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

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Received 18 May 1995/Accepted 18 August 1995

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 two orientations in a recombinant plasmid able to replicate in moderate halophiles and, in particular, within the sequence of its pH15 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 Pbla (the promoter of the β-lactamase gene of Escherichia coli) and Ppol (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 subglauciescola, 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.

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Modestly 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 high degree of resistance in hypersaline environments (22), have been studied extensively with respect to their taxonomy (26) and physiology (11). Recently, moderate halophiles have gained 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, pH515, 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 non-pathogenic 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 subglauciescola, Halomonas
meridiana, Halomonas halodurans, Volcaniella eurhaliina, and Deleva halophilia, subcloned in a suitable shuttle vector (25). The \textit{inaZ} gene was expressed and quantified in all moderate halophiles tested under the control of two different heterologous promoters. Native promoter activity for \textit{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. \textit{E. coli} cells were grown on Luria broth by standard methodology (23). The host for subcloning and maintenance of recombinant plasmids was \textit{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 salts solution and their concentrations indicated: ampicillin, 50 μg/ml; chloramphenicol, 30 μg/ml; streptomycin, 10 μg/ml; tetracycline, 25 μg/ml; origin of vegetative replication; Apr, resistance to ampicillin (\textit{bla} gene); \textit{Sm}, chloramphenicol; Sm, streptomycin; Tc, tetracycline; Ice +, ice nucleating; Tra +, conjugal transfer functions; Mob +, mobilization functions; oriT, origin of transfer replication; r, resistant.

![Partial restriction and genetic map of \textit{inaZ} reporter plasmids](https://example.com/fig1.png)

**TABLE 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Plasmid</th>
<th>Relevant plasmid markers</th>
<th>Plasmid reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{E. coli}</td>
<td>HB101</td>
<td>pRK600</td>
<td>Smr, Tra +, Mob +</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>pH5α</td>
<td>pHS15</td>
<td>Ap +, Smr, oriT</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>pDS3154-inaZ</td>
<td>pHS17</td>
<td>Cm +, oriT, Ice +</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>pPTTZ-inaZ</td>
<td>Ap +, Tc +, oriT, Ice +</td>
<td>2, 21</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pHS18</td>
<td>Ap +, oriT, Ice +</td>
<td>Present work</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pHS19</td>
<td>Ap +, oriT, Ice +</td>
<td>Present work</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pHS20</td>
<td>Ap +, oriT, Ice +</td>
<td>Present work</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pHS21</td>
<td>Ap +, oriT, Ice +</td>
<td>Present work</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pHS22</td>
<td>Ap +, oriT, Ice +</td>
<td>Present work</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pHS23</td>
<td>Ap +, oriT, Ice +</td>
<td>Present work</td>
<td></td>
</tr>
<tr>
<td>\textit{H. elongata}</td>
<td>ATCC 33174</td>
<td>pHS17–pHS23</td>
<td>Ap +, oriT, Ice +</td>
<td>Present work</td>
</tr>
<tr>
<td>\textit{H. meridiana}</td>
<td>DSM 5425</td>
<td>pHS18</td>
<td>Ap +, oriT, Ice +</td>
<td>Present work</td>
</tr>
<tr>
<td>\textit{H. subglaciescina}</td>
<td>UOM 2927</td>
<td>pHS18</td>
<td>Ap +, oriT, Ice +</td>
<td>Present work</td>
</tr>
<tr>
<td>\textit{H. halodurans}</td>
<td>ATCC 29696</td>
<td>pHS18</td>
<td>Ap +, oriT, Ice +</td>
<td>Present work</td>
</tr>
<tr>
<td>\textit{V. eurhaliina}</td>
<td>ATCC 49336</td>
<td>pHS17–pHS23</td>
<td>Ap +, oriT, Ice +</td>
<td>Present work</td>
</tr>
<tr>
<td>\textit{D. halophilia}</td>
<td>CCM 3662</td>
<td>pHS18</td>
<td>Ap +, oriT, Ice +</td>
<td>Present work</td>
</tr>
</tbody>
</table>

* 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.

**RESULTS**

Construction of \textit{inaZ} plasmids suitable for moderate halophiles. A promoterless version of the ice nucleation gene \textit{inaZ} of \textit{P. syringae} (12) was excised from plasmid pUZ19 (1) as a 3.7-kb \textit{PstI} or \textit{EcoRI} fragment and was subcloned in the \textit{PstI} or \textit{EcoRI} site of plasmid vector pHS15, respectively. The absence of the \textit{inaZ} promoter in the subcloned fragment has been proved by previous work of other researchers (1, 12, 30). Furthermore, when the promoterless \textit{inaZ} was cloned in the opposite orientation downstream from the \textit{P}_{bla} and \textit{P}_{pde} 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 \textit{BamHI-BglII} fusion of the natural plasmid pHE1 of \textit{H. elongata} (25) in a recombinant product of pBluecript and pKSIoriT (25) (Fig. 1). This plasmid contains single

![Deleval halophilia](https://example.com/fig2.png)

PstI and EcoRI 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 (Pbla) and pyruvate decarboxylase of Z. mobilis (Ppdc) promoters was excised from plasmids pDS3154-inaZ (2) and pPTZ3-inaZ (2, 21) as 4.5-kb and 4.0-kb EcoRI fragments, respectively. These fragments were subcloned in the EcoRI 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 digoxigenin-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 Pbla), or pHS23 (inaZ under the control of Ppdc). 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 pKSorIT sequences. Pbla and Ppdc 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°C 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 (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 Pbla of E. coli and Ppdc 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 Pbla and Ppdc 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. 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%.
expression 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 pH18 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 pH18 to these strains was accomplished by pRK600-assisted conjugation as described above. Here again, in all cases, streptomycin-resistant transconjugants were isolated, indicating that pH15 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 pH15 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., 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 pHEI of H. elongata should contain at least two promoters, one located on the left of the Psrl site and the other on the right of the EcoRI site (Fig. 1), controlling transcription of opposite orientations. This conclusion comes from the fact that the orientations of inaZ in pH18 and pH19 confer more than 35-fold- and 15-fold-higher ice nucleation activity, respectively, than the opposite ones. In support of this hypothesis, the EcoRl-Psrl-Psrl fragment from pH18 was subcloned in pBR325 double-restricted with EcoRI-Psrl. This restriction removes the promoters of β-lactamase and chloramphenicol acetyltransferase, allowing expression of the inaZ gene only by promoters located within the EcoRI-Psrl fragment of pH1E. The pBR325-inaZ-pHE1 construct, verified by restriction analysis, produced E. coli DH5α transforms 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 pH17 and pH21 is probably due to distant promoters of either pHE1 or vector sequences. The putative native promoter located on the left of the Psrl site of the plasmid pHE1 (in pH18) was found to confer high levels of expression in a wide range of moderately halophilic species (Fig. 4), thus confirming the broad promiscuity of pH15 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.

ACKNOWLEDGMENTS

We wish to thank N. J. Panopoulos (Institute of Molecular Biology and Biotechnology-FORTH, Heraklion, Greece) for helpful and encouraging discussions.

This work was supported financially by the EC Generic Project “Biotechnology of Extremophiles” (contract BIO-CT93-02734), the Spanish Ministerio de Educacion y Ciencia (PB 92-0670, BIO94-0846-CE, and PB93-0920), and the Junta de Andalucia.

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