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An ice nucleation reporter gene system: identification of inducible pathogenicity genes in *Pseudomonas syringae* pv. *phaseolicola*

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We have constructed derivatives of the transposon Tn*3* that allow an ice nucleation gene (*inaZ*) to be used as 'reporter' of the transcriptional activity of genes into which it is inserted. In these derivatives (Tn*3*-Ice and Tn*3*-Spice), the *lacZYA* sequences of transposon Tn*3*-HoHo1 were replaced with *inaZ* lacking its native promoter. The ice nucleation activity of *virB*::*inaZ* fusions in the correct transcriptional orientation was inducible by acetosyringone, a plant metabolite which activates the *vir* operon of *Agrobacterium tumefaciens* Ti plasmids, while fusions in the opposite orientation were unresponsive to the inducer. Tn*3*-Spice was also used to investigate the expression of a cluster of genes (*hrp*) which control pathogenicity and hypersensitivity elicited by *Pseudomonas syringae* pv. *phaseolicola*. An inducible region was identified which is expressed at low levels *in vitro* but becomes activated when the bacteria come into contact with the susceptible host, bean. Activation of this region occurred within 2 h post-inoculation and was nearly complete by the time the bacteria began to multiply in the leaf tissue. The *inaZ* reporter appears to be at least 10^5-fold more sensitive than *lacZ* in *P.s.phaseolicola*. Thus, the *inaZ* fusion system provides a sensitive, convenient and inexpensive tool for the study of bacterial gene expression, particularly during plant pathogenesis, and should be generally useful as a reporter gene system in Gram-negative bacteria.

Key words: inaZ reporter / Tn*3*-Ice / Tn*3*-Spice / *virB/hrp* cluster/hypersensitive reaction

Introduction

Gene and protein fusions have been instrumental in the study of gene regulation, protein processing and export and other aspects of gene function. Such fusions are especially useful (i) when a gene does not encode a protein (e.g. r-RNA, t-RNA, antisense RNA genes), (ii) when the protein product of a gene has no known or easily assayable function, (iii) when duplicate or multiple gene copies are present in the genome, (iv) when complex regulatory mechanisms involving pre- and post-translational steps are to be analyzed or (v) when two or more interacting organisms (e.g. a pathogen and its host) possess the same gene or enzyme activity.

All reporter gene systems in current use involve genes that encode an enzymatically active protein. The sensitivity of these systems varies according to the properties of the reporter enzyme, the nature and quality of the available assays and the presence or absence of interfering activities in the cell type or tissue under study. Sensitivity becomes especially important when a gene is expressed at very low levels or when gene activity must be measured in samples that contain a small number of cells, for example in the study of bacterial gene expression during the early stages of symbiosis or pathogenesis. In *Rhizobium*–plant interactions, the number of infectible sites on the plant root surface is small (Djordjevic *et al.*, 1987) and, by necessity, so is the number of bacterial cells that will establish symbiotically significant interactions with the plant. With bacterial pathogens, inoculum concentrations below $\sim 5 \times 10^6$ c.f.u./ml (i.e. 2.5×10^4 c.f.u./cm^2 leaf) are generally advised for inoculation of susceptible hosts because artificial reactions may arise with more concentrated inocula (Klement, 1982). The number of cells per unit area in symbiotic or pathogenic reactions does not begin to increase until several hours post-inoculation. The study of defense responses such as the hypersensitive reaction (HR), occurring on resistant host cultivars and/or non-host plants, usually involves more concentrated inocula. However, little or no bacterial multiplication occurs by the time the reaction is complete, although events crucial to the plant–bacterium interaction occur as early as 20–30 min post-inoculation (Klement, 1982). Such early events may also occur in susceptible interactions.

In the present work we describe a new gene expression assay system that affords high sensitivity, is rapid and simple, and requires no radioactive substrates or expensive equipment. An ice nucleation gene (*inaZ*) devoid of its native promoter serves as a reporter gene. The expression of ice nuclei produced by target gene–*inaZ* fusion is easily and quantitatively measured by a droplet freezing assay performed on sheets of aluminum foil that are cooled on a refrigerated alcohol bath set at a suitable sub-zero temperature. No sample processing other than grinding and dilution is required and the results are obtained within minutes. The utility of this system was assayed by analyzing the ice nucleation activity of *virB*::*inaZ* fusions in *Agrobacterium tumefaciens* grown in the presence and absence of acetosyringone, an inducer of the *vir* operon carried by Ti plasmids. The *inaZ* fusion system has enabled us to identify an inducible region in a previously described cluster of genes (*hrp*) of *Pseudomonas syringae* pv. *phaseolicola* which controls a very early step(s) in pathogenesis.

Results

Expression of ice nucleation activity in P.syringae pv. phaseolicola, A.tumefaciens and Rhizobium meliloti

In order to choose a suitable model system to evaluate the utility of the ice nucleation reporter gene in studies involving

plant–bacterium interactions, we initially sought to establish whether ice nucleus formation occurred in three non-ice nucleating bacterial species representative of different types of such interactions: *P.syringae* pv. *phaseolicola* (leaf spot and hypersensitive reaction symptoms), *A.tumefaciens* (crown gall) and *R.meliloti* (symbiotic nodulation). The *iceC* gene from *P.syringae* strain Cit7, carried on the broad host range plasmid pICE1 (Orser *et al.*, 1985), was transferred to *A.tumefaciens* C58, *R.meliloti* RM1021, and *P.s.phaseolicola* NPS3121. Functional ice nuclei were formed in all species tested but the quantitative and qualitative expression of ice nucleation differed in these bacterial hosts (Figure 1). *A.tumefaciens* and *R.meliloti* expressed ice nucleation much less efficiently than *P.syringae* Cit7 or *P.s.phaseolicola*. The threshold nucleation temperature, i.e. the warmest temperature in which ice nucleus formation could be detected, was −3°C for *A.tumefaciens* C58(pICE1) and −3.5°C for *R.meliloti* RM1021(pICE1), which was 1–1.5°C lower than the nucleation threshold of *P.syringae* Cit7 or *P.s.phaseolicola* NPS3121(pICE1). Ice nucleation frequency increased with decreasing temperature to about −9°C when little or no further change in nucleation activity occurred. Maximum nucleation frequencies (measured at or below −10°C) of Cit7 and NPS3121(pICE1) were similar. (The slightly higher activities observed with the latter may reflect the higher copy number of plasmid-borne *iceC* gene.) However, maximum nucleation frequencies of C58(pICE1) and RM1021(pICE1) were considerably lower at all assay temperatures. The basis for the observed differences in both threshold temperature and maximum ice nucleation activity is unknown and may reflect a combination of factors, such as differential stability and assembly of ice protein monomers into nucleation sites, different promoter recognition and other factors.

Construction of inaZ transposons

Genes may be fused to one another either *in vitro*, by cloning appropriate DNA fragments into a promoter–probe plasmid, or *in vivo*, by insertional mutagenesis with specially modified transposons. In the present study, we explored the *in vivo* approach by constructing two modified Tn*3*-based transposons, Tn*3*-Ice and Tn*3*-Spice (Figure 2; see Materials and methods). In these final constructs, the inverted repeats IR_L and IR_R of Tn*3* were retained intact. Plasmid pTn*3*-Ice (~11.5 kb) carries the *inaZ* gene (Green and Warren, 1985) such that when the element is inserted in the proper orientation transcription initiating within the target gene would proceed through IR_L into *inaZ*. The ATG initiation codon and Shine–Dalgarno sequence (GAGGA) of *inaZ* are proximal to the left inverted repeat (IR_L) of Tn*3*. A multi-linker segment containing the recognition sequences for *Eco*RI, *Sma*I(*Xma*I), *Bam*HI, *Sal*I and *Pst*I is present between IR_L and *inaZ*. The sequence between nucleotide number 1 (G) of IR_L and the ATG of *inaZ* (nt 150–152) contains three stop codons in the frame in which *inaZ* is translated; therefore, Tn*3*-Ice is not expected to produce translational fusions between *inaZ* and the target gene.

Many bacterial strains are naturally resistant to high levels of β-lactam antibiotics. Therefore, to increase the usefulness of the *inaZ* transposon, the Omega fragment (Prentki and Krish, 1984) carrying the Sp^r/Sm^r gene from R100 was cloned into the *Cla*I site on pTn*3*-Ice. The resulting plasmid, pTn*3*-Spice, is ~13.5 kb in size, of which 10.5 kb comprise

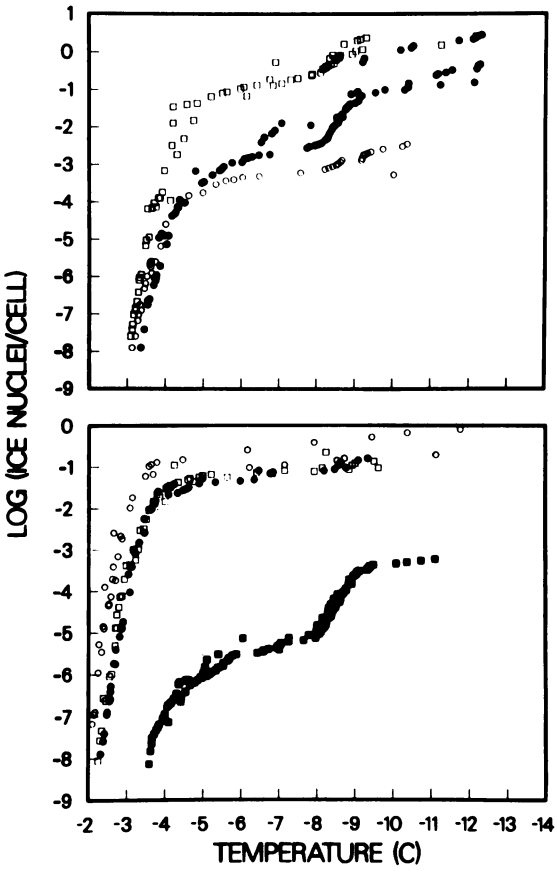


Fig. 1. Top frame: ice nucleation activity of *A.tumefaciens* C58(pICE1) (open circles) and C58(Tn*3*-Spice 11) grown in the presence (open squares) and absence (full circles) of acetosyringone. **Bottom frame**: ice nucleation activity of *R.syringae pv. phaseolicola* NPS3121(pICE1) (open circles), NPS3121(pPL11::Tn*3*-Spice 45) (open squares) and *R.meliloti* RM1021(pICE1) (full squares). Bacterial growth conditions were as described in the text.

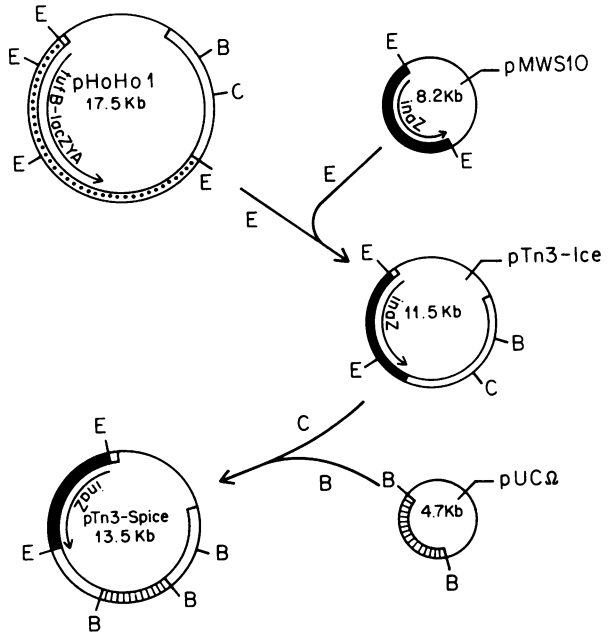
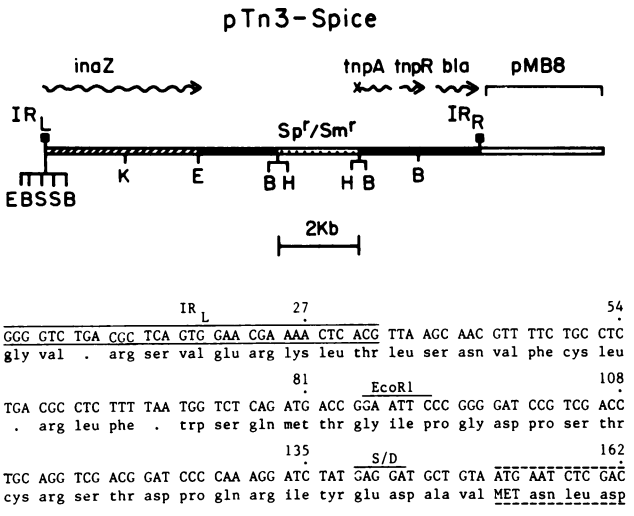


Fig. 2. Construction of Tn*3*-Spice transposons. The restriction enzymes used at each step are designated as E (*Eco*RI), B (*Bam*HI), and C (*Cla*I). Other abbreviations: Sm, streptomycin; Sp, spectinomycin. Note: plasmid and segment sizes are approximate.



IR$_L$ 27 54

GGG GTC TGA CGC TCA GTG GAA CGA AAA CTC ACG TTA AGC AAC GTT TTC TGC CTC
gly val . arg ser val glu arg lys leu thr leu ser asn val phe cys leu

81 EcoRI 108

TGA CGC CTC TTT TAA TGG TCT CAG ATG ACC GGA ATT CCC GGG GAT CCG TCG ACC
. arg leu phe . trp ser gln met thr gly ile pro gly asp pro ser thr

135 S/D 162

TGC AGG TCG ACG GAT CCC CAA AGG ATC TAT GAG GAT GCT GTA ATG AAT CTC GAC
cys arg ser thr asp pro gln arg ile tyr glu asp ala val MET asn leu asp

Fig. 3. Physical map of Tn*3*-Spice. Wavy arrows indicate the location and direction of transcription of relevant genes. IR_L, IR_R: left and right inverted repeats of Tn*3* respectively. Solid regions are Tn*3*-HoHo1 sequences; cross-hatched region is the Omega fragment. Sp, spectinomycin; Sm, streptomycin; *bla*, β-lactamase; *tnp*A, Tn*3* transposase (inactive; *tnp*R, Tn*3* resolvase; E, *Eco*RI; B, *Bam*HI; S, *Sal*I; H, *Hin*dIII; K, *Kpn*I. A *Pst*I site is also present between the two multilinker *Sal*I sites and three others in pMB8-derived segments. There are also three *Sal*I sites, but no *Bam*HI, *Pst*I, *Eco*RI or *Hin*dIII sites in the *ina*Z coding region. The nucleotide sequence of the IR_L–*inaZ* junction is shown as predicted from the Tn*3lac* (Stachel *et al.*, 1985a) and the pMWS10 sequence (Wolber *et al.*, 1986), beginning at nucleotide no. 1 of IR_L. Only the *Eco*RI site of the pMWS10 multilinker sequence used in the construction is shown. The SD (Shine–Dalgarno) sequence (position 139–143) and translation initiation codon (position 151–153) are those of *inaZ*. The reading frame shown is that in which the *inaZ* sequence is translated.

the transposable segment, Tn*3*-Spice, and the remaining portion the pMB8 plasmid sequences present in pHoHo1 (Stachel *et al.*, 1985a). Like their progenitor element Tn*3*HoHo1, both Tn*3*-Ice and Tn*3*-Spice retain the ability to transpose in *Escherichia coli* hosts containing the Tn*3*-transposase (*tnpA*) encoding plasmid pSShe. The frequency of transposition was $\sim 10^{-4}$ (data not included), which is similar to that obtained with Tn*3*HoHo1. The insertions appeared to be stable since no secondary transpositions have been seen in the absence of *tnpA* plasmids either in *E.coli* or in other bacteria used thus far in our studies.

Ice nucleation activity of virB::inaZ fusion

To determine whether the *inaZ* transposons were faithful reporter gene elements, we determined whether the expression of ice nucleation by Tn*3*-Spice insertions in an inducible target gene was orientation and inducer dependent. The *vir* region of the *A.tumefaciens* Ti plasmids contain several loci that are transcriptionally activated by acetosyringone, a plant signal molecule (Stachel *et al.*, 1985a,b; Stachel and Nester, 1986; Stachel and Zambryski, 1986). The *virB* locus is highly inducible and was selected as a test locus. The cosmid clone pCK257 (Stachel and Zambryski, 1986) consists of *Sal*I fragments 43 through 7 from the *vir* region of pTiA6 cloned into the broad host range vector pVCK102 (Figure 4). The insert contains the entire *virB* locus, as well as *virA* and *virG* which are required for *virB* induction by acetosyringone (Stachel *et al.*, 1986). One hundred and eighty independent Tn*3*-Spice insertions in pVCK257 were selected in *E.coli*.

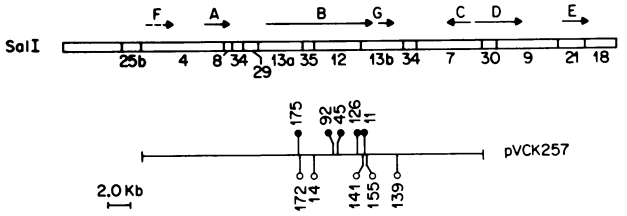


Fig. 4. Physical map and transcriptional organization of the *vir* region of plasmid pTiA6. The *Sal*I restriction fragments are numbered as in Stachel and Nester (1986). The arrows indicate the location and transcriptional direction of the *pinF*, *virA*, *virB*, *virG*, *virC*, *virD* and *virE* loci. The sites of Tn*3*-Spice insertions on plasmid pVCK257 are shown by vertical lines. Insertions in which transcription of *inaZ* is from left to right are shown above the horizonal line marked with solid circles to designate acetosyringone-mediated induction and those in the opposite orientation below the line with open circles to indicate lack of induction.

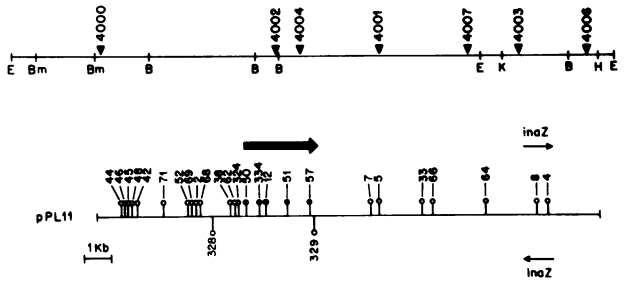


Fig. 5. Physical map of the Hrp region of *P.syringae* pv. *phaseolicola* NPS3121 showing the location of genomic Tn*5* mutants (triangles) and Tn*3*-Spice insertions in pPL11 (vertical lines). Highly inducible insertions are marked with closed circles. For insertions shown above the line, *inaZ* transcription is from left to right, for those below the line, from right to left. The thick arrow marks the highly inducible region. E, Bg, Bm, H, and K designate *Eco*RI, *Bgl*II, *Bam*HI, *Hin*dIII and *Kpn*I respectively.

Nine insertions were mapped unambiguously within *virB* based on single and/or double digestions with restriction endonucleases *Eco*RI, *Sal*I and *Bam*HI. In five of these (no. 175, 92, 45, 126 and 11), the *inaZ* gene had the same transcriptional orientation as *virB* (left to right in Figure 4) and in the other four (no. 172, 14, 141 and 155) the opposite orientation (right to left). An additional insertion (no. 139) in the intergenic region between *virG* and *virC* and three others (not shown in Figure 4) located at undefined points within the vector sequences, were also retained for further study. The above plasmids were introduced into *A.tumefaciens* strain C58 and the ice nucleation activities of the resulting transconjugants were determined after cultivation in the presence and absence of acetosyringone. although all insertions gave detectable levels of ice nucleation activity, only those within *virB* that had the proper transcriptional orientation (left to right in Figure 4) gave inducer-dependent activity (Table I). All other insertions were not induced by acetosyringone. Similarly, strain C58(pICE1) in which the ice gene is expressed from its native promoter exhibited no significant induction. Thus, acetosyringone-mediated induction of INA requires that *inaZ* be fused within the inducible *virB* region and in the same transcriptional orientation as the target locus. A gradient of induction with increasing distance from the *virB* promoter was observed with *virB*::*inaZ* (data not shown) as with *virB*::*lac* fusions (Stachel and Nester, 1986).

Table I. Ice nucleation activity (INA) of *A.tumefaciens* C58 carrying different pVCK257::Tn*3*-Spice plasmids and pICE1 grown in the presence and absence of acetosyringone (AS)

Direction of *inaZ* and *virB* transcription	INA units[a]/cell (−9°C)		Induction (fold)
	−AS	+AS	
Same (5)[b]	1.34×10^{-2}	0.85	63.2
Opposite (4)	2.12×10^{-4}	2.16×10^{-4}	NS
NA (5)[c]	Variable[d]	Variable	NS

[a]Mean ice nuclei/cell for 13 strains carrying transposon insertions within or outside *virB* and *A.tumefaciens* C58(pICE1). Values are the average ice nucleation activity from three separate experiments.
[b]Five different insertions in the same and four in opposite transcriptional orientations to *virB* were used (nos. 175, 92, 45, 126, 11 and 192, 14, 141, 155 respectively). These are diagrammed in Figure 4. The values given are the averages of the mean value from three separate experiments for each insertion.
[c]These include the values for strains carrying pICE1 and four different pVCK257::Tn*3*-Spice derivatives with the insertions in the vector portion of the plasmid.
[d]Values differed according to the insertion, but for each insertion the values were reproducibly similar between experiments.
NA, not applicable; NS, not significant.

Insertions in *virB* that were oppositely oriented with respect to *virB* transcription gene showed, on average, 63-fold lower activity compared to the uninduced levels of those oriented similarly to *virB* transcription (average activity 2.12×10^{-4} versus 1.34×10^{-2} nuclei/cell at −9°C) and nearly 4000-fold lower activity compared to acetosyringone-induced levels (average 2.16×10^{-4} versus 0.85 nuclei/cell at −9°C). The activity of these opposite-oriented insertions in *virB* is not due to spontaneous freezing of water or to endogenous nucleating factors in *Agrobacterium* cells, since neither C58 nor C58(pVCK257) gave any detectable nucleation activity ($< 10^{-7}$nuclei/cell at −9°C). Evidently, this activity reflects the inherent 'noise' of the system and in the case of pVCK257 may originate from several possible sources, e.g. incomplete termination of transcription of *virC*, which lies adjacent and is oriented opposite to *virB* (Figure 4), vector promoters or artificial promoter-like sequences generated at the *virB*::Tn*3*-Spice fusion points.

Analysis of expression of hrp::inaZ fusions

In previous studies (Lindgren *et al.*, 1986, 1988), a group of genes (*hrp*) that are required for pathogenicity and for the elicitation of the hypersensitive reaction by *P.s.phaseolicola* and other closely related phytopathogenic *Pseudomonas* spp. on plants was described. Attempts to define the transcriptional organization of this region by Tn*3*1*ac* mutagenesis were unsuccessful (P.B.Lingren and N.J.Panopoulos, unpublished) since these insertions, which included some that were properly oriented and mapped within the regions now known to be inducible (see below), gave no detectable β-galactosidase activity *in vitro*. Endogenous ONPG-hydrolyzing activity in green leaves also interfered with efforts to analyze gene expression *in planta*. To circumvent these problems, we employed Tn*3*-Spice to mutagenize the *hrp* region of *P.s.phaseolicola*. Plasmid pPL6 (Lindgren *et al.*, 1986) contains most of this region from strain NPS3121 but was deemed unsuitable for Tn*3*-Spice analysis because the P*lac* promoter on the pLAFR3 vector was directed toward the insert. To avoid interference from this promoter, we subcloned the 19 kb *Bam*HI−*Hin*dIII segment of pPL6 into another broad host range vector, pWB5a, to make plasmid pPL11. This segment covers nearly the entire interval between the outermost *hrp*::Tn*5* insertions previously mapped in the *hrp* region of NPS3121 (Lindgren *et al.*, 1986) and restored the wild-type phenotype (Hrp^+) to all mutants except NPS4000 and NPS4006, in which the Tn*5* is located very near the border sites of the 19 kb insert (Figure 5).

Tn*3*-Spice insertions distributed across the entire length of the pPL11 insert were obtained, and these plasmids were introduced into wild-type *P.s.phaseolicola* NPS3121. The ice nucleation activity of each strain grown *in vitro* was compared to the *in planta* activity. Several insertions gave significantly greater ice nucleation activity after inoculation than in culture. We refer to these insertions as 'inducible', even though mechanisms other than induction *per se* (e.g. decreased InaZ protein turnover or increased mRNA stability or translatability) may have brought about the same result. Several highly inducible insertions were observed in the central region of the pPL11 insert (Figure 5). All were oriented from left to right, and mapped in a region ~2.5 kb (from insertions no. 50−57). Based on statistical mean comparison tests, several other insertions were also inducible, but to a lower degree, indicating that other inducible loci may be present. Several insertions gave lower activity *in planta* than *in vitro* and for three of them (Table II) the differences were statistically significant. Very few insertions oriented from right to left in the pPL11 insert were obtained in our study. Although the reason for this is not clear, orientational preference for Tn*3* has been noted previously (S.E.Stachel, personal communication). One such insertion that was unambiguously mapped in the highly inducible region and another lying further to the left gave no significant induction *in planta* (Table II). The source of the 'constitutive' activity associated with these insertions is likely similar to that which was observed with right-to-left oriented insertions in *virB* (see above).

Quantitative relationship between ice nucleation activity and amount of ice protein

The availability of pPL11:Tn*3*-Spice fusions expressing different levels of ice nucleation activity permitted the assessment of the relationship between ice protein and ice nucleation activity in the same genetic and physiological background. Twelve such fusion plasmids giving different levels of ice nucleation activity in *P.s.phaseolicola* NPS3121 were grown under identical cultural conditions and ice protein content of these cells was quantified by immunoblotting. Ice nucleation activity and relative amount of InaZ protein increased non-linearly with the concentration of InaZ protein (Figure 6). Of several methods for plotting the data that were tried, the double logarithmic plot (Figure 6) gave a straight-line relationship. The ice nucleation activity values varied over a 500-fold range (from 7×10^{-3} to 4×10^{-1} nuclei/cell; strains with lower values did not produce a detectable band in our immunoblots); the corresponding values for the relative amount of InaZ protein varied over a 40-fold range. The slope of the straight line in the double logarithmic plot (Figure 6) is very close to 2, i.e., ice nucleation activity increased according to the second power of the increase in the amount of InaZ protein.

A non-linear relationship between ice nucleation activity and relative amount of InaZ protein was also observed in *A.tumefaciens* carrying *virB*::Tn*3*-Spice insertions in

Table II. *In vitro* versus *in planta* ice nucleation activity of *P.syringae* pv. *phaseolicola* NPS3121(pPL11::Tn*3*-Spice) strains

Plasmid insertion no.	Mean log ice nuclei/cell		Ratio of activities[c]
	In vitro[a]	*In planta*[b]	
pICE1	−0.86 DEFG[d]	−0.66 ABCD	1.57
44	−2.15 STUVW	−2.20 CDEFGHIJK	0.893
46	−2.40 RST	−2.65 BCDEFG	0.57
45	−0.99 AZY	−1.78 EFGHIJK	0.16
48	−2.96 MNOPQ	−4.31 AB	0.04
42	−2.57 RSTU	−2.64 BCDEFGH	0.86
71	−1.86 UVW	−3.55 ABCDE	0.02
70	−3.06 KLMN	−1.44 FGHIJKL	41.7*[e]
52	−3.19 JKLM	−3.20 BCDEF	0.97
69	−2.71 NOPQR	−0.87 HIJKLM	71.6*
2	−2.24 RST	−1.07 GHIJKL	15.0*
68	−2.38 RSTU	−1.30 GHIJKL	11.8*
38	−3.14 JKLM	−1.62 FGHIJK	34.3*
62	−2.97 LMNO	−1.93 DEFHIJK	11.6*
324	−3.84 CDE	−1.11 GHIJKL	54.1*
50	−2.32 QRST	0.98 NO	2010*
334	−3.12 IJKLM	0.92 NO	11 324*
51	−2.09 STUVW	0.85 MNO	881*
57	−1.78 WX	0.75 MNO	358*
7	−2.59 OPQR	−4.24 AB	0.02
5	−3.32 IJKLM	−5.00 A	0.02*
33	−2.19 TUVW	−2.83 BCDEFG	0.23*
66	−0.66 A	−1.49 FGHIJKL	0.15*
64	−2.67 NOPQR	−3.75 ABC	0.09
8	−2.41 QRST	−2.60 BCDEFGH	0.64
4	−5.05 B	−5.00 A	1.12
328	−4.99 B	−5.04 A	0.8
329	−3.99	−3.79	1.5

[a]*In vitro* activity is the average of three separate experiments.
[b]*In planta* activity represents one experiment of two replicates.
[c]Ratio of activities = *in planta* activity/*in vitro* activity.
[d]Since some variability of estimates of ice nucleation is expected between different samples of a given bacterial strain, statistical tests of the significance of differences between mean ice nucleation activities exhibited by different strains tested *in vitro* or in *planta* were performed. Values within each column which are followed by the same letter were not significantly different in the Waller−Duncan *K*-ratio *T*-test.
[e]Asterisks indicate that *in planta* activity was different from the corresponding *in vitro* activity as determined by Duncan's multiple range test.

pVCK257 and grown in the presence and absence of acetosyringone (data not shown). A straight line relationship was obtained in the double logarithmic plot, but with a slightly higher slope (2.3) than in *P.s.phaseolicola*. We note that the *A.tumefaciens* data included very high ice nucleation activity values expressed by acetosyringone-induced cultures (see Table I). Since the present method cannot measure more than one nucleus/cell, the slightly different slopes of the log−log plots for the two bacteria may reflect the inherent limitation of the present assay at high ice nucleation activity values or a slightly different quantitative relationship between ice nucleation frequency and InaZ protein content in different bacterial hosts.

It is interesting to note that a non-linear concentration−activity relationship was also reported recently for *E.coli* expressing the InaZ protein from different plasmid promoters (Southworth *et al.*, 1988). As these authors point out, the non-linear relationship between InaZ protein content and ice nucleation activity implies co-operativity between

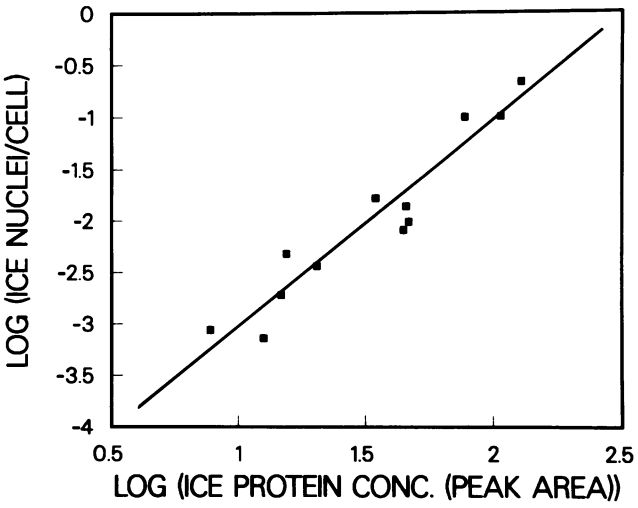


Fig. 6. Quantitative relationship between ice nucleation activity and amount of immunologically detectable *inaZ* protein in *P.s.phaseolicola* NPS3121 carrying pPL11:Tn*3*-Spice plasmids that gave different *in vitro* levels of ice nucleation activity (double logarithmic plot). Relative amount of *inaZ* protein is expressed as the area under peaks of this protein band on a densitometer tracing as determined gravimetrically (in mg). The slope of the straight line is 2.0.

InaZ protein monomers in the ice nucleus assembly process, which is further discussed later.

Relative sensitivity of the inaZ and lacZ reporter assays

To obtain a measure of the sensitivity of the *inaZ* reporter relative to a conventional reporter gene, such as *lacZ*, we determined the ice nucleus and β-galactosidase content of two *Pseudomonas* strains that expressed the two reporters from the same promoter. Plasmids p*avrB'−inaZ* and p*avrB'−lacZ* carry the 0.6 kb promoter-containing fragment of *avrB*, an avirulence gene cloned from *P.syringae* pv. *glycinea* (Staskawicz *et al.*, 1987). The *avrB* promoter fragment is positioned upstream of the *inaZ* and *lacZ* coding regions such that the promoter drives their transcription (details given in T.V.Huynh *et al.*, submitted). The complete sequence of *avrB* and the characterization of its promoter are described elsewhere (Tamaki *et al.*, 1988; T.V.Huynh *et al.*, submitted). The β-galactosidase and ice nucleation activities obtained with actively growing mid-log phase cultures of *P.s.phaseolicola* and *P.s.glycinea* are given in Table III. In this and several other similar experiments, the INA values were $1-6 \times 10^5$ times higher than β-galactosidase when expressed on a per-cell basis. The INA activity could be readily quantitated after 10^4-10^6 dilution of the cultures. By contrast, β-galactosidase activities determined in 0.1 ml aliquots of these cultures ranged from 30 to 87 units and, thus, would be barely detectable in >10-fold diluted cultures (Weinstock *et al.*, 1983; Carni *et al.*, 1988). It appears that the *inaZ* reporter assay is at least 10^5 times more sensitive than the *lacZ* reporter in *Pseudomonas* strains. Similar tests could not be carried out in *E.coli* since the *avrB* promoter is expressed at a very low level in this host (D.Staskawicz and B.J.Dahlbeck, unpublished data) and other suitable reporter constructs were not available.

Time-course of hrp gene activation in planta

The suitability of *inaZ* fusions for studying gene expression during the early stages of plant pathogenesis was investigated

Table III. Relative sensitivity of the *inaZ* and *lacZ* reporters

Host	Reporter activity[a]		
	inaz[b]	*lacZ*[b]	Ratio (*inaZ*/*lacZ*)
P.s.phaseolicola HBF	5.0×10^{-3}	4.1×10^{-8}	1.2×10^{5}
P.s.glycinea Psg0	3.5×10^{-3}	8.1×10^{-8}	4.3×10^{5}

[a]Activities were determined in mid-log phase shake cultures grown at 24°C in test tubes containing 5–8 ml King's B medium with vigorous shaking (250 r.p.m.). The values are averages of four assays, two in each of duplicate cultures.
[b]*inaZ* and *lacZ* reporter activity was determined in transconjugants carrying the p*avrB'-inaZ* and p*avrB'-lacZ* plasmids respectively. To facilitate comparison, data are expressed as units/cell. The β-galactosidase activity values measured were 32 and 59 units/ml for HBF and Psg0 respectively. β-galactosidase activity in the host strains lacking the plasmids or carrying the pLAFR6-*lacZ* construct without the *avrB* promoter fragment was undetectable (T.V.Huynh *et al.*, submitted; our data, not shown). The host strains lacking the plasmids had no detectable ice nucleation activity. The pLARF6-*inaZ* construct lacking the *avrB* promoter fragment gave 4–5 orders of magnitude lower ice nucleation activity in HBF and Psg0 strains respectively.

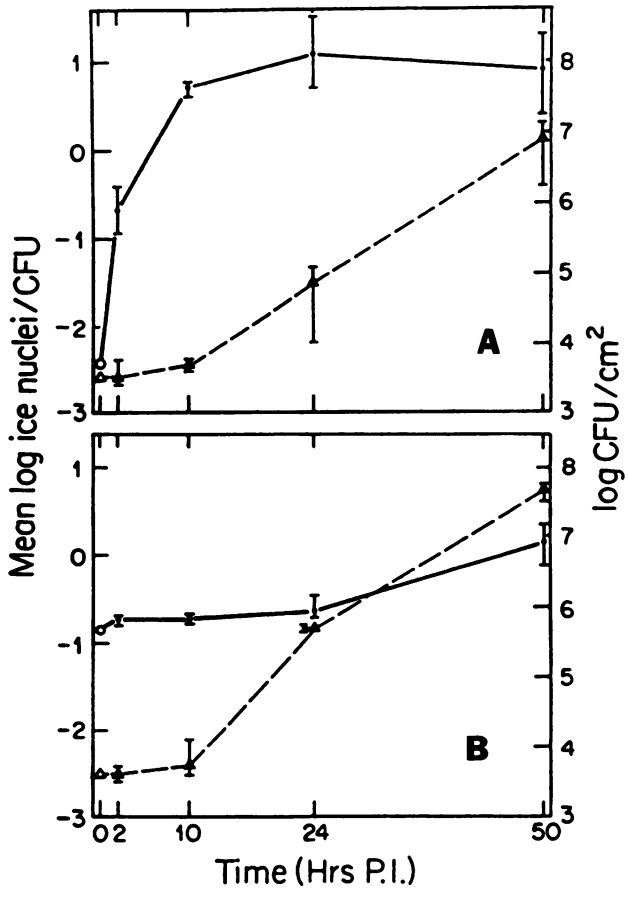


Fig. 7. Time-course of induction of INA (continuous line) and bacterial multiplication (dashed line) for *P.s.phaseolicola* NPS3121(pPL11-Tn*3*-Spice 12) (**A**) and NPS3121(pICE1) (**B**) after inoculation onto Red Kidney bean leaves. The inoculum contained ~10^5 c.f.u./ml, which corresponds to ~5×10^2 c.f.u./cm^2 leaf area. Each sampling point is the average of three replicates. Vertical bars show actual minimum and maximum values. Zero time values represent the *in vitro* INA of each strain.

by inoculating bean leaves with strain NPS3121 carrying a highly inducible pPL11:Spice insertion plasmid. Activation of *inaZ* expression in this strain was very rapid (Figure 7A).

Table IV. Complementation analysis of the inducible region

Plasmids[a]	Genomic mutants (Hrp$^-$)			
	MEX50[b]	MEX12	4002[c]	MEX57
38	+[d]	+	+	+
62	+	+	+	+
50	–	–	–	–
12	–	–	–	–
51	–	–	–	–
57	–	–	–	–
7	+	+	+	+
5	+	+	+	+

[a]All plasmids are Tn*3*-Spice insertion derivatives of pPL11, designated as in Table II.
[b]MEX designations stand for marker exchange mutants that correspond to the Tn*3*-Spice insertion plasmids with the same numerical notation. All MEX mutants were phenotypically Hrp$^-$.
[c]NPS4002 is a genomic Hrp$^-$ Tn*5* insertion mutant (Lindgren *et al.*, 1986).
[d]+, – refer to ability and inability of the plasmids to complement the Hrp$^-$ phenotype of the mutants.

Ice nucleation activity increased 60-fold over the *in vitro* level within 2 h after inoculation. This is long before the bacteria began to multiply in the bean leaves (Figure 7A). By 10 h, the ice nucleation activity was several-hundred-fold above the activity in culture. A high level of ice nucleation activity was maintained during the next 40 h. By contrast, ice nucleation activity of NPS3121(pICE1) remained essentially constant and equal to the *in vitro* activity throughout the first 24 h after inoculation and increased only slightly thereafter (Figure 7B). This increase may reflect the greater metabolic activity of the actively dividing cells at later stages after inoculation.

Genetic characterization of the highly inducible hrp region

The highly inducible region in pPL11 was examined genetically in two respects. First, complementation analysis was carried out to determine whether the target locus was in fact involved in the expression of the Hrp phenotype (Table IV). Eight Spice insertion plasmids were introduced into mutant NPS4002 (whose Tn*5* insertion maps in this region) (Figure 5) and the merodiploid strains were tested for their pathogenicity on bean and ability to elicit HR on tobacco. All plasmids carrying highly inducible insertions failed to complement this mutant in regard to either phenotype. In addition to the *trans* complementation tests with the mutant NPS4002, three pPL11:Tn*3*-Spice insertions which defined the inducible region were marker exchanged into the chromosome of the wild-type strain NPS3121 (MEX mutants). All three marker exchange mutants were Hrp$^-$ when assayed on bean and tobacco plants. Eight pPL11::Tn*3*-Spice plasmids were introduced into the three MEX mutants to give a more detailed complementation analysis of the region (see Table IV). The left boundary of the inducible region appears to lie between insertions 50 and 62 and the right boundary between insertions 57 and 7.

Discussion

We describe the construction of a new reporter gene system and its utility for studying bacterial gene expression during plant pathogenesis. The system is based on the use of an

ice nucleation gene devoid of its native promoter placed near the left inverted repeat of transposon Tn*3*. The transposable elements T*3*-Ice and Tn*3*-Spice allow for the construction of target gene−*inaZ* fusions by insertional mutagenesis. Because the carrier replicon, pMB8, is *polA* dependent, the target plasmid to be mutagenized must have *polA*-independent replication to allow for the isolation of target plasmid::Tn*3*-Spice derivatives by direct selection in *polA* strains. Like Tn*3*-HoHo1, these transposons do not encode an active Tn*3* transposase (i.e. the *tnp*A gene product). This function is provided in *trans* by the plasmid pSShe, which is *polA* dependent and thus not inherited in strain C2110 or in non-enteric hosts where the mutant plasmids may subsequently be transferred. This is a useful feature in the experimental use of these transposons, both in preventing the occurrence of secondary insertions in the recipient strains and in providing biological containment of the transposons.

The nucleotide sequence 5′ of *inaZ* in Tn*3*-Spice and Tn*3*-Ice contains three translational stop codons in phase with the *inaZ* initiation codon (Figure 4). Thus, these transposons should normally generate transcriptional but not translational fusions. This potentially simplifies the interpretation of *inaZ* fusion data since N-terminal extension of the *inaZ* protein may affect ice nucleus stability of activity. We note that a potential −10 'consensus' sequence occurs upstream of *inaZ* (nucleotides 64−69) and has the proper spacing (8 nucleotides) from an ATG codon (nucleotides 78−81) upstream of the *inaZ* reading frame (Figure 3). However, there is no recognizable −35 consensus sequence present upstream of the −10 sequence. Furthermore, the acetosyringone-dependent ice nucleation activity of *virB*::*inaZ* fusions and the occurrence of very low and very high activity *hrp*::*inaZ* fusions (Tables I and II and other data not shown) suggest that the above region does not function as a promoter in *A.tumefaciens*, *P.s.phaseolicola* or *E.coli*.

The most useful feature of the ice nucleation reporter system for measuring gene expression is its sensitivity. Comparison between ice nucleation and β-galactosidase activities expressed by strains carrying *inaZ* and *lacZ* fused to the same promoter suggest at least five orders of magnitude greater sensitivity for *inaZ*. This sensitivity derives from the fact that fewer InaZ protein molecules than β-galactosidase molecules are needed to generate a measurable signal. Based on the mol. wt of ice nucleation proteins (118−120 kd, predicted from sequence data; see Warren, 1987 and references cited therein) and the *in situ* size of ice nuclei active at −9°C (620 kd, determined by γ-ray inactivation; Govindarajan and Lindow, 1988), we calculate an upper value of five protein monomers/nucleus for nuclei active at −9°C. Since the number of monomers needed to generate an active β-galactosidase enzyme is four, the InaZ protein is not inherently more active than β-galactosidase. However, as many as 10^{11} molecules of β-galactosidase would be needed to generate an easily measurable signal by ONPG hydrolysis (A_{420} = 0.1; 50 units; Carni *et al.*, 1987), although levels as low as 1 unit (i.e. 2×10^9 molecules) can also be detected (Weinstock *et al.*, 1983). Based on our calculations, the sensitivity of ice nucleation reporter assays appears to be at least 10^5-fold greater than β-galactosidase. This is comparable to the difference in sensitivity between bacterial luciferase and β-galactosidase (Carni *et al.*, 1987).

The *in situ* size of bacterial ice nucleation sites active at different temperatures has been measured by γ-ray inactivation experiments (Govindarajan and Lindow, 1988). The estimated size of ice nuclei varied from 620 kd for nuclei that are active at −9°C to 19 000 kd for nuclei that are active at −2°C. Chemical cross-linking experiments (A.Govindarajan and S.E.Lindow, unpublished) further suggest that ice protein molecules mostly have other such molecules as their nearest neighbors. These data and the markedly non-linear relationship between InaZ protein content and ice nucleation activity determined in this study and also reported by others (Southworth *et al.*, 1988; see below) suggest co-operativity between ice protein monomers during ice nucleus assemby. Such co-operative interactions are also in line with proposed models for ice nucleus conformation (Warren *et al.*, 1986; Warren, 1987).

Southworth *et al.* (1988) reported a non-linear relationship between InaZ protein content and ice nucleation frequency in *E.coli* expressing *inaZ* from promoters of different strengths. Their study covered a broader range of ice nucleation activities than ours and several different assay temperatures (from −3 to −11°C), although only a few experimental points fell in the range of values encountered in our study. The authors calculate slightly higher slopes (from double logarithmic plots) for the concentration−activity relationships at −9°C in the activity range encountered in our study and a slope very close to 2 for activites almost two orders of magnitude lower than ours. As they point out, the different slopes in different parts of these plots could be an artefact of relatively minor errors in the estimation of protein concentrations, which would also apply to our data. It may be significant that when their data for the −9°C activity in *E.coli* were plotted together with ours for *P.s.phaseolicola*, the straight lines obtained in the double logarithmic plot were superimposable (not shown). It appears likely that a square power relationship, or a relationship very close to it, may apply over a wide range of ice nucleation activity values measured at −9°C (from $\sim 10^{-7}$ to ~0.4 nuclei/cell) and in more than one bacterial host. However, a different (possibly linear) relationship may apply to values approaching one nucleus/cell where a high proportion of cells are likely to contain more than one active nucleus (Southworth *et al.*, 1988).

From a practical point of view it is desirable to relate ice nucleation activity data to target gene transcription. Although our study did not address this point directly, the data of Southworth *et al.* (1988) showed that the amount of InaZ protein does correlate with promoter strength in *E.coli*. The non-linear concentration−activity relationship between ice protein content and ice nucleation activity makes the interpretation of ice nucleation frequency data in terms of promoter strength or gene transcriptional activity less straightforward than with conventional reporters. However, the square power relationship discussed above provides a basis for comparing gene expression levels for a given fusion in different situations (e.g. gene induction) over a wide range of values. Whether this relationship applies to bacterial hosts other than *P.s.phaseolicola* and *E.coli* is not certain.

Because of the non-linearity, the *inaZ* reporter is particularly sensitive to various perturbations, such as vector promoter effects upon cloned genes, vector copy number, transcriptional termination efficiency, degree of polarity within operons, physiological state of the cells, generation of artificial promoters at fusion points, stability and

translatability of mRNA and cellular turnover of the ice nucleation protein. These perturbations constitute an 'aggregate noise' of the system which is likely to differ in each situation. In our experience, the use of single-copy constructs (marker exchange Tn*3*-Spice mutants) substantially eliminates the noise encountered with pPL11::Tn*3*-Spice plasmids (L.G.Rahme, M.N.Mindrinos and N.J. Panopoulos, in preparation). Another limitation of the system is the effect of growth temperature on ice nucleation activities (Lindow, 1982). Growth at 24−25°C has been shown to optimize the expression of the ice nucleation activity of most wild-type ice nucleating bacterial strains (Lindow *et al.*, 1982) and the same holds true for *E.coli* (Orser *et al.*, 1985) and other naturally non-ice nucleating species expressing ice nucleation genes (our unpublished data).

If ice nucleation sites are cell bound, as is the case with *P.syringae* strains (Maki *et al.*, 1974), the ice nucleation activity values measured in intact cells should not normally exceed one ice nucleus/cell. However, values several-fold higher than this were obtained with the highly inducible *hrp*::Tn*3*-Spice insertions *in planta* (Table II, Figure 7A). A likely reason for this is plasmid loss during bacterial multiplication in the leaves since the pLAFR3 replicon is not very stable in *P.s.phaseolicola* in Tc-free medium. Only Tc^r cells were counted in our study and ice nucleation sites may persist for some time after the cells have lost the plasmid. Another possible explanation is the release of ice nuclei in membranous vesicles shed by the cells, as reported for some strains of *Erwinia herbicola* (Phelps *et al.*, 1986). Finally, although cell death is not normally expected to occur on susceptible host plants in early stages of bacterial infection, it is an untested possibility and may be encounterd in other situations (e.g. in the hypersensitive response).

The *inaZ* 'reporter' system may be generally useful for gene expression studies in bacteria that are devoid of endogenous ice nucleation activity (phenotypically Ice^-), which is true of most prokaryotes. With naturally occurring Ice^+ strains, mutational inactivation or removal of the endogenous gene would be necessary. Furthermore, ice nuclei must be capable of being assembled in the host bacterium once it acquires the ability to synthesize the ice nucleation protein by gene transfer. These nuclei must be active at temperatures at which spontaneous freezing of water droplets is negligible (e.g. above −12°C for 10-μl droplets) and which can be easily provided by cooling baths. Most Gram-negative species should be capable of forming such nuclei, since naturally occurring Ice^+ strains occur in diverse taxonomic groups, e.g. *P.syringae, Pseudomonas fluorescens, Xanthomonas translucens, E.herbicola* (Lindow, 1983). In addition, *E.coli, P.s.phaseolicola, P.s.glycinea, Xanthomonas campestris* pv. *versicatoria, Rhizobium* and *Agrobacterium*, which are naturally INA^-, express ice nucleation activity readily upon transfer of cloned ice nucleation genes (Green and Warren, 1985; Orser *et al.*, 1983, 1985; this study and B.J.Staskawicz, unpublished). However, different threshold nucleation temperatures and nucleation frequencies are found among natural Ice^+ strains and such differences are observed in pICE1 transconjugants of *P.s.phaseolicola*, *A.tumefaciens* and *R.meliloti*, suggesting that quantitative variations in expression and, thus, in sensitivity will occur when the *inaZ* system is used in different genetic backgrounds.

Expression of ice nucleation activity becomes less frequent in Ice^+ strains as the assay temperature is increased (Figure 1). Appropriate assay temperatures (where ice nucleation activity does not change greatly with temperature) can be selected to ensure that the number of ice nuclei expressed is lower than the number of independently sorting particles on which they reside. The relative abundance of ice nuclei expressed at assay temperatures below −9°C is apparently constant in the bacterial species used in our study (Figure 1) and the same is true of *E.coli* (Orser *et al.*, 1983, 1985) and other naturally ice nucleating species (Lindow, 1982). This feature also suggests a way to minimize possible effects of salt and other solutes on ice nucleation. In our experience, these effects are very small: concentrations of 0.3 M NaCl or 3% sucrose reduced ice nucleation frequency at −9°C 2- to 3-fold, but did not have a significant effect at −11°C (data not shown). At 0.03 M NaCl or 0.5% sucrose, the ice nucleation frequency of a given culture was the same as in distilled water.

The preliminary analysis of the *hrp* region from *P.s.phaseolicola* presented here allowed us to define a highly inducible region that is involved in the expression of the Hrp phenotype and to determine its transcriptional orientation. Several insertions to the left of this region also gave higher nucleation activities *in planta* than in culture, indicating that another inducible locus(i) might be found in the *hrp* cluster. The apparent presence of a repressive locus(i) and, possibly, of other inducible loci that may be transcribed in the leftward direction or occur in sparsely mutagenized parts of the *hrp* cluster is presently under investigation.

The time-course data indicate that the inducible region was activated very soon (within 2 h) after inoculation and was maximally expressed by the time bacteria had begun to multiply in susceptible plants. This induction time is much earlier than any reported event associated with infection by *P.s.phaseolicola*. Rapid activation was also observed in non-hosts (M.N.Mindrinos and N.J.Panopoulos, unpublished; T.V.Huynh *et al.*, in preparation), as would be expected from the short induction time reported for hypersensitive necrosis in various heterologous plant−bacterium combinations (Klement *et al.*, 1982).

Plant-induced genes have been reported in several other phytopathogenic bacteria (e.g. Stachel *et al.*, 1985b; Osburn *et al.*, 1987) and in plant symbiotic *Rhizobium* (e.g. Downie and Johnston, 1986). Specific plant-derived chemical signals mediate the induction of the *Agrobacterium vir* and *Rhizobium nod* genes (Stachel *et al.*, 1985b; see Downie and Johnston, 1986). In our study acetosyringone did not exert any effect on the ice nucleation activity of *P.s.phaseolicola* carrying inducible *hrp*::*inaZ* fusions (data not shown). Recent studies suggest that the level of expression of this *hrp* locus is influenced by the nutritional status of the cells (L.G.Rahme, M.N.Mindrinos and N.J.Panopoulos, in progress), as in the case of the *avrB* gene of *P.s.glycinea* T.V.Huynh *et al.*, submitted).

Materials and methods

Bacterial strains

Bacterial strains, plasmids and their relevant characteristics are designated in Table V.

Culture media and techniques

Agrobacterium tumefaciens, *R.meliloti* and *P.s.phaseolicola* were routinely grown in King's medium B (KB) (King *et al.*, 1954) at 30°C and *E.coli*

Table V. Bacteria and plasmids

Strain or plasmid	Relevant characteristic[a]	Reference or source
E.coli		
HB101	*rpsL20* (Sm^r)	Boyer and Roulland-Dussoix (1969)
C2110	Nal^r *polA*	Stachel *et al.* (1985a)
A.tumefaciens		
C58	Rif^r	B.J.Staskawicz
B.meliloti		
RM1021	Rif^r	Sharon Long
P.syringae		
Cit7	Rif^r, Ice^+	Orser *et al.* (1985)
P.s.glycinea		
Psg0	Rif^r	B.J.Staskawicz
P.s.phaseolicola		
HBF	Race 2	N.J.Panopoulos
NPS3121	Rif^r, race 2	Peet *et al.* (1986)
NPS4002	*hrp*::Tn*5*, mutant of NPS3121	Lindgren *et al.* (1986)
Plasmids		
pHoHo1	Ap^r *lacZYA*, pMB8 replicon	Stachel *et al.* (1985a)
pSShe	$tnpA^+$, pACYC184 replicon; Cm^r	Stachel *et al.* (1985a)
pMSW10	pKK223.3-*inaZ*, Ap^r	Wolber *et al.* (1986)
pUC8::Omega	pUC8 with Omega fragment (Prentki and Krish, 1984); Sp^r Sm^r Ap^r	G.Warren
pRK2013	IncP TraRK2$^+$△*rep* RK2 *rep*E1$^+$$Km^r$	Ditta *et al.*, 1980)
pPL6	pLAFR3 containing the *hrp* cluster of NPS3121; Tc^r	Lindgren *et al.* (1986)
pWB5A	pRK290 with πVX polylinker (Maniatis *et al.*, 1982); Tc^r	B.Buikema and F.M.Ausubel
pPL11	pWB5A carrying the 19 kb *Bam*HI−*Hin*dIII fragment from pPL6; Tc^r	This study
pICE1	pLAFR1 cosmid containing *iceC* from Cit7; Tc^r	Orser *et al.* (1985)
pVCK257	pVCK102 containing the *vir* region from pTiA6; Km^r	Stachel and Zambryski (1986)
pTn*3*-Ice	$inaZ^+$; Ap^r	This study
pTn*3*-Spice	$inaZ^+$; Ap^r Sp^r Sm^r	This study
p*avrB'-lacZ*	pRK290 derivative carrying an *avrB* promoter-*lacZ* fusion; Tc^r	T.V.Huynh *et al.* (submitted)
p*avrB'-inaZ*	pRK290 derivative carrying an *avrB* promoter-*inaZ* fusion; Tc^r	T.V.Huynh *et al.* (submitted)

[a]Ap^r, Cm^r, Sp^r, Sm^r, Km^r, Tc^r, Rif^r: resistant to ampicillin, chloramphenicol, spectinomycin, streptomycin, kanamycin, tetracycline, and rifampicin respectively.

on either Luria (Miller, 1972) or KB at 30°C or 37°C, except as otherwise stated. The concentrations of antibiotics (μg/ml) used in genetic selections were: rifampicin (Rif), 100; tetracycline (Tc), 15; streptomycin (Sm), 20; ampicillin (Ap), 50; kanamcyin (Km), 30; spectinomycin (Sp), 20; nalidixic acid (Nal), 50; and chloramphenicol (Cm), 20. In studies of the induction of the *virB* region of *A.tumefaciens*, acetosyringone (Aldrich Chemical) was incorporated into KB at a concentration of 400 μM.

Construction of Tn3-Spice

The plasmids pHoHo1 and pMSW10 have been described previously (Stachel *et al.*, 1985a; Wolber *et al.*, 1986). Plasmid pHoHo1 was digested to completion with *Eco*RI and treated with alkaline phosphatase. The 3.8 kb *Eco*RI fragment containing the promoterless *inaZ* gene was isolated after *Eco*RI digestion of pMSW10, electrophoresis and extraction from low-melting agarose. The fragment was then joined by T4 DNA ligase to pHoHoI DNA above to make pTn*3*-Ice. Plasmid pUC8::Omega carries a 2.0 kb *Bam*HI fragment (Omega) specifying Sp/Sm resistance (Prentki and Krisch, 1984) cloned into the *Bam*HI site of pUC8. After digestion with *Bam*HI and incubation with DNA polymerase (Klenow fragment) in the presence of dATP, dTTP, dCTP and dGTP, the 2.0 kb Omega fragment was recovered from low-melting agarose. The pTn*3*-Ice was digested to completion with *Cla*I, the restriction site filled in with Klenow polymerase in the presence of dCTP and dGTP, and ligated to the Omega fragment. The *Bam*HI sites were regenerated in this process but the *Cla*I site was destroyed. The resulting plasmid was designated pTn*3*-Splice.

Construction of plasmid pPL11

Plasmid pPL6 (Lindgren *et al.*, 1986) was digested to completion with the restriction endonucleases *Bam*HI and *Hin*dIII and the *Bam*HI−*Hin*dIII fragment (~19 kb) was isolated from low-melting agarose. Plasmid pWB5a was digested to completion with *Bam*HI and *Hin*dIII, treated with alkaline phosphatase and ligated to the 19 kb *Bam*HI−*Hin*dIII fragment above to give plasmid pPL11.

Genetic techniques

The *E.coli* host for all plasmid constructions was HB101. For insertional mutagenesis, the target plasmids were transformed into HB101 containing the transposon-donor plasmids and pSShe, which carries the Tn*3* transposase gene (*tnpA*, Stachel *et al.*, 1985a). Plasmids pVCK257 and pPL11 contain the *virB* region of *A.tumefaciens* and the *hrp* region of *P.s.phaseolicola* respectively (Lindgren *et al.*, 1986; Stachel and Nester, 1986). Transformants of the transposon donor strain, HB101(pTn*3*-Spice, pSShe), carrying these plasmids were mated with the conjugational helper HB101(pRK2013) and C2110, the selected recipient. The mating mixture was plated on Luria agar containing Nal, Sp, and Km or Tc, to select for C2110(pVCK257::Tn*3*-Spice) or C2110(pPL11::Tn*3*-Spice) transconjugants respectively. Plasmid DNA was isolated as described in Ish-Horowicz and Burke (1981) and the position and orientation of the transposon within the target sequences was mapped by single and double digestions with various restriction endonucleases. The techniques for marker exchange mutagenesis and merodiploid construction have been detailed elsewhere (Lindgren *et al.*, 1988). Other recombinant DNA techniques were according to Maniatis *et al.* (1982).

Measurement of ice nucleation activity

Ice nucleation activity of bacterial suspensions was routinely quantified at −9°C by a droplet freezing technique similar to that described previously (Lindow *et al.*, 1982). Bacterial strains were grown for 48 h at 24°C on

KB agar medium, supplemented with the appropriate antibiotic to minimize plasmid loss. Bacteria were suspended in 0.1 M potassium phosphate buffer, pH 7.4, containing 0.1% Bacto peptone and the cell concentrations were determined from turbidity measurements (OD_{600}). Ten-fold serial dilutions were made in phosphate buffer and 40 10-μl droplets of each dilution were placed on an aluminium foil 'boat' which was floating on a circulating alcohol bath maintained at −9°C. The aluminium foil was pre-coated with paraffin by spraying with a 3% solution of paraffin in xylene, and heating at 65°C in an oven to remove the solvent. By increasing the surface tension, the paraffin coat forces the droplets to assume a round shape and refractile appearance. After freezing, the droplets become opaque and slightly distorted in shape, and thus are easily distinguished from unfrozen ones. Individual droplets freeze within a few seconds once ice formation within them is initiated. The final count of frozen droplets is generally done after 3−5 min since few, if any, droplets freeze thereafter. If desired, the assay may be repeated at the same or at a different temperature by removing the foil, placing it for 1−2 min on a bench surface to allow the droplets to thaw and re-floating it on the alcohol bath. The fraction, f, of frozen droplets was recorded 3 min after the transfer of the last droplets to the aluminium foil. The ice nucleation activity of cultures, N, at a given temperature, T, was calculated according to Vali (1971) from the volume, V, of droplets (in ml), and the number, D, of 10-fold serial dilutions represented by a given sample as:

$$N(T) = \ln \frac{1}{(1-f)} \cdot \frac{10^D}{V}$$

The number of ice nuclei was normalized for the cell concentration, C, in each sample, to obtain the nucleation frequency, which was calculated as:

$$\frac{N(T)}{C}$$

Ice nucleation activity spectra of bacterial suspensions

The ice nucleation of bacterial suspensions at different assay temperatures, was determined by the procedure described by Lindow *et al.* (1982). Bacterial strains containing pICE1, pVCK257::Tn*3*-Spice or pPL11::Tn*3*-Spice derivatives were grown, harvested and suspended in buffer as above. The top of a hollow aluminium block was the temperature-controlled working surface. The block was surrounded with styrofoam for insulation. The working surface was coated with paraffin by spraying with a 3% solution of paraffin in xylene, and the solvent removed by circulating warm water (65°C) through the block. Forty 10-μl droplets of each test suspension were placed on the working surface. The temperature of the block was decreased at ~0.2°C/min by circulating refrigerated ethanol through the block. The temperature of the block was measured continuously with a thermodiode and the freezing temperature of each drop determined visually.

Plant inoculations

Pathogenicity and hypersensitivity tests were conducted as described in Lindgren *et al.* (1988). For *in planta* induction experiments, bacterial suspensions (~5 × 10^5 c.f.u./ml) were infiltrated under vacuum into the intercellular spaces of the primary leaves of 9−12 day old 'Red Kidney' bean plants, which were transferred to a growth chamber at 24°C, with a 14 h light/dark cycle, Leaf disks (11 mm diameter) were periodically removed and ground in 5.0 ml (final volume) of 0.1 M potassium phosphate buffer. Ice nucleation activity was determined following serial dilution (10-fold) in the same buffer and colony forming units (c.f.u.) were determined by plating on Tc-supplemented KB agar plates.

Immunochemical detection of inaZ protein

Antibodies to an *iceC−lacZ* fusion protein were prepared and affinity purified as described elsewhere (A.Govindarajan *et al.*, in preparation). Immunoblotting (Western blot) was carried out essentially as described by Burnette (1981). SDS−PAGE of the total cell lysates (100 μg protein/lane) was carried out according to Laemmli (1970) and electrotransferred to nitrocellulose paper. After saturating the free binding sites of nitrocellulose paper with 10 mg/ml BSA or 2% non-fat dry milk in PBS−Tween (0.05%), the antigen was incubated with fusion protein antibodies at 1:1000 dilution and probed with affinity-purified alkaline phosphatase-conjugated goat anti-rabbit IgG (BioRad) as a second antibody. The blot was scanned in a Zeineh soft laser scanning densitometer (SL504XL Model, Biomed Instruments Inc., Fullerton, CA, USA) and the peak area was estimated gravimetrically. The proportionality of estimates of ice protein content was verified using a reference immunoblot prepared with different concentrations of antigen.

Assay of β-galactosidase

β-Galactosidase activity of strains containing *lacZ* was assayed in chloroform 1% SDS-treated bacteria at 28°C, pH 7.0 as described by Miller (1972) except that the buffer was supplemented with 100 μg/ml bovine serum albumin (T.V.Huynh *et al.*, submitted). Reactions were initiated by the addition of 0.2 ml *o*-nitrophenyl-*b*-D-galactopyranoside (ONPG, 4 mg/ml) and activity was calculated from the rate of change in A_{420} (1 cm light path) using the formula: units/ml = 666 × ΔA_{420}/min × 1/(sample volume in ml). According to this relationship, 1 unit of enzyme hydrolyzes 1 nmole of substrate/min (Struck *et al.*, 1985). Enzyme activity calculated according to Miller (1972) (units = [A_{420} − (1.72 × A_{550})] × 1/time (min) × 1/vol (ml) × 1/A_{600}) was 1.4−1.5 times higher than the values obtained from the above formula.

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References

Boyer,H.W. and Roulland-Dussoix,D. (1969) *J. Mol. Biol.*, **41**, 459−472.
Burnette,W.N. (1981) *Anal. Biochem.*, **112**, 195−203.
Carmi,O.A., Stewart,G.S.A.B., Ulitzur,S. and Kuhn,J. (1987) *J. Bacteriol.*, **169**, 2165−2170.
Ditta,D.W., Stanfield,S., Corbin,D. and Helinski,D.R. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 7347−7351.
Djordjevic,M.A., Gabriel,D.W. and Rolfe,B.G. (1987) *Annu. Rev. Phytopathol.*, **25**, 145−168.
Downie,J.A. and Johnston,A.W.B. (1986) *Cell*, **47**, 153−154.
Green,R.L. and Warren,G.J. (1985) *Nature*, **317**, 645−648.
Govindarajan,A. and Lindow,S.E. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 1334−1338.
Ish-Horowicz,D. and Burke,J.D. (1981) *Nucleic Acids Res.*, **9**, 2989−2998.
King,E.O., Wood,M.K. and Raney,D.E. (1954) *J. Lab. Clin. Med.*, **44**, 301−307.
Klement,Z. (1982) In Mount,M.S. and Lacy,G.H. (eds), *Phytopathogenic Prokaryotes*. Academic Press, New York, Vol. 2, pp. 149−177.
Laemmli,U.K. (1970) *Nature*, **227**, 680−685.
Lindgren,P.B., Peet,R.C. and Panopoulos,N.J. (1986) *J. Bacteriol.*, **168**, 512−522.
Lindgren,P.B., Panopoulos,N.J., Staskawicz,B.J. and Dahlbeck,D. (1988) *Mol. Gen. Genet.*, **211**, 499−506.
Lindow,S.E. (1982) In Mount,M.S. and Lacy,G.H. (eds), *Phytopathogenic Prokaryotes*. Academic Press, New York, Vol. 2, pp. 335−362.
Lindow,S.E. (1983) *Annu. Rev. Phytopathol.*, **21**, 363−384.
Lindow,S.E., Arny,D.C. and Upper,C.D. (1982) *Plant Physiol.*, **70**, 1084−1089.
Maki,L.R., Galyon,E.L., Chang-Chien,M. and Caldwell,D.R. (1974) *Appl. Microbiol.*, **28**, 458−459.
Maniatis,T.E., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
Miller,H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Orser,C.S., Staskawicz,B.J., Loper,J., Panopoulos,N.J., Dahlbeck,D., Lindow,S.E. and Schroth,M.N. (1983) In Puhler,A. (ed.), *Molecular Genetics of the Plant−Bacterial Interaction*. Springer-Verlag, Berlin, pp. 353−361.
Orser,C., Staskawicz,B.J., Panopoulos,N.J., Dahlbeck,D. and Lindow,S.E. (1985) *J. Bacteriol*, **164**, 359−366.
Osburn,A.E., Barber,C.E. and Daniels,M.J. (1987) *EMBO J.*, **6**, 23−28.
Peet,R.C., Lindgren,P.B., Willis,D.K. and Panopoulos,N.J. (1986) *J. Bacteriol.*, **166**, 1096−1105.
Phelps,P., Giddings,T.H., Prochoda,M. and Fall,R. (1986) *J. Bacteriol.*, **167**, 496−502.
Prentki,P. and Krisch,H.M. (1984) *Gene*, **29**, 303−313.

Southworth,M.W., Wolber,P.K. and Warren,G.J. (1988) *J. Biol. Chem.*, **263**, 15211–15216.
Stachel,S.E. and Nester,E.W. (1986) *EMBO J.*, **5**, 1445–1454.
Stachel,S.E. and Zambryski,P.C. (1986) *Cell*, **46**, 325–333.
Stachel,S.E., An,A., Flores,C. and Nester,E. (1985a) *EMBO J.*, **4**, 891–898.
Stachel,S.E., Messens,E., Van Montague,M. and Zambryski,P. (1985b) *Nature*, **318**, 624–629.
Stachel,S.E., Nester,E.W. and Zambryski,P.C. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 379–383.
Struck,D.K., Maratea,D. and Young,R. (1985) *J. Mol. Appl. Genet.*, **3**, 18–25.
Staskawicz,B.J., Dahlbeck,D., Keen,N.T. and Napoli,C. (1987) *J. Bacteriol.*, **169**, 5789–5794.
Tamaki,S., Dahlbeck,D., Staskawicz,B.J. and Keen,N.T. (1988) *J. Bacteriol.*, **170**, 4846–4854.
Vali,G. (1971) *J. Atmos. Sci.*, **28**, 402–406.
Warren,G.J. (1987) *Biotechnol. Genet. Engng. Rev.*, **5**, 107–135.
Warren,G., Gorotto,L. and Wolber,P. (1986) *Nucleic Acids Res.*, **14**, 8047–8060.
Weinstock,G.M., Berman,M.L. and Silhavy,T.J. (1983) In Pappas,T.S., Rosenberg,M. and Chirikjian,J.G. (eds), *Gene Amplification and Analysis, Vol. 3: Expression of Cloned Genes in Prokaryotic and Eukaryotic Cells*. Elsevier, New York, Chapter 2, pp. 27–64.
Wolber,P.K., Deininger,C.A., Southworth,M.W., Vanderkerckhove,J., van Montagu,M. and Warren,G.J. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 7256–7260.

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