Development of a Gene Reporter System in Moderately Halophilic Bacteria by Employing the Ice Nucleation Gene of *Pseudomonas syringae*

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The expression of the ice nucleation gene *inaZ* of *Pseudomonas syringae* in several moderate halophiles was investigated to establish its utility as a reporter for promoter activity and gene expression studies in these biotechnologically and environmentally important bacteria. A promoterless version of *inaZ* was introduced in two different restriction sites and at both orientations in a recombinant plasmid able to replicate in moderate halophiles and, in particular, within the sequence of its pHE1 part, a native plasmid of *Halomonas elongata*. One orientation of both recombinant constructs expressed high levels of ice nucleation activity in *H. elongata* and *Volcaniella eurihalina* cells, indicating that *inaZ* was probably introduced in the correct orientation downstream of putative native promoters. A recombinant construct carrying a tandem duplication of *inaZ* at the same orientation gave significantly higher ice nucleation activity, showing that *inaZ* is appropriate for gene dosage studies. The ice nucleation gene was also expressed in *H. elongata* and *V. eurihalina* under the control of P_{bla} (the promoter of the β-lactamase gene of *Escherichia coli*) and P_{pdc} (the promoter of the pyruvate decarboxylase gene of *Zymomonas mobilis*). One of the *inaZ* reporter plasmids expressing high levels of ice nucleation activity under the control of a native putative promoter was also transferred in *Halomonas subglaciescola*, *Halomonas meridiana*, *Halomonas halodurans*, and *Deleya halophila*. In all cases, Ice$^+$ transconjugants were successfully isolated, demonstrating that *inaZ* is expressed in a wide spectrum of moderately halophilic species.

Moderately halophilic bacteria constitute a very heterogeneous group of microorganisms which grow best in media containing 3 to 15% NaCl (11). These extremophiles, which play an important ecological role because of their abundance in hypersaline environments (22), have been studied extensively with respect to their taxonomy (26) and physiology (11). Recently, moderate halophiles have gained an increased biotechnological attention, because they are very good sources for halophilic enzymes (i.e., amylases, proteases, and nucleases) (8) as well as protecting agents for both enzymes and whole cells (compatible solutes) (4, 5). Additionally, they are potentially useful in enhanced oil recovery and degradation of industrial residues or toxic chemicals that can pollute hypersaline habitats (27). On the other hand, since they exhibit a wide salt tolerance among prokaryotes, they are excellent tools to study the molecular biology of osmoregulation processes. In spite of these potentialities, knowledge concerning the genetics of moderate halophiles is very limited. For example, studies on the isolation of mutants (10, 17) or the detection of indigenous plasmids (3, 25) are very scarce. Very recently, the first shuttle vector for moderate halophiles, pHS15, was developed from a small cryptic plasmid of *Halomonas elongata* (25). This cloning vector was successfully mobilized from *Escherichia coli* to a number of *Halomonas* strains, showing that genetic transfer between nonhalophilic and moderately halophilic bacteria is possible via conjugation. However, genes of moderate halophiles have not been cloned to date, and other useful tools in molecular biology studies have not yet been developed. These include transformation procedures, transposon mutagenesis, and the use of reporter genes to study gene regulation, protein processing, or protein export. When reporter genes are used, the presence of cellular intrinsic activity leads to interferences in the assay interpretation. In this respect, many bacteria, including some moderate halophiles (6, 19, 20, 28), have intrinsic β-galactosidase or phosphatase activities, two of the most widely used reporter systems. On the other hand, although it was shown recently that resistance to common antimicrobial agents can be used in some moderate halophiles as a genetic marker (17), the majority of these halophiles are unsusceptible to antimicrobial agents as a result of the high salt concentration usually required for optimal growth (17). Therefore, the search for alternative selective markers or reporter genes, which could be used for moderate halophiles, is very important.

The utility of the ice nucleation gene *inaZ* of the plant pathogen *Pseudomonas syringae* (7) has been assessed and proved to be very useful in other gram-negative bacteria (2, 12). Its activity can be measured by a very sensitive assay such as a droplet-freezing assay, and its expression can be quantified by very simple methods (12, 18, 29). Apart from its usefulness for gene expression studies, the ice nucleation gene may also have various applications in frozen food and artificial snow industries (15). Therefore, its transfer and expression in nonpathogenic bacteria also attract considerable economic attention.

The present study deals with the transfer of the ice nucleation gene *inaZ* to various moderately halophilic bacteria such as *Halomonas elongata*, *Halomonas subglaciescola*, *Halomonas*

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TABLE 1. Bacterial strains and plasmids

Species	Strain	Plasmid	Relevant plasmid markers[a]	Plasmid reference
E. coli	HB101	pRK600	Sm^r, Tra$^+$, Mob$^+$	9
	DH5α	pHS15	Ap^r, Sm^r, oriT	25
	DH5α	pDS3154-*inaZ*	Cm^r, oriT, Ice$^+$	2
	DH5α	pPTZ3-*inaZ*	Ap^r, Tc^r, oriT, Ice$^+$	2, 21
	DH5α	pHS17	Ap^r, oriT, Ice$^+$	Present work
	DH5α	pHS18	Ap^r, oriT, Ice$^+$	Present work
	DH5α	pHS19	Ap^r, oriT, Ice$^+$	Present work
	DH5α	pHS20	Ap^r, oriT, Ice$^+$	Present work
	DH5α	pHS21	Ap^r, oriT, Ice$^+$	Present work
	DH5α	pHS22	Ap^r, oriT, Ice$^+$	Present work
	DH5α	pHS23	Ap^r, oriT, Ice$^+$	Present work
H. elongata	ATCC 33174	pHS17–pHS23	Ap^r, oriT, Ice$^+$	Present work
H. meridiana	DSM 5425	pHS18	Ap^r, oriT, Ice$^+$	Present work
H. subglaciescola	UQM 2927	pHS18	Ap^r, oriT, Ice$^+$	Present work
H. halodurans	ATCC 29696	pHS18	Ap^r, oriT, Ice$^+$	Present work
V. eurihalina	ATCC 49336	pHS17–pHS23	Ap^r, oriT, Ice$^+$	Present work
D. halophila	CCM 3662	pHS18	Ap^r, oriT, Ice$^+$	Present work

[a] Abbreviations: Ap, ampicillin; Cm, chloramphenicol; Sm, streptomycin; Tc, tetracycline; Ice$^+$, ice nucleating; Tra$^+$, conjugal transfer functions; Mob$^+$, mobilization functions; oriT, origin of transfer replication; r, resistant.

meridiana, *Halomonas halodurans*, *Volcaniella eurihalina*, and *Deleya halophila*, subcloned in a suitable shuttle vector (25). The *inaZ* gene was expressed and quantified in all moderate halophiles tested under the control of two different heterologous promoters. Native promoter activity for *inaZ* was also detected in the pHE1 sequence of the shuttle vector pHS15.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. Bacterial strains, plasmids, and their relevant characteristics are listed in Table 1. *E. coli* cells were grown on Luria broth by standard methodology (23). The host for subcloning and maintenance of recombinant plasmids was *E. coli* DH5α (Bethesda Research Laboratories, Gaithersburg, Md.). The moderately halophilic strains used in this study were spontaneous rifampin-resistant mutants isolated from the corresponding culture collection strains. All moderate halophiles were grown in a saline medium (SWYE medium) containing 10% (wt/vol) total salts and 0.5% (wt/vol) yeast extract (Difco). The ingredients of the salt solution and their concentrations (in grams per liter) were the following: NaCl, 81; MgCl$_2$, 7; MgSO$_4$, 9.6; CaCl$_2$, 0.36; KCl, 2; NaHCO$_3$, 0.06; and NaBr, 0.0026 (16). The pH of the media was adjusted to 7.2. Solid media were obtained by adding 2% (wt/vol) Bacto Agar (Difco). Incubation took place at 37°C, and liquid cultures were shaken at 200 rpm in an orbital shaker. The following filter-sterilized antibiotic solutions were added when required for genetic selections or plasmid maintenance at the concentrations indicated: ampicillin, 50 μg ml^{-1}; rifampin, 25 μg ml^{-1}; and streptomycin, 40 μg ml^{-1}.

Recombinant DNA techniques. Plasmid DNA was isolated from moderate halophiles by a modified alkaline-lysis method described previously (25). Restrictions, ligations, and Southern blotting were carried out by standard methodology (23). Hybridizations were performed under high-stringency conditions with a nonradioactive labeled probe (digoxigenin-11-dUTP) with a DNA labeling and detection kit, nonradioactive (catalog no. 1093 657), from Boehringer Mannheim as described in the instructions of the manufacturer. The *Pst*I fragment of *inaZ* from pDS3154-*inaZ* (2) cut from the agarose gel and purified by GENE-CLEAN II kit of Bio 101 (La Jolla, Calif.) was used as a probe for labeling and hybridization experiments.

Bacterial conjugations. Constructed plasmids were mobilized from *E. coli* to moderately halophilic strains by triparental matings in which the RK2 *tra* genes were provided in *trans* by the helper plasmid pRK600 (9). One-hundred-microliter volumes of logarithmic-phase cultures of each of the donor, helper, and recipient strains were mixed on a nitrocellulose filter (0.22-μm pore diameter) on a plate of a modified SWYE complex medium (SW-2), in which the final percentage of the total salt solution was decreased to 2% (wt/vol) to permit the growth of *E. coli*. Filters were incubated overnight at 37°C, and the transconjugants of moderate halophiles were selected on SW-2 containing rifampin (to counterselect the donor strains) and streptomycin.

Ice nucleation tests. For maximum ice nucleation activity, all cultures were grown at 24°C for 20 h. *E. coli* was grown in Luria broth. The salt concentration of cultures growing on SWYE medium (10% [wt/vol] total salts) did not cause any apparent inhibition of the ice nucleation activity. For this reason, the moderately halophilic strains were grown in this usual medium. Antibiotic selection was routinely employed to ensure plasmid maintenance. Ice nucleation activity

was quantified by droplet-freezing assays as described previously (12, 24). Whole cultures were diluted 10-fold serially up to 10^{-8} with distilled H$_2$O, and 10-μl droplets from each dilution (total of 20) were placed on the surface of an aluminum foil sheet (spray coated with a 2% solution of paraffin in xylene and heat dried to remove the solvent) floating on an antifreeze bath set at −9°C. Ice nucleation activity was calculated by the equation of Vali (24) by use of a software program (13) and was expressed as the logarithm of ice nuclei per CFU [log(ice nuclei/cell)] of the whole cultures.

RESULTS

Construction of *inaZ* plasmids suitable for moderate halophiles. A promoterless version of the ice nucleation gene *inaZ* of *P. syringae* (12) was excised from plasmid pUZ119 (1) as a 3.7-kb *Pst*I or *Eco*RI fragment and was subcloned in the *Pst*I or *Eco*RI site of plasmid vector pHS15, respectively. The absence of the *inaZ* promoter in the subcloned fragment has been proved by previous work of other researchers (1, 12, 30). Furthermore, when the promoterless *inaZ* was cloned in the opposite orientation downstream from the P$_{bla}$ and P$_{pdc}$ promoters (in plasmids pDS3154 and pPTZ3, respectively), it did not express ice nucleation activity (2). The pHS15 vector (12.25 kb) is the result of a *Bam*HI-*Bgl*II fusion of the natural plasmid pHE1 of *H. elongata* (25) in a recombinant product of pBluescript and pKSΩoriT (25) (Fig. 1). This plasmid contains single
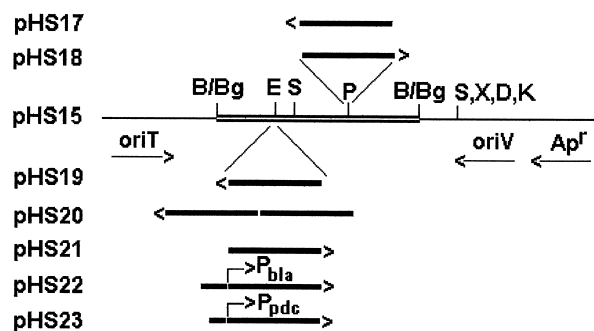

FIG. 1. Partial restriction and genetic maps of *inaZ* reporter plasmids. Abbreviations and symbols: B, *Bam*HI; Bg, *Bgl*II; D, *Dra*I; E, *Eco*RI; K, *Kpn*I; P, *Pst*I; S, *Sal*I; Sm, *Sma*I; X, *Xho*I; oriT, origin of transfer replication; oriV, origin of vegetative replication; Ap^r, resistance to ampicillin (β-lactamase gene); ⇄, orientation of replication in oriT or oriV; <, orientation of transcription. —, pBluescript-pKSΩoriT; ═, pHE1; ▬, *inaZ*. Maps are not drawn to scale.
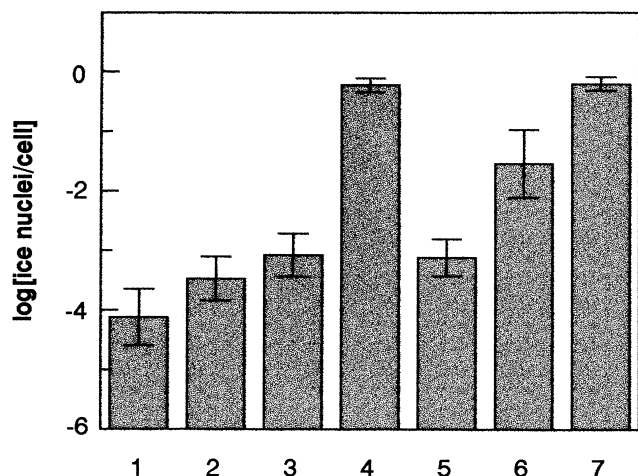
FIG. 2. Ice nucleation activity in *E. coli* DH5α cells harboring *inaZ* reporter plasmids. Bars: 1, pHS17; 2, pHS18; 3, pHS19; 4, pHS20; 5, pHS21; 6, pHS22; 7, pHS23. The negative control *E. coli* DH5α harboring pHS15 did not express ice nucleation activity [log(ice nuclei/cell) → −∞]. Each value represents the mean of five replicas. Standard errors were calculated by common numerical analysis software with a confidence coefficient of 99%.

*Pst*I and *Eco*RI sites, which are located in the pHE1 part. Insertion of *inaZ* was accomplished at both orientations, producing recombinant plasmids pHS17, -18, -19, and -21. A recombinant construct, named pHS20, carrying two copies of *inaZ* at the same orientation as that in pHS19, was also generated (Fig. 1). Furthermore, the *inaZ* gene under the control of the β-lactamase of *E. coli* (P_{bla}) and pyruvate decarboxylase of *Z. mobilis* (P_{pdc}) promoters was excised from plasmids pDS3154-*inaZ* (2) and pPTZ3-*inaZ* (2, 21) as 4.5-kb and 4.0-kb *Eco*RI fragments, respectively. These fragments were subcloned in the *Eco*RI site of pHS15, producing the recombinant constructs pHS22 and pHS23, respectively (Fig. 1). All of the recombinant plasmids described above were isolated from *E. coli* DH5α transformants and characterized by restriction enzyme digestion and hybridization with the digoxygenin-11-dUTP-labeled *inaZ* fragment as a probe. All *inaZ*-carrying constructs expressed ice nucleation activity in *E. coli* cells (Fig. 2). The highest nucleation activity was obtained with transformants carrying pHS20 (two copies of *inaZ*), pHS22 (*inaZ* under the control of P_{bla}), or pHS23 (*inaZ* under the control of P_{pdc}). However, ice nucleation activity was expressed by all other constructs, indicating that *inaZ* was expressed under the control of native promoters in pHE1 or more distant promoters in the pBluescript and pKSΩoriT sequences. P_{bla} and P_{pdc} activity was verified by comparing the log (ice nuclei/cell) values conferred by pHS21, pHS22, and pHS23, in which *inaZ* has the same orientation. In pHS22 and pHS23 transformants, the ice nucleation activity was about 10- and 20-fold higher, respectively, as compared with the activity assessed in pHS21 transformants (Fig. 2). *E. coli* DH5α cells transformed by pHS15 did not exhibit any ice nucleation activity.

Transfer and expression of *inaZ* in moderately halophilic bacteria. *H. elongata* and *V. eurihalina* cells grown on a modified SWYE (5% total salts) or the usual SWYE (10% total salts) liquid medium at 24°C were tested for native ice nucleation activity. Droplet freezing was not observed after screening 100 droplets (10 μl each) from dilutions of 10^0, 10^{-1}, and 10^{-2} at −9 and −14°C. It was then concluded that none of the moderately halophilic strains expressed native ice nucleation activity. The pHS-*inaZ* plasmid constructs were transferred

from *E. coli* to *H. elongata* and *V. eurihalina* by pRK600-mediated conjugation. In all cases, streptomycin-resistant transconjugants were isolated. The presence of the recombinant plasmids in the transconjugant cells was confirmed by plasmid DNA preparation, retransformation of *E. coli* DH5α, and enzyme digestion analysis. This strategy, which is longer than a direct digestion of the DNA isolated from the moderately halophilic transconjugants, was selected because plasmid DNA preparations from many of these strains are often not clean enough to permit direct digestion with endonucleases. In all cases, the restriction profiles of the recombinant plasmids isolated from retransformed *E. coli* cells corresponded to those of the pHS15 derivatives shown in Fig. 1, indicating that no rearrangements or modifications of the constructed plasmids occurred in the moderate halophiles for at least 80 cell divisions under selective conditions.

All moderately halophilic transconjugants harboring a pHS15 derivative expressed ice nucleation activity (Fig. 3). However, unlike that in *E. coli*, one orientation of the *inaZ* constructs gave much higher nucleation activity. Specifically, pHS18 and pHS19 conferred considerably higher activity than pHS17 and pHS21 did, respectively, in both *H. elongata* and *V. eurihalina* (Fig. 3), indicating that *inaZ* in pHS18 and pHS19 is possibly expressed under the control of native pHE1 promoters. It is noteworthy to point out here that pHS20 carrying a tandem duplication of *inaZ* at the same frame as that in pHS19 (Fig. 1) gave significantly higher ice nucleation activity than its single-copy equivalent, pHS19 (Fig. 3). *inaZ* was also transferred and expressed in *H. elongata* and *V. eurihalina* under the control of the promoters P_{bla} of *E. coli* and P_{pdc} of *Zymomonas mobilis*. Ice nucleation activity was comparable to that obtained from the expression of the native putative promoters (Fig. 3). Here again, expression of the P_{bla} and P_{pdc} promoters in *H. elongata* and *V. eurihalina* is verified by comparing the log (ice nuclei/cell) values obtained by plasmids pHS21, pHS22, and pHS23, in which *inaZ* has the same orientation. Ice nucleation activity conferred by pHS22 and pHS23 is about 35- and 30-fold higher than that conferred by pHS21 in *H. elongata* and *V. eurihalina*, respectively (Fig. 3). This significant differ-
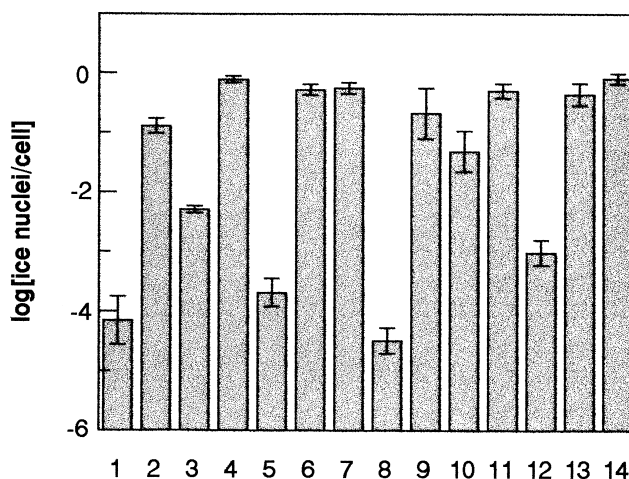

FIG. 3. Ice nucleation activity in *H. elongata* (bars 1 to 7) and *V. eurihalina* (bars 8 to 14) cells harboring *inaZ* reporter plasmids. Bars: 1 and 8, pHS17; 2 and 9, pHS18; 3 and 10, pHS19; 4 and 11, pHS20; 5 and 12, pHS21; 6 and 13, pHS22; 7 and 14, pHS23. Each value represents the mean of five replicas. Standard errors were calculated by common numerical analysis software with a confidence coefficient of 99%. The negative controls *H. elongata* and *V. eurihalina* did not express ice nucleation activity [log(ice nuclei/cell) → −∞].
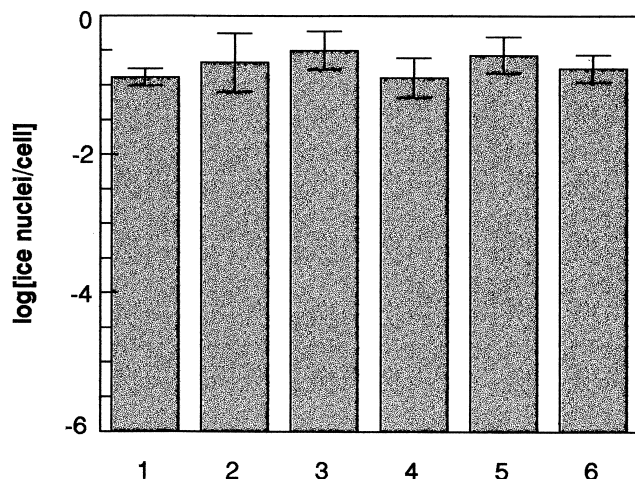
FIG. 4. Comparison of ice nucleation activities conferred by plasmid pHS18 in various moderately halophilic bacteria. Bars: 1, *H. elongata*; 2, *V. eurihalina*; 3, *H. subglaciescola*; 4, *H. meridiana*; 5, *H. halodurans*; 6, *D. halophila*. Each value represents the mean of five replicas. Standard errors were calculated by common numerical analysis software with a confidence coefficient of 99%. The negative controls *H. subglaciescola*, *H. meridiana*, *H. halodurans*, and *D. halophila* did not express ice nucleation activity [log(ice nuclei/cell) → −∞].

ence should be due to promoter activity of P_{bla} and P_{pdc}, respectively, indicating that foreign promoters can be functional in moderate halophiles.

To further examine the possibility of the expression of *inaZ* in a broader host range of moderate halophiles, plasmid pHS18 carrying *inaZ* under the control of a putative strong native promoter was transferred from *E. coli* to other species of moderate halophiles like *H. subglaciescola*, *H. meridiana*, *H. halodurans*, and *D. halophila*. As in the case of *H. elongata* and *V. eurihalina*, none of the strains described above and tested as described before can express native ice nucleation activity. Transfer of pHS18 to these strains was accomplished by pRK600-assisted conjugation as described above. Here again, in all cases, streptomycin-resistant transconjugants were isolated, indicating that pHS15 was able to replicate not only in *H. elongata* but also in other moderate halophiles. All transconjugants had ice nucleation activity (Fig. 4). The highest levels of activity were observed in *V. eurihalina*, *H. subglaciescola*, and *H. halodurans*. Differential expression could be due to either different promoter strength or function of the ice nucleation protein. This point requires further investigation. Nevertheless, the results described above verify that vector pHS15 can replicate and *inaZ* can be expressed in a broad spectrum of moderately halophilic bacteria.

DISCUSSION

The lack of suitable genetic tools has hampered the biotechnological exploitation of moderately halophilic bacteria, a group of extremophiles which offer very interesting potentialities for both industrial and detoxification purposes. Shuttle vectors and DNA transfer procedures have been developed only very recently (25). However, no reporter genes for these halophiles are available to date. In this work, the utility of the ice nucleation gene *inaZ* of the plant-pathogenic bacterium *P. syringae* as a potential reporter in moderate halophiles was investigated. Expression of *inaZ* has been reported for many gram-negative bacteria, including *E. coli* (14), *Pseudomonas* spp., *Pseudomonas* spp., *Agrobacterium* and *Rhizobium* spp. (12, 18), *Z. mobilis* (2),

and plants (1). However, this is the first report of the expression of a reporter gene for moderately halophilic bacteria. The differential expression of *inaZ* under the control of native as well as heterologous promoters demonstrates that ice nucleation can be utilized to detect promoter activity and to monitor levels of gene expression in various moderately halophilic species. Moderate halophiles lack intrinsic ice nucleation activity. This fact, together with the easy, sensitive, and quantifiable assay for ice nucleation activity, makes *inaZ* a very useful tool to study gene regulation in this group of extremophiles.

The data presented herein indicate that the native plasmid pHE1 of *H. elongata* should contain at least two promoters, one located on the left of the *Pst*I site and the other on the right of the *Eco*RI site (Fig. 1), controlling transcription of opposite orientations. This conclusion comes from the fact that the orientations of *inaZ* in pHS18 and pHS19 confer more than 35-fold- and 15-fold-higher ice nucleation activity, respectively, than the opposite ones. In support of this hypothesis, the *Eco*RI-*Pst*I-*Pst*I fragment from pHS18 was subcloned in pBR325 double-restricted with *Eco*RI-*Pst*I. This restriction removes the promoters of β-lactamase and chloramphenicol acetyltransferase, allowing expression of the *inaZ* gene only by promoters located within the *Eco*RI-*Pst*I fragment of pHE1. The pBR325-*inaZ*-pHE1 construct, verified by restriction analysis, produced *E. coli* DH5α transformants expressing ice nucleation activity (unpublished results). The occurrence of promoter sequences in pHE1 are under further investigation at present as part of a separate project including maxicell, nucleotide sequence, and deletion analyses. Because the subcloned *inaZ* reporter gene does not contain its own promoter and the moderately halophilic strains lack intrinsic ice nucleation activity, the low ice nucleation activity still observed with the opposite orientation in the case of plasmids pHS17 and pHS21 is probably due to distant promoters of either pHE1 or vector sequences. The putative native promoter located on the left of the *Pst*I site of the plasmid pHE1 (in pHS18) was found to confer high levels of expression in a wide range of moderately halophilic species (Fig. 4), thus confirming the broad promiscuity of pHS15 and, hence, its usefulness in genetic studies of these biotechnologically important bacteria. Moreover, the significant increase of ice nucleation activity in Ice⁺ transconjugants carrying two copies of *inaZ* shows that the ice nucleation gene can be used to study gene dosage in moderate halophiles.

Two heterologous promoters, P_{bla} of *E. coli* and P_{pdc} of *Z. mobilis*, were also examined for expression by employing the *inaZ* gene. They were both found to yield high levels of expression in *H. elongata* and *V. eurihalina* that were comparable to the level of expression obtained by the putative native promoters. These activities are very comparable to the native ice nucleation activity expressed naturally in *P. syringae* pv. phaseolicola [log (ice nuclei/cell) = −0.86] (12), which is the source of the *inaZ* gene used here. Expression of *inaZ* in moderate halophiles under the control of its own promoter was not possible because such a DNA fragment containing both (the promoter and the coding sequences) was not available. Expression of heterologous promoters ensures that foreign genes can be introduced independently and expressed in moderate halophiles, facilitating their genetic improvement and strain construction plans.

In conclusion, the results presented here demonstrate that the ice nucleation gene of *P. syringae* probably has no constraints for its expression in a very broad spectrum of moderate halophiles. Apart from the versatile uses of *inaZ* in genetic studies, Ice⁺ moderate halophiles can also be used as alternative sources of bacterial ice nuclei, provided that these extremophiles are not pathogenic for plants and animals, offering

a perspective of yet another application for these industrially attractive bacteria.

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