 16-residue unit is repeated three times to make a 48-residue unit, which itself is repeated 19 times (along with eight more 16 amino acid repeats).

per cell between -4.2 and -6.3), which surpassed that of the controls (Fig. 2). Based on these functional assays, the gene sequence has been designated *inaPb*.

DISCUSSION

Survival in a surface tundra community demands adaptations to long cold winters. Reports list more than a dozen microbes that appear to dominate low-temperature consortia and these include *Pseudomonas* species (Wynn-Williams, 1983; Shivaji *et al.*, 1989). Some *Pseudomonas* strains are known to degrade hydrocarbon spills, particularly in the Arctic (Stallwood *et al.*, 2005), valuable 'cold enzymes' have been isolated from others (Villeret *et al.*, 1997; Cavicchioli, 2002), and membranes of some strains have enhanced fluidity at low temperatures (Kumar *et al.*, 2002). Strains of *P. syringae* are well known for their INPs, and this phenotype might also be considered a low-temperature adaptation. '*P. borealis*' DL7 originated from tundra soils and was recovered by ice-affinity selection, an isolation that we now show appears to have been partially due to its ability to retain high viability after freezing, as well as its modest ice affinity. Indeed, because this strain is easily cultivated at temperatures just a few degrees above 0 °C and did not divide at 37 °C, it can be classified as a psychrophile. '*P. borealis*' DL7 showed typical pseudomonad properties, including its cell and colony morphology, but in addition it had INA. This activity is apparent above -5 °C (Figs 1 and 2), allowing the '*P. borealis*' INP to be classified as a high-activity, type I nucleator (Phelps *et al.*, 1986).

INA has been described in certain strains of several evolutionarily diverse plant pathogens and epiphytic bacteria, and the genes encoding the INPs are homologous (Abe *et al.*, 1989; Warren & Corotto, 1989; Edwards *et al.*, 1994). Since '*P. borealis*' is considered a beneficial soil bacterium (Raaijmakers *et al.*, 2002), it was of interest to determine the sequence of its INP. Cloning the corresponding gene was challenging due to the large number of internal repeats (GenBank accession EU573998; Fig. 3), with alternative cloning strategies yielding disappointing results, but eventually the sequence was isolated using PCR and chromosome-walking techniques. To ensure that the INP had been correctly identified, the entire gene was assembled from amplified fragments and used to transform *E. coli*. The resulting recombinant protein showed modest, but consistent INA (Figs 1 and 2). Similarly, when INP genes from other bacteria are expressed in *E. coli*, the resulting INA is not as high as in the originator cells (Wolber *et al.*, 1986).

Although we can only speculate that INP might confer some freeze protection to '*P. borealis*', the identification of the sequence in this soil bacterium and its divergence has prompted us to consider its structure and function. The '*P. borealis*' INP is composed of a repetitive core and is flanked by non-repetitive hydrophobic amino- and hydrophilic carboxyl-terminal regions, similar to other INPs. The INP genes from *Pantoea ananatis*, *Pseudomonas syringae*, *Pseudomonas fluorescens* and *X. campestris* are highly conserved, with the main difference being the number of 16-residue repeats. The hydrophobic amino-terminal

portion of the '*P. borealis*' shows no significant identity to other sequences in the database and the carboxyl terminal region was also divergent, with more polar and fewer hydrophilic residues than other INPs, but with similar numbers of charged residues to the *P. fluorescens* protein. When the InaPb sequence with its overall average 66% amino acid identity to other INPs was compared in phylogenetic trees, again it was more divergent than sequences from the plant epiphytes, but appeared more closely related to *P. fluorescens*, another soil microbe (Fig. 3). It is not known if *inaPb* is a result of horizontal gene transfer from an as-yet-unknown donor or if the sequence differences in '*P. borealis*' and *P. fluorescens* reflect an adaptation to a soil habitat.

Because of the divergence, *inaPb* should be useful for the discovery of functional residues in INPs. Previously, *P. syringae* INP has been modelled as a β -fold structure, with 16 residues per turn forming an ice-like surface (Wolber & Warren, 1989; Kajava & Lindow, 1993; Graether & Jia, 2001). Experimental evidence for this model has been provided by structural characterization of a six-turn, truncated polypeptide (Kobashigawa *et al.*, 2005). It is notable that the '*P. borealis*' sequence can also be modelled with similar folds and similarly has a reiterated TxT (Fig. 3), a motif that has been identified as the flat, ice-adsorption face in the β -helix antifreeze proteins (AFPs) from moths and beetles (Tyshenko *et al.*, 1997; Graham *et al.*, 1997). A second flat surface on the opposite side of the InaPb β -fold is predicted to derive from xLi/t motifs, where x is frequently Ser and the Leu residues project inward. Although a model for a grass AFP shows two flat, putative ice-adsorption faces on opposite sides of the β -roll (Kuiper *et al.*, 2001), we do not believe that this additional motif would have primarily evolved as a second ice-binding surface in INPs. Rather, we speculate that this second face functions to provide a complementary surface for the 'interdigitation' of the INPs (Kajava & Lindow, 1993). Since INPs are displayed on the membrane surface, two flat surfaces on either side of the β -folds would allow close inter-protein association along the faces, thus permitting INPs to assemble as three-dimensional 'stairs' consisting of overlapping INP molecules. Since less energy is required for the addition of water molecules to steps (Pruppacher & Klett, 1997), the propagation of ice along these discontinuities would be greatly facilitated, resulting in proteins that are second only to ice itself in the heterogeneous nucleation of water.

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