The EMBO Journal vol.5 no.2 pp.231–236, 1986

Ice nucleation activity of *Pseudomonas fluorescens*: mutagenesis, complementation analysis and identification of a gene product

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Communicated by N.E.Murray

A DNA fragment of 7.5 kb from *Pseudomonas fluorescens* MS1650 confers an ice nucleation phenotype when cloned in *Escherichia coli*. This DNA encodes a protein with an apparent mol. wt of 180 kd, which is found in both inner and outer membrane fractions of transformed *E. coli* cells. Insertion mutations throughout a 3.9-kb region cause deficiency in ice nucleation, and eliminate the 180-kd protein. Complementation is not observed between any pair of mutations, suggesting that the nucleating phenotype is encoded by a single transcriptional unit. Mutations in most parts of the 3.9-kb region are not completely deficient in phenotype: they still generate ice nuclei at low frequency. One insertion mutation was found to generate pseudowild revertants, which had undergone deletions of the entire insertion and some of the adjacent sequence; these could account for the incomplete deficiency. These deletions displayed depressed nucleation temperatures, but their nucleation frequencies were close to that of the wild-type gene.

Key words: bacterial membrane/heterologous expression/bacterial genetics

Introduction

The ability of certain bacteria to nucleate ice formation has been implicated as a factor contributory to the injury of plants by frost (Arny *et al.*, 1976; Lindow *et al.*, 1978; Lindow, 1982). Within the genus *Pseudomonas*, certain strains of *P. syringae* and *P. fluorescens* are active in ice nucleation (Maki *et al.*, 1974). Molecular analysis of these strains was facilitated considerably by the demonstration that a single small region of the chromosome, cloned from *P. syringae*, could confer ice nucleation activity (INA) on *Escherichia coli* (Orser *et al.*, 1985). The nucleotide sequence of such a region from *P. syringae* was found to contain only one open reading frame of significant length (Green and Warren, 1985). This suggested that the product of a single gene from *P. syringae* (named *inaZ*) can enable a heterologous bacterium such as *E. coli* to nucleate ice formation. The open reading frame in the *P. syringae* sequence is highly repetitive, which has significant implications for the gene product's mode of action. Such repetitiveness may also predispose a gene to display some unusual genetic properties.

This report concerns the genetic analysis of the INA from a different species of bacterium. Its origin is a strain of *P. fluorescens* which was isolated from strawberry blossoms, and shown to be capable of blossom re-colonization (J.Lindemann, in preparation). Such a strain is of novel ecological interest because the combination of epiphytic growth habit and ice nucleation ability have not previously been described in *P. fluorescens*. Our interest in this strain stems both from the desirability of understanding the genetic basis of ice nucleation in more than one species, and from the practical consideration that manipulation of *P. fluorescens* may be necessary to provide frost protection to certain plant systems. In particular, we wished to determine genetically the number of genes necessary to encode the ice nucleation phenotype, and to identify the corresponding gene product(s).

Results

Cloning of INA

A cosmid library containing ~1000 clones of *P. fluorescens* MS1650 DNA was constructed in vector pRK7813 (J.Jones and

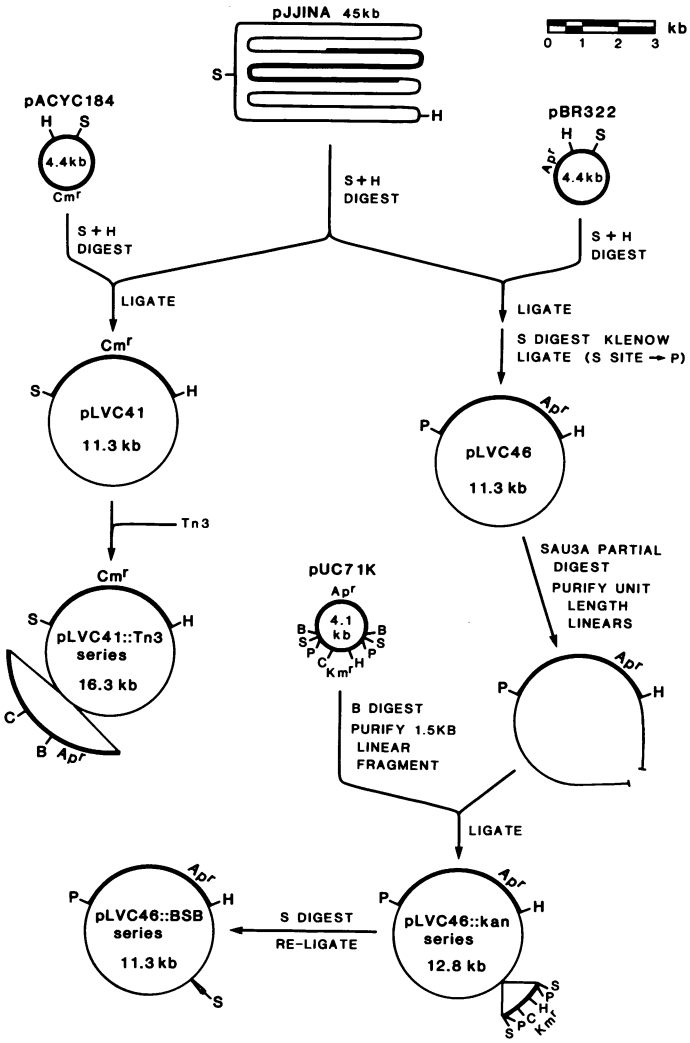

Fig. 1. Construction of plasmids used in the complementation tests. B, *Bam*HI; C, *Cla*I; H, *Hin*dIII; P, *Pvu*I; S, *Sal*I. Ap^r, Cm^r, Km^r: genes encoding resistance to ampicillin, chloramphenicol and kanamycin, respectively. Heavy lines indicate vector sequences.

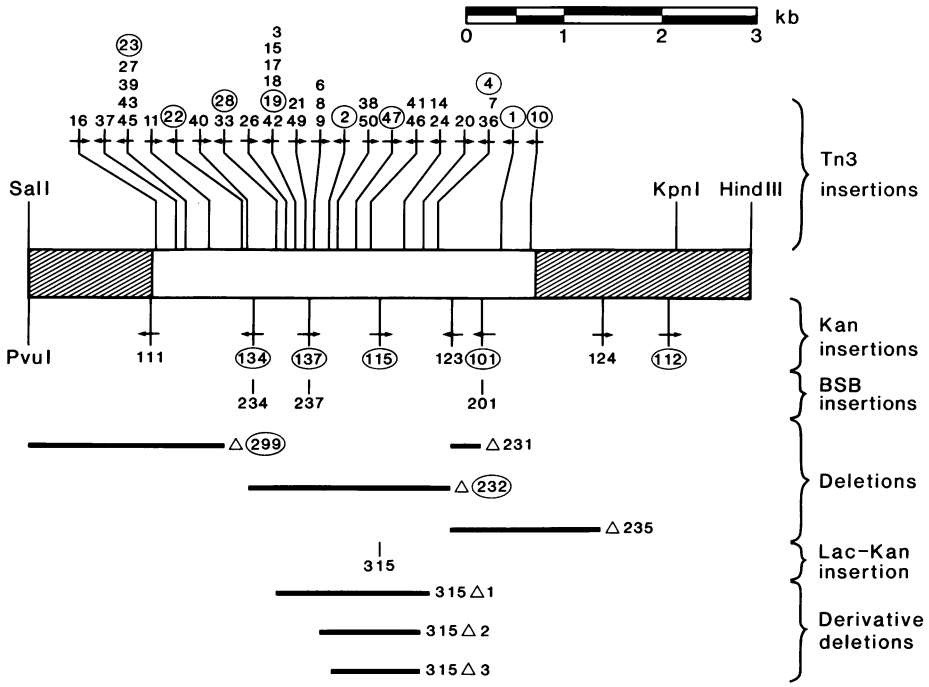

Fig. 2. Map of insertion mutations in the region of DNA encoding ice nucleation. Above the central bar: lines indicate positions of Tn*3* insertions into the region cloned in pACYC184. Allele numbers are shown for each position; orientation of insertion is denoted by an arrow pointing in the direction of *bla* transcription. Below the bar: allele numbers of *kan* insertions, BSB insertions and deletions in the same region cloned in pBR322. Orientation of each *kan* insertion is denoted by an arrow pointing in the direction of *kan* transcription. Heavy horizontal lines show the regions removed by their corresponding deletions. The circling of an allele number indicates its use in the complementation tests in Results. Within the central bar: shaded sections denote regions which are not shown to encode INA.

N.Gutterson, in preparation) and INA was detected in a single clone in *E. coli*. A 7.5-kb *Hin*dIII-*Sal*I fragment was subcloned from this into vectors pACYC184 (Chang and Cohen, 1978) and pBR322 (Bolivar *et al.*, 1977). (A schematic of these and subsequent constructions is given in Figure 1.) These vectors were chosen to permit the stable co-maintenance of their derivatives in subsequent complementation tests. The *Sal*I site of the pBR322 derivative was now removed (incidentally generating a *Pvu*I site) by cutting with *Sal*I, filling in the recessed ends with polymerase I Klenow fragment, and re-ligating the resultant blunt ends.

The INA of the *Hin*dIII-*Sal*I subclones was as high as that of the parent cosmid (data not shown), suggesting that all relevant genetic information was included in the subcloned fragment. It still remains possible that the subclones lacked some information significant to ice nucleation, and that this lack was compensated by their increased copy number, relative to the original cosmid clone.

Insertional mutagenesis

Mutagenesis was performed by introducing three types of insertion into the cloned sequences: (i) the 4957-bp element Tn*3* was allowed to transpose into the pACYC184 derivative, pLVC41; (ii) a 1496-bp kanamycin resistance-conferring restriction fragment (*kan*) was inserted into *Sau*3A sites of the pBR322 derivative, pLVC46; (iii) a 12-bp insertion ('BSB') was derived from certain of the 1496-bp *kan* insertions, by excision of a 1484-bp *Sal*I fragment, followed by re-ligation. Each type of insertion is shown schematically in Figure 1. The Tn*3* and *kan* insertions were expected to create polar effects on the transcription of genes downstream, as well as causing severe disruption of any protein into whose coding sequence they insert. By contrast, the 12-bp insertions, which cannot create a termination codon in any frame, were expected to cause little or no polarity, and comparatively minor disruption of protein structure (by the addition of four amino acids). Four deletions (shown in Figure 2) were obtained incidentally (see Materials and methods) and retained for comparison with the other types of mutation. For convenience of description, we shall refer to all such mutations as alleles of *inaW*, until further subdivision of the cloned region is called for.

We mapped all insertions of *kan* and BSB, but eliminated duplicated alleles (i.e. insertions at the same *Sau*3A site) from further analysis. Of the Tn*3* insertions, however, we only mapped those which reduced ice nucleation below detectable frequency at −5.0°C in a preliminary test; they constituted 42% of the total number. All these Tn*3* mutations made a coherent grouping on the map (see Figure 2), suggesting that ice nucleation is encoded by one contiguous segment of DNA within the cloned fragment.

INA was assayed in two ways for each of the Tn*3*, *kan* and BSB insertion mutations. One method was to test for reduction in the frequency of INA. Cultures were diluted to ~10^2 cells/ml and three 10 ml aliquots of each were cooled to −9.0°C to test for nucleation. Since ~10^3 cells are tested per replicate, and since ~50% of cells which contain a wild-type sequence can nucleate at −9.0°C, a negative result indicates an approximate frequency reduction of >500-fold. The results of this frequency threshold test are recorded by the shading of the bar in Figure 2. All insertions and deletions affecting the unshaded portion of the map gave negative results (indicating deficiency); the remaining three, and the pLVC41 and pLVC46 controls, were positive in this test.

The second method of testing the insertion mutations was a more sensitive assay to measure residual levels of INA, which we shall describe because, contrary to expectation, nearly all the insertions retained detectable (but low-frequency) activity. Cultures were diluted to ~10^7 c.f.u./ml and three 10 ml aliquots of each were gradually cooled from −3°C to −11.5°C. The temperature at which freezing took place represented the warmest

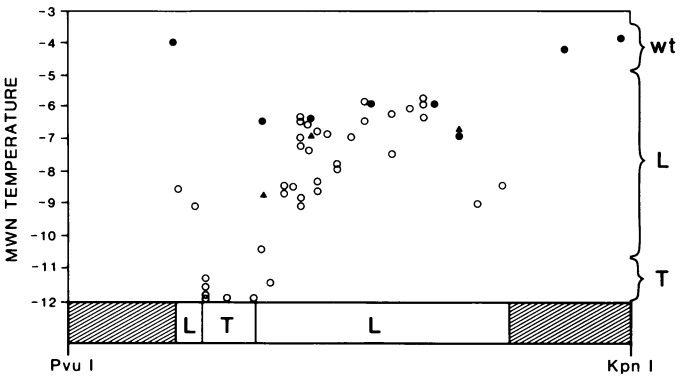

Fig. 3. Correlation of insertion position to nucleation phenotype. MWN: *m*ean *w*armest *n*ucleation temperature. Open circles represent data points obtained from Tn*3* insertions; solid circles represent *kan* insertions; solid triangles represent BSB insertions. The symbols L and T refer to observations of mutant phenotype discussed in Results. They denote leaky and tight deficiency phenotypes, respectively.

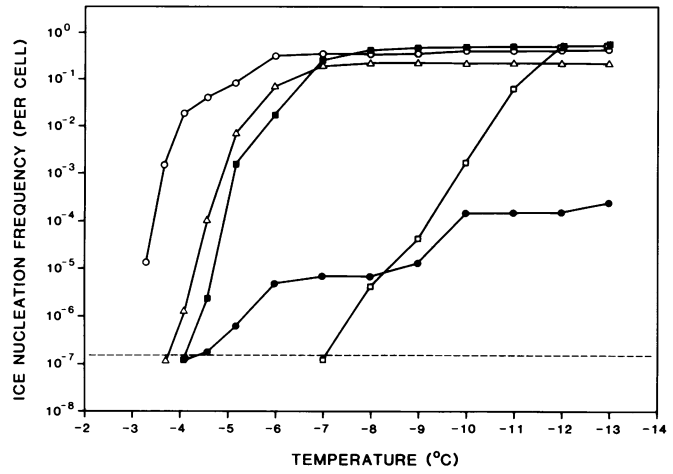

Fig. 4. Cumulative ice nucleation frequency plots for the wild-type *inaW* gene in pLVC46, *kan-lac* insertion mutant *inaW*315 and three pseudowild revertants. ○–○ *inaW*$^+$. ●–● *inaW*315. △–△ *inaW*315Δ3. ■–■ *inaW*315Δ2. □–□ *inaW*315Δ1.

temperature at which an aliquot displayed one active nucleus: therefore we call the means of these measurements the 'mean warmest nucleation (MWN) temperatures'. They are plotted in Figure 3, and show a remarkable pattern: there appears to be some correlation between the position of an insertion and its residual activity ('leakiness'). However, deletions Δ299, Δ232 and Δ235 were completely deficient ('tight') by this test, even though the latter two are deleted for regions in which all insertions are 'leaky'(Δ231 was slightly more deficient than BSB insertion *inaW*201). We sought to understand the reason for the leakiness of insertion mutants in order to interpret the results of these and further genetic experiments.

Cause of incomplete deficiency in ice nucleation

In seeking to understand this phenomenon we first considered polar effects on downstream transcription. This seemed inadequate as an explanation, because (i) the orientation of Tn*3* or *kan* inserts did not correlate with their leakiness, (ii) the non-polar BSB insertions were no more leaky than their *kan* insertion counterparts and (iii) the deletions Δ232 and Δ235 were completely deficient. For similar reasons we did not favour the proposition that leakiness was due to the production of partially active,

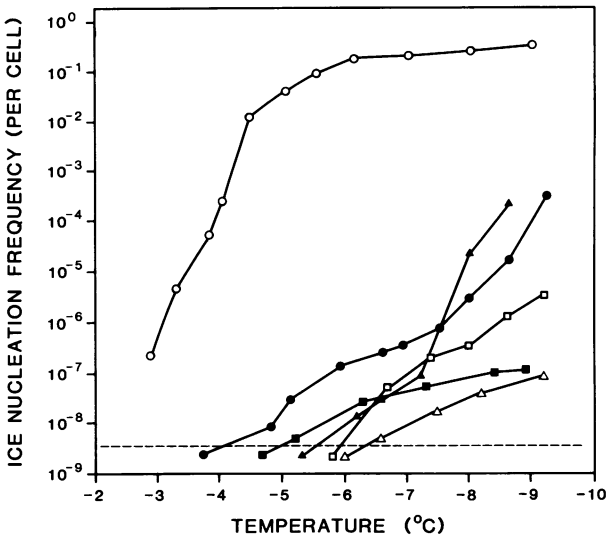

Fig. 5. Cumulative ice nucleation frequency plots of the wild-type *inaW* gene in pLVC41, and five Tn*3* insertion alleles whose membrane proteins were extracted and examined. ○–○ *inaW*$^+$. ●–● *inaW*16. □–□ *inaW*10. ■–■ *inaW*19. △–△ *inaW*22. ▲–▲ *inaW*47.

truncated proteins. An alternative possibility is that apparent leakiness is due to the occurrence of revertants at low frequency each time a mutant is cultured.

To test this explanation it was necessary to isolate such revertants from a culture of a leaky INA$^-$ insertion mutant. We reasoned that revertants would probably lose all or part of the inserted material, and therefore could also be detected (and thereby isolated) by loss of any phenotype encoded by the insertion. Therefore we cloned a *lac* operon into the site of the most leaky *kan* insertion mutation, *inaW*115. The new construct (*inaW*315) contained both *lac* and *kan* genes and was as leaky in its INA phenotype as its progenitor. Plasmid DNA was extracted from a culture containing the new plasmid and re-transformed at low DNA concentration, so that individual transformants would represent clones of plasmids present in the original culture. The *lac* gene permitted us to infer that loss of the insertion had occurred from ~1 in 10^4 of the original plasmids when transformants were cultured on Lactose-MacConkey agar. Eight of the Lac$^-$ clones were isolated and tested for INA; three out of eight possessed an elevated frequency of ice nucleation at −12.0°C. Their temperature/frequency spectra were determined and are presented in Figure 4. This showed that the revertants had gained in plateau frequency by three orders of magnitude, relative to *inaW*315, yet they were distinct from wild-type in their phenotype. Hence we designate them 'pseudowild'.

Plasmid DNA was extracted from the revertants and mapped to determine its alteration relative to *inaW*315. In each case the plasmid mapped as though a single deletion had removed the entire *lac-kan* insertion, and a varying amount of the *inaW* sequence to either side of it. The revertant alleles are referred to as *inaW*315-Δ1, Δ2 and Δ3: the extents of their deletions are shown in Figure 2. It is noteworthy that the larger its deletion, the lower the temperature at which a revertant became active in ice nucleation (compare Figures 2 and 4).

These observations persuaded us that revertants can occur with sufficient frequency to account for the leakiness of the insertion mutations. (It is also possible to account for the apparent relationship between mutation position and degree of leakiness by the quite reasonable postulate that frequency of deletion is related

Table I. Complementation between six mutant alleles in pLVC46 and 10 mutant alleles in pLVC41

Allele	134	137	115	101	Δ299	Δ232	112
16	***	***	**–	***	N.T.	N.T.	+ + +
23	– * –	– – –	– – –	– – –	– – –	– – –	+ + +
22	– – –	– – –	– – –	– – –	– – –	– – –	+ + +
28	– – –	– – –	– – –	– – –	– – –	– – –	+ + +
19	– – –	– – –	– – –	– – –	– – –	– – –	+ + +
2	– – –	– – –	– – –	– – –	– – –	– – –	+ + +
47	– – –	– – –	– – –	– – –	– – –	– – –	+ + +
4	– – –	* – –	– – –	– – –	– – –	– – –	+ + +
1	– – –	– * –	– – –	– – –	– * –	– – –	+ + +
10	– – –	– – –	– – –	– – *	* – –	– – –	+ + +
pLVC41	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +

Positive controls are provided by the non-deficient insertion allele 112 and by pLVC41 itself. '+': active above −6.0°C; '*': active above −9.0°C but not above −6.0°C (i.e. intermediate deficiency); '−': not active above −9.0°C.

to insertion position.) Therefore the insertions themselves may actually possess a completely deficient phenotype. This strengthens the inference that a structural gene encoding ice nucleation extends over the unshaded region of the map in Figure 2. It also indicates that complementation experiments should use a high threshold frequency for detection; otherwise revertants, as well as inter-molecular recombinants, may obscure the results. In the Discussion, we will suggest an interpretation for this reversion phenomenon, in terms of the known sequence of a different ice nucleation gene.

Complementation analysis

To determine genetically the number of transcription groups which encode the nucleation activity, complementation tests were performed between certain of the insertion mutants — those whose allele numbers are circled in Figure 2. Seven derivatives of the pBR322 clone (pLVC46) were first transformed into the *recA*$^-$ strain JC10291, by selection for their ampicillin resistance (Ap^r) marker. Next, each of the seven transformed strains was re-transformed with the pACYC184 clone (pLVC41) and each of 10 pLVC41 derivatives, with selection for the pLVC41 chloramphenicol-resistance (Cm^r) marker. Although pLVC41 and pLVC46 are compatible plasmids, we minimised the time during which either plasmid might be lost by inoculating the cultures for the nucleation tests directly from the selective transformation plates.

We assayed all cultures in triplicate for nucleation above −6.0°C, and above −9.0°C. The assays were performed at a cell dilution which would have permitted the detection of ice nuclei at 1/500 of their frequency in a 'wild-type' culture. The results (in Table I) were interpreted to mean that no complementation had taken place: in no case was −6.0°C activity restored, and only in the case of *inaW*16 was activity at −9.0°C seen in more than one of the replicates. The lack of complementation implies that all mutations affect the same transcriptional unit, and possibly the same gene.

Identification of an ice nucleation gene product

Because the cell membrane seemed the likely location of a bacterial ice nucleus (Maki *et al.*, 1974; Sprang and Lindow, 1981), we isolated total membranes from a strain containing pLVC41 and from a plasmid-free control. A band corresponding to a mol. wt of 180 kd was observed in the membranes from the pLVC41 strain, when their proteins were separated by electrophoresis on SDS-polyacrylamide gels and stained with Coomassie blue. To test whether this band might correspond to the product of an ice nucleation gene, five INA$^-$ mutants (Figure 5) were subjected to a similar analysis. As may be seen from Figure 6, the 180-kd band was absent from all of them. Thus the band at 180 kd correlates well with INA, and therefore it may contain the molecule which constitutes the template for ice crystallization. Its characterization will be of interest; from experimenting with extraction protocols our preliminary results indicate that its properties are very similar to those of an ice nucleation gene product from *P. syringae* (P.Wolber, C.Deininger and G.Warren, unpublished).

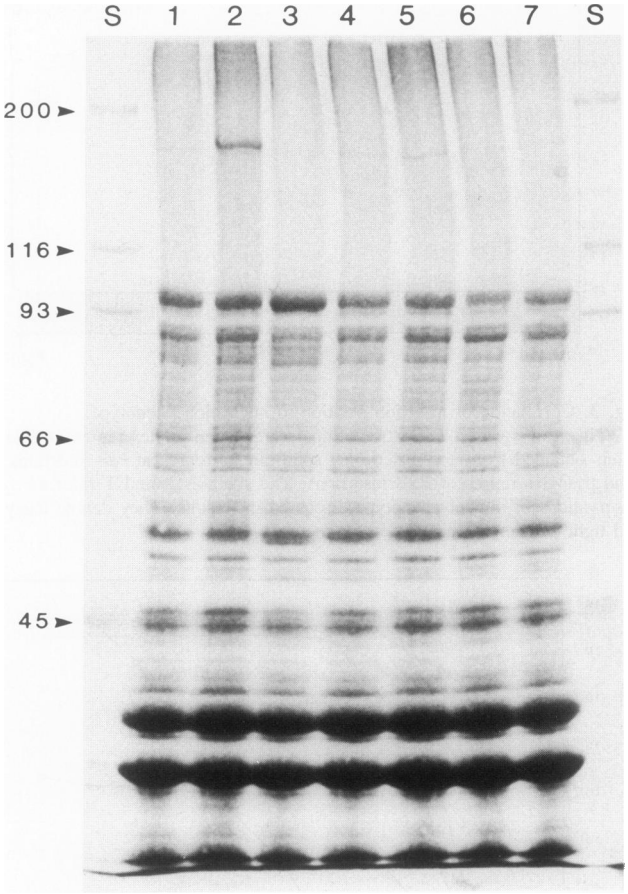

Fig. 6. SDS-polyacrylamide gel electrophoresis of total membranes from *E. coli* (JC10291) strains harbouring the following plasmids.
Lane 1: pACYC184 (vector control). **Lane 2:** pLVC41 (INA$^+$ subclone). **Lanes 3–7:** INA$^-$ pLVC41::Tn*3* plasmids, with alleles as follows: **lane 3,** *inaW*16; **lane 4,** *inaW*22; **lane 5,** *inaW*19; **lane 6,** *inaW*47; **lane 7,** *inaW*10. Lanes marked 'S' contain mol. wt standards whose mol. wts (kd) are shown along the left margin.

The five mutations examined in Figure 6 were chosen because they map at positions scattered throughout the 3.9-kb region; two of them lie at the opposite extremes of this region. The simplest interpretation of the results is that all mutations affect the same transcriptional unit, which encodes a protein (p180) whose apparent mol. wt is 180 kd. It remains possible that other, smaller ice nucleation gene products are synthesized but are masked on SDS-PAGE by normal components of the membranes. If this were the case, the gene encoding p180 would be likely to lie downstream of the other genes, since INA$^-$ mutations throughout the region cause the loss of its product. A rather less plausible

interpretation would be that certain INA$^-$ mutants lack other gene product(s), which are necessary to stabilize p180 and thereby permit its detection.

Discussion

The evidence from complementation analysis agrees with that from protein fractionation in suggesting that a single transcriptional unit encodes INA in *P. fluorescens*. The observed mobility of a protein encoded by the cloned DNA indicates a mol. wt of 180 kd, which would (for a protein of average amino acid composition) require a coding region of 4.2 kb. The minimum and maximum possible extents of the region, as defined genetically by insertion mutants, are 3.9 and 4.6 kb, respectively. Therefore, it is likely that few, if any, gene products beside p180 are required to confer an INA$^+$ phenotype on *E. coli*.

The leakiness of most INA$^-$ insertion mutations is interesting. It could be due to either (i) the occurrence of revertants at low frequency or (ii) to the ability of the disrupted region to encode ice nuclei much less efficiently than those of wild-type. We prefer the former explanation for its simplicity. In one case we have shown that revertants can be formed at a frequency consistent with the degree of apparent leakiness. A mechanism for the reversion is suggested by the sequence analysis of an ice nucleation gene from another species, *P. syringae*. This gene contains repetitive sequence elements through much of its length (Green and Warren, 1985). If the *P. fluorescens* gene possessed a similar structure it would be possible for homologous recombination between repeats to cause deletion of the insert together with several of the repeats flanking it. This agrees with the nature of the deletions present in the observed revertants. The phenotypes of these revertants are also consistent with the consequent prediction that they should make normal quantities of a protein with ice nucleation properties inferior to wild-type: the onset of nucleation is at a lower temperature than in the wild-type, but the plateau level of nucleation (~0.5 nuclei/cell) is similar. Ability to revert (by recombination between repeats) would vary with the flanking repeats' mutual homology and extent: therefore this reversion mechanism could also explain the correlation between the position of an insertion and its degree of apparent leakiness. Our revertant plasmids were isolated from a strain deleted for *recA* (namely JC10291: Willis *et al.*, 1981). Therefore, it would be necessary to postulate that reversion could occur by *recA*$^+$-independent recombination.

Since it has not yet been demonstrated that reversion is the sole cause of the mutants' incomplete deficiency, we note that the alternative explanation raised above suggests some interesting possibilities. For example, different domains of the original protein might still be produced and associate together, giving a low level of activity, when an insertion separates them. However, it is difficult to see how this could be true for all of the many insertions at different positions. Another unconventional hypothesis, made more attractive by the possibly repetitive nature of the sequence, is that RNA transcribed from the upstream side of an insertion could re-prime (at very low efficiency) on the downstream side. Because it causes a self-propagating effect, the ice nucleation gene product(s) can be detected with extremely high sensitivity, and therefore it could permit the detection of extremely rare events in gene expression.

Materials and methods

Bacterial strains

Except for *P. fluorescens* MS1650 (described in the Introduction), the bacterial strains were derivatives of *E. coli* K12, with genotypes as follows. JC10291, *ara*, *galK*, *his*, *lacY*, *leuB*, *mtl*, *proA*, *rpsL*, *supE*, *thi*, *thr*, *tsx*, Δ(*srl-recA*) 303 (Willis *et al.*, 1981). JM83, *ara*, Δ(*lac-pro*), *rpsL*, *thi*, φ80d*lacZ* ΔM15 (Vieira and Messing, 1982). SK1592, *hsdR*, *thi*, *gal*, *endA*, *sbcB*, *tonA*. HB101, *hsdR*, *hsdM*, *leu*, *pro*, *recA* (Bolivar *et al.*, 1977).

Plasmid constructions

Refer to Figure 1. Chromosomal DNA was isolated from *P. fluorescens* MS1650 and subjected to partial digestion with endonuclease *Sau*3A. Cosmid vector pRK7813 was digested by *Bam*HI, mixed with the *Sau*3A-cut fragments and treated with T4 DNA ligase. [pRK7813 (J.Jones and N.Gutterson, in preparation) is a *cos*-containing derivative of pRK404 (Ditta *et al.*, 1985); its choice for use here was arbitrary.] The ligation mixture was packaged into phage coats (Hohn and Murray, 1977) and transfected into HB101. Approximately 1000 transfectants were screened for INA and pJJINA was isolated from the only positive clone.

Plasmid pLVC41 was obtained by ligating together *Sal*I/*Hin*dIII digests of pJJINA and pACYC184, followed by selection for Cm^r and screening for INA among the resulting clones. Similarly, *Sal*I/*Hin*dIII fragments were subcloned into pBR322, selecting Ap^r: the INA$^+$ subclone contained a DNA fragment identical to that in pLVC41. Plasmid pLVC46 was derived from this pBR322 subclone by digestion with *Sal*I, treatment with DNA polymerase Klenow fragment and deoxyribonucleoside triphosphates and re-ligation; thus in pLVC46, a *Pvu*I site has been generated and has replaced the *Sal*I site previously present (GTCGAC became GTCGATCGAC).

Mutagenesis by transposon Tn*3* was performed as follows. A Tn*3*-containing derivative of R64*drd*11 (Meynell and Datta, 1967) was transferred by conjugation to strain SK1592 containing pLVC41. After outgrowth at 25°C for ~30 generations, plasmid DNA was extracted and re-transformed into SK1592, with selection for Ap^r and Cm^r. After preliminary screening (see Results) plasmid DNA was extracted from the INA$^-$ clones and the site of Tn*3* insertion into pLVC41 was mapped using *Cla*I and *Bam*HI+*Hin*dIII.

Insertion of a Km^r-conferring element ('*kan*') into pLVC46 was performed by manipulation *in vitro*, as follows. After partial *Sau*3A digestion of pLVC46, DNA fragments were separated by electrophoresis on a 1% agarose gel, and a band corresponding to single scission of the circular pLVC46 genome was identified. The band was excised and DNA re-isolated. A 1.5-kb *Bam*HI fragment of plasmid pUC71K (Vieira and Messing, 1982) was similarly recovered from an agarose gel. The recovered DNA species were mixed, treated with ligase and transformed into SK1592, with selection for Ap^r and Km^r. The various *kan* inserts thus obtained were mapped by digestion with *Cla*I and *Hin*dIII.

Insertions of a 12-bp element ('BSB') into *inaW* were obtained by derivation from certain of the *kan* insertions. The *kan* element contains a *Sal*I site just proximal to each of its terminal *Bam*HI sites: therefore digestion by *Sal*I and re-ligation left BSB as the residue. Deletions Δ231, Δ232 and Δ235 were obtained in the same way as the BSB insertions, except that they derive from plasmids where the insertion of *kan* was accompanied by the deletion of some adjacent DNA. Deletion Δ299 was obtained after *Pvu*II digestion and re-ligation: its rightmost end point lies at the single *Pvu*II site present in the cloned DNA.

Allele number 315 was derived from allele 115, as follows. A 7.1-kb *lacZY*-containing fragment was excised from plasmid pMC871 (Casadaban *et al.*, 1980) by digestion with *Bam*HI+*Sal*I. The plasmid containing allele 115 was partially digested by *Sal*I and ligated together with the fragment from pMC871. After transformation into JM83, an Ap^r Km^r Lac$^+$ clone was obtained: mapping with *Sal*I confirmed that the *lacZY* fragment was inserted immediately adjacent to the *kan* insertion.

DNA preparation, manipulation and electrophoresis on agarose gels

The DNA preparation method of Holmes and Quigley (1981) was followed. Restriction enzyme cleavage, ligation and transformation were as described by Mizuuchi *et al.* (1982). Agarose gel electrophoresis was performed according to Warren and Green (1985).

Preparation of bacterial membranes and SDS-PAGE

Membranes were isolated from *E. coli* strain JC10291 (harbouring various plasmids) by the method of Osborn and Munson (1974). Cultures (600 ml in L-broth) were grown at 24°C in the presence of 50 μg/ml chloramphenicol, to an A_{600} between 0.5 and 0.6. Only the total membrane fraction was isolated; bacterial inner and outer membranes were not separated. Membranes were resuspended in 1 ml 25% (w/w) sucrose, 5 mM EDTA (pH 7.5) and stored frozen at −20°C until use.

Polyacrylamide gel electrophoresis in the presence of SDS was performed with a discontinuous buffer system, according to Laemmli (1970). Stacking and separation gels (1 mm thick) were 5% and 8% (w/v) acrylamide, respectively. Samples were boiled for 10 min in sample buffer immediately before electrophoresis. Gels were stained with 0.1% (w/v) Coomassie brilliant blue in 7% (v/v) acetic acid, 50% (v/v) methanol, and destained in 7% (v/v) acetic acid, 20% (v/v) methanol, 3% (v/v) glycerol. Molecular weight standards (myosin, β-galactosidase, phosphorylase, bovine serum albumin, ovalbumin) were from Bio-Rad.

Ice nucleation assays

Cumulative ice nucleation frequency/temperature spectra were measured using a drop-freezing assay, as described by Vali (1971). Thirty or 40 replicates of 10 μl drops at each dilution were monitored while reducing their temperature stepwise. Dilutions covered the 10^{-1}–10^{-7} range in 10^{-1} increments. Droplets were held on nominally nucleus-free, paraffin-coated aluminium foil trays; temperature control was achieved by floating the trays on the surface of a refrigerated constant temperature ethylene glycol bath.

Acknowledgements

The cosmid pJJINA was isolated by Jonathan Jones and Julianne Lindemann. J.Bedbrook, P.Dunsmuir, R.Jorgensen and K.Steinback provided constructive criticism on the manuscript. We thank S.Lindow, N.Panopoulos and K.Willis for useful discussions, and our colleagues for their help and advice.

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Received on 4 November 1985; revised on 17 December 1985