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Cloning, Sequencing, and Characterization of the *nucH* Gene Encoding an Extracellular Nuclease from *Aeromonas hydrophila* JMP636

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An *Escherichia coli* clone expressing activity on DNase agar was obtained by cloning chromosomal DNA of *Aeromonas hydrophila* JMP636 into plasmid pUC19. Examination of the clone's nuclease activity on a sodium dodecyl sulfate (SDS)-polyacrylamide gel containing DNA as a substrate revealed an activity band at approximately 100 kDa. Subsequently, subcloning localized the gene, designated *nucH*, to a 3.6-kb DNA fragment (pJP9521). Southern blotting of the *nucH* gene against chromosomal DNA of JMP636 confirmed that it had originated from this strain and demonstrated that it was present in a single copy, although additional faint bands were also detected. Analysis of the subclone using in vivo transcription and translation revealed only a single polypeptide of approximately 110 kDa. Sequencing of pJP9521 predicted an open reading frame of 3,213 bp encoding a protein of 1,070 amino acids and having a molecular mass of 114 kDa. Comparison of the deduced nucleotide sequence and the NucH predicted protein sequence with relevant databases indicated that no known homologs have previously been identified. A signal sequence was predicted from these data, and cellular fractionation of a *nucH* clone in *E. coli* indicated that the protein was able to be processed to the periplasm. An activity similar in size was detected in an extracellular protein sample of JMP636, while inactivation of the *nucH* gene resulted in loss of this activity band. By native SDS-polyacrylamide gel electrophoresis, NucH substrate specificity, cofactor requirements, and sensitivity to denaturing agents were assessed.

Aeromonas hydrophila is noted for its production of a wide range of extracellular enzymes and toxins, which has aided in the study of protein secretion in this organism. Most of these proteins are synthesized as precursors with an N-terminal leader sequence, which is removed on translocation through the inner membrane, presumably by a signal peptidase. While this pathway has not yet been characterized for *A. hydrophila*, it is believed to be similar to the *secA*-dependent pathway of *Escherichia coli* (40). This idea is supported by observations that cloned gene products from *A. hydrophila* are processed to the periplasm in *E. coli* with removal of the N-terminal sequence (7, 20) while similar processing also occurs in *A. hydrophila* mutants which are defective in protein secretion (21). Proteins processed by this pathway in *E. coli* are believed to remain periplasmic; however, those of *A. hydrophila* can be further secreted to the external environment. Genes which encode this terminal branch of the general secretory pathway in *A. hydrophila* have been cloned and characterized (22, 24, 26, 27).

Among the extracellular proteins produced by *A. hydrophila* are several classes of degradative enzymes. Amylases (7, 16), chitinases (9, 45), lipases (2, 23), proteases (43, 44), and a DNase (8) have previously been identified. While many of these are perceived to play a nutritional role in the acquisition of carbon compounds, the function of an extracellular DNase is seemingly more ambiguous. As the nucleic acid by-products are rich in both carbon and nitrogen, DNases have also been considered as possible nutritional enzymes (12). This proposal is supported by the observation that in some fungi, DNases can be regulated by phosphate levels (28, 29). A further possible function in disease genesis has been postulated, although this has not been substantiated experimentally (12). What is obvious, however, is the effect of DNase-producing strains in a laboratory situation, and as such a protective role for these enzymes has been considered. Previously, DNases have been shown to be a barrier to the entry of foreign DNA by transformation and electroporation, presenting problems in the genetic manipulation of bacteria (12, 30, 32, 35, 42). Another consequence is the difficulty in isolation of intact plasmid or chromosomal DNA (35). Also, PCR products can become degraded when amplified from template prepared from a cell lysate, as seen with *Yersinia enterocolitica* and *Salmonella* spp. (14, 37).

Although extracellular DNase activity is well recognized, relatively few genes which encode these enzymes have been cloned. For the gram-negative eubacteria, such as *A. hydrophila*, studies have also been performed with genes cloned from *Serratia marcescens*, *Erwinia chrysanthemi*, *E. coli*, and *Vibrio cholerae* (4, 8, 11, 25, 34, 38). Most of the predicted proteins have homology at the amino acid level, with the exception of the nuclease of *S. marcescens*, which is homologous to some eukaryotic enzymes (13). A common feature of most cloned genes is the relatively small size of the protein, ranging from 25 to 30 kDa. One notable exception is the product of the *xds* gene from *V. cholerae*, which was shown to encode a protein with an approximate molecular mass of 100 kDa (38). No nucleotide sequence data were available for this gene, and so its relationship to any of the other studied nucleases is uncertain, although its distribution across a number of *Vibrio* species was evident (12).

In this paper, we report on the cloning, sequencing, and characterization of the gene *nucH* encoding an extracellular nuclease from *A. hydrophila* JMP636. The deduced open reading frame predicts a protein of 114 kDa, and this is in agreement with results obtained by both nuclease gel assay and

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant genotype, phenotype, and/or description	Source or reference
Strains		
A. hydrophila		
JMP636	Wild type	16
JMP636	Rif^r mutant	23
JMP5513 and JMP5514	JMP636::pJP9527 *nucH*	This study
E. coli		
JM109	*recA1 endA1 hsdR17* Δ(*lac-proAB*)	52
JM109λpir	JM109 lysogenized with λpir	39
S17-1λpir	Pro$^-$ Res$^-$ Mod$^+$; integrated plasmid RP4-Tc::Mu-Kan::Tn*7*	39
DH1	*recA1 endA1 hsdR17*	17
DH5α	*recA1 endA1 hsdR17* Δ*lacU169*(ϕ80*lacZ*ΔM15)	48
K38	HfrC (*lam*)	47
JMP5511	DH1 pUC19	This study
JMP5512	DH1 pJP9521	This study
Plasmids		
pUC19	Ap^r *lacZ*	52
pGP1-2	Km^r; T7 RNA polymerase	S. Tabor
pT7-3 to -6	Ap^r; T7 ϕ10 promoter	S. Tabor
pJP5603	Km^r *lacZ*; R6K origin	39
pJP9520	Ap^r; 8.2-kb *nucH* insert in pUC19	This study
pJP9521	Ap^r; 3.6-kb *nucH* insert in pUC19	This study
pJP9522	Ap^r; 3.3-kb *nucH* insert in pUC19	This study
pJP9523 to pJP9526	Ap^r; 3.6-kb *nucH* insert in pT7-3 to -6	This study
pJP9528	Km^r; 1.8-kb internal *nucH* insert in pJP5603	This study

exclusive protein labelling studies. The predicted protein sequence contains an N-terminal leader sequence, while the cloned gene product could be shown to be processed to the periplasm in *E. coli*. In *A. hydrophila*, inactivation of *nucH* results in the loss of a band of extracellular DNase activity. Studies of the gene product NucH indicate that it has sensitivity to heat treatment; resistance to 2-mercaptoethanol; and an ability to degrade double-stranded DNA, single-stranded DNA, and RNA. No nucleotide homology could be identified among previously sequenced genes, while the predicted protein sequence was also unique. This indicates that *nucH* is both a novel nuclease and a novel extracellular enzyme produced by *A. hydrophila.*

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids used in this study are described in Table 1.

All bacteria were routinely cultured in Z medium (16). Antibiotics were incorporated into media at the following final concentrations: ampicillin, 100 μg/ml; rifampin, 100 μg/ml; and kanamycin, 50 μg/ml for *E. coli* strains and 100 μg/ml for *A. hydrophila*. DNase agar was purchased from Oxoid and supplemented when required with 0.1% toluidine blue O. MacConkey agar was purchased from Becton-Dickinson. *E. coli* was grown at 37°C, while *A. hydrophila* was cultured at 32°C unless otherwise stated. Conjugational transfer of plasmid DNA between strains utilized an overnight mating (23).

Chromosomal DNA extraction. A loopful of culture grown overnight was suspended in 0.4 ml of saline-EDTA (150 mM NaCl, 100 mM EDTA) containing 25 μg of lysozyme per ml and 50 μg of RNase per ml. Following incubation at 65°C for 15 min, proteinase K was added to a concentration of 250 μg/ml and then 10 μl of a 25% sodium dodecyl sulfate (SDS) solution was added. After a further 15-min incubation at 65°C, the tube was examined for cell lysis. If the tube was insufficiently clear, an additional aliquot of SDS was added and the incubation was repeated. The resulting lysate was chilled briefly on ice and then extracted twice with chloroform. Each step employed vigorous shaking to mix and then breaking of the emulsion by centrifugation at 13,200 rpm in an IEC MicroMax microcentrifuge for 20 min, with removal of the aqueous layer to a fresh tube. The final extract was dialysed overnight at 4°C against Tris-EDTA (10 mM:1 mM) buffer, pH 7.5.

Cloning, subcloning, and DNA manipulations. Restriction enzymes and T4 DNA ligase were purchased from Promega. All manipulations were performed according to standard procedures (48). Plasmids were extracted from *E. coli* strains by alkaline lysis (5). Transformation employed the one-step procedure of Chung et al. (10). Isolation of DNA bands from agarose gels was routinely performed with a Prep-a Gene kit as per the manufacturer's instructions (Bio-Rad), while isolation from low-melting-point gels utilized agarase (Boehringer-Mannheim).

Preparation of protein samples. Crude cell-free protein samples and concentrated supernatant samples were produced from 30 ml of culture which was grown overnight at 200 rpm in an environmental shaker (Lab-Line) and centrifuged at 13,200 rpm and 4°C for 10 min in an IEC MicroMax centrifuge. Aliquots of culture supernatant were concentrated by adding trichloroacetic acid to a concentration of 5% and then incubating the mixtures on ice for 30 min and centrifuging them at 13,200 rpm for 15 min in an IEC MicroMax centrifuge. The recovered pellet was suspended in 50 μl of Tris-glycine (15 mM:150 mM) buffer. Crude cell extracts were produced by suspension of the cell pellet in 5 ml of Tris-glycine buffer and disruption by two passages through a French pressure cell (Aminco). Ultracentrifugation at 20,000 rpm for 20 min at 4°C in a Beckman model L8-M with a 50 Ti rotor was performed to remove debris, and the final extracts were stored at −20°C.

Native SDS-PAGE. Gels were prepared essentially as originally described by Rosenthal and Lacks (46), except that substrate nucleic acid was contained only in the separating gel. To test DNase activity, herring sperm DNA (Promega), either denatured for 10 min by boiling (single-stranded DNA [ssDNA]) or not treated (double-stranded DNA [dsDNA]), was added at a concentration of 25 μg/ml. RNase activity was tested by using *E. coli* 23S and 16S rRNA (Boehringer-Mannheim) as suggested by Muro-Pastor et al. (36). Sample buffer contained 2% SDS, 10% glycerol, 0.001% bromophenol blue, and 0.0625 M Tris-HCl (pH 6.8). Protein samples were diluted 1:1 in sample buffer but not usually subjected to boiling, and typically 10 to 20 μl was loaded for activity determinations. Gels were run at 4°C, with removal of SDS, development of activity, and staining occurring as originally described (46) at room temperature. For sizing of proteins, broad-range prestained SDS-polyacrylamide gel electrophoresis (PAGE) standards (Bio-Rad) were also run. Nuclease gels used for determining cofactor requirements were prepared similarly, and activity was developed in cation buffers as described by Muro-Pastor et al. (36).

Southern blot analysis. Transfer of DNA to nylon membrane (Amersham) and hybridization conditions were in accordance with standard methods (48). Probe DNA was labelled by nick translation with biotin-14-dATP (GIBCO-BRL) and visualized with streptavidin-alkaline phosphate conjugate activity on nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (from Promega).

In vivo transcription and translation. The pT7 promoter vector system of Tabor and Richardson was used (49), as has been done in this laboratory previously and as described in detail by Gobius and Pemberton (16).

Sequencing. Deletion banks were produced for sequencing from both ends of pJP9501 using the unidirectional exonuclease III procedure of Henikoff (19) to enable double-stranded sequencing. Clones were constructed in the bacterial

host strain DH5α. Template DNA for sequencing was extracted by the alkaline lysis-polyethylene glycol procedure recommended by Applied Biosystems, Inc. Reactions utilized the PRIZM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit of Applied Biosystems. Sequencing primers, both pUC forward and reverse 17-mers, were purchased from Promega. Thermocycling utilized a Perkin-Elmer model 480, and analysis was performed on the Applied Biosystems model 373A DNA sequencing system at the Joint Sequencing Facility, University of Queensland, St. Lucia, Australia.

Computer sequence analysis. Searches for nucleotide and protein sequence similarity were conducted with the Blast programs (1, 15) at the National Center for Biotechnology Information, available through the Australian National Genome Information Service. The Prosite database, also accessed through the Australian National Genome Information Service, was used to identify any protein motifs present in the predicted sequence (3).

Cellular fractionation. Periplasmic and cytoplasmic cellular fractions were prepared according to the spheroplast protocol of Randall and Hardy (41). These were assayed for the marker enzymes alkaline phosphatase (periplasmic) and β-galactosidase (cytoplasmic) as previously described by Ball et al. (4). The absorbance values obtained in these assays were added, and the results for each fraction were expressed as a percentage of the total activity obtained for that enzyme. DNase activity was assessed comparatively by running 10 μl of each fraction on a native SDS-PAGE gel.

Nucleotide sequence accession number. The nucleotide sequence of the *nucH* gene from clone pJP9521 has been assigned the GenBank accession number L76304.

RESULTS

Cloning of *nucH*. As DNase activity could not be detected in a clone bank previously prepared in this laboratory (16), a "shotgun" cloning method was employed. *A. hydrophila* chromosomal DNA was digested with various restriction endonucleases and electrophoresed in low-melting-point agarose gels to isolate 4- to 6-kb fragments. These fragments were ligated into similarly digested pUC19 DNA and transformed into *E. coli* JM109, and bacteria were then plated on DNase-toluidine blue O agar and incubated at 32°C. After 3 to 4 days, a clone originating from *Sph*I-digested DNA which possessed a faint pink halo, indicative of DNase activity, was identified. However, most notable was an intense dark blue-indigo color of the clone, which was subsequently designated pJP9520.

Clone phenotype. Before subcloning was performed, nuclease activity was assessed by native SDS-PAGE. In this method, protein samples are loaded on nucleic-acid-containing polyacrylamide gels in the presence of SDS, such that proteins migrate according to approximate molecular weight but are not irreversibly denatured. After electrophoresis, the SDS is washed out of the gel and a band of degradation appears where nuclease activity is present after staining with ethidium bromide and visualization using a UV transilluminator. Parallel running of stained molecular weight markers enables an approximation of protein size. By using this system, a total protein extract from JM109 containing pJP9520 was found to have a band of nuclease activity corresponding to approximately 100 kDa, while control JM109 did not demonstrate any activity (Fig. 1).

Subcloning and chromosomal copy number. Extraction of pJP9520 revealed an 11-kb plasmid which, when digested with *Sph*I, produced fragments of 4.3 and 3.9 kb in addition to the vector. Restriction mapping was used to identify unique *Eco*RI, *Kpn*I, and *Sma*I sites within the 8.2-kb insert region. To subclone the gene, designated *nucH*, pJP9520 was digested individually with one of these enzymes and religated, with selection of positive clones on DNase agar supplemented with ampicillin. Analysis of clones produced from these experiments showed that *nucH* had been subcloned to a 3.6-kb *Sph*I-*Kpn*I insert (pJP9521) and that activity was also retained on a smaller 3.3-kb *Sph*I-*Sma*I fragment (pJP9522). On the basis of the approximation that 1 kb of DNA would encode a protein of 37 kDa, the 100-kDa nuclease would require approximately 3 kb, and hence no further subcloning was attempted. By using

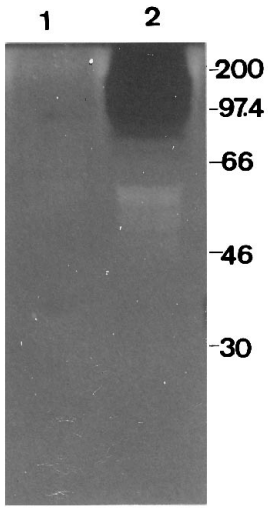

FIG. 1. Analysis of nuclease activity of clone pJP9520 on dsDNA. Lanes: 1, protein sample prepared from JM109; 2, protein sample prepared from JM109 containing pJP9520. Molecular masses of protein standards are given on the right in kilodaltons.

the smaller fragment as a probe against chromosomal digests of *A. hydrophila*, *nucH* was found to have originated from this organism and to be present in a single copy (Fig. 2). However, faint bands also appeared on the blot, which may indicate that the gene has homology with other regions of the chromosome. These bands were detected on a number of blots which were performed, all with posthybridization stringency washes of 90 to 100%, and were not believed to have resulted from partial digestion or nonspecific binding of the probe.

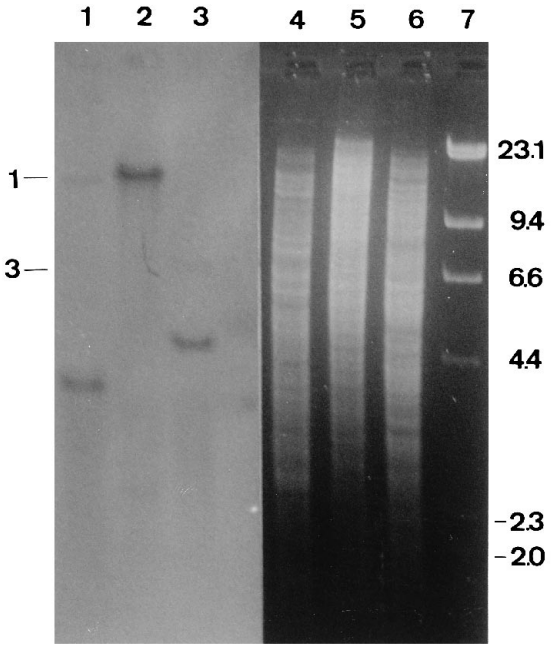

FIG. 2. Southern blot analysis of chromosomal DNA from JMP636 using a 3.3-kb *nucH* probe. Lanes 1 to 3 are the blots, while restriction digests are shown in lanes 4 to 6 to demonstrate completeness. Lanes: 1 and 4, *Sma*I digest; 2 and 5, *Kpn*I digest; 3 and 6, *Sph*I digest. Lane 7 is the marker, *Hin*dIII-digested lambda DNA, with molecular sizes of bands given in kilobases on the right. The faint bands observed in lanes 1 and 3 are marked on the left.

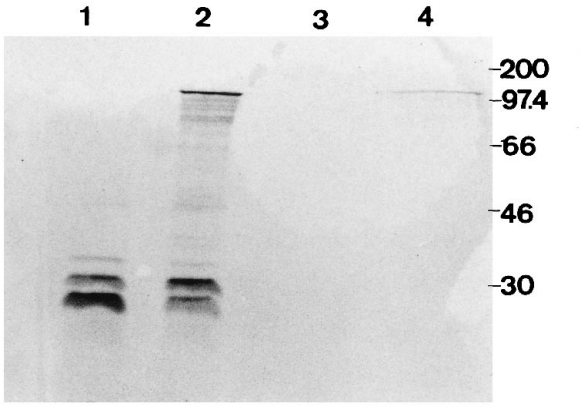

FIG. 3. pT7 analysis of the 3.6-kb *nucH* insert. Lanes: 1, pJP9523; 2, pJP9524; 3, pJP9525; 4, pJP9526. Control β-lactamase forms are present in lanes 1 and 2, while the NucH product is present in lanes 2 and 4. The molecular masses of protein standards are given on the right in kilodaltons.

In vivo transcription and translation. In order to confirm the presence of the *nucH* open reading frame, the pT7 system of Tabor and Richardson (49) was employed. The 3.6-kb *nucH* insert was isolated from pJP9521 after electrophoresis following digestion with *Hin*dIII and *Eco*RI. This insert was directionally cloned into the similarly digested pT7-3, pT7-4, pT7-5, and pT7-6 vectors. Recombinants were selected on DNase agar supplemented with toluidine blue O and ampicillin, and positives were subsequently extracted and digested to ensure that the correct insert was present. The resultant clones pJP9523, pJP9524, pJP9525, and pJP9526 were transformed into the expression host *E. coli* K38 containing pGP1-2. This vector encodes the rifampin-resistant T7 RNA polymerase, which is repressed at 30°C. After initial growth of pT7 clones at this low temperature, they were transferred to 42°C in the presence of rifampin. The native *E. coli* polymerase is knocked out and the T7 polymerase becomes functional under these conditions. Transcription can occur only from the genes under the control of the ϕ10 T7 promoter in pT7 vectors. Pulsing at this time with [^{35}S]methionine results in exclusive labelling of vector-produced proteins. From Fig. 3, it can be seen that a 110-kDa labelled protein has been produced from the vectors pT7-4 and pT7-6. This indicates that the gene is transcribed from *Hin*dIII to *Eco*RI, and therefore in pUC19 (pJP9521) it would be in the same orientation as the *lacZ* gene. Control β-lactamase production was also evident in pT7-3 and pT7-4, while a background of labelled proteins was also present, predominantly in the pT7-4 track. This may have resulted from degradation of the labelled NucH protein at Asp-Pro peptide bonds due to boiling of the samples prior to loading in a Tris buffer solution as suggested by Kubo (31).

Sequencing. To make an exonuclease deletion bank, it is necessary to identify two unique restriction enzyme sites—one producing a 3′ overhang closest to the vector and, internal to this site, one which leaves a blunt end or 5′ overhang to initiate degradation by exonuclease III. Ideally, these sites can be provided by the multiple cloning site of the vector; however, this was not possible for pJP9521. Therefore, for sequencing of the first strand, the unique *Kpn*I site was used as a protective site, while a unique *Sma*I site located approximately 300 bp away (and used in the construction of pJP9522) was used to produce deletions. This region was able to be sequenced directly from pJP9521. To confirm the data obtained, a deletion bank of the second strand was also produced. While *Sph*I could be used as a protective site, it was necessary to use an *Nru*I site located 200 bp away to create deletions. This site was predicted to be affected by *dam* methylation and only partial digestion occurred, but this partial digestion still enabled the bank to be made. The sequence obtained, the predicted open reading frame, and associated features are presented in Fig. 4.

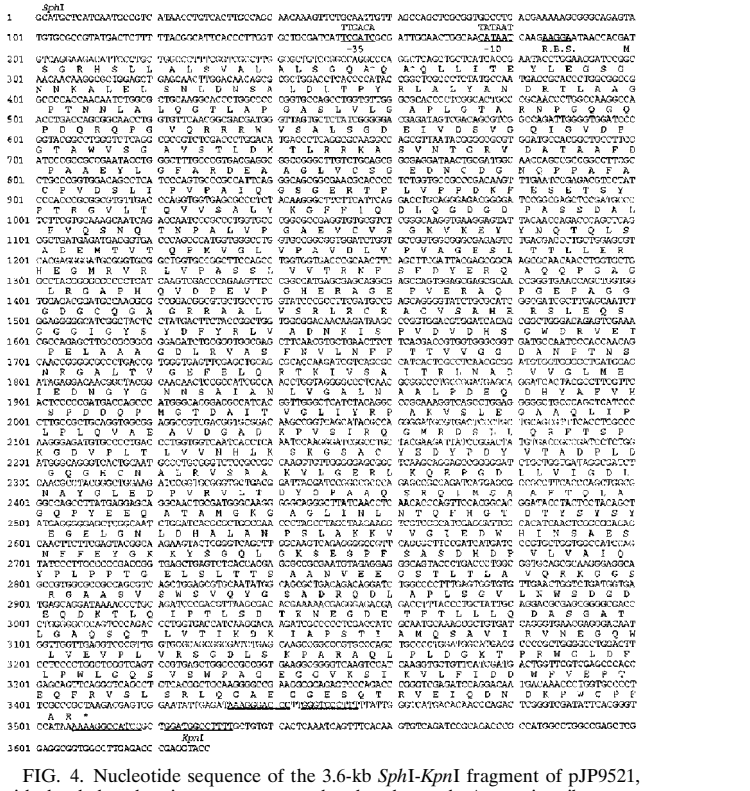

FIG. 4. Nucleotide sequence of the 3.6-kb *Sph*I-*Kpn*I fragment of pJP9521, with the deduced amino sequence translated underneath. A putative ribosome-binding site (R.B.S.) has been detected upstream from the start codon, which occurs 198 bp from the beginning of the sequence. Also upstream is a potential *E. coli* σ^{70} promoter region, marked as −10 and −35, with consensus sequences provided above for comparison (18). Underlined are two regions of self-complementarity which may act as transcriptional terminators following the stop codon (*) at position 3409. Also indicated are two potential signal sequence cleavage sites occurring after Ala-20 and Ala-22 (^).

DNA sequence and predicted protein analysis. No regions of extensive DNA homology could be detected when the *nucH* sequence was compared with those in the GenBank and EMBL databases. Searches for weaker homology using a six-frame translation of the sequence did not reveal any homologs, nor did comparison of the predicted protein sequence with relevant databases. A search of the Prosite database revealed a putative leucine zipper motif from Leu-44 to Leu-65, although this is not considered a specific sequence and its presence may not be significant. The criteria of von Heijne (50, 51) were used to assess the N terminus of the predicted protein region for a signal sequence. Two such possible cleavage sites, after Ala-20 and Ala-22, were identified, with the weight-matrix method (51) determining that the one after Ala-22 was more likely. Examination of the amino acid composition showed that only eight reactive cysteine residues were present in the active protein, with the potential for only four disulfide bridges. A large number of aliphatic residues are present, however, with leucine, valine, and alanine each comprising over 10% of the number of residues in NucH.

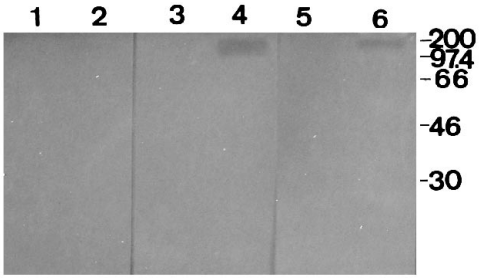

FIG. 5. DNase activities of samples prepared by cellular fractionation. Lanes: 1, JMP5511 supernatant; 2, JMP5512 supernatant; 3, JMP5511 periplasmic fraction; 4, JMP5512 periplasmic fraction; 5, JMP5511 cytoplasmic fraction; 6, JMP5512 cytoplasmic fraction. Molecular mass markers are given on the right in kilodaltons. No DNase activity is observed for JMP5511, while NucH activity is seen in both periplasmic and cytoplasmic fractions.

Cellular fractionation. *E. coli* DH1 was chosen as a host strain for fractionation studies, and this strain was transformed separately with pUC19 and pJP9521. The resulting strains, designated JMP5511 and JMP5512, were grown to the exponential phase, and periplasmic and cytoplasmic fractions were prepared. Marker enzyme assays were performed, and these were used to ensure the integrity of the fractions prepared. No significant alkaline phosphatase or β-galactosidase activity was detected in the culture supernatants. Alkaline phosphatase activity in the periplasmic fraction was determined to be 86% for JMP5511 and 90% for JMP5512 (averages of two determinations). Cytoplasmic β-galactosidase activity was shown to be 84% for JMP5511 and 94% for JMP5512. These fractions were then tested for nuclease activity by native SDS-PAGE. DNase activity was not evident for JMP5511 extracts, while JMP5512 displayed DNase activity in both periplasmic and cytoplasmic fractions (Fig. 5). Therefore, it is apparent that NucH is capable of being processed to the periplasm in *E. coli.*

Inactivation of the *nucH* gene. pJP9521 was digested with *Sac*I and electrophoresed to isolate an internal 1.8-kb *nucH* fragment. This DNA was ligated into pJP5603 which had been digested with the same enzyme, and the plasmid was transformed into the host JM109λpir. Selection on MacConkey agar containing kanamycin was used to identify recombinants through the *lacZ* screening system available for this vector. A clone containing the required insert, designated pJP9527, was transformed into the delivery host S17-1λpir, and this strain was mated overnight with rifampin-resistant JMP636. Transconjugants were selected on agar containing rifampin and kanamycin. The expected loss of DNase activity was tested on DNase agar, although no reduction in halo size could be detected. To confirm the insertion of pJP9527, Southern blotting using the 1.8-kb *Sac*I fragment as a probe was performed on two separate clones, JMP5513 and JMP5514. When *Bam*HI-digested DNA extracted from these clones is compared with that extracted from JMP636, disruption of the wild-type positive band is observed, indicating insertion into *nucH* (Fig. 6). Extracellular protein samples were prepared from JMP636 and JMP5513, and these were run on a native SDS-PAGE gel. This demonstrated the loss of a band of DNase activity corresponding to a protein similar in size to NucH, in addition to the presence of two additional bands of extracellular DNase activity (Fig. 7). Therefore, the inability to detect the inactivation of *nucH* on DNase agar can be attributed to the presence of these additional extracellular activities, and it also appears that NucH is not the major activity of JMP636.

Nuclease analyses. A total protein sample was prepared from *E. coli* DH5α containing pJP9521. This host strain was

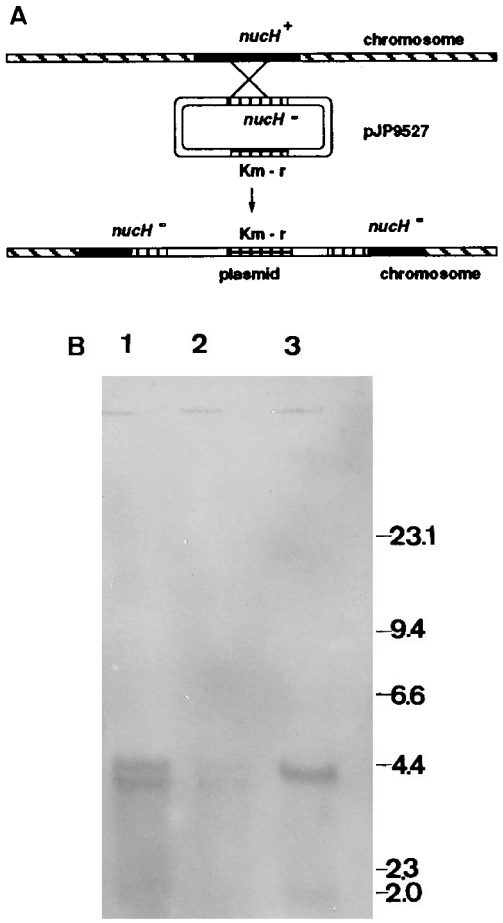

FIG. 6. (A) Inactivation of NucH by homologous recombination with pJP9527. (B) Southern blot demonstrating disruption of the wild-type *nucH* gene in JMP636 after suicide of pJP9527. All lanes are *Bam*HI digests. Lanes: 1, mutant strain JMP5513; 2, mutant strain JMP5514; 3, wild-type JMP636. Molecular sizes are given on the right in kilobases. The single band observed for the parental strain JMP636 has been disrupted by the insertion of pJP9527 to produce two distinct bands seen in lanes 1 and 2.

chosen as it has been used in previous studies and is devoid of the periplasmic *E. coli* EndA activity (36, 48). Extracts were run on a native SDS-PAGE gel after boiling for 5 min in sample buffer or mixing with sample buffer containing 2-mercaptoethanol at a final concentration of 10%. Lanes were separated after running and developed overnight. When they were compared with a control, one which had received neither of these pretreatments, activity was seen to have been reduced by preboiling of the sample and to a lesser degree by 2-mercaptoethanol (Fig. 8). To test the substrate specificity of NucH, which was already known to degrade dsDNA, extracts were run on gels containing ssDNA and RNA, and in both cases degradation occurred (Fig. 9). Cofactor requirements were also tested with extracts, with Mg^{2+} being the most effective in producing NucH activity and Ca^{2+} and Mn^{2+} also capable but with limited activity observed with Zn^{2+} (Fig. 10).

DISCUSSION

In this paper, we have reported a novel nuclease from *A. hydrophila* which has no known homologs on the basis of its nucleotide or predicted protein sequence. This is only the

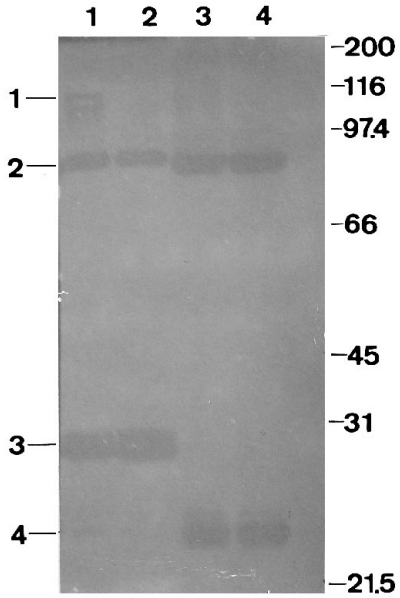

FIG. 7. Nuclease gel showing the loss of DNase activity in protein samples from JMP636 after inactivation with pJP9527. Lanes: 1, concentrated supernatant sample from JMP636; 2, concentrated supernatant sample from JMP5513; 3, crude cell protein sample from JMP636; 4, crude cell protein sample from JMP5513. Molecular mass markers are given on the right in kilodaltons. NucH activity present only in lane 1 is marked on the left (1), as are two additional extracellular activities (2 and 3). Activities present in the cell protein samples are marked also (2 and 4), with no NucH activity evident in these lanes.

second time that an extracellular gram-negative bacterial nuclease of such a size has been studied—Newland et al. (38) reported a 100-kDa nuclease from *V. cholerae*, although the cloned *xds* gene encoding this protein was not sequenced. Focareta and Manning previously examined the distribution of the *xds* gene and also the *dns* gene encoding the smaller 25-kDa DNase in members of the family *Vibrionaceae* by Southern blotting (12). They found that both genes were present in a number of species within the genus *Vibrio* but that neither was detected when hybridized against DNA of *A. hydrophila*. While the stringency used in these experiments is not known,

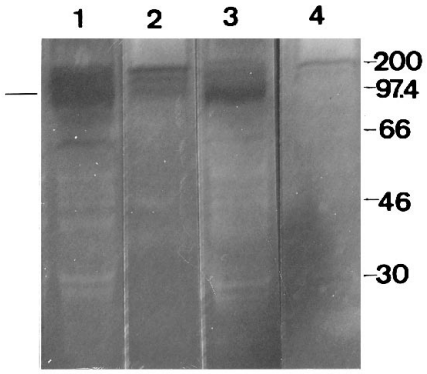

FIG. 8. Activity of NucH on dsDNA after treatment with denaturing agents. Lanes: 1, no treatment (control sample); 2, protein sample boiled for 5 min in sample buffer prior to loading; 3, protein sample loaded with buffer containing 2-mercaptoethanol; 4, sample boiled and treated with 2-mercaptoethanol. Molecular mass markers are given on the right in kilodaltons. Lanes run with 2-mercaptoethanol were developed separately from the remainder of the gel. NucH activity is marked on the left.

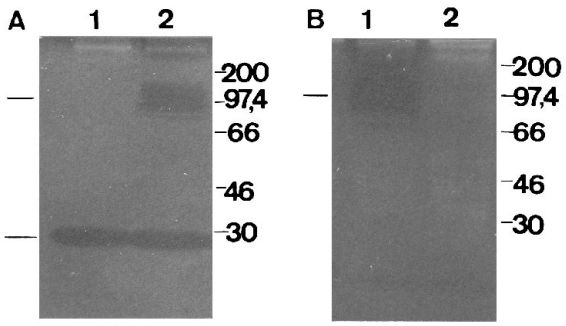

FIG. 9. (A) Activity of NucH on RNA. Lanes: 1, JMP5511; 2, JMP5512. Molecular mass markers are given on the right in kilodaltons. NucH activity is marked on the left, as is *E. coli* RNase activity visible in both lanes. (B) Activity of NucH on ssDNA. Lanes: 1, JMP5512; 2, JMP5511. Molecular mass markers are given on the right in kilodaltons, with NucH activity marked on the left.

this result may be due to a lack of homology at the DNA level or to the absence of these genes from the strain used. The subsequent cloning of a *dns* homolog from *A. hydrophila* displayed only a 60% homology at the DNA level (8); hence, it is also entirely possible that *xds* would not have been detected. Therefore, while no homologs of *nucH* have previously been identified through sequencing, given the overall relationship between these organisms it is possible that *xds* is related to *nucH*, and this supposition could be confirmed by sequencing of *xds.*

As well as showing that it is a novel enzyme with nuclease activity, these results also indicate NucH is an addition to the range of extracellular enzymes known to be produced by strains of *A. hydrophila*. When *nucH* was introduced into *E. coli*, the protein product was shown to be processed only to the periplasm, as has previously been observed by other groups; this has been attributed to a deficient secretion system in *E. coli*. This result indicates, though, that the existence of an N-terminal signal peptide as predicted from sequence data is likely. However, an extracellular location for NucH in *A. hydrophila* was confirmed by the inactivation of the gene and comparison of the DNase activity in a supernatant sample with that of wild-type JMP636. No cell-associated NucH activity could be detected by using the native SDS-PAGE system. Inactivation of the *nucH* gene also showed that its product is not an essential enzyme, at least when *A. hydrophila* is grown in rich media. A reduction in extracellular DNase activity was not observed on agar medium, indicating that NucH is not the major activity present in JMP636. Focareta and Manning (12) have also shown that of the two DNase activities present in *V. cholerae*, the smaller Dns enzyme has a stronger activity than does the 100-kDa Xds protein. Examination of the extracellu-

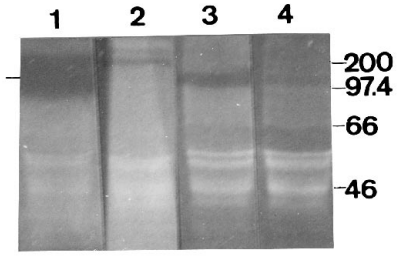

FIG. 10. Development of NucH activity on dsDNA in the presence of various cofactors. Lanes: 1, Mg^{2+}; 2, Ca^{2+}; 3, Mn^{2+}; 4, Zn^{2+}. Molecular mass markers are given on the right in kilodaltons.

lar DNase profiles of JMP636 and the *nucH*-deficient JMP5513 indicated that at two additional regions of activity appear to be present in the latter strain. One of these is similar in size to the previously described Dns of *A. hydrophila*, while to our knowledge no reports of an 80-kDa gram-negative bacterial DNase have previously been made. The inactivation of *nucH* without loss of the 80-kDa band indicates that this band represents a separate activity and not a truncated form of NucH. Further studies are required to examine the other nucleases in this strain in order to elucidate the function of three enzymes with apparently similar activities.

Examination of the determined DNA sequence of the *nucH* clone has identified the predicted ribosome-binding site as AAGGA. This site is identical to that predicted for an amylase from this strain (16) and also to that predicted for the previously studied DNase from *A. hydrophila* (8). As *A. hydrophila* has a high moles percent G+C content, this can be expected to be reflected in the nucleotide composition of *nucH*. Overall, this gene's moles percent G+C content was 64%, with a value of 78% determined for the third position of each codon. This latter value supports the open reading frame predicted for *nucH*, while both values are within the range previously observed (7). Sequencing of a large open reading frame can provide useful insight into the codon usage of *A. hydrophila*. The bias for the G or C in the last position is evident for all amino acids except asparagine. There are 72 of these residues predicted to occur in NucH, with 38 residues encoded by GAT and 34 encoded by GAC. Examination of sequence data for other extracellular enzymes did not demonstrate a bias for usage either, although fewer residues were present in the genes examined (7, 16).

These preliminary studies do not show any differences between the function of NucH and those of previously described nucleases. Cation requirements have been studied only for the *Anabaena* sp. NucA, with similar results obtained for both this and NucH (36). Substrate specificity has also not been studied for some enzymes. Gene products from *V. cholerae* (Dns and Xds) and *A. hydrophila* (Dns) were isolated on DNase media and were not reported to have been tested for RNase activity (8, 11, 38). EndA from *E. coli* has been shown to be inhibited by RNA (25), while its homolog NucM from *E. chrysanthemi* can degrade this substrate (33).

The obvious difference between NucH and previously studied nucleases is its large molecular mass. Also, it contains large numbers of hydrophobic residues, in contrast to the mainly hydrophilic smaller nucleases (4, 8). Both NucH and the previously described 25-kDa Dns protein from *A. hydrophila* have eight cysteine residues in the mature protein (8). Whereas Dns is affected by 2-mercaptoethanol, NucH does not show an apparent loss of activity. Boiling has been shown to reduce NucH activity, while in contrast, a heat-stable nuclease activity has been detected in other gram-negative bacteria and is also present in *A. hydrophila* (14, 37; also our unpublished data). Among well-studied nucleases, only NucM has been tested for heat stability, and it was found to be relatively resistant to heat inactivation (33).

Two regions of self-complementarity are observed downstream of the predicted *nucH* stop codon, the second of which was predicted by using the algorithm of Brendell and Trifonov (6). These two regions may form terminators for divergent genes, although we have sequenced downstream of *nucH* and this did not predict homology to a recognized coding region. Similarly, analysis of the 200-bp region preceding *nucH* did not predict homology to existing sequence data. Random sequences from *A. hydrophila* JMP636 have been able to identify significant homology to coding regions from the well-sequenced *E. coli* and *Haemophilus influenzae* (our unpublished data). Other studies have also demonstrated homology of *A. hydrophila* unidentified open reading frames to *E. coli* sequences (24).

We have cloned the gene encoding a novel nuclease from *A. hydrophila* and begun preliminary characterization of its protein product. The main differences observed between *nucH* and previously cloned and sequenced bacterial nuclease genes are the predicted size of the protein product and its amino acid composition, resulting in different in vitro susceptibilities. This work will provide a starting point for examination of nuclease activity in this organism—the function of NucH may be better understood when the additional DNase activities observed in the strain are also studied.

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