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Cloning and Expression of Bacterial Ice Nucleation Genes in *Escherichia coli*

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Epiphytic populations of *Pseudomonas syringae* and *Erwinia herbicola* are important sources of ice nuclei that incite frost damage in agricultural crop plants. We have cloned and characterized DNA segments carrying the genes (*ice*) responsible for the ice-nucleating ability of these bacteria. The *ice* region spanned 3.5 to 4.0 kilobases and was continuous over this region in *P. syringae* Cit7R1. The cloned fragments imparted ice-nucleating activity in *Escherichia coli*. Substantial increases in the nucleating activity of both *E. coli* and *P. syringae* were obtained by subcloning the DNA fragments on multicopy plasmid vectors. Southern blot analysis showed substantial homology between the *ice* regions of *P. syringae* and *E. herbicola*, although individual restriction sites within the *ice* regions differed between the two species.

Water in the liquid state can be supercooled to several degrees below the melting point (0°C) of its solid state, ice. Supercooling temperatures as low as −40°C can be reached with small volumes of pure water before ice nuclei form spontaneously (3). However, in the presence of suitable catalysts (ice nucleation-active substances), the liquid-to-solid phase transition (freezing) will occur at temperatures only slightly below 0°C. Such catalysts may be ice crystals themselves or a variety of organic or inorganic heterogeneous ice nucleation agents (24, 28, 29). Certain bacterial species produce ice nucleation-active substances with relatively high threshold nucleation temperatures (ca. −1.5 to −1.8°C). These bacteria include several pathovars (7) of *Pseudomonas syringae* van Hall and certain strains of *Erwinia herbicola* (Lohnis) Dye, *Pseudomonas fluorescens*, and *Xanthomonas campestris* pathovar *translucens* (Migula) (1, 9, 15, 19, 21, 22, 32; D. Sands, S. E. Lindow, and C. S. Orser, submitted for publication). In the presence of these ice nucleation-active bacteria, plant tissues cannot reach their normal supercooling point (usually −6 to −8°C) and thus cannot avoid freezing injury (5, 11, 12, 15, 16, 19). Because these bacterial species are common plant epiphytes (18), they are important incitants of frost injury in a variety of agricultural crops at relatively warm subzero temperatures (0 to −6°C) (16, 18–20, 32). The role of bacterial ice nuclei in plant frost injury has recently been reviewed (15).

The ice nucleation-active substances associated with these bacteria have been studied in several laboratories, but their exact composition is still unclear. Nucleation activity is nondiffusible and associated with the bacterial cell itself. The ice nucleation-active substance of *P. syringae* is proteinaceous and appears to be localized in the outer cell membrane (M. I. Sprang and S. E. Lindow, Phytopathology **71**:256, 1981; M. I. Sprang and S. E. Lindow, manuscript in preparation). The ice nucleation site(s) of *P. syringae* and *E. herbicola* apparently also contains carbohydrates (14) and lipids (A. G. Govindarajan and S. E. Lindow, Plant Physiol. **75**:43, 1984). The minimum size of these nucleation sites is approximately 140 kilodaltons (kDa) as determined from gamma radiation inactivation kinetics (Govindarajan and Lindow, Plant Physiol. **75**:94, 1984). Genetic analysis of the ice nucleation property was initiated previously (S. E. Lindow and B. J. Staskawicz, Phytopathology **71**:237, 1981), but the mutants previously obtained were not biochemically characterized. The cloning and molecular characterization of DNA segments containing ice nucleation genes from *P. syringae* and *E. herbicola* are described here.

(Preliminary accounts of this work have appeared previously [25a; C. S. Orser, B. J. Staskawicz, N. J. Panopoulos, and S. E. Lindow, Phytopathology **72**:1000, 1982]).

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used are described in Table 1. Since all *P. syringae* strains used in this study were nonpathogenic on all indicator plants tested, no pathovar designation (7) for this species will be used. The initial cloning and subcloning and other vector constructions are shown in Fig. 1 and described in the text.

Media, antibiotics, and other chemicals. *P. syringae* strains were routinely grown on King B agar (13) or broth at 20°C or as otherwise indicated. *E. herbicola* and *Escherichia coli* strains were routinely grown in Luria broth or Luria agar (25) at 30 and 37°C, respectively, or as stated in the text and the figure legends.

The antibiotics and their concentrations (in micrograms per milliliter) were as follows unless otherwise stated: ampicillin, 100; kanamycin, 20; tetracycline, 15; rifampin, 100; and streptomycin, 15, 50, and 100 (for selection in *P. syringae*, *E. herbicola*, and *E. coli*, respectively). Ampicillin was not used in crosses involving *P. syringae* as the donor because the strains used had high tolerance levels for the antibiotic. All antibiotics were purchased from Sigma Chemical Co., St. Louis, Mo.

Restriction endonucleases, T4 DNA ligase, and DNA polymerase I were purchased from Bethesda Research Laboratories, Gaithersburg, Md., and used as recommended by the supplier. The sources of other chemicals and supplies were: CsCl, Gallard-Schlessinger, Carle Place, N.Y.; X-ray film and ethyl methanesulfonate (EMS), Eastman Kodak, Rochester, N.Y.; [α-^{32}P]ATP Amersham Corp., Arlington Heights, Ill.; agarose (low M_r), Bio-Rad Laboratories, Richmond, Calif.

DNA extraction and fractionation and gel electrophoresis. Small-scale extractions of plasmid DNA were done by the

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TABLE 1. Bacterial strains and plasmids[a]

Strain or plasmid	Genotype or relevant properties	Source or reference
E. coli		
HB101	*recA rpsL pro leu lacY*	23
JM83	Δ(*lacZM15 pro*) *rpsL*	J. Messing (30)
HB102	HB101 *rpoA*	Rif^r derivative of HB101 (this laboratory)
BHB2688	N205 *recA* γ *imm*^{434} *c*Its *b2 red E*am *S*am/γ	10
BHB2690	N205 *recA* γ *imm*^{434} *c*Its *b2 red D*am *S*am/γ	10
P. syringae		
Cit7R1	Ice$^+$	Rif^r derivative of Cit7R1, this laboratory
31R1	Ice$^+$	Rif^r derivative of strain 31R1
E. herbicola		
26SR6-2	Ice$^+$	Str^r derivative of *E. herbicola* 26 (17), this laboratory
Plasmids		
pLAFR1	Tc^r *cos*$^+$ *incP rlx*$^+$ Tra$^-$	8
pBR322	Tc^r *bla*$^+$	23
pBR325	Tc^r *bla*$^+$ *cat*$^+$	
pUC8, pUC9	*bla*$^+$ *lacZ'*	J. Messing (30)
RSF1010	Tra$^-$ Sm^r Tp^r *incQ*	This laboratory
pBPW1::Tn*5*	Tra$^+$ *aph*$^+$ RepTs	This laboratory
pRK2013	Tra$^+$ Δ(*rep*^{RK2}) *rep*$^{+E1}$ *aph*$^+$	6
pRKTV5	pRK2013 *aph*::Tn*7*	This laboratory
λ::Tn*5*	*c*I857 *b221 rex*::Tn*5*	R. Reidel

[a] Genetic symbols for *E. coli* strains and plasmids are standard. Ice$^+$, Ice nucleation-positive phenotype. Resistance to rifampin in *P. syringae* and *E. herbicola* is phenotypically designated Rif^r because the genetic basis of this phenotype in these bacteria has not been established.

method of Birnboim and Doly (4). Preparative isolation of total genomic and plasmid DNAs was accomplished through two cycles of cesium chloride-ethidium bromide density gradient centrifugation (Beckman Ti50 rotor, 12 h, 242, 000 × *g*, or Beckman Ti65 rotor, 6 to 8 h, 400,000 × *g*) in a Beckman L-8 centrifuge at 20°C. Size fractionation of DNA fragments was performed in linear sucrose gradients (5 to 20% in 50 mM Tris buffer, pH 7.5) run for 2 h in a Beckman SW50.1 rotor at 5°C. Agarose gels (0.7%) were run at either 1.2 V/cm for 8 to 10 h or 2 to 3 V/cm for 3 to 4 h in Tris-borate buffer (23). DNA bands were stained either by incorporating ethidium bromide (0.1 mg/ml) into the gel or by soaking the gel in a 0.5-mg/ml ethidium bromide solution for 10 to 15 min (23).

Cosmid cloning. Genomic libraries were constructed with total DNA from *P. syringae* 31R1 and Cit7R1 and from *E. herbicola* 26SR6-2, partially digested with *Eco*RI (0.2 U/μg of DNA for 1 h), and size fractioned (18 to 25-kilobase [kb] fragments). The cosmid vector pLAFR1 (8) was digested to completion with *Eco*RI, ligated to the DNA fragments (1:3 to 1:4 ratio) and packaged in vitro with freeze-thaw and sonication extracts of phage lambda, prepared after heat induction of lysogen strains BHB2688 and BHB2690 as described previously (23). Packaged molecules were transduced into *E. coli* HB101 essentially as described previously (23). Transductants were selected on Luria agar plates supplemented with streptomycin and tetracycline. The colonies were screened for ice nucleation activity (Ice$^+$) after 1 to 2 days of incubation at 30°C by a replica-freezing technique described previously (18). Bacterial colonies were transferred to velvet pads as in routine replica-plating procedures, and the pads were subsequently printed onto sheets of aluminum foil coated with paraffin. The foil sheets were placed on circulating alcohol baths adjusted to −5 or −9°C and sprayed with a fine mist of sterile water. Microdroplets contacting Ice$^+$ colonies froze rapidly under these conditions, and the colonies gained a frosty appearance. Several Ice$^+$ colonies from each library were purified and stored for further characterization.

Tn*5* mutagenesis. Recombinant plasmids in *E. coli* hosts were mutagenized with transposon Tn*5* by infecting maltose (0.2%)-induced cells with phage λ *c*I857 *b*221 *rex*:Tn*5* (λ::Tn*5*) at a ratio of 2:1 and plating on Luria agar supplemented with kanamycin. Plasmid DNA was extracted from Kan^r colonies by the method of Birnboim and Doly (4) and used to transform *E. coli* HB101, selecting for Kan^r. Alternatively, a Tn*5*-containing derivative (N. J. Panopoulos, unpublished data) of the thermosensitive plasmid pBPW1

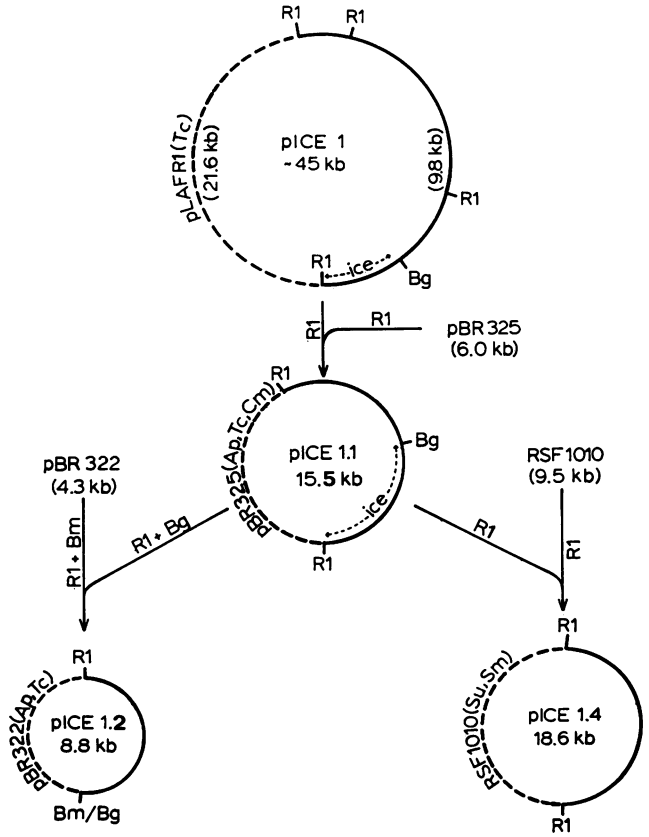

FIG. 1. Pedigrees of some plasmids carrying the *P. syringae* Cit7R1 *ice* region. Abbreviations: S, *Sal*I; R1, *Eco*RI; Bg, *Bgl*II; Bm, *Bam*HI; Tc, tetracycline; Ap, ampicillin; Cm, chloramphenicol; Sm, streptomycin; Su, sulfonamide; Bm/Bg, *Bam*HI-*Bgl*II fusion site. The enzymes used to digest each plasmid are indicated on the arrows. The Ice phenotype of each plasmid is indicated in Fig. 6 or discussed in the text.

(26) was introduced by conjugation and subseqently eliminated from the transconjugants by streaking on Luria agar containing kanamycin and tetracycline and incubating at 42°C. A survivor carrying Tn*5* in its chromosome was then used in triparental matings with two other suitably marked *E. coli* strains, one lacking any plasmids and one carrying pRKTV5, as described below. Selection for cotransfer of a vector marker (e.g., Amp^r) and Kan^r to the recipient gave almost exclusively Tn*5* derivatives of the recombinant plasmid. The Tn*5* insertion sites were mapped by restriction analysis of plasmid DNA extracted as described above.

Restoration of the Ice$^+$ phenotype in Ice$^-$ mutants. *P. syringae* 31R1 cells were treated with EMS to a 90% kill level as described by Miller (25), allowed to grow for 2 h, and plated on King B agar (ca. 100 colonies per plate). Ice nucleation-deficient (Ice$^-$) colonies were identified among the survivors by a replica-freezing technique (18). Recombinant plasmid pICE1.4, conferring ice nucleation properties on *E. coli* HB101, was transferred to Ice$^-$ mutants of *P. syringae* 31R1 by pRK2013-assisted mobilization (6), selecting for Str^r Rif^r. Transconjugants were screened for ice nucleation activity and for the presence of plasmid DNA by agarose gel electrophoresis as described above.

Other techniques. Subcloning of DNA fragments and transformation with plasmid DNA were done by standard procedures (23). Southern blot hybridization on nitrocellulose membranes was done by the method of Wahl et al. (31), with nick-translated DNA from a mixture of the two *Sst*II restriction fragments internal to the cloned *P. syringae* Cit7R1 *ice* DNA insert (see Fig. 3) used as the ^{32}P-labeled probe. Recombinant plasmids were mobilized by triparental matings, with plasmid pRK2013 (6) or its Kan^s derivative pRKTV5 as the conjugation-proficient element. Appropriate antibiotic selections were used according to vector and host resistance markers.

Measurement of ice nucleation activity. Ice nucleation activity of bacterial suspensions was routinely quantified at two temperatures, −5 and −9°C, by a droplet freezing technique (28) described previously (17, 20). Cells were grown for 48 h on nutrient agar supplemented with 25 g of glycerol per liter and 15 μg of tetracycline per ml or 100 μg of ampicillin per ml for strain HB101 carrying Tet^r and Amp^r recombinant plasmids, respectively, or 20 μg of streptomycin per ml for strains carrying pICE1.4. Forty 10-μl droplets of serially diluted bacterial suspensions were placed on paraffin-coated sheets of aluminum foil, which were then transferred to circulating alcohol-water baths adjusted to −5 and −9°C. The nucleation frequencies were expressed as the ratio of the number of cells per ice nucleus or the inverse of this ratio. The ratio of frozen to unfrozen droplets was recorded at 3 min after transfer of the aluminum sheets to the alcohol bath (28). The number of CFU per milliliter was determined from the turbidity of undiluted suspensions based on a CFU versus OD_{600} reference plot. Ice nucleation spectra (nucleation frequencies at different assay temperatures from 0 to −12°C) was determined as described above, except that an ice nucleation spectrometer was used as described previously (19).

RESULTS

Cloning of DNA fragments carrying the *ice* region. Ice$^+$ colonies were readily identified among Tet^r transductants of *E. coli* HB101 obtained with the in vitro-packaged DNA from all three genomic libraries tested by replica freezing at −9°C. All such transductants contained recombinant cosmids having in common a 21.6-kb *Eco*RI fragment (pLAFR1) and one or more additional fragments. Representative of the plasmids isolated from *E. coli* HB101 Ice$^+$ clones were pICE1 and pICE7, originating from genomic libraries of *P. syringae* Cit7R1 and *E. herbicola* 26SR6-2, respectively. The fragments that carried the *ice* genes were subsequently identified by subcloning and Tn*5* insertion analysis and by transfer to Ice$^-$ mutants of *P. syringae* (see below). Southern blot hybridization of total DNA from strain Cit7R1 digested with various restriction enzymes and probed with ^{32}P-labeled DNA from subcloned *ice* inserts (Fig. 2; other data not shown) demonstrated colinearity of the cloned fragment with the chromosomal *ice* region of this strain.

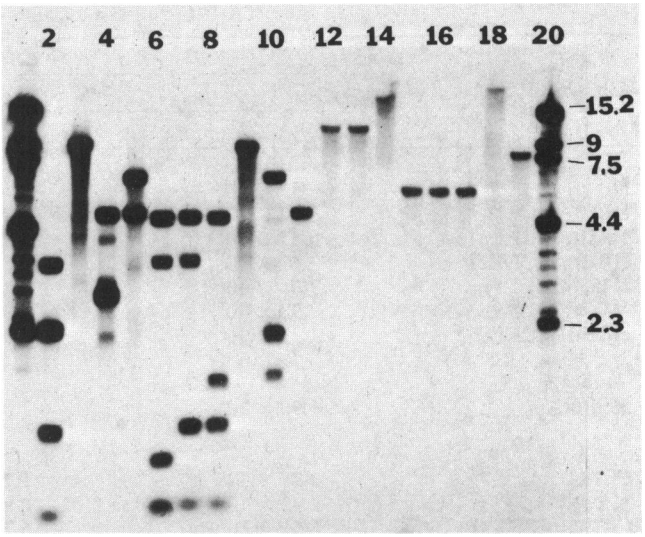

FIG. 2. Southern blot hybridization between ^{32}P-labeled internal *Sst*II *ice* fragments of pICE1.2 and restriction digests of *P. syringae* Cit7R1 (lanes 2 through 10) and *E. herbicola* 26SR6-2 (lanes 11 through 19) (each lane contained 4.0 μg of DNA). Digestion: lanes 10 and 11, *Eco*RI and *Pvu*II; lanes 9 and 12, *Eco*RI; lanes 8 and 13, *Eco*RI and *Sal*I; lanes 7 and 14, *Sal*I; lanes 6 and 15, *Sal*I and *Pvu*II; lanes 5 and 16, *Pvu*II; lanes 4 and 17, *Pvu*II and *Bgl*II; lanes 3 and 18, *Bgl*II; lanes 2 and 19, *Bgl*II and *Sal*I. Lanes 1 and 20, Marker fragments. Sizes are indicated in kilobases. Marker fragments included pICE1.1 and pICE1.21; *Bgl*II-digested pICE1.1 and pICE1.21; *Pvu*II-digested pICE1.2; and 2.3- and 1.2-kb *ice* fragments isolated from *Sst*II-digested pICE1.2.

Location and mapping of the *ice* region. The *ice* region from *P. syringae* Cit7R1 was further characterized by a combination of subcloning, deletion, and Tn*5* insertion analysis. A 9.5-kb *Eco*RI fragment from plasmid pICE1, which was common to all Ice$^+$ cosmids from the Cit7R1 genomic library, was subcloned into the *Eco*RI site of plasmid pBR325 to give plasmid pICE1.1. The ability of *E. coli* HB101(pICE1.1) transformants to nucleate ice suggested that pICE1.1 carried all or part of the *ice* region of *P. syringae* Cit7R1. Based on restriction site information (see below and Fig. 3), pICE1.1 DNA was subsequently digested with *Eco*RI plus *Bgl*II, and the fragments were ligated to plasmid pBR322 which had been digested with *Eco*RI plus *Bam*HI. A plasmid, designated pICE1.2, was obtained which contained a 4.5-kb insert and conferred ice nucleation activity on *E. coli* HB101, suggesting that the *ice* region was contained wholly or in part within this fragment.

Similar experiments were carried out with plasmid pICE7, isolated from the *E. herbicola* library. A 5.8-kb *Eco*RI-*Bgl*II fragment was subcloned from this plasmid into pBR322 and

TABLE 2. Ice nucleation frequencies of bacterial strains

Bacterial strain	Ice nucleation activity[a] (no. of cells/ice nucleus) at indicated temp after growth at:			
	21°C		24°C	
	−5°C	−9°C	−5°C	−9°C
E. coli				
HB101	ND[b]	ND	ND	ND
HB101(pICE1)	1.22×10^{2} *i*	9.96×10^{1} *i*	7.33×10^{5} *f*	6.17×10^{3} *fg*
HB101(pICE1.1)	5.57×10^{1} *jkl*	1.78×10^{1} *jkl*	4.43×10^{3} *h*	4.27×10^{2} *h*
HB101(pICE1.2)	3.67×10^{1} *jklm*	5.83×10^{0} *lmnop*	7.74×10^{2} *i*	4.71×10^{1} *ij*
JM83(pICE1.8)	7.05×10^{1} *jk*	5.49×10^{0} *lmnop*	5.81×10^{3} *h*	4.31×10^{1} *ij*
JM83(pICE1.9)	7.98×10^{1} *jk*	7.93×10^{0} *klmno*	6.95×10^{3} *h*	4.51×10^{1} *ij*
HB101(pICE7)	1.21×10^{5} *g*	8.94×10^{3} *fg*	6.52×10^{6} *cde*	1.00×10^{5} *e*
E. herbicola				
26SR6-2	6.96×10^{4} *g*	7.03×10^{2} *h*	2.28×10^{6} *ef*	1.69×10^{4} *f*
P. syringae				
Cit7R1	2.75×10^{1} *klmn*	1.29×10^{1} *klm*	2.5×10^{1} *klm*	1.10×10^{1} *kl*
31R1	2.42×10^{1} *klmno*	8.99×10^{0} *klmn*	3.01×10^{1} *klm*	9.77×10^{0} *klmn*
Cit7R1(pICE1.4)	6.51×10^{0} *pq*	5.72×10^{0} *lmnop*	4.31×10^{0} *pqr*	1.62×10^{0} *pq*
31R1 (pICE1.4)	6.56×10^{0} *nopqr*	4.29×10^{0} *mnop*		
31R1-12	3.96×10^{8} *s*	7.25×10^{7} *t*		
31R1-12(pICE1.4)	1.52×10^{0} *r*	1.01×10^{0} *q*		
31R1-13	3.41×10^{7} *t*	4.54×10^{6} *cd*		
31R1-13(pICE1.4)	1.37×10^{1} *lmnop*	5.39×10^{0} *lmnop*		
31R1-26	4.41×10^{7} *t*	4.69×10^{6} *cd*		
31R1-26(pICE1.4)	5.83×10^{0} *opqr*	2.63×10^{0} *nopq*		
31R1-28	5.07×10^{8} *s*	5.07×10^{8} *s*		
31R1-28(pICE1.4)	3.50×10^{0} *pqr*	3.54×10^{0} *mnopq*		
31R1-29	1.55×10^{5} *g*	3.43×10^{3} *g*		
31R1-29(pICE1.4)	2.67×10^{0} *qr*	1.79×10^{0} *pq*		
31R1-31	4.67×10^{6} *de*	1.59×10^{6} *d*		
31R1-31(pICE1.4)	3.34×10^{0} *pqr*	2.13×10^{0} *opq*		
31R1-32	2.19×10^{7} *ct*	5.89×10^{6} *c*		
31R1-32(pICE1.4)	2.37×10^{1} *klmno*	1.02×10^{1} *klm*		
31R1-33	6.61×10^{5} *f*	5.85×10^{4} *e*		
31R1-33(pICE1.4)	9.00×10^{0} *mnopq*	3.76×10^{0} *mnop*		
31R1-11	1.52×10^{7} *cdt*	4.28×10^{6} *cd*		
31R1-11(pICE1.4)	4.33×10^{0} *pqr*	3.47×10^{0} *mnopq*		

[a] All values followed by the same superscript letter (*c* through *t*) did not differ significantly ($P = 0.05$) by Duncan's multiple-range test.
[b] ND, Not detected (below detection limit of 10^{9} CFU per ice nucleus. *E. coli* HB101 with no plasmid and with plasmid vectors pLAFR1, pBR325, pBR322, pUC8, pUC9, and RSF1010 had no nucleation activity.

shown to be sufficient to impact an Ice$^+$ phenotype to *E. coli* HB101.

Restriction sites for several endonucleases were identified and mapped within the subcloned *ice* inserts from *P. syringae* Cit7R1 and *E. herbicola* 26SR6-2. The restriction map of *P. syringae* Cit7R1 (Fig. 3) showed little similarity to that of *E. herbicola* 26SR6-2.

Several Tn*5* insertion mutants of plasmid pICE1.2 were isolated after mutagenesis of strain HB101 (pICE1.2) and scoring for the Ice$^-$ phenotype by the replica freezing technique. The sites of Tn*5* insertion were mapped in each mutant by single or double digestions with *Hin*dIII, *Eco*RI, and other suitable enzymes (Fig. 3). All mutants scored as Ice$^-$ had Tn*5* insertions within the 4.5-kb insert. The locations of 15 such insertions are shown in Fig. 3. All mutants having a fully Ice$^+$ phenotype had Tn*5* insertions within 500 base pairs of either end of this cloned 4.5-kb insert. It appears that the *ice* region of *P. syringae* Cit7R1 spans ca. 3.5 to 4.0 kb and is completely contained in this region.

Ice nucleation parameters. The frequency of ice nucleation, threshold nucleation temperature, and ice nucleation spectra were quantified in several *E. coli* Ice$^+$ recombinants and compared with those of the DNA source strains (Table 2). Nucleation frequency (i.e., the fraction of cells in a population containing active ice nucleation sites at any given time) was generally low for both *P. syringae* and *E. herbicola* (less than 10^{-2} and 10^{-5}, respectively, at −5°C), although the values differed greatly between strains. Nucleation frequency is also determined by growth conditions, particularly growth temperature, the growth medium, and the temperature at which nucleation activity is measured (20). The ice nucleation activity of *P. syringae* 31R1 and Cit7R1 was generally highest when cells were grown on nutrient agar medium containing 2.5% glycerol for 2 days at 24°C. *E. herbicola* 26SR6-2 expressed maximum nucleation frequency when grown at temperatures below 15°C. The conditions for maximizing nucleation frequency in *E. coli* strains harboring recombinant plasmids were established in this study for purposes of comparison.

E. coli Ice$^+$ transductants carrying cosmid clones, such as pICE1 or pICE7, expressed ice nucleation activity with frequencies similar to those of the DNA source strains when each was grown at the respective optimum temperature (Table 2; Fig. 4 and 5) and assayed at either −5 or −9°C. However, the optimum growth temperature differed; *P. syringae* Cit7R1, the source strain for pICE1, had highest nucleation activity when grown at 24°C, whereas *E. coli* HB101(pICE1) showed a comparable maximum when grown

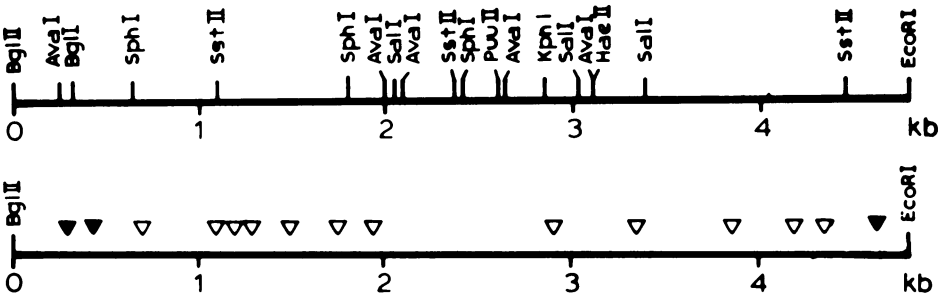

FIG. 3. Restriction site and Tn*5* insertion map of the *ice* regions of *P. syringae* Cit7R1 (from pICE1.2). Ice$^-$ (△) and Ice$^+$ (▲) Tn*5* insertions are indicated.

at 21°C. With *E. coli* HB101(pICE7) and *E. herbicola* 26SR6-2, the source strain for pICE7, the nucleation frequency at −5°C was substantially lower than that of *P. syringae* Cit7R1 and *E. coli* HB101(pICE1). Large reductions in nucleation activity were observed with both *E. coli* HB101(pICE1) and HB101(pICE7), as with their respective DNA source strains, at growth temperatures above and, to a lesser extent, below their respective temperature optima (data not shown). The maximum ice nucleation frequency of these *E. coli* strains at −9°C was slightly higher than those of the DNA source strains when growth temperatures were optimized.

The ice nucleation spectrum and the threshold nucleation temperatures of *E. coli* strains carrying pICE1 and pICE7 were similar to those of the respective DNA source strains (Fig. 4 and 5). The difference in ice nucleation spectra profiles of *P. syringae* and *E. herbicola* were maintained in *E. coli* strains carrying their respective cloned fragments.

Amplification of ice nucleation activity. In view of the low number of cells expressing ice nucleation activity in both the *E. coli* recombinants and the DNA source strains, cloning the *ice* genes in high-copy-number vectors or downstream from plasmid promoters was used to increase their level of expression. The copy number of pLAFR1, although not precisely known, is believed to be low (S. Long, personal communication). Plasmids pICE1.1, carrying a 9.5-kb *ice* insert from strain Cit7R1 cloned into pBR325, and pICE1.2, pICE1.8, and pICE1.9, carrying the 4.5-kb *ice* insert cloned into the high-copy-number vector pBR322, pUC8, or pUC9, respectively (Fig. 6) were introduced into strains HB101 and

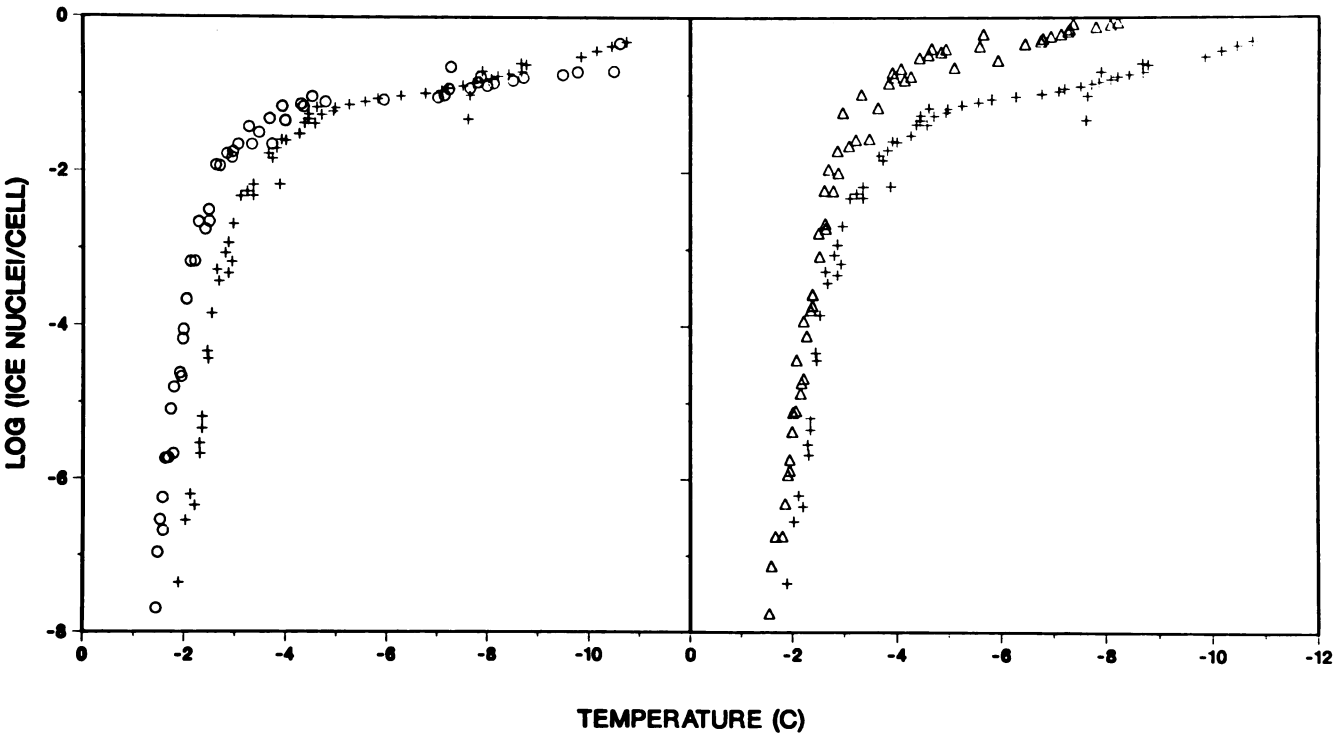

FIG. 4. Ice nucleation of *P. syringae* Cit7R1 (+), *E. coli* HB101(pICE1) (○), and *E. coli* HB101(pICE1.1) (△). Forty 10-μl droplets of appropriate serial dilutions of bacterial cultures were placed on an ice nucleation spectrometer (19). The rate of decrese in temperature, measured with a Digitec Thermistor thermometer, was 0.1°C/min. The freezing of individual droplets was recorded as the temperature decreased. The number of ice nuclei per milliliter of the suspensions was determined as described previously (19, 28). Bacterial growth conditions were as described in the text.

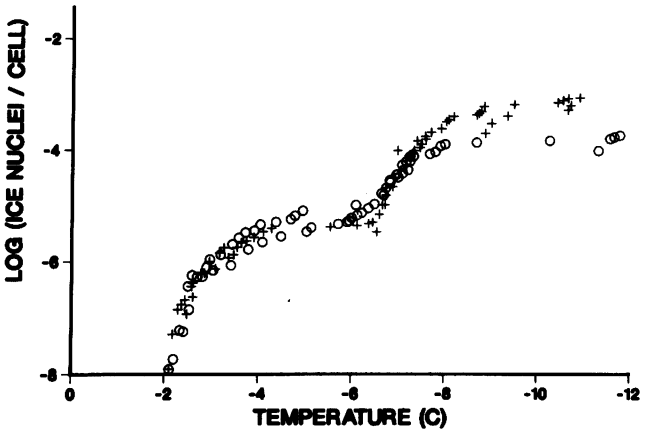

FIG. 5. Ice nucleation spectra of *E. herbicola* 26SR6-2 (+) and *E. coli* HB101(pICE7) (○). Assay and growth conditions were as described in the legend to Fig. 4.

JM83 by transformation. Plasmid pICE1.4, carrying the 9.5-kb insert cloned into the *Eco*RI site of RSF1010, was also introduced into *P. syringae* Cit7R1 by mobilization. The nucleation frequencies of the resulting strains (Table 2) were significantly greater than those of *E. coli* HB101(pICE1). When assayed at −9°C, approximately 20% of all HB101 cells carrying high-copy-number *ice* plasmids were Ice$^+$ (taking into account the presence of dead cells generally produced in *recA* strains). By contrast, the threshold nucleation temperature of strains with cloned ice fragments was similar to those of the DNA source strains (Fig. 4 and 5).

Equivalent nucleation frequencies were measured for strains JM83(pICE1.8) and JM83(pICE1.9) (Table 2). The *Bgl*II-*Eco*RI-*ice* fragment in these plasmids is inserted in opposite orientations downstream from the *lacZ* promoter of pUC8 and pUC9 (30). Furthermore, the nucleation frequency of HB101(pICE1.2) cloned on pBR322 was similar to that of the other strains with *ice* genes cloned on high-copy-number vectors. There are no known promoters on the pBR322 vector located upstream from either end of the *ice* insert in this plasmid (27) (Fig. 6), suggesting that the strain Cit7R1 *ice* genes were maximally expressed from their own promoter(s).

Restoration of the Ice$^+$ phenotype in *P. syringae* Ice$^-$ mutants. To determine whether genes outside the cloned *ice* region were involved in the ice nucleation activity of *P. syringae*, plasmid pICE1.4 was transferred to nine EMS-induced Ice$^-$ mutants of *P. syringae* 31R1. Ice nucleation activity was restored in all cases. The presumptive merodiploids were further shown to contain plasmids identical, by restriction endonuclease criteria, to pICE1.4 (data not shown). The higher nucleation frequencies of all pICE1.4 merodiploid strains compared with the parental strain *P. syringae* 31R1 (Table 2) presumably reflects genetic amplification resulting from the high copy number of the vector, RSF1010. Small quantitative differences in nucleation frequency were observed among the merodiploids (Table 2), but ice nucleation activity measured at −5°C did not differ significantly between strains, and only *P. syringae* 31R1-12 was significantly different from a merodiploid of the parental strain in ice nucleation activity at −9°C.

Homology between *P. syringae* Cit7R1 and *E. herbicola ice* regions. DNA sequences internal to the cloned *P. syringae* Cit7R1 *ice* region hybridized to total genomic DNA from *E. herbicola* (Fig. 2). The intensity of the hybridization signal produced by *E. herbicola* DNA was lower than that observed with DNA from the homologous *P. syringae* strain. The number and location of restriction sites within or near the *ice* region of these two species also differed considerably. DNA conferring the Ice$^+$ phenotype was localized on different-sized *Eco*RI restriction fragments in *P. syringae* and *E. herbicola*. Similarly, the *ice* region in *E. herbicola* did not contain restriction sites for *Sal*I or *Pvu*II, whereas these enzymes produced multiple restriction fragments of the *P. syringae* Cit7R1 *ice* region.

DISCUSSION

The genes responsible for bacterial ice nucleation activity in *P. syringae* and *E. herbicola* are clustered in a small genomic region. Furthermore, they are expressed in *E. coli*, normally devoid of ice nucleation activity, and could be detected in *E. coli* clones without the need to perform complementation tests with Ice$^-$ mutants of the DNA source strain. Data from subcloning and deletion and insertion mutagenesis indicate that the genetic region required for expression of ice nucleation activity in *E. coli* is ca. 3.5 to 4.0 kb long. Thus, the *ice* DNA segment has potential coding capacity for either one very large or more than one average-size polypeptides. Preliminary data from Tn*5* insertion analysis, gamma radiation target size estimates cited earlier, and preliminary studies with maxi- and mini cell labeling experiments (A. G. Govindarajan and S. E. Lindow, Abstr. Annu. Meet. Am. Phytopathol. Soc., abstr. no. 805, 1985) are consistent with the first prediction. The functional organization (number and location of transcripts or cistrons) and translation products of the *ice* region are presently under investigation.

The cloned DNA segment from *P. syringae* Cit7R1 restored all nine EMS-induced Ice$^-$ mutants of *P. syringae* 31R1 to Ice$^+$. The small quantitative differences in restoration of the Ice$^+$ phenotype observed among the pICE1.4 merodiploids of the mutants suggest that genes outside the 9.5-kb region carried by this plasmid may be involved in the expression of ice nucleation activity by whole bacterial cells, but their effect is insignificant compared with that of the cloned *ice* gene(s). Other studies (Sprang and Lindow, Phytopathology **71**:256, 1981; Govindarajan and Lindow, Plant Physiol. **75**:43, 1984) have indicated that membrane fatty acids and other membrane components may be associated with the ice nucleation site(s) or affect its expression as a functional catalytic site, since their removal reduces the ice nucleation activity of bacterial membrane fractions. Alternatively, mutations at other sites may alter the protein or its transport to the membrane, thus indirectly affecting the expression of proteinaceous ice-nucleating components.

Quantitative differences in nucleation frequency among *E. coli* strains carrying different recombinant plasmids reflected differences in vector copy number but were not related to the insert location with respect to plasmid vector promoters. For example, all recombinant plasmids constructed with high-copy-number vectors, regardless of the size of the *ice* DNA insert (e.g., pICE1.1 with a 9.5-kb insert and pICE1.2, pICE1.8, and pICE1.9 with a 4.5-kb insert), gave roughly equal nucleation frequencies in the same host strain (HB101). Furthermore, the orientation of the 4.5-kb insert with respect to the *lacZ* promoter in pUC8 was opposite that in pUC9, but there was little or no difference in nucleation frequency between strains JM83(pICE1.8) and JM83(pICE1.9). In pICE1.2, which had an ice nucleation frequency similar to that of other high-copy-number plasmids, there are no known vector (pBR322) promoters di-

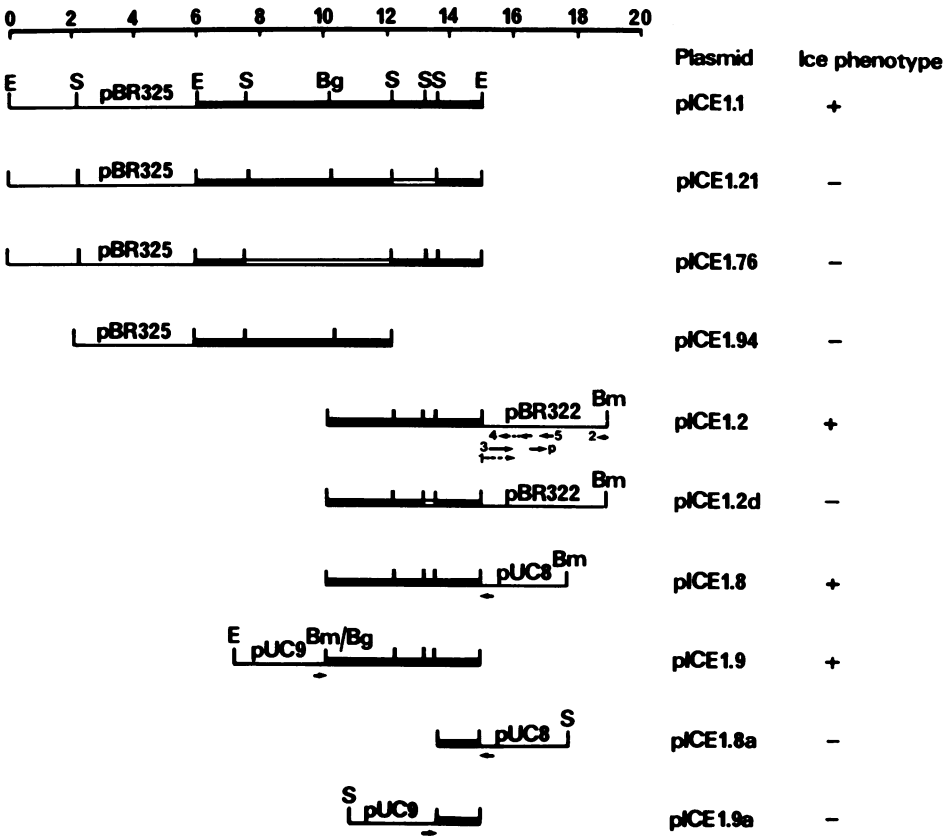

FIG. 6. Deletion and subcloning analysis of the *ice* region of *P. syringae* Cit7R1. Only some restriction endonuclease sites are shown. Each site is designated once in the map of the plasmid in which it appears first, and all vertically aligned sites are the same. All plasmids are shown as linearized molecules aligned at the right-most *Eco*RI end of their *ice* inserts (except pICE1.94, in which it is deleted). Deletions are shown as open boxes and vectors as thin lines. Abbreviations: E, *Eco*RI; S, *Sal*I; Bm, *BamH*I; Bg, *Bgl*II; Bm/Bg, *Bam*HI-*Bgl*II fusion site. Arrows indicate the location, direction, and approximate range of influence (downstream) of various vector promoters discussed in the text, based on published information (27, 30). Promoters 1, 2, 3, 4, 5, and p on pICE1.2 correspond to P1, P2, P3, P4, P5, and Pp, respectively, in pBR322 (27). The lactose promoter (30) on pUC8- and pUC9-derived plasmids is shown by an arrow below each of the maps. Dashed-line arrows indicate the read through activity of the P4 and the P1 and P2 promoters of pBR322, which were assumed to be deleted during the construction of pICE1.2 and pICE1.2d. The deletion in pICE1.94 includes the pBR325 segment between the *Eco*RI and *Sal*I sites in the Tc^r gene.

rected towards the *ice* insert (27). Therefore, the observed levels of ice nucleation activity must reflect expression of the *ice* gene(s) from its own promoter(s). These observations suggest that the 4.5-kb *Eco*RI-*Bgl*II *ice* DNA fragment from *P. syringae* Cit7R1 apparently carries one or more *cis*-acting regulatory elements directing its in vivo expression.

The restriction site maps of *P. syringae* Cit7R1 and 31R1 were similar if not identical (data for 31R1 not shown), but differed substantially from the corresponding map of the *E. herbicola ice* DNA segment (Fig. 3). The ice nucleation spectra curves of *E. coli* HB101 carrying *ice* genes from the two bacteria were also different (Fig. 4 and 5). Nevertheless, there was substantial DNA sequence conservation of the regions determining ice nucleation in these taxonomically distant bacteria, as evidenced by Southern blot hydridization (Fig. 2). The evolutionary origin and selective advantage conferred by the *ice* regions in these two dissimilar bacterial species remain to be determined.

The cloning of ice nucleation genes reported here is potentially significant for many basic and applied investigations. It should greatly assist in isolation and characterization of the elusive ice nucleation proteins and elucidation of the biochemical and biophysical properties of the ice nucleation site(s) on bacterial membranes. It has also permitted the construction of *P. syringae* strains carrying deletions of specific portions of the *ice* region (C. S. Orser, R. Lotstein, E. Lahue, D. K. Willis, N. J. Panopoulos, Phytopathology **74**:798, 1984; C. S. Orser, R. Lotstein, S. E. Lindow, and N. J. Panopoulos, manuscript in preparation). Such strains may be useful as biological control agents of frost injury to agricultural plants.

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