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Relatedness of a Periplasmic, Broad-Specificity RNase from *Aeromonas hydrophila* to RNase I of *Escherichia coli* and to a Family of Eukaryotic RNases

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The isolation, sequencing, and characterization of a periplasmic RNase gene from *Aeromonas hydrophila* AH1133 is described. Following subcloning of the gene on a 2.7-kb *Pst*I fragment, its direction of transcription and approximate location were determined. Analysis of the nucleotide sequence reveals that the gene is 645 bp long, coding for 215 amino acid residues with a total molecular weight of 24,215. A typical leader sequence is present at the beginning of the corresponding protein. Computer analysis revealed strong local similarities to *Escherichia coli* RNase I and to the active site of a family of eukaryotic RNases. Expression studies indicate that the RNase natural promoter functions poorly in *E. coli*. In this organism, the enzyme is mainly localized in the cytoplasm and periplasm, although high levels of expression lead to significant release into the extracellular medium. Functional and physical characterizations further indicate that the periplasmic and cytoplasmic enzymes of *A. hydrophila* are likely to be the counterparts of *E. coli* RNase I and its cytoplasmic form RNase I*: as for the *E. coli* enzymes, the *A. hydrophila* RNase forms have similar sizes and show broad specificity, and the periplasmic form is more active towards natural polymer RNA than its cytoplasmic counterpart. Both forms are relatively thermosensitive and are reversibly inactivated by up to 0.6% sodium dodecyl sulfate. Southern hybridization revealed homology to *E. coli* K-12 and *Shigella* sp. genomic DNA, a finding which correlates with the presence of secreted RNases in these organisms. In contrast, species of phylogenetically closer genera, such as *Vibrio* and *Plesiomonas*, did not hybridize to the *A. hydrophila* RNase gene.

The export of bacterial proteins through the cytoplasmic membrane, a feature of most gram-negative and gram-positive organisms, has been the focus of intensive research in the past 20 years, leading to a good understanding of the basic process. Exported proteins are generally synthesized as precursor molecules possessing an amino-terminal sequence (leader sequence) which is cleaved by a so-called signal peptidase upon translocation through the membrane to generate the mature product. The latter either remains as a component of the inner membrane or is directed to the periplasm, to the outer membrane, or to the surrounding environment. Although much is known about the passage of proteins across lipid bilayers, the ensuing localization process remains obscure, despite some attempts to define structural features characteristic of proteins localized in different compartments (51; for reviews, see references 11, 35, 44, 50).

Aeromonas hydrophila, a human enteropathogen and a member of the *Vibrionaceae* family, excretes a large number of proteins, including various virulence factors and degradative enzymes (4, 8, 14), into the medium (18) and is, therefore, a useful organism in the study of extracellular secretion and targeting of excreted products. The genes encoding two such extracellular products, an aerolysin (14, 15) and an amylase (8), have recently been isolated, sequenced, and expressed in *Escherichia coli*, where they were found to remain in the periplasm (8, 14). The characterization of periplasmic proteins of *A. hydrophila* should be helpful in identifying structural features important for protein localization in this organism.

We report here the isolation, mapping, and sequencing of a gene from *A. hydrophila* coding for an RNase I-like RNase and the characterization of its product.

MATERIALS AND METHODS

Strains and media. The strains and plasmids used in this study are listed in Table 1. All *E. coli* strains were grown in L broth (LB) (34) either in liquid or on solid medium containing 2% agar. Plasmids were selected in the presence of 100 μg of ampicillin per ml or 50 μg of spectinomycin per ml. Selection of RNase-positive clones and radial diffusion assays were performed on LB-RNA plates, which contained 2 g of yeast RNA (BDH Chemicals, Ltd., Poole, England) per liter and 50 μg of toluidine blue O (Sigma Chemical Co., St. Louis, Mo.) per ml (45). Non-*E. coli* strains were grown in either liquid or solid brain heart infusion medium (Difco, Basel, Switzerland).

Screening of RNase-proficient clones. Transformation mixtures containing RNase-producing clones were plated onto LB-RNA plates and incubated overnight at 37°C. RNase-producing clones could be readily detected by the pink halo that formed around the colony. Detection of very weakly expressing colonies could normally be enhanced by flushing the plate with a few milliliters of 1 N HCl, which revealed around the colony a clear halo in an opaque background.

Radial diffusion assay of RNase and β-lactamase activity. (i) RNase. Typically, 10 to 20 μl of enzyme suspension was loaded into holes punched in LB-RNA agar in petri dishes. Whenever samples from different clones were compared, the

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Characteristic(s)	Source or origin
Strains		
A. hydrophila		
AH1133	Wild type	S. C. Sanyal, Varanasi, India
AH1	Wild type	S. C. Sanyal, Varanasi, India
AH22	Wild type	S. C. Sanyal, Varanasi, India
CD172B	Wild type	S. C. Sanyal, Varanasi, India
E. coli K-12		
DH5α	F^- *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lacZ* M15	1
LE392	F^- *hsdR514 supE44 supF58 lacY1 galT22 metB1 trpR55*	37
E. coli O9:K9(B):H12	Wild type	ATCC 23505
S. sonnei 53GII	Phase II variant	55
S. flexneri 4b	Wild type	ATCC 12024
S. boydii type 1	Wild type	ATCC 9207
S. typhimurium LT2	*polA2 ara-9*	SSVI[a] strain collection
S. typhi Ty2	Wild type	SSVI strain collection
K. pneumoniae BL1151	Wild type	SSVI strain collection
P. aeruginosa PA103-29	Wild type	SSVI strain collection
P. putida KT2442	*hsdR1 hsdM*$^+$	7
V. cholerae Ogawa El Tor 3083	Wild type	SSVI strain collection
V. cholerae Inaba El Tor	Wild type	SSVI strain collection
V. cholerae Inaba CVD103Hg-R	*ctxA hlyA*::mer	27
P. shigelloides M51	Wild type	SSVI strain collection
Plasmids		
pHC79	Ap^r Tc^r *cos*	12
pUC8	Ap^r, high copy number	54
pMTL23	Ap^r, high copy number	5
pCL1920	Spc^r, low copy number	26
pING1	Ap^r P_{araB}	19
pJLA602	Ap^r P_L *atpE* TIR[b]	48
pDF1	pHC79 $RNase^+$	This study
pDF2	pUC8 $RNase^+$	This study
pSSVI48-1	pMTL23 $RNase^+$	This study
pSSVI48-18	pCL1920 $RNase^+$	This study
pSSVI48-20	pCL1920 $RNase^+$	This study
pSSVI48-21	pING1 $RNase^+$	This study
pSSVI48-22	pJLA602 $RNase^+$	This study

[a] SSVI, Swiss Serum and Vaccine Institute.
[b] TIR, translational initiation region.

exact amounts loaded were normalized to the optical density at 600 nm (OD_{600}) of the cultures at the time of sampling. The plates were then incubated at 37°C for a given time, and the diameter of each pink halo upon hydrolysis was measured. These measurements were taken as a reflection of the RNase concentration in the sample, as observed by Schill and Schumacher (49), who found a linear relationship between the diameter of the hydrolysis zones and the RNase concentration using the radial diffusion assay, so long as all subclones yielded the same specific activity.

(ii) β-Lactamase. The procedure used was essentially that of Kuo and Feng, with slight modifications (24). The enzyme suspension (10 μl) was loaded into holes punched in LB agar containing 1% starch and incubated overnight at 37°C. The plate was then flushed with 5 ml of Lugol solution (Hoechst, Darmstadt, Germany) containing 0.2% benzylpenicillin (Sigma Chemical Co., Basel, Switzerland), which was removed after 10 s. The plate was then rinsed with water and incubated at room temperature in the dark until clear halos developed.

Enzymatic assay of RNase. RNase specificity and kinetics were assayed by using the method of Postek et al. (43), as described for the OD_{295} assay except that reaction volumes were reduced to 250 μl. The determination of enzyme kinetics with either 0.1 mM uridylyl(3′-5′)adenosine (UpA), cytidylyl(3′-5′)adenosine (CpA), guanosyl(3′-5′)adenosine (GpA), or adenylyl(3′-5′)adenosine (ApA) as the substrate was based on the initial velocity rates at room temperature, by using partially purified periplasmic (0.03 U) or cytoplasmic (0.4 U) RNases, in the presence of 2.5 U of adenosine deaminase, 0.1 U of xanthine oxidase, and 0.64 U of nucleoside phosphorylase (all from Sigma Chemie, Buchs, Switzerland). The formation of uric acid from the dinucleoside monophosphate substrates was monitored at OD_{295} as a function of time. One unit of RNase activity is defined as the activity that produces an OD_{295} change of 1.0/min at room temperature, with 0.1 mM GpA as the substrate.

Partial purification of cytoplasmic and periplasmic forms of the *A. hydrophila* RNase. The method used is essentially that of Meador et al. (30), with minor modifications and omission of the last mono-S column chromatography. An overnight culture (10 ml) of the overproducing strain DH5α(pSSVI48-

22) or the control strain DH5α(pJLA602) grown in LB medium containing 100 μg of ampicillin per ml at 30°C was added to 1 liter of the same medium, and the culture was induced with shaking at 39°C to an OD_{600} of 0.6. This cell density proved to be optimal in order to avoid premature lysis of spheroplasts in subsequent steps. The culture was centrifuged, and the pellet was resuspended in 300 ml of spheroplasting buffer (20% sucrose, 30 mM Tris-HCl [pH 7.5], 1 mM EDTA). Freshly prepared lysozyme was added at 50 μg/ml, and the suspension was left on ice for 20 min, during which time more than 90% of the cells were converted to spheroplasts (33). The spheroplasts were harvested at 4°C for 10 min at 5,000 rpm in an HB-4 Sorvall rotor. The supernatant was clarified by a 15 min centrifugation at 10,000 rpm in a GSA Sorvall rotor and dialyzed twice against 4 liters of 20 mM morpholineethanesulfonic acid (MES) buffer (pH 6.1). This dialysate was designated as the periplasmic fraction. The pellet was resuspended in 30 ml of distilled water, and the cells were disrupted by sonication. The lysate was centrifuged for 15 min at 15,000 rpm in an SS-34 Sorvall rotor; the supernatant was filtered through a 0.45-μm-pore-size membrane filter and dialyzed twice against 2 liters of 20 mM MES buffer (pH 6.1). The dialysate constituted the cytoplasmic fraction. RNases from both fractions were then chromatographed in turn with an S-Sepharose fast flow column (courtesy of K. E. Schnorf, Pharmacia, Dübendorf, Switzerland) equilibrated with 20 mM MES buffer (pH 6.1) and eluted with a gradient composed of 40 ml each of MES buffer and MES buffer containing 1 M NaCl. Fractions containing RNase activity were then pooled, dialyzed twice against 2 liters of 50 mM potassium phosphate buffer, (pH 7.2), and stored at −20°C.

Recombinant DNA techniques. Recombinant DNA techniques were carried out in accordance with standard procedures (46). Nucleotide sequencing was performed with denatured double-stranded plasmid DNA in accordance with the procedure of Sanger et al. (47), with Sequenase (United States Biochemicals, Cleveland, Ohio).

Hyperexpression of RNase. An overnight culture of DH5α containing plasmid pSSVI48-20, -21 or -22 was diluted to an OD_{600} of 0.1 and grown to an OD_{600} of 0.4, at 30°C for pSSVI48-22 or at 37°C for pSSVI48-20 and -21. The cultures were then split into two parts. One part was left to grow as before, whereas the other part was induced. DH5α (pSSVI48-20) and DH5α(pSSVI48-21) cultures were induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside and 1% arabinose, respectively, and incubation was continued for 2 h at 37°C. DH5α(pSSVI48-22) cells were induced by a temperature shift from 30 to 41°C, and samples were taken at specified time intervals. The OD_{600} of the cultures was measured after induction.

Cell fractionation. Our procedure is similar to that of Oliver and Beckwith (40). Each step was carried out at 4°C. Each 1.5- to 5-ml culture, processed as described above, was centrifuged, and the supernatant was kept as the extracellular fraction. The pellet was resuspended in 0.1 volume of a 20% sucrose–10 mM Tris (pH 7.5) solution and kept on ice for 10 min, after which the cells were pelleted. The pellet was resuspended in 0.1 volume of H_2O and placed on ice for an additional 10 min. This suspension was centrifuged as described above, and the supernatant was kept as the periplasmic fraction. The corresponding pellet was again resuspended in 0.1 volume of H_2O and sonicated. The final lysate was then clarified by a 2-min centrifugation in a microcentrifuge; the resulting supernatant was designated as the cytoplasmic fraction and was stored at 4°C. In certain cases, the membrane fraction was separated from the cytoplasmic fraction by centrifugation for 20 min at 18,000 rpm in an SS-34 Sorvall rotor. The supernatant represented the cytoplasmic fraction, and the pellet was resuspended in 1 volume of phosphate-buffered saline (PBS) and stored at 4°C as the membrane fraction.

Alternatively, the procedure of Osborn and Munson was used as described previously (42). Both fractionation procedures gave essentially the same results.

Colony hybridization. Overnight cultures (3 μl) of relevant strains were spotted onto a nitrocellulose filter and air dried. Subsequent cell lysis, DNA denaturation, and fixation were done as described by Montenegro et al. (36). Nonradioactive labeling of the DNA probe with digoxigenin (Boehringer GmbH, Mannheim, Germany), hybridization, and detection were carried out in accordance with the manufacturer's instructions.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli, with 12.5 to 15% acrylamide (25). Silver staining was performed with a silver-staining kit (Bio-Rad Laboratories, Glattbrugg, Switzerland), following the manufacturer's recommendations.

Nucleotide sequence accession number. The accession number X67054 was given to the nucleotide sequence of the RNase gene by the EMBL data library in Heidelberg, Germany.

RESULTS

Isolation, characterization, and sequencing of the *A. hydrophila* RNase gene. A cosmid gene bank of *A. hydrophila* AH1133 genomic DNA created in vector pHC79 and established in *E. coli* LE392 (4) was screened on LB-RNA agar for the production of RNase. One positive clone (pDF1) was used for further work. A 2.7-kb *Pst*I fragment from pDF1 subcloned into plasmid pUC8 directed full RNase activity. A restriction map of this clone, named pDF2, was subsequently constructed (Fig. 1A). The direction of transcription of the RNase gene within pDF2 was determined by fusion to Tn*phoA* following infection of pDF2-containing cells with λTn*phoA* (28). PhoA-positive clones had Tn*phoA* inserted to the right of the unique *Eco*RV site and transcribed from left to right on the map shown in Fig. 1A. Deletion analysis allowed further subcloning of the RNase gene as an *Xho*I-*Pst*I 2.1-kb fragment into pMTL23 to give pSSVI48-1. Lac promoter-driven RNase expression of various fragments from pSSVI48-1 subcloned into pCL1920 allowed further localization of the RNase gene. The *Eco*RV-*Cla*I fragment conferred a fully positive RNase-producing phenotype, whereas the *Nco*I, *Bam*HI, and *Xho*I-*Fsp*I fragments were negative (Fig. 1A). By using the subclones generated for the above analysis, both strands of the RNase gene and flanking regions were sequenced. A total of 1,073 bp of the 1,272 bp sequenced are presented in Fig. 1B. Computer analysis of the translated sequence identified a 645-bp open reading frame starting at bp 143 with a coding capacity for a 24,215 Da protein (215 amino acids). This open reading frame overlaps the region bearing the RNase gene as defined by deletion and subcloning analyses. It is transcribed in the same direction as indicated by Tn*phoA* analysis. The first 19 to 22 amino acids of the corresponding protein sequence have the characteristics of a typical leader sequence: residues 2 and 3 are positively charged (Lys-Lys) and are followed by a long stretch of hydrophobic amino acids (4 to 17). Two serine residues precede one potential cleavage site

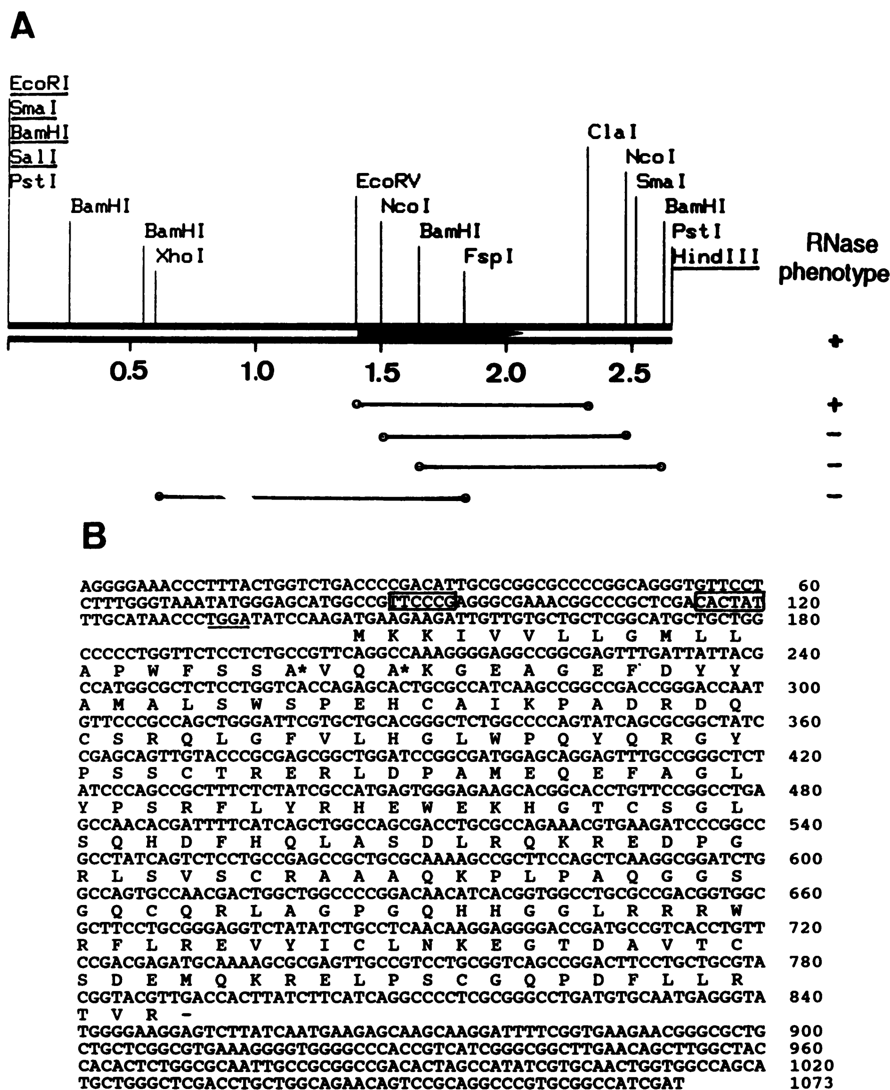

FIG. 1. (A) Restriction map of pDF2 insert. The pUC8 vector is not shown, except for the underlined restriction sites which are part of the polylinker. The closed area with a point depicts the location of the RNase gene and its direction of transcription. The lines underneath the arrow represent sequences subcloned into pCL1920 via pDF2 or pSSVI48-1 and tested for their RNase phenotype, indicated by "+" or "−" signs. (B) Nucleotide sequence of the *A. hydrophila* RNase gene. The putative Shine-Dalgarno sequence is underlined and putative −10 and −35 sequences are boxed. The asterisks (*) after the 19th and 22nd amino acids indicate the two potential leader peptide cleavage sites.

between residues 19 and 20 (Ala-Val). A second possible cleavage site is located between residues 22 and 23 (Ala-Lys). Taken together, these data indicate that the 645-bp open reading frame most probably corresponds to the RNase gene.

Putative −10 and −35 sequences were detected upstream from the initiator codon of the RNase gene with, for both sequences, 3 of 6 bases matching those of the consensus promoter in *E. coli*. The two sequences are separated by 21 bp, a suboptimal spacing for in vivo function in *E. coli*. A possible Shine-Dalgarno sequence is also found 8 bp upstream from the start codon (TGGA).

Codon usage for the RNase gene correlates well with those from other genes of *A. hydrophila* (Table 2). It is noteworthy that the stop codon is TGA, as for the aerolysin (15) and the phospholipid-cholesterol acyltransferase genes (53). In *A. hydrophila*, the sequence TGA is, in general, heavily favored over TAA or TAG (data not shown). It is therefore likely that TGA is the preferred stop codon for this organism, in contrast to the overall tendency in prokaryotes which is biased towards TAA (17, 23).

Amino acid sequence similarity to other RNases. The amino acid sequence of the *A. hydrophila* RNase deduced from the DNA sequence presented above was compared with those of 152 RNases present in the SWISSPROT 20 data base. The results are presented in Fig. 2. The highest degrees of similarity were found with the periplasmic RNase I of *E. coli* (31) and the excreted RNases M of *Aspergillus saitoi* (57), T2 of *Aspergillus oryzae* (22), and Rh of *Rhizopus niveus* (13). By using the shared motif HGLWP, a search of the SWISSPROT 20 data base allowed the further identification of RNase LE of *Lycopersicon esculentum* (20), a tomato; MC of *Momordica charantia* (16), a bitter gourd species; and the stylar glycoprotein 2 of *Nicotiana alata* (winged tobacco)

TABLE 2. Comparison of codon usage in *A. hydrophila* genes encoding RNase, amylase, and aerolysin

AA[a]	Codon[b]	No. of codons in[c]:			AA	Codon	No. of codons in:		
		rna	*amy*	*aer*			*rna*	*amy*	*aer*
Ala	GCU	3	3	4		CUC	6	14	6
	GCC*	11	25	17	Leu	CUA	1	1	1
	GCA	0	0	2		**CUG***	13	25	21
	GCG	4	9	11	Lys	**AAA**	2	2	5
Arg	**CGU**	3	1	7		AAG*	7	20	20
	CGC*	9	11	11	Met	AUG	5	9	7
	CGA	3	2	3	Phe	UUU*	4	3	2
	CGG	3	4	1		UUC	3	12	9
	AGA	0	2	1	Pro	CCU	1	3	1
	AGG	0	1	1		CCC	5	11	6
Asn	AAU	0	2	8		CCA	2	0	5
	AAC*	1	20	20		**CCG***	7	7	13
Asp	**GAU**	5	16	10	Ser	UCU	2	1	3
	GAC	5	19	23		UCC*	7	3	13
Cys	UGU	4	2	4		UCA	2	0	4
	UGC*	5	2	0		UCG	0	4	1
Gln	CAA	7	5	10		AGU	1	2	3
	CAG*	9	25	13		**AGC**	4	12	15
Glu	**GAA**	1	2	9	Thr	ACU	0	1	3
	GAG*	12	13	9		**ACC***	4	10	23
Gly	**GGU**	2	3	12		ACA	0	0	3
	GGC*	10	30	27		ACG	1	2	2
	GGA	3	3	0	Trp	TGG	5	11	18
	GGG	4	9	9	Tyr	UAU*	6	10	15
His	CAU	3	2	3		UAC	1	16	6
	CAC*	5	7	3	Val	**GUU**	2	0	2
Ile	**AUU**	1	0	5		GUC*	3	14	14
	AUC*	2	15	11		GUA	1	1	1
	AUA	0	1	9		**GUG**	2	11	17
Leu	UUA	0	1	1	STOP	**UAA**	0	1	0
	UUG	1	0	3		UGA*	1	0	1
	CUU	2	3	2		UAG	0	0	0

[a] AA, amino acid.
[b] Codons in bold type correspond to those most frequently used in *E. coli*. The asterisks (*) emphasize the preferred codons in the *A. hydrophila* gene encoding RNase.
[c] *rna*, gene encoding RNase; *amy*, gene encoding amylase; *aer*, gene encoding aerolysin.

(29), all of which are excreted proteins, as belonging to the same family. Two global regions of similarity were detected, one extending from Leu-61 to Pro-76 and the other from Glu-102 to Gly-111 (*A. hydrophila* sequence coordinates). These clusters include His-53 and His-115 of RNase T2 of *A. oryzae* and Glu-93 of RNase Rh of *R. niveus*, all of which are essential for RNase activity (21, 22). Furthermore, complete identity was found at Cys-43 and Cys-76, which are not part of these clusters, and Cys-109, which is part of the last cluster. Cys-68 and Cys-118 of the RNase T2, which correspond to Cys-76 and Cys-109 of the *Aeromonas* RNase, have been suggested to form a disulfide bond which would bring the two reactive His residues into close proximity (21). Pairwise alignment with the *A. hydrophila* RNase reveals further similarities, especially at the N terminus of *E. coli* RNase I (Ala-19 to Trp-38) and at the C termini of the eukaryotic proteins (Asp-116 to Glu-129 and Val-178 to Cys-181). In contrast to the above eukaryotic RNases, no potential glycosylation sites were found in either of the two prokaryotic proteins. Interestingly, the extracellular amylase of *A. hydrophila* also bears some similarity to the cognate enzyme of *A. oryzae* (8), one of the mold strains tested.

Hyperexpression and subcellular localization. The expression of the *A. hydrophila* RNase gene in *E. coli* was studied by using various vectors, and the production of RNase and its localization were assayed. Plasmid pSSVI48-18 consists of the *Pst*I fragment from pDF2 cloned into the corresponding site of the low-copy-number vector pCL1920, which is based on the pSC101 replicon and is thus present at about five copies per cell (26). Plasmid pSSVI48-20 has the *Eco*RV-*Cla*I fragment of pDF2 cloned into the *Sma*I site of pCL1920. In pDF2, pSSVI48-18, and pSSVI48-20, the RNase gene is controlled by the Lac promoter. Finally, the *Eco*RV fragment from pSSVI48-1 (one of the *Eco*RV sites belongs to the pMTL23-derived polylinker and is not shown in Fig. 1A) was subcloned into the *Sma*I site of pING1, on one hand, and into the blunted *Nco*I site of pJLA602, on the other hand. The latter plasmids, named pSSVI48-21 and -22, respectively, are present at about 20 copies per chromosome. Each plasmid was transformed into DH5α cells. Cultures from each strain were then grown and induced for 1 h (pSSVI48-22) or 2 h (pSSVI48-20). The various cellular compartments were then fractionated as described in Materials and Methods and analyzed for RNase activity by the radial diffusion assay.

Results of the radial diffusion assay are shown in Fig. 3. Control DH5α cells without plasmid displayed very limited RNase activity in each compartment with respect to the RNase clone-containing cells. The levels of RNase activity directed by pSSVI48-18 indicate that the natural RNase promoter is only poorly expressed in *E. coli*. The presence of

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                                                             *
1. MK----KIVVL-LGMLLAPWFSSAVQAKGEAGEFDYYAMALSWSPEHCAI   45
2. MKAFWRNAALLAVSLLPFSSANALALQAKQYGDFDRYVLALSWQTGFCQS   50
3. -------------------EFPSCP------KDIPFSCQNSTAVADSCCF   25
4. -------------------TIDTCS------SDSPLSCQ--TDNEASCCF   23
5. MK----A--VLALATLIGSTLASSC------SSTALSCSNSAN-SDTCCS   37
6. AK----D-----------------F------DFFYFVQ---QWPGSYCDT   20
7. ------------------------F------DSFWFVQ---QWPPAVCSF   17
8. MS----KSQLTSVFFILLCALSPIY------GAFEYMQLVLTWPITFCRI   40

                            .*****       .
1. KPA------------DRDQCSRQLGFVLHGLWPQYQRG-YPS--------   74
2. QHDRNRNERDECR-LQTETTNKADFLTVHGLWPGLPKSVAARGVDERRWM   99
3. NSPGGALLQTQFWDTNPPSG-PSDSWTIHGLWPDNCDGSYGQF-------   67
4. NSPGGSLLQTQFWDYDPSDG-PSDSWTIHGLWPDNCDGSYQEY-------   65
5. PEYGLVVLNMQW---APGYG-PDNAFTLHGLWPDKCSGAYAPSG------   77
6. KQSCCY----------PTTGKPAADFGIHGLWPNNNDGTYPSN-------   53
7. QKSGSC----------PGSGLRT--FTIHGLWPQGS-GTSLTN-------   47
8. KHCERT----------PT------NFTIHGLWPD-NHTTMLNY-------   66

     *  ..                     .    ..
1. --SCTRER----------------LDPAMEQEFAGLYPSR--------FL   99
2. RFGCA-TRPIPNLPEARASRMCSSPETGLSLETAAKLSEVMPGAGGRSCL  148
3. ---CDKSREYSNITAILQEQGRTELLSYMKKYWPNYEGDD----EE--FW  108
4. ---CDDSREYSNITSILEAQDRTELLSYMKEYWPDYEGAD----EDESFW  108
5. --GCDSNRASSSIASVIKSKD-SSLYNSMLTYWPSNQGNN----N--VFW  118
6. ---CDPNSPYDQSQI-------SDLISSMQQNWPTLACPSGSG---STFW   90
7. ---CPQGSPFDITKI-------SHLQSQLNTLWPNVL--RANN---QQFW   82
8. ---CDRSKPYNMFTD-------GKKKNDLDERWPDLTKTKFDSLDKQAFW  106

     *. ***.* .                     .   .   .
1. YRHEWEKHGTCSGLSQH-------------DFHQLASDLRQKREDPGRLS  135
2. ERYEYAKHGACFGFDPDAYFGTMVRLNQEIKESEAGKFLADNYG--KTVS  196
3. E-HEWNKHGTCINTIEPSCYKDYSPQKEVGDYLQKTVDLFKGLDSYKALA  157
4. E-HEWNKHGTCINTIDPSCYTDYYAQEEVGDFFQQVVDLFKTLDSYTALS  157
5. S-HEWSKHGTCVSTYDPDCYDNYEEGEDIVDYFQKAMDLRSQYNVYKAFS  167
6. S-HEWEKHGTCAE-------SVLTNQHA---YFKKALDLKNQIDLLSILQ  129
7. S-HEWTKHGTCSE-------STF-NQAA---YFKLAVDMRNNYDIIGALR  120
8. KD-EYVKHGTCCS-------DKFDREQ----YFDLAMTLRDKFDLLSSLR  144

       .      .                                  .
1. VSCRAAAQKPLPAQGGSGQCQRLAGPGQH-----HGGLRRRWRFLREVYI  180
2. RRDFDAAFAKSWGKENVKAV----------KLTCQGNPA----YLTEIQI  232
3. KAGIVPDSSKTYKRSEIESALAAIHDGKKPYISCEDGALNEIWYFYNIKG  207
4. DAGITPSEDATYKLSDIEDALAAIHDGYPPYVGCEDGALSQLYYYFNVKG  207
5. SNGITPGG--TYTATEMQSAIESYF-GAKAKIDCSSGTLSDVALYFYVRG  214
6. GADIHPDGE-SYDLVNIRNAIKSAI-GYTPWIQCNVD-QSGNSQLYQVYI  176
7. PHAAGPNGR-TKSRQAIKGFLKAKF-GKFPGLRCRTDPQTKVSYLVQVVA  168
8. NHGI-SRGF-SYTVQNLNNTIKAIT-GGFPNLTC-----SRLRELKEIGI  186

            .           *
1. CLNKEGTDA-VTCSDEMQKRELPSCGQPDFLL---RTVR  215
2. SIKADAINAPLSANSFLPQPHPGNCGKT---FVIDKAGY  268
3. --NAITGEY-QPIDTLTS----PGCSTSGIKYLPKKSEN  239
4. --SAIGGTY-VASERLED----SNCKGSGIKY-PPKSSS  238
5. --R---DTY-VITDALST----GSCSG-DVEY----PTK  238
6. CVDGSGSSL-IECPIFPG----GKC-GTSIEFP---TF-  205
7. CFAQDGSTL-IDCTR-------DTC-GANF------IF-  191
8. CFDETVKNV-IDCPN--P----KTC-KPTNKGV---MFP  214
```

FIG. 2. Amino acid sequence similarity between various RNases. The number at the beginning of each line identifies the RNase: 1, *A. hydrophila* RNase I-like, 2, *E. coli* RNase I; 3, *A. oryzae* RNase T2; 4, *A. saitoi* RNase M; 5, *R. niveus* RNase Rh; 6, *L. esculentum* RNase LE; 7, *M. charantia* RNase MC; and 8, *N. alata* S2 stylar glycoprotein. The asterisks and dots above the sequences highlight shared and similar amino acids, respectively. Shared amino acids between the *Aeromonas* RNase and any of the other enzymes are underlined. The number at the end of each line indicates the position of the last amino acid in the line within the sequence.

the gene on a high-copy-number plasmid, such as pDF2, affords a much higher level of expression, as did expression from the Lac promoter in pSSVI48-20. The highest levels of expression were obtained from the λp_L promoter present in plasmid pSSVI48-22. The results obtained with pSSVI48-21 were similar to those obtained with pSSVI48-22 (data not shown). Total activity was contributed by the various compartments in the same way for all constructs except pSSVI48-18, for which the periplasmic fraction had about twice as much activity as the cytoplasmic fraction. For all other constructs, activity was equally divided between the periplasmic and the cytoplasmic fractions; in addition, 0 to 30% of total activity was recovered from the culture supernatants, the highest percentage correlating with the highest total activity. Membrane fractions did not contain any detectable RNase activity (data not shown). Increasing the

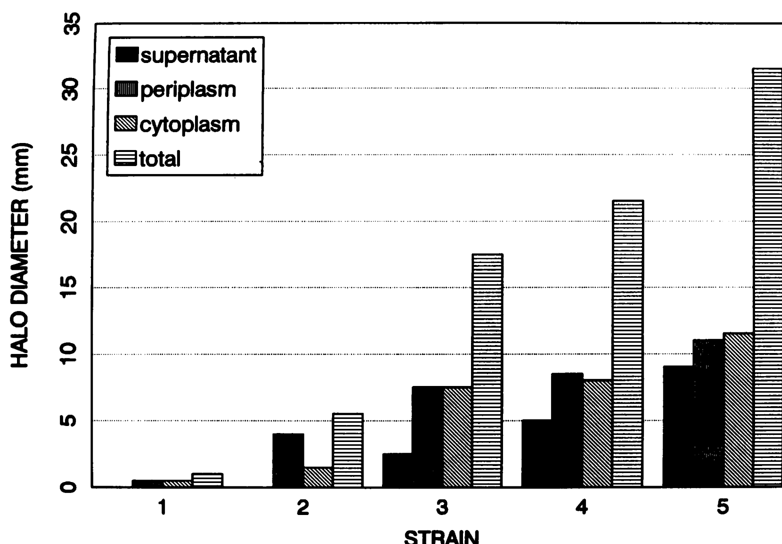

FIG. 3. Radial diffusion assay of RNase from the supernatant, periplasm, or cytoplasm fractions from recombinant RNase clones carried by *E. coli* DH5α. Cultures were processed at the end of the log phase. The volumes of extract assayed correspond in all cases to roughly 10^7 cells. Halo diameters were measured after 8 h at 37°C. In three successive experiments, halo diameters for the samples were within 15% of each other. DH5α strain plasmids: 1, control (no plasmid); 2, pSSVI48-18; 3, pSSVI48-20; 4, pDF2; 5, pSSVI48-22.

induction time of DH5α (pSSVI48-22) from 60 to 120 or 240 min resulted in an enrichment of cytoplasmic activity relative to that in the periplasm (data not shown). Since it is known that overproduction can lead to an aberrant fractionation pattern, the most realistic results in terms of the proportion of RNase in the various compartments may be those shown for pSSVI48-18, i.e., the amount of enzyme secreted in the periplasm may be about twice that remaining in the cytoplasm, whereas no or very little enzyme may be excreted from the cell into the supernatant. On the other hand, the presence of cellular RNA in the cytoplasmic crude extract may limit the amount of cytoplasmic RNase detected in the assay. Caution must therefore be exercised in attempting to derive the relative RNase concentrations in the various compartments.

As controls, the subcellular locations of β-lactamase, a periplasmic enzyme, and lactate dehydrogenase (LDH), a cytoplasmic enzyme, were assayed. β-Lactamase was recovered exclusively from the periplasmic fraction, whereas LDH was found mainly in the cytoplasm. Supernatants of induced DH5α(pSSVI48-22) cultures contained less than 2.5% total LDH activity (data not shown).

Partial purification of cytoplasmic and periplasmic forms of the RNase. Cultures of DH5α(pJLA602) and DH5α (pSSVI48-22) were induced at 40°C and fractionated as described in Materials and Methods. The cytoplasmic and periplasmic compartments were then individually separated by cation-exchange chromatography on S-Sepharose, and the collected fractions were assayed by radial diffusion for 4 h at 37°C on LB-RNA plates. Periplasmic RNase activity from the overproducing strain DH5α(pSSVI48-22) eluted at around 0.55 M NaCl. In successive purifications, we sometimes noted weak RNase activity in the flowthrough. In contrast, no RNase activity could be detected from the corresponding samples of the control strain DH5α(pJLA602) under identical assay conditions. More RNase could be eluted at 0.55 M NaCl when aliquots of the flowthrough were rechromatographed. Detected flowthrough activity is therefore likely to stem from supersaturation of the gel matrix with bound material. As for its periplasmic counterpart, the cytoplasmic form of the RNase eluted around 0.55 M NaCl. No significant RNase activity could be detected after chromatography of the cytoplasmic material from the control strain. Following dialysis in 50 mM phosphate buffer, aliquots of the purified enzymes and corresponding fractions from the control strain were analyzed by SDS-PAGE and silver staining of the gel. As can be seen in Fig. 4, both enzymes appear to be greater than 90% pure, with some faint bands appearing only below and above the cytoplasmic RNase. Both forms of the RNase appear to have the same apparent M_r of 26,500. Interestingly, SDS-PAGE of over-

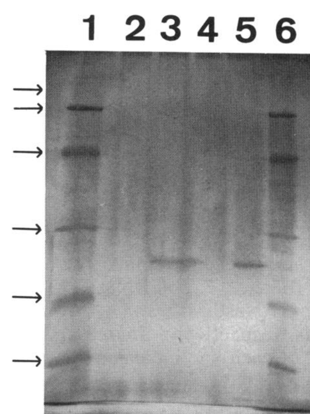

FIG. 4. Silver-stained SDS-polyacrylamide gel of purified RNases. Lanes: 1, molecular weight standards (shown by arrows on the left) with sizes (in thousands) of 97.4, 66.2, 45.0, 31.0, 21.5, and 14.4 (the largest marker did not respond to the silver stain); 2, periplasmic fraction of DH5α(pJLA602); 3, periplasmic fraction of DH5α(pSSVI48-22) containing ca. 500 ng of protein, amounting to 0.05 U of periplasmic RNase; 4, cytoplasmic fraction of DH5α (pJLA602); 5, cytoplasmic fraction of DH5α(pSSVI48-22) containing ca. 750 ng of protein, amounting to 0.2 U of cytoplasmic RNase.

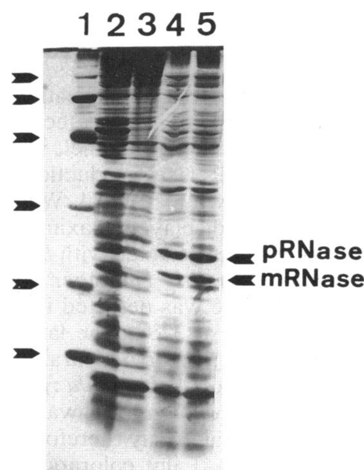

FIG. 5. SDS-PAGE of whole-cell extracts of DH5α(pJLA602) and DH5α(pSSVI48-22). Cultures (20 ml) were grown at 30°C to an OD_{600} of 0.6. One-half was then induced at 42°C, whereas the other half was left to grow at a low temperature to the early stationary phase. After the indicated induction times or at suitable cell densities, 1-ml aliquots were removed and centrifuged, and the pellets were resuspended in 200 μl of sample buffer. Aliquots (20 to 60 μl) were loaded per lane, depending on the final OD_{600} value. Lanes: 1, molecular weight standards (see the legend to Fig. 3 for sizes); 2, DH5α(pJLA602); 3, DH5α(pSSVI48-22), uninduced; 4 and 5, DH5α(pSSVI48-22) induced at 41°C for 60 and 120 min, respectively. pRNase, precursor RNase; mRNase, putative mature RNase. In a separate experiment, the protein(s) contained in a gel slice corresponding to the upper band labeled "pRNase" was eluted in buffer and shown to possess RNase activity (data not shown).

produced RNase from whole-cell extract of DH5α(pSSVI48-22) (Fig. 5) revealed putative RNase bands at positions consistent with M_rs of 24,000 and 26,500. If these bands correspond, respectively, to the mature and precursor forms of the RNase, it follows that the purified RNases are recovered as native enzymes. Note that similar sizes ($M_r \approx$ 27,000) (3) corresponding to the unprocessed state (31) were also found for the purified RNase I and its counterpart, RNase I*, in *E. coli*. We do not know why the purified enzyme recovered from the periplasmic fraction is apparently unprocessed.

Enzymatic properties. Since it is obvious from amino acid sequences that the *A. hydrophila* RNase is related to RNase I of *E. coli*, we sought to characterize further its enzymatic properties. We took advantage of the newly developed assay by Postek et al. (43), which allows the determination of substrate specificity and enzyme kinetics by using partially purified enzymes from the cytoplasmic and periplasmic fractions. Figure 6A and B show a double reciprocal plot depicting the kinetics of both forms of the RNase that was used in the determination of their V_{max} and K_m values. These data together with those shown in Table 3 demonstrate that both enzymes are capable of efficiently cleaving all four dinucleoside monophosphate substrates. The K_m values are higher for the periplasmic RNase than for its cytoplasmic counterpart, indicating weaker substrate affinity. Both enzymes display similar K_m values for all substrates except ApA, for which the K_m is somewhat lower. The rates of substrate cleavage as measured by V_{max} are comparable for all four substrates for each enzyme form, with the lowest and highest values attained with the ApA and UpA substrates, respectively. As a control, the K_m and V_{max} values for the RNase A were determined. As expected from previously published data (58), RNase A cleaved only UpA and CpA substrates and showed a marked preference for the CpA over the UpA substrate. The K_m value for RNase A with the UpA substrate is similar to the corresponding value for the cytoplasmic *A. hydrophila* RNase, whereas that with the CpA substrate is about 10-fold lower. These results demonstrate broad specificity for both forms of the *A. hydrophila* RNase, with the highest activity with respect to the dinucleoside monophosphate substrates exhibited by the cytoplasmic form. What is the situation with respect to natural polymer RNA? In order to answer this question, aliquots of bacterial or baker's yeast RNA were incubated for 90 min at 37°C with samples from the periplasmic or cytoplasmic fractions of a DH5α(pSSVI48-21) induced culture, and the mixtures were then analyzed on a 1% agarose gel. Figure 7 shows that the cytoplasmic RNase is not as active as its periplasmic counterpart, especially towards yeast RNA. Cytoplasmic RNase activity towards bacterial RNA varied from low to very high in successive experiments (data not shown). Taken together, these results suggest that the cytoplasmic form of RNase has reduced activity towards natural RNA. These results are consistent with those of Cannistraro and Kennel (3), who showed that *E. coli* RNase I, but not RNase I*, is able to degrade natural RNA. The fact that the activity of the cytoplasmic RNase against total yeast RNA was clearly seen in radial diffusion assays might be due to refolding of the enzyme into a more active conformation when presented to the plate medium. The heavy band in lane 3 and that above the RNA band in lane 6 are derived from the cytoplasmic fractions used in the assay (data not shown) and probably represent cellular DNA.

Sensitivity to denaturing agents. (i) Heat. The rate of heat inactivation of both forms of the *A. hydrophila* RNase was measured. Aliquots of the partially purified enzymes were boiled at pH 7.5 for the indicated times and enzymatically assayed with ApA as the substrate. Figure 8 shows that 50% inactivation is reached after 90 s of boiling for the periplasmic form, in contrast to 3.5 min for the cytoplasmic form. For both forms, however, about 20% of the total activity is resistant to prolonged boiling. These results differ from those found for *E. coli* RNase I, which is highly heat resistant (3). In contrast, the cytoplasmic form of RNase I, RNase I*, shows a rate of heat inactivation at neutral pH similar to that of the cytoplasmic form of the *A. hydrophila* RNase.

(ii) SDS. Samples (10 μl) of periplasmic or cytoplasmic fractions of overexpressed DH5α(pSSVI48-22) cells were mixed with SDS at a 0.1, 0.2, 0.4, 0.6, 0.8, 1, or 5% final concentration in a 20-μl volume. Controls without SDS were included. The solutions were loaded into wells punched in an LB-RNA agar plate, which was incubated at 37°C. Results were the same for both the periplasmic and cytoplasmic fractions. After 1.5 h of incubation at 37°C, only the samples in 0.1% SDS showed activity identical to that of the control; samples in 0.2 or 0.4% SDS showed activity characterized by having the same halo size as the 0.1% SDS samples but with much weaker coloration. After 6.5 h, the 0.2% samples showed the same color intensity as the control and the 0.1% SDS samples. The 0.6% SDS samples presented a pale halo with the same size as those containing less SDS. Following overnight incubation, the 0.4% SDS samples had acquired the same color intensity as the controls, whereas the 0.6% SDS samples remained pale. No significant level of activity could be detected in samples containing more than 0.6% SDS. These results indicate that RNase is resistant to SDS concentrations up to 0.1% and is reversibly inhibited by SDS

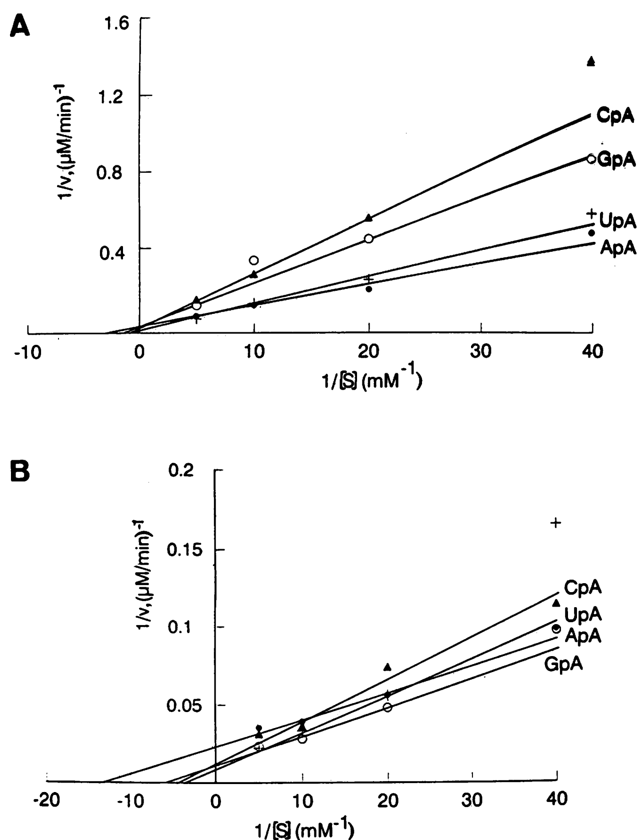

FIG. 6. Kinetics of periplasmic (A) and cytoplasmic (B) *A. hydrophila* RNases with different dinucleoside monophosphate substrates. The formation of uric acid from the substrates was determined from initial velocity rates. The substrate concentration ranged from 0.025 to 0.2 mM. Values of $-1/K_m$ and $1/V_{max}$ are determined by the intercepts with the abscissa and ordinate, respectively. ●, ApA; ▲, CpA; ○, GpA; +, UpA.

up to 0.6%. In the above assay, dilution of SDS by diffusion into the agar presumably allows the RNase to regain its active conformation when the local SDS concentration falls below a certain critical level. It is unclear whether the presence of more than 0.6% SDS irreversibly inhibited the enzyme or whether the local SDS concentration never fell below that level needed for the activity to be regained.

Homology to other members of the family *Enterobacteriacae* and production of secreted RNase. We performed dot blot hybridization with small amounts of cultures of a battery of bacterial strains from various genera. Four independent isolates of *A. hydrophila* were also tested, including the positive control AH1133. The probe was the *Eco*RV-*Cla*I fragment from pDF2. Stringent hybridization and washing conditions were employed in order to detect only significant homology to the RNase gene. In parallel, we tested all the strains for extracellular RNase production on LB-RNA plates. The results are shown in Table 4. Whereas all tested strains of *A. hydrophila* displayed maximal hybridization signals, a weaker signal was detected with *E. coli* DH5α and with all three *Shigella* strains. No other strain gave any signal. Extracellular RNase was detected in various strains after overnight incubation at 37°C. The largest halos were produced by the four *A. hydrophila* strains, followed by *Vibrio cholerae* Inaba El Tor, *Salmonella typhi* Ty2, *Salmonella typhimurium* LT2, *V. cholerae* Ogawa El Tor 3083, and *Shigella flexneri*. These strains may therefore encode one or more extracellular RNases. Faint coloration was also seen for *E. coli* DH5α, *Shigella sonnei*, and *Klebsiella pneumoniae*. In contrast, there were no halos around colonies of *Plesiomonas shigelloides*, *V. cholerae* CVD103Hg-R, *S. boydii*, *E. coli* 09, *Pseudomonas aeruginosa*, or *Pseudomonas putida*.

DISCUSSION

In this paper, we report the subcloning, sequencing, and characterization of a gene coding for a periplasmic RNase from *A. hydrophila* and the characterization of its product. The generation of active gene fusions between Tn*phoA*, which encodes the mature part of alkaline phosphatase, and the subcloned *A. hydrophila* RNase gene showed that the RNase leader sequence is efficiently recognized and processed by the *E. coli* export machinery, permitted determination of the direction of transcription of the gene, and, combined with deletion and subcloning analyses, allowed approximate delineation of its boundaries, thereby facilitating sequencing of the gene.

Computer analysis of sequenced regions upstream of the RNase gene revealed putative promoter sequences. Three of six nucleotides of the putative −35 and −10 regions are homologous to the corresponding regions in the *E. coli* consensus, and in each case, homologous nucleotides correspond to those highly conserved in the consensus hexamers (10). Moreover, four of six nucleotides of the −35 region are homologous to those of the *A. hydrophila* aerolysin promoter (15), and four of six are homologous to the −10 region of the *A. hydrophila argT* promoter (9). Thus, we consider it likely that these sequences can serve as promoters in vivo, although the spacing of 21 nucleotides is not optimal for function in *E. coli*. A potential Shine-Dalgarno sequence

TABLE 3. K_m and V_{max} values of *A. hydrophila* cytoplasmic and periplasmic RNases with different dinucleoside monophosphate substrates[a]

Substrate	Periplasmic RNase		Cytoplasmic RNase		RNase A	
	K_m (M)	V_{max} (μM/min)	K_m (M)	V_{max} (μM/min)	K_m (M)	V_{max} (μM/min)
ApA	4×10^{-4}	37	8×10^{-5}	45	ND[b]	ND
GpA	8×10^{-4}	37	2×10^{-4}	100	ND	ND
CpA	1×10^{-3}	50	2×10^{-4}	83	3×10^{-5}	59
UpA	1×10^{-3}	83	3×10^{-4}	125	2×10^{-4}	80

[a] K_m and V_{max} values for the *A. hydrophila* RNases were calculated from the data shown in Fig. 5. The corresponding data for 200 ng of RNase A (from bovine pancreas; Fluka, Buchs, Switzerland) as the control are not shown. Since, for practical reasons, the amount of periplasmic or cytoplasmic RNase or RNase A used in the assay was different, V_{max} values can be compared for each type of RNase but not between different types.

[b] ND, not determined.

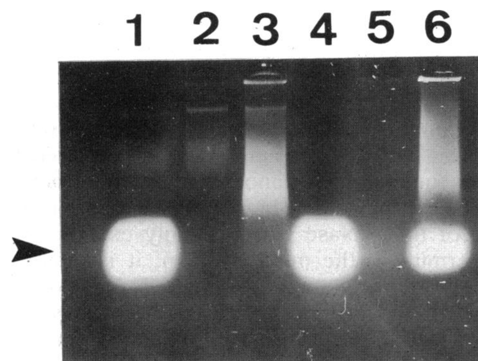

FIG. 7. Activity of periplasmic and cytoplasmic forms of RNase towards polymer RNA. Crude periplasmic or cytoplasmic fractions (5 μl) from induced DH5α(pSSVI48-21) cultures were incubated with 2 μg of RNA for 90 min at 37°C, and the samples were run through a 1% agarose gel containing 1 μg of ethidium bromide per ml. Lanes: 1, control, untreated bacterial RNA from DH5α; 2, periplasmic RNase-treated bacterial RNA from DH5α; 3, cytoplasmic RNase-treated bacterial RNA from DH5α; 4, control, untreated yeast RNA; 5, periplasmic RNase-treated yeast RNA; 6, cytoplasmic RNase-treated yeast RNA. The arrowhead indicates the position of RNA.

TABLE 4. *A. hydrophila* RNase gene homology with other members of the family *Enterobacteriaceae* and correlation with production of exported RNase

Bacterium	RNase activity[a]	DNA homology[b]
A. hydrophila		
AH1133	+++	++
AH1	+++	++
AH22	+++	++
CD172B	+++	++
V. cholerae Inaba El Tor	+++	−
V. cholerae 3083	++	−
S. typhi	++	−
S. typhimurium	++	−
S. flexneri	++	+
S. sonnei	+	+
E. coli DH5α	+	+
K. pneumoniae	+	−
P. shigelloides	−	−
V. cholerae CVD103Hg-R	−	−
S. boydii	−	+
E. coli O9	−	−
P. aeruginosa	−	−
P. putida	−	−

[a] Strains were grown on brain heart infusion agar containing 1% RNA and 50 mg of toluidine blue O per ml as short streaks. The sizes of pink halo zones were compared after overnight growth at 37°C with that produced by *A. hydrophila* AH1133.

[b] Hybridization signal: ++, as strong as that of control strain AH1133; +, positive but weaker than that of the control; −, negative (no signal).

(TGGA) is located 8 bp upstream of the initiation codon, in agreement with consensus rules for these sequences (52). Probably as a result of its suboptimal sequence features, the RNase promoter is only weakly active in *E. coli*. High levels of expression resulting from the replacement of the natural RNase promoter by strong *E. coli* promoters, such as in pSSVI48-20, -21, and -22, indicate that the codon usage of the *A. hydrophila* RNase gene is compatible with the expression in *E. coli*. However, as with other *A. hydrophila* genes, it exhibits a preference for a number of codons (including the stop codon) which are nonoptimal for *E. coli*. The codons preferred often contain a higher percentage of G's or C's (e.g., GAG for GAA, AAG for AAA, or TGA for TAA), consistent with the fairly high G+C content of *A. hydrophila* DNA (ca. 60% versus ca. 50% for *E. coli*) (for a discussion, see reference 41).

The putative leader sequence of RNase is rather typical, consisting of the N-terminal methionine, two positively charged residues at positions 2 and 3 followed by a core of 13 hydrophobic residues, and, finally, two potential cleavage sites between residues 19 and 20 and residues 22 and 23. These cleavage sites fit the criteria established by von Heijne (56), with, in our case, a slight preference for the second site because of the polar residue (Gln) at position −2.

Similar amino acid sequences were found between the *A. hydrophila* RNase and other RNases, including *E. coli* RNase I. For the RNase I, three clusters with a high degree of similarity have been identified, two of which are aligned with similar clusters in the RNases from three mold strains and three species belonging to higher plants. The identical clusters (underlined) (. . .HGLWP. . . and . .HEWEKHG TC. . .) include amino acids essential for RNA catalysis in this family of eukaryotic RNases, namely, all three His residues (39) and the first Glu residue in the second cluster. Thus, it is very likely that they define the active site in their prokaryotic counterpart as well. This is consistent with a previous suggestion that one or more His residues of *E. coli* RNase I are part of the active site of the enzyme because of its structural and functional analogies with the pancreatic RNase A (31). This notion is further supported by the presence of four identical Cys residues, two of which are supposedly involved in the spatial arrangement of the active clusters. Note also that His-102 is not conserved in *E. coli* RNase I and in the S2 gene product of *N. alatia*. This is in agreement with the conclusions of Ohgi et al. (39), who found that, in the case of the RNase Rh, this His residue is

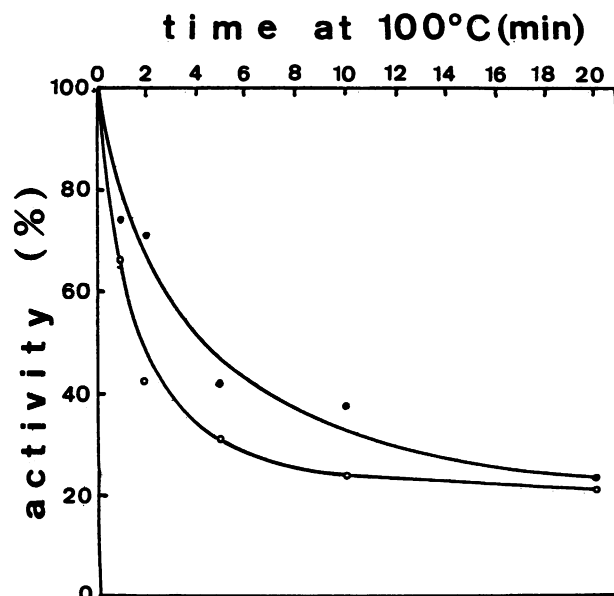

FIG. 8. Heat inactivation of RNases. Aliquots of partially purified RNases were boiled for the indicated times and enzymatically assayed for activity by using 0.1 mM ApA as the substrate. Each assay was carried out with, respectively, 0.03 and 0.4 U of the periplasmic (○) and cytoplasmic (●) enzymes.

not as critical to the catalytic activity as the other two. Similar amino acids, clustered or isolated, are also found throughout the sequences suggesting that there are other regions of functional significance such as binding sites. For example, whereas the leader peptides and C termini of the prokaryotic RNases show no obvious similarities, the putative N termini of the mature proteins, from residues 19 to 38 (by *A. hydrophila* RNase numbering) are 60% identical. On the other hand, the *A. hydrophila* enzyme shows significant C-terminal (e.g., residues 116 to 127 and 177 to 194), but not N-terminal, similarity to the eukaryotic proteins. It is therefore possible that the N termini of the prokaryotic RNases encode a similar function, which might be irrelevant or differently executed in eukaryotes. It is noteworthy that, contrary to most of the eukaryotic RNases examined in this study, the prokaryotic proteins do not contain any potential glycosylation site. This might indicate that the latter either have appeared in the eukaryotic lineage soon after the divergence of prokaryotes and eukaryotes or were lost during prokaryotic evolution. Our data bank search with the HGLWP sequence detected further limited but possibly significant similarities between *A. hydrophila* RNase and other prokaryotic and eukaryotic enzymes with no known RNase activity, such as the cellulase of *Bacillus polymyxa* (2) (comparison not shown). Taken together, the *Aeromonas* RNase and its family can be labeled as "ancient," first-edition proteins, in the classification by Doolittle et al. (6). It would be of great interest to further compare these features with those of their counterparts in archaebacteria and mycoplasmas.

RNase activity was mostly recovered from the cytoplasmic and periplasmic fractions, a significant percentage being also present in the supernatant in some cases (Fig. 3, pDF2, pSSVI48-20, and pSSVI48-22). LDH and β-lactamase subcellular locations showed that cell lysis alone cannot account for these results. The presence of a large plasmid-borne RNase pool in the cytoplasm is unlikely to reflect inefficiency in the secretion process. Therefore, the plasmid-borne RNase activity found in the cytoplasmic and periplasmic compartments is likely to reflect different forms of the same enzyme, in a manner analogous to *E. coli* RNase I (periplasmic) and RNase I* (cytoplasmic), which appear to be isoforms of the same enzyme apparently encoded by the same gene (3). Indeed, in addition to obvious protein sequence similarities, various lines of evidence strongly suggest that the two forms of the *A. hydrophila* enzyme are the counterparts of RNase I and RNase I* in *E. coli*. (i) All of them are broad-specificity RNases, being able to cleave phosphodiester bonds after any nucleotide. Furthermore, as for the suggestion that RNase I* utilizes oligonucleotides or homopolymers more efficiently than does RNase I, the RNase I*-like enzyme also appears to have more activity towards, and more affinity for, the dinucleotide monophosphate substrates than does the RNase I-like form. (ii) Cytoplasmic forms from both organisms are less active towards natural polymer RNA than are their periplasmic counterparts. It was hypothesized for the *E. coli* enzyme that the cytoplasmic form may be unable to form disulfide bonds in the highly reducing environment of the cytoplasm. Indeed, it was found to contain free sulfhydryl groups (3), suggesting a conformation for RNase I* different from that of RNase I. The latter enzyme contains eight Cys residues with the potential to form four disulfide bridges, whereas the *A. hydrophila* enzyme contains nine Cys residues and thus various conformations may be possible. A similar argument was presented in the case of other exported degradative enzymes in *E. coli*, such as alkaline phosphatase, which is activated upon export from the cytoplasm (32). (iii) They all have very similar migration rates on SDS-PAGE, with apparent molecular masses of ca. 27 kDa. It is somewhat puzzling that this value for the *A. hydrophila* enzyme appears to be about 10% higher than that predicted from the DNA sequence. Since amino acid similarity between the protein sequences of the *A. hydrophila* enzyme and other members of this RNase family mainly extends from near their N termini to the middle region, it is likely that the discrepancy involves some unexpected event occurring at the 3′ end of the gene, such as frameshifting, which would affect the location of transcription termination or translational read-through. Amino- and carboxy-terminal amino acid sequencing will probably be needed in order to settle the issue. So far, the only obvious functional difference between the enzymes from the two organisms pertains to heat stability. In *E. coli*, RNase I is very heat stable compared with RNase I* (3), whereas the RNase I-like enzyme is inactivated by heat at an even faster rate than the cytoplasmic form. In addition, however, we note that in agreement with other RNases of this RNase family (39), both isoforms of the *Aeromonas* enzyme show a preference for adenylic acid, whereas RNase I of *E. coli* seems to show some preference for cytidylic or guanylic acid (30).

The hybridization signal obtained with *E. coli* K-12 and the three *Shigella* species genomic DNAs probed with the *A. hydrophila* RNase gene indicates possible relatedness of their RNases. It was, however, surprising that phylogenetically more closely related strains of *V. cholerae* (38) did not bind the probe.

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