The gene encoding a periplasmic deoxyribonuclease from *Aeromonas hydrophila*

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Abstract

A gene encoding a deoxyribonuclease, *dnsH*, was cloned from *Aeromonas hydrophila* JMP636. The predicted mature protein was very similar to the previously described extracellular Dns from this organism and an N-terminal region corresponding to a large putative signal sequence was predicted for the JMP636 protein. Inactivation of *dnsH* demonstrated that the DnsH protein was not present extracellularly in this strain. As DnsH degraded plasmid DNA and was believed to have a periplasmic location, a *dnsH* mutant was constructed to determine whether electroporation of *A. hydrophila* with plasmid DNA could be achieved. No transformants were detected. From SDS-PAGE studies, at least two additional DNases remain to be characterised from *A. hydrophila* JMP636. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: *Aeromonas hydrophila*; Deoxyribonucleases; Gene inactivation; Periplasmic enzyme

1. Introduction

Genetic manipulation of bacteria can be hampered by the presence of extracellular and periplasmic DNases. Previous studies involving *Aeromonas hydrophila* JMP636 have not been successful in transformation of this strain, with genetic transfer accomplished by conjugation [1,2]. Poor transformation of organisms by foreign DNA has previously been observed in other Gram-negative bacteria such as the related *Vibrio cholerae*. Two distinct genes which encode extracellular DNases have been recognised in *Vibrio*, while the inactivation of both results in a strain with increased transformability [3]. Heat-inactivation of a *Serratia marcescens* nuclease also produced a strain which could be transformed more efficiently [4], as did the removal of a periplasmic DNase of *Vibrio alginolyticus* by osmotic shock [5]. In addition, when cloned DNase genes from *V. cholerae* and *Erwinia chrysanthemi* are introduced into *Escherichia coli*, transformation efficiency of this host organism also decreases [6,7].

Initial studies on DNase activity in *A. hydrophila* JMP636 identified the novel nuclease gene *nucH* and detected additional activities [2]. A gene for extracellular DNase, *dns*, which is genetically distinct to *nucH* has previously been cloned from *A. hydrophila* CHC-1 strain [8]. However, the possible effect of this enzyme on transformation was not examined. Homologues of this gene in *E. coli* (*endA*) and *E. chrysanthemi* (*nucM*) are periplasmic proteins which are
capable of degrading plasmid DNA [7,9]. Here we report of the characterisation and inactivation of a homologue of dns from JMP636. In contrast to previous studies in A. hydrophila, this enzyme is found to have a periplasmic location and examine its potential to affect transformation of this organism by foreign DNA.

2. Materials and methods

2.1. Bacterial strains, plasmids and media

Cultures and constructs used in this study are listed in Table 1. E. coli was grown in Z medium [1] at 37°C supplemented with ampicillin (100 μg ml⁻¹), chloramphenicol (50 μg ml⁻¹), kanamycin (50 μg ml⁻¹) and tetracycline (20 μg ml⁻¹) as required. A. hydrophila was cultured in Z medium at 32°C supplemented with chloramphenicol (20 μg ml⁻¹), kanamycin (100 μg ml⁻¹), rifampicin (100 μg ml⁻¹) and tetracycline (10 μg ml⁻¹) when needed. Other media used were MacConkey agar (Becton-Dickinson) and DNase agar (Oxoid), which was supplemented with 0.1% Toluidine blue O. Conjugational transfer of plasmid DNA between strains utilised an overnight mating [2], while electroporation was performed with a Bio-Rad pulse controller [15].

2.2. DNA manipulations

Standard DNA manipulations were used throughout this study [16]. Plasmid DNA extraction of clones containing full length dnsH were prone to degradation and were treated with chloroform prior to pelleting of plasmid DNA. Chromosomal DNA extraction from A. hydrophila was performed as described previously, while Southern hybridisations used biotin-labelled probes [2]. Clones for sequencing were generated using an exonuclease procedure and sequenced using conditions recommended by Applied Biosystems [2]. PCR was performed using standard buffing conditions with 1.5 mM Mg²⁺ and an annealing temperature of 48°C [16]. Primers

Table 1

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or phenotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. hydrophila</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JMP636</td>
<td>wild-type</td>
<td>[1]</td>
</tr>
<tr>
<td>JMP636</td>
<td>Rif⁺ mutant</td>
<td>[2]</td>
</tr>
<tr>
<td>JMP5501</td>
<td>Rif⁺ Km⁺ JMP636::pJP9509 dnsH</td>
<td>This study</td>
</tr>
<tr>
<td>JMP5513</td>
<td>Rif⁺ Km⁺ nucH</td>
<td>[2]</td>
</tr>
<tr>
<td>JMP5515</td>
<td>Rif⁺ Km⁺ Tc⁺ JMP5501::pJP9530 dnsH nucH</td>
<td>This study</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH1</td>
<td>supE44 recA1 endA1 hsdR17 gyrA96</td>
<td>[10]</td>
</tr>
<tr>
<td>DH5α</td>
<td>supE44 recA1 endA1 hsdR17 gyrA96 ΔlacU169(Δ80lacZΔM15)</td>
<td>[10]</td>
</tr>
<tr>
<td>S17-1pir</td>
<td>S17-1 (endA1 hsdR17 supE44 integrated RP4-Te::Mu-Kan::Tn7) lysogenized with λpir</td>
<td>[11]</td>
</tr>
<tr>
<td>JEM5502</td>
<td>DH1 (pJP9508)</td>
<td>This study</td>
</tr>
<tr>
<td>JEM5511</td>
<td>DH1 (pUC19)</td>
<td>[2]</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUC19</td>
<td>Ap⁺ lacZ⁺ cloning vector</td>
<td>[12]</td>
</tr>
<tr>
<td>pPR510</td>
<td>Cm⁺ lacZ⁺ cloning vector</td>
<td>[13]</td>
</tr>
<tr>
<td>pJP5603</td>
<td>Km⁺ lacZ⁺ orR6K mobRP4</td>
<td>[11]</td>
</tr>
<tr>
<td>pJP5608</td>
<td>Tc⁺ lacZ⁺ orR6K mobRP4 pJP5603 with kan replaced by tet</td>
<td>[14]</td>
</tr>
<tr>
<td>pJP9501</td>
<td>4.2-kb KpnI fragment of JMP636 DNA in pUC19 containing dnsH</td>
<td>This study</td>
</tr>
<tr>
<td>pJP9508</td>
<td>1.5-kb SalI-SphI dnsH fragment from pJP9501 in pUC19</td>
<td>This study</td>
</tr>
<tr>
<td>pJP9509</td>
<td>Sau3A1 internal fragment of dnsH in pJP5603</td>
<td>This study</td>
</tr>
<tr>
<td>pJP9521</td>
<td>3.6-kb fragment from JMP636 containing nucH in pUC19</td>
<td>[2]</td>
</tr>
<tr>
<td>pJP9530</td>
<td>pJP5608 containing a 1.8-kb internal SauI fragment of nucH</td>
<td>This study</td>
</tr>
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</table>
were designed to amplify the structural *dns* gene [8], and corresponded to nucleotides +226 to +245 and the complement of +922 to +941. These oligonucleotides were: F-5′-CCAAGGATATTCATGTTTCG; and R-5′-GTCAAATGGGGTGTTAGCA.

### 2.3. Construction of *dnsH* and *dnsH nucH* mutants

An internal *dnsH* fragment was produced by Sau3A1 cleavage of the PCR amplified *dnsH* gene and was cloned into *Bam*HI-digested pJP603. The desired construct (pJP9509) was mobilised into rifampicin-resistant JMP636 using S17-1pir, producing the *dnsH* strain JMP5501. Disruption of the wild-type fragment in JMP636 was confirmed by Southern hybridisation, although a DNase-positive phenotype on agar media remained. An internal 1.8-kb *SacI* fragment of *nucH* used previously for inactivation of this gene [2] was cloned into pJP5608 to produce pJP9530. S17-1pir containing this construct was mated with JMP5501 and transconjugants were selected on media containing kanamycin, rifampicin and tetracycline, to produce the *dnsH nucH* strain JMP5515. No difference in DNase activity was detected on agar media, with SDS-PAGE used to confirm the absence of activities identified as DnsH and NucH.

### 2.4. Protein studies

Periplasmic and cytoplasmic cellular fractions of *E. coli* cultures were prepared from sphaeroplasts and assayed for the marker enzymes alkaline phosphatase and β-galactosidase to ensure fraction integrity [2]. Concentrated supernatant and cellular samples of *A. hydrophila* were prepared as described previously [2] and run on SDS-PAGE gels containing 25 μg ml⁻¹ herring sperm DNA [2]. Degradation of plasmid DNA was detected by agarose gel electrophoresis following the incubation of cellular samples from *E. coli* clones [2] with pUC19 DNA for 30 min at 37°C.

### 3. Results

#### 3.1. Cloning *dns* from JMP636

A PCR product of approximately 700 bp was obtained following amplification of JMP636 DNA using primers designed against *dns* from *A. hydrophila* CHC-1. This DNA was used as a Southern hybridisation probe against JMP636 DNA cleaved with various restriction endonucleases to identify clonable segments. DNA fragments of approximately 4.0-kb generated with *KpnI* were ligated to compatibly digested pUC19 and transformed into DH5α with selection on DNase media. After 2 days incubation at 32°C, a clone producing a pink halo indicative of DNA degradation was identified. Southern hybridisation confirmed homology between this clone, designated pJP9501, and the amplified PCR product, while subcloning localised the gene to a 1.5-kb *SalI*-*SphI* fragment (pJP9508).

#### 3.2. Sequencing and analysis

The entire nucleotide sequence of both strands of the insert of pJP9508 was determined (GenBank accession number L78266). An open reading frame of 711 nucleotides predicts a protein of 237 amino acids with a molecular mass of 27.4 kDa. The motif ‘AAGGA’ which has been proposed as a ribosome binding site for other *A. hydrophila* genes [2,8] is located five nucleotides upstream from the predicted start site. DnsH is seven amino acids larger than Dns from CHC-1 strain [8], with a ClustalW alignment [17], indicating the unmatched residues occur in the

![Fig. 1. ClustalW alignment of the first 50 amino acids of the putative DnsH protein with the homologous region of Dns (GenBank accession number M99491). Identical (+), similar (−) and unmatched (−) residues are indicated. The site of the predicted signal sequence cleavage site is marked for DnsH.](image-url)
N-terminal region (Fig. 1). Using the weight-matrix method of von Heijne [18], a signal sequence of 27 amino acids is predicted, which is larger than that of CHC-1 and also larger than the optimal procaryotic signal sequence length of 21–23 residues. The predicted mature proteins produced by JMP636 and CHC-1 are of identical size (210 amino acids) and contain 92% amino acid similarity and 89% amino acid identity.

3.3. Activity and localisation of recombinant DnsH

Protein samples prepared from JMP5502 (DH1 pJP9508) and JMP5511 (DH1 pUC19) were tested using SDS-PAGE for degradation of ssDNA and RNA [2], with the utilisation of both substrates indicating DnsH is a general nuclease rather than specific DNase (not shown). Activity on plasmid pUC19 DNA was tested using diluted protein samples (Fig. 2), with the absence of plasmid following incubation in the presence of DnsH indicating this enzyme has an endonuclease activity.

To determine the cellular location of DnsH in *E. coli* cultures, JMP5502 and JMP5511 were grown to exponential phase and cellular fractions were prepared and assayed for periplasmic and cytoplasmic enzymes (not shown). Similar low levels of β-galactosidase and alkaline phosphatase activity were detected in the supernatant of JMP5502, but not JMP5511 cultures, which is indicative of some cell lysis in the strain expressing DnsH. Protein samples from cellular fractions were run on an SDS-PAGE gel (Fig. 3). Although results are not quantitative, the presence of DnsH activity in the periplasm indicates that the predicted N-terminal signal sequence was likely to be functional in *E. coli*. The low levels of DnsH activity seen in the supernatant are consistent with cell lysis.

3.4. Analysis of dnsH mutant strains

Cellular protein and concentrated supernatant samples were prepared from JMP636 and JMP5501 and analysed by SDS-PAGE (Fig. 4). No difference in extracellular protein profiles was detected, although a band of cellular activity with an approximate molecular mass of 25 kDa is absent from JMP5501. As DnsH is capable of being processed to the periplasm in *E. coli*, it is likely that in JMP636 the enzyme is also located periplasmically or is membrane associated, but is not secreted. The largest band of extracellular activity has been shown to be NucH [2], leaving two additional areas of extracellular activity and one area of cell-associated activity uncharacterised.

To determine whether DnsH or NucH played a role in preventing transformation of JMP636, the electroporation of mutants deficient in either or both of these proteins and wild-type *A. hydrophila* was attempted using pPR510. Viable cell counts of

![Fig. 2. Activity of DnsH on plasmid DNA. Lanes: 1, 10× dilution of JMP5502 sample; 2, undiluted JMP5511 sample. The three forms of plasmid DNA in lane 2 are marked at the right.](image1)

![Fig. 3. DNase activities of cellular fractions from JMP5502. Lanes: 1, cytoplasmic fraction; 2, periplasmic fraction; 3, supernatant sample. Apparent molecular masses are given at the left in kDa.](image2)
the electroporation mixes were in the order of $10^9$, while time constants of 4.3–4.4 ms were obtained from all experiments. No transformants were detected for any of the A. hydrophila strains, despite a control E. coli DH5α having a transformation efficiency of 1 in $10^5$ cells per μg DNA and comparable viable cell counts. Similar results were obtained from 3 separate experiments, indicating that factors other than the periplasmic DnsH and extracellular NucH prevent plasmid uptake or maintenance in JMP636.

4. Discussion

The DNase activity of A. hydrophila JMP636 on agar media has been shown to result from the presence of multiple proteins, with the inactivation of two genes (dnsH and nucH) producing no visible change to this phenotype. A previous description of dns from A. hydrophila found that its protein product could be secreted in E. coli during cellular fractionation studies without leakage of marker enzymes [8], leading to the classification of Dns as an extracellular enzyme. In contrast, these studies have indicated that a higher percentage of both periplasmic and cytoplasmic enzyme activity was detected in the supernatant of E. coli producing DnsH than a control strain. This may reflect cell lysis and indicate that high-level expression of dnsH in E. coli DH1 has a detrimental effect on the host cell. The specific inactivation of dnsH produced no changes in the extracellular DNase profile of JMP636, which indicates that DnsH is not secreted. In E. coli and E. chrysanthemi, homologues of DnsH (EndA and NucM) have been determined to be periplasmic enzymes [7,9]. While in E. coli, this may have been attributed to a deficient secretion system, E. chrysanthemi is known to produce multiple extracellular enzymes and has a well-characterised general secretory pathway [19]. In addition to differing cellular locations, the other major difference between DnsH and Dns is the length of the N-terminal signal sequence. While the mechanism by which some proteins remain in the periplasm and others are secreted has not been elucidated, the signal sequence is believed to facilitate transport of the protein through the inner membrane only [20].

Despite the capacity for DnsH to break down plasmid DNA, no transformants were obtained during this study even when DnsH was knocked out, indicating that other DNases may be more of a barrier to the entry or maintenance of plasmid DNA in A. hydrophila. Previous work has demonstrated that in organisms with a restriction-modification system, the frequency of transformation can be increased when modified plasmids are recovered and used to retransform the bacteria [21]. While the presence of restriction-modification has been detected previously in some A. hydrophila strains [22], the presence of such a system in JMP636 has not been confirmed by assay due to the abundant non-specific DNases (unpublished observation). In addition, early work [23] into the transformation of E. coli identified that mutations of the recBC genes encoding an ATP-dependent DNase results in higher frequencies of transformation. In related organisms, the presence of DNases has been shown to affect transformation by foreign DNA; however, a similar occurrence in A. hydrophila JMP636 could not be confirmed with additional DNases and additional factors likely to affect this process.
Acknowledgments

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References


