

Halovibrin, Secreted from the Light Organ Symbiont *Vibrio fischeri*, Is a Member of a New Class of ADP-Ribosyltransferases

KARL A. REICH¹ AND GARY K. SCHOOLNIK^{1,2*}

Howard Hughes Medical Institute¹ and Department of Microbiology,²
Stanford University School of Medicine, Stanford, California 94305

Received 30 August 1995/Accepted 26 October 1995

The purification, cloning, and deduced amino acid sequence of an ADP-ribosyltransferase secreted from the marine bacterium *Vibrio fischeri* (*V. fischeri* ADP-r) is described. This enzyme was purified from culture supernatant, and partial amino acid sequence obtained from the purified protein was used to design a degenerate oligonucleotide probe that was used to clone a cross-hybridizing DNA fragment from *V. fischeri* genomic DNA. Recombinant *Escherichia coli* clones harboring this fragment possessed ADP-ribosyltransferase activity. The DNA fragment was sequenced, and deletion analysis localized the ADP-ribosyltransferase activity to one of the three possible open reading frames in the fragment; the deduced amino acid sequence from this open reading frame matched the amino acid sequence obtained from the purified protein. *V. fischeri* ADP-r has no significant homology (DNA or amino acid) with other known ADP-ribosyltransferases. This enzyme appears to require neither proteolytic cleavage nor a reducing agent for enzymatic activity. The cloned gene is expressed but not secreted in *E. coli*; however, it is secreted from a heterologous marine *Vibrio* species. We have named this enzyme halovibrin.

Vibrio fischeri is a luminescent, halophilic, gram-negative marine organism and the bacterial symbiont of a number of luminescent fish and squid (24). This ecological niche brings it into direct and intimate contact with a eucaryotic host (16). The specificity and anatomy of this interaction bear a striking resemblance to the interaction of *Vibrio cholerae* and the human intestinal tract (19, 20). This analogy can be further strengthened by the observation that *V. fischeri* has a functional homolog of the transcriptional activator *toxR*, which in *V. cholerae* regulates the production of cholera toxin (CTX), an ADP-ribosyltransferase (5, 23). We reasoned that *V. fischeri* might have a homolog of CTX; therefore, we searched for ADP-ribosyltransferase activity in *V. fischeri* using an artificial substrate (17) and found evidence for such an enzyme in spent media from cultures of *V. fischeri*.

Bacterial ADP-ribosyltransferases constitute a diverse group of enzymes that share surprisingly little amino acid sequence homology (6). These proteins can be highly toxic (e.g., diphtheria toxin, exotoxin A, and mosquitocidal toxin) or modulatory (e.g., CTX and pertussis toxin) or have effects on host cells that are less well understood (e.g., clostridial C3 toxin and exoenzyme S). All of the targets of this family of enzymes are eucaryotic proteins.

The squid host of *V. fischeri* hatches without a symbiont and must acquire a specific strain of *V. fischeri* from the environment in order to initiate the developmental program of its light organ (20, 24). These observations suggest that the bacterial symbiont provides some signal to its host (20, 24), and our hypothesis is that this signal could be, by analogy to host-pathogen systems, an ADP-ribosyltransferase. In this report, we describe the purification, cloning, sequencing, and initial characterization of an ADP-ribosyltransferase secreted from the luminescent marine bacterium *V. fischeri* (*V. fischeri* ADP-r).

* Corresponding author. Mailing address: Stanford University Hospital, Beckman Center, Rm. B241, Stanford, CA 94304-5428. Phone: (415) 723-8158. Fax: (415) 723-1399.

MATERIALS AND METHODS

ADP-ribosyltransferase assay. ADP-ribosyltransferase activity (17) was assayed by using 1 mg of poly-L-arginine (Sigma) per ml in 25 mM sodium phosphate (pH 7.0)–20 mM dithiothreitol (DTT)–10 μ Ci of [³²P]NAD (1,000 mCi/mmol; Amersham) per ml. Reaction mixtures (100 μ l) were incubated for 3 h at 30°C. At this time, 50 μ l was spotted onto individually numbered trichloroacetic acid-treated 3MM (Whatman) paper squares. These were washed twice with 5% trichloroacetic acid, dried in methanol, and counted in fluor (Ready-Safe; Beckman). Reactions were run in duplicate with authentic CTX (Sigma) as the standard.

Preparation of *V. fischeri* culture supernatant. Overnight cultures of *V. fischeri* MJ-A1 were grown in Luria-Bertani (LB)–salt medium (7), washed, diluted 25-fold in fresh prewarmed medium (500 ml in a 2-liter flask), and grown at 28°C with vigorous aeration (200 rpm). After 3.5 h, cultures were harvested by centrifugation (5,000 \times g for 30 min). The volume of the supernatant was measured, and 198 g of solid (NH₄)₂SO₄ (Ultrapure; ICN) per liter was added to the spent media. This solution was filtered through Whatman 3MM, and the filtrate could be stored for several days without significant loss of activity.

Hydrophobic-interaction chromatography. A phenyl-Sepharose column (5 by 10 cm) was equilibrated in 50 mM Tris-Cl (pH 7.5) containing 1.5 M (NH₄)₂SO₄ and 5 mM DTT, and the prepared culture supernatant described above was loaded on the column at 1.5 liter/h, and 7-ml fractions were collected and assayed for ADP-ribosyltransferase activity. Salt concentrations were estimated with a conductivity meter (Fisher Scientific).

NAD-agarose affinity chromatography. A 4-ml column (1.5 by 2.5 cm) of NAD-agarose was prepared in 25 mM 2-(N-morpholino)ethanesulfonic acid (MES; pH 5.8) containing 2 mM DTT. The concentrated, buffer-exchanged phenyl-Sepharose eluate was loaded at 10 ml/h, and the column was washed with 3 volumes of the initial start buffer and developed with a NaCl step gradient from 0 to 0.4, 0.4 to 1, and 1 to 2 M, with each step containing 25 mM MES (pH 5.8)–2 mM DTT.

High S fast protein liquid chromatography (FPLC) cation exchange. A 5-ml High S column (Bio-Rad) was equilibrated in 25 mM MES (pH 5.5) and eluted with a 25-ml linear NaCl gradient (0 to 0.5 M) at a flow rate of 1 ml/min. Fractions (1 ml) were collected and assayed for ADP-ribosyltransferase activity; a single symmetrical peak of *V. fischeri* ADP-r activity reproducibly eluted at 0.2 M NaCl.

Southern hybridization. Genomic (or plasmid) DNA was digested with the appropriate restriction enzyme; separated by agarose gel electrophoresis; transferred to a nylon membrane (Vacuum Blotter; Bio-Rad); probed with a ³²P-labeled degenerate oligonucleotide probe in 0.5 M sodium phosphate (pH 7.0) containing 7% sodium dodecyl sulfate (SDS), 1 mM EDTA, and 1% bovine serum albumin (9); and washed at 42°C with 2 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS.

DNA sequencing. Dideoxy DNA sequencing was performed on double-

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
Bacterial strains		
<i>V. fischeri</i> MJ-A1	Light organ symbiont	8
<i>V. fischeri</i> MJ-1	Light organ symbiont	25
<i>V. fischeri</i> ES114	Light organ symbiont	3
MVT606	Str ^r variant of <i>Vibrio</i> sp. strain 60	11
MVT1203	Secretion mutant of MVT606	11
S17.1	<i>E. coli</i> , RP4 <i>tra</i> functions	26
DH5 α F'	<i>E. coli</i> cloning host	1
Plasmids		
pUC19	Amp ^r , cloning vector	Pharmacia
pBSKSII ⁺	Amp ^r , cloning vector	Stratagene
pMMB208	Cm ^r , conjugative expression vector	21
pUC19/ <i>Eco</i> RI	<i>Eco</i> RI fragment cloned into pUC19	This study
pMMB208/ <i>Eco</i> RI	<i>Eco</i> RI fragment cloned into pMMB208	This study
Recombinant strains		
A and B	DH5 α F' harboring pUC19/ <i>Eco</i> RI; orientation 1	This study
C and D	DH5 α F' harboring pUC19/ <i>Eco</i> RI; orientation 2	This study
DH5 α F'/pMMB208/ <i>Eco</i> RI	<i>E. coli</i> host harboring <i>V. fischeri</i> ADP-r clone	This study
MVT606/pMMB208	MVT606 harboring pMMB208	This study
MVT606/pMMB208/ <i>Eco</i> RI	MVT606 harboring <i>V. fischeri</i> ADP-r clone	This study
MVT1203/pMMB208/ <i>Eco</i> RI	MVT1203 harboring <i>V. fischeri</i> ADP-r clone	This study
DH5 α F'/pBSKSII ⁺ / <i>Eco</i> RI- <i>Eco</i> RI	<i>E. coli</i> host harboring <i>Eco</i> RI fragment cloned in pBSKSII ⁺	This study
DH5 α F'/pBSKSII ⁺ / <i>Hind</i> III- <i>Eco</i> RI	<i>E. coli</i> host harboring <i>Hind</i> III subclone in pBSKSII ⁺	This study
DH5 α F'/pBSKSII ⁺ / <i>Sal</i> I- <i>Eco</i> RI	<i>E. coli</i> host harboring <i>Sal</i> I subclone in pBSKSII ⁺	This study
DH5 α F'/pBSKSII ⁺ / <i>Sac</i> I- <i>Eco</i> RI	<i>E. coli</i> host harboring <i>Sac</i> I subclone in pBSKSII ⁺	This study
DH5 α F'/pBSKSII ⁺ / <i>Pst</i> I- <i>Eco</i> RI	<i>E. coli</i> host harboring <i>Pst</i> I subclone in pBSKSII ⁺	This study

stranded templates with Sequenase (U.S. Biochemicals), universal primers, and oligonucleotides derived from the sequence.

Amino acid sequencing. Purified protein was processed for tryptic digestion, reverse-phase high-pressure liquid chromatography (HPLC) separation, sequencing of selected peptides, and N-terminal amino acid analysis by the Beckman Center Protein and Nucleic Acid Facility, Stanford, Calif.

Molecular biological techniques. The degenerate oligonucleotide was designed on the basis of sequences obtained from tryptic fragments of the purified ADP-ribosyltransferase. The oligonucleotide was labeled with [γ -³²P]ATP and purified over a 1-ml column of P2 resin (Bio-Rad). DNA sequences were analyzed by using DNA Strider and Genetics Computer Group and IG suites. Standard conditions (1) were used for restriction enzyme digestions, plasmid isolation, ligation reactions, subcloning, kinasin, and transformations.

Conjugation. Conjugations were performed by mixing equal volumes (150 μ l) of washed overnight cultures of *E. coli* S17.1 (26) harboring the appropriate plasmid and *Vibrio* sp. strain 60 (11). Cells were pelleted, resuspended in a minimal volume (15 μ l), incubated on LB-1% NaCl agar from 4 h to overnight, resuspended in 600 μ l of LB-1% NaCl, vortexed thoroughly, and spread on six LB-1% NaCl plates containing 800 μ g of ampicillin per ml and 5 μ g of chloramphenicol per ml. Exconjugants were reisolated, and the presence of the appropriate plasmid was confirmed by restriction digestion.

Cell sonicates. The supernatant was quantitatively removed from samples of centrifuged bacterial cultures (1 ml) and assayed for ADP-ribosyltransferase activity. The bacterial pellet was resuspended (300 μ l) in TE buffer (10 mM Tris-Cl [pH 8.0] containing 1 mM EDTA). The resuspended bacterial pellet was sonicated twice (10 s each) with a probe sonicator at maximum power. This lysate of the bacterial pellet was centrifuged for 10 min, and the supernatant (1 to 10 μ l) was assayed for *V. fischeri* ADP-r activity.

Media, growth conditions, and antibiotics. The strains and plasmids used in this study are listed in Table 1. *V. fischeri* was grown in LB-salt medium (7) at 28°C. *E. coli* strains were grown in LB at 37°C with an appropriate antibiotic (ampicillin, 100 μ g/ml; chloramphenicol, 25 μ g/ml). *Vibrio* sp. strain 60 was grown in LB-1% NaCl at 30°C (11) and, as required, with chloramphenicol (5 μ g/ml). All bacteria were grown with shaking (200 rpm).

Nucleotide sequence accession number. The halovibrin DNA sequence has been assigned GenBank accession number U38815.

RESULTS

Supernatants from cultures of *V. fischeri* possess ADP-ribosyltransferase activity. We assayed spent culture media from strains of *V. fischeri* (3, 8, 25) and obtained evidence (Table 2)

that this species secretes an ADP-ribosyltransferase activity. This activity was heat labile, protease sensitive, could be assayed directly from the supernatants of exponentially growing cultures, and gave a peak of activity at the end of the exponential phase of growth (data not shown). Our initial attempt to clone the gene coding for this activity was based on a low-stringency Southern blot technique (1) using a DNA probe consisting of the coding region of the enzymatic moiety of CTX (the A1 peptide). We cloned and sequenced a CTX cross-hybridizing DNA fragment from *V. fischeri* genomic DNA and unfortunately could find no significant sequence homology to CTX, and no ADP-ribosyltransferase activity was produced by these recombinant clones (data not shown). Therefore, we used the more traditional approach of first purifying the enzyme and using the amino acid sequence data derived from the

TABLE 2. ADP-ribosyltransferase activities^a

Source and organism	Southern positive	Activity (cpm)
Supernatant		
<i>V. fischeri</i> MJ-A1	Yes	27,871
<i>V. fischeri</i> MJ-1	Yes	24,345
<i>V. fischeri</i> ES114	Yes	17,230
Cell sonicate		
Clone A	Yes	5,897
Clone B	Yes	3,761
Clone C	Yes	4,664
Clone D	Yes	4,827
Clone E	No	790
Clone F	No	810
DH5 α F'	No	756
DH5 α F'/pUC19	No	856

^a Assays were done in duplicate (<10% variation), and the means without background subtraction are reported.

purified protein to clone and identify the gene coding for the *V. fischeri* ADP-r.

Purification of the ADP-ribosyltransferase secreted from *V. fischeri*. Several strains of *V. fischeri* were assayed for ADP-ribosyltransferase activity by using the artificial substrate poly-L-arginine and [³²P]NAD, as described in Materials and Methods. One strain, MJ-A1 (Table 1), was found to consistently produce the highest level of activity, though all of the strains tested positive in our assay. We developed a three-step purification protocol that permits the repetitive processing of 6-liter batches of culture supernatant (~five batches). Hydrophobic-interaction chromatography was used to initially concentrate the sample and remove the bulk of medium-derived proteins. This was followed by an affinity chromatography step on NAD-agarose and, for final purification, FPLC cation exchange (Fig. 1).

Cultures of *V. fischeri* MJ-A1 were grown to early stationary phase, and bacteria were removed by centrifugation. The supernatant was brought to 1.5 M (NH₄)₂SO₄ and chromatographed on phenyl-Sepharose. The column was washed with 2.5 bed volumes of start buffer and developed with a 1-liter linear gradient [1.5 to 0 M (NH₄)₂SO₄, 50 mM Tris-Cl (pH 7.5) containing 5 mM DTT]. As high concentrations of (NH₄)₂SO₄ inhibited the *V. fischeri* ADP-r assay, column fractions were desalted by ultrafiltration buffer exchange (Centricon 10; Amicon). The ADP-ribosyltransferase consistently eluted at 0.15 M (NH₄)₂SO₄ (Fig. 1A). The active fractions were pooled and concentrated by ultrafiltration in a stirred cell concentrator (Amicon) with a YM10 membrane. The concentrated, pooled phenyl-Sepharose eluate was buffer exchanged through a pre-equilibrated PD-10 column (Pharmacia) into 25 mM MES (pH 5.8) containing 2 mM DTT. This material was loaded onto a NAD-agarose column (Sigma). The ADP-ribosyltransferase activity bound tightly to this resin and was best eluted with a step gradient of NaCl (Fig. 1B). Fractions were again desalted before being assayed for ADP-ribosyltransferase activity. Purity was assessed by SDS-polyacrylamide gel electrophoresis (PAGE) and silver staining; less pure fractions were retained and rechromatographed. Peak fractions were pooled, concentrated by ultrafiltration, and chromatographed by High S FPLC (Fig. 1C), as described in Materials and Methods. The protein profiles of the purification steps are shown in Fig. 2. The molecular masses of the ADP-ribosyltransferase determined by FPLC gel permeation chromatography (data not shown) and SDS-PAGE were the same. Thus, this ADP-ribosyltransferase appears to function as a single polypeptide under our assay conditions. The purified material was digested with trypsin and separated by reverse-phase HPLC. Selected tryptic peptides were subjected to automated Edman degradation.

Genomic Southern analysis. Partial amino acid sequences from several tryptic peptides were determined as described above, and on the basis of one such sequence (NH₃-Asp-Tyr-Asn-Asp-Thr-Ser-Gln-Asp-Gly-COOH), a degenerate oligonucleotide (5'-GAY TAY AAY GAY ACI YSI CAR GAY GG-3') was synthesized. No codon usage bias was used in its design, but at positions of fourfold degeneracy, the neutral base inosine was incorporated. This oligonucleotide was radio-labeled, as described in Materials and Methods, and used as a probe for Southern blot analysis of *V. fischeri* genomic DNA digested with a variety of restriction enzymes. The results (data not shown) revealed a single cross-hybridizing band for each restriction enzyme tested. On the basis of the results of this analysis, we cloned the *EcoRI* cross-hybridizing DNA fragment from *V. fischeri* MJ-A1.

Cloning of the *V. fischeri* ADP-r gene. A size-selected (4 to 6

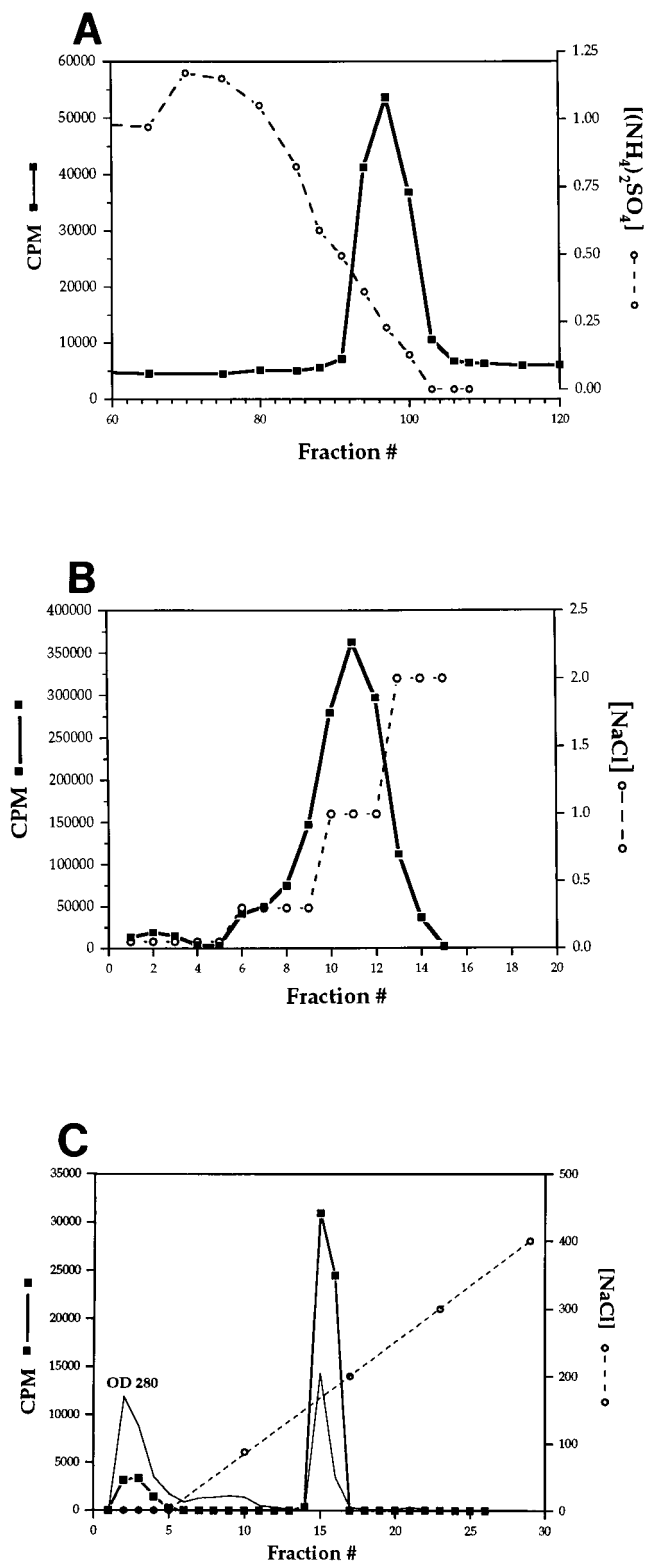


FIG. 1. Purification of *V. fischeri* ADP-r: column elution profiles of hydrophobic-interaction, affinity, and cation exchange chromatography. (A) Phenyl-Sepharose elution profile; (B) NAD-agarose elution profile; (C) High S FPLC elution profile. OD280, optical density at 280 nm.

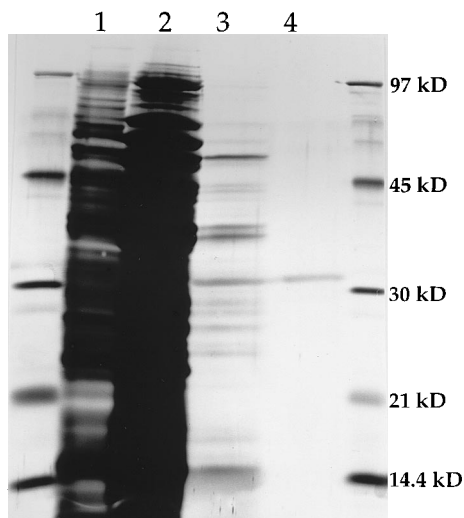


FIG. 2. Protein profile of *V. fischeri* ADP-r purification. Silver-stained SDS-PAGE. Outside lanes, molecular mass (in kilodaltons [kD]) standards; lane 1, spent culture supernatant; lane 2, phenyl-Sepharose eluate-concentrated pool; lane 3, NAD-agarose eluate pool; lane 4, High S FPLC eluate pool.

kb) library of *EcoRI*-digested *V. fischeri* genomic DNA was constructed in pUC19 and DH5 α F' (Pharmacia). One thousand recombinant clones were individually picked and screened in pools of 50. Twenty pools were grown overnight, and plasmid DNA was extracted, digested with *EcoRI*, and separated by agarose gel electrophoresis (Fig. 3A). The DNA was transferred to a nylon membrane (Hybond N⁺; Amersham) and probed with the radiolabeled degenerate oligonucleotide described above. Four recombinant pools (no. 2, 11, 12, and 14) contained cross-hybridizing DNA (Fig. 3B). These four pools were subdivided into smaller pools and rescreened by Southern hybridization. This process was repeated until the four individual recombinant clones containing DNA that cross-hybridized to the degenerate oligonucleotide were identified. Restriction digestion analysis revealed that all four *EcoRI* fragments were identical and that this DNA fragment had been cloned in both possible orientations.

Southern hybridization-positive recombinant clones possess ADP-ribosyltransferase activity. Whole-cell sonicates from the four Southern hybridization-positive clones and two other random clones containing similarly sized inserts were tested in the ADP-ribosyltransferase assay. The four recombinant clones (Table 2) possessed ADP-ribosyltransferase activity (clones A, B, C, and D), while the clones containing the vector alone or the vector with inserts that do not cross-hybridize with the degenerate oligonucleotide (clones E and F) possessed only background activity. One clone (clone A) was chosen for further study, and the DNA sequence of its insert was determined.

Deletion analysis and identification of an ADP-ribosyltransferase gene. By using convenient restriction sites within the *EcoRI* cross-hybridizing DNA fragment, four subclones were constructed in pBSKSII⁺ (Stratagene) (Table 1), and cell sonicates of *E. coli* strains harboring the respective subclones were assayed for ADP-ribosyltransferase activity (Fig. 4). These restriction sites interrupt the open reading frames in this DNA fragment and unambiguously eliminate the first two possible reading frames as coding regions for the ADP-ribosyltransferase. The *HindIII*, *SalI*, and *SacI* subclones possessed ADP-r activity, but the *PstI* clone was not active in our assay. This

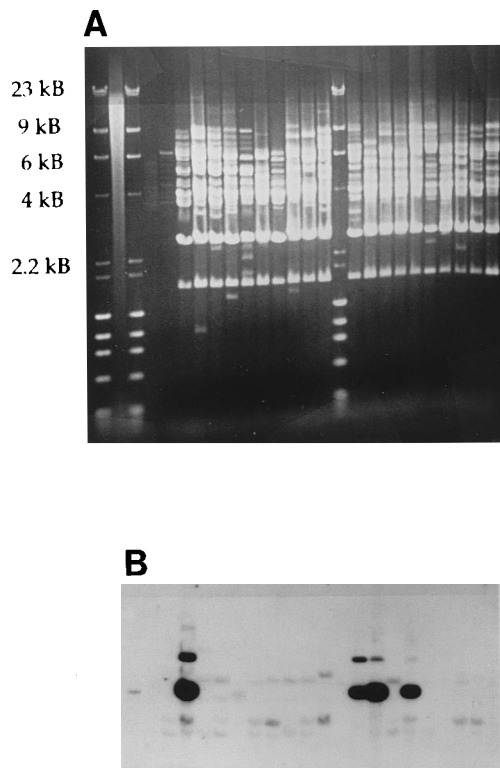


FIG. 3. Cloning strategy for *V. fischeri* ADP-r. (A) Ethidium-stained agarose gel. Lanes: 1, 3, and 16, DNA size (in kilobases [kB]) standards; 2, *EcoRI*-digested *V. fischeri* genomic DNA; lanes 6 to 15 and 17 to 26, *EcoRI*-digested pools from size-selected library. Visible bands are released *EcoRI* fragments, vector bands, and bands of undigested material. (B) Autoradiogram of same gel transferred to a nylon membrane and probed with the radiolabeled degenerate oligonucleotide. Lane 2, genomic DNA showing the cross-hybridizing band; pools 2, 11, 12, and 14 contain this same DNA fragment.

series of experiments localized the ADP-ribosyltransferase activity to the rightmost (and smallest) of the deduced open reading frames.

DNA and deduced amino acid sequences of *V. fischeri* ADP-r. The DNA sequence and corresponding deduced amino acid sequence of the open reading frame identified by deletion analysis and discussed above are shown in Fig. 5. The peptide sequence used in the design of the degenerate oligonucleotide (DYNDTSQDG) was readily identified in the deduced amino acid sequence (Fig. 5 [bold]). The carboxy-terminal amino acid of this peptide, glycine, was determined by Edman degradation. However, the deduced amino acid at this position is a cysteine (on the basis of the DNA sequence). This was confirmed by repeated DNA sequencing of this region. As cysteine residues are poorly recovered from automatic amino acid sequencing, we assume that the chromatograph of the PTH derivative of this residue was incorrectly interpreted and that the deduced amino acid sequence is the correct one. Two other short peptide sequences derived from the purified protein were also found in this open reading frame.

This constellation of observations that (i) clones harboring the cross-hybridizing DNA fragment possess ADP-ribosyltransferase activity, (ii) deletion analysis of the cloned DNA identifies one reading frame as having ADP-ribosyltransferase activity, and (iii) the deduced amino acid sequence of the open reading frame identified by deletion analysis has peptide sequences identical to those obtained from the purified protein

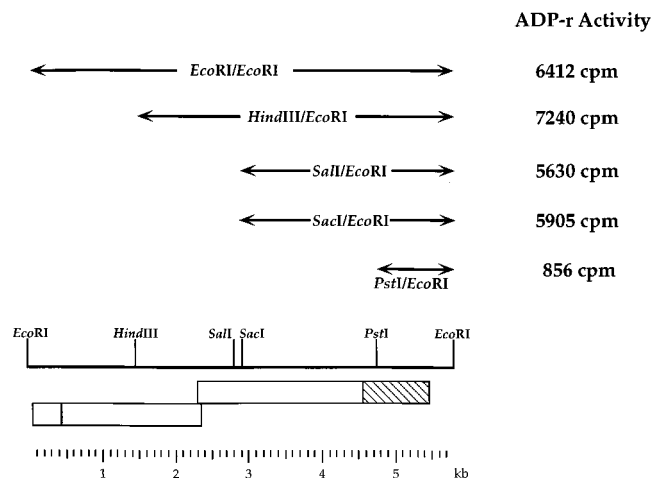


FIG. 4. Deletion analysis of the *EcoRI* fragment. The sizes and restriction sites of subcloned fragments (double-headed arrows) and the ADP-r activities assayed from cell sonicates of the respective clones are shown. A partial restriction map, the positions of the open reading frames within the *EcoRI* fragment, and the position of *hvn* (hatched box) are also indicated.

constitutes formal proof that we have cloned an ADP-ribosyltransferase gene from *V. fischeri*.

This gene codes for a protein of 33,307 Da, with a calculated pI of 6.05. This correlates well with the molecular weight determined by SDS-PAGE (Fig. 2) and FPLC size exclusion chromatography (data not shown) and with the experimental pI of 6.8 determined by isoelectric focusing (data not shown). Kyte and Doolittle hydropathy profiles (13) of this protein revealed no significant regions of strong hydrophobic or hydrophilic character (data not shown) nor was a signal peptide motif noted.

***V. fischeri* ADP-r is exported by *Vibrio* sp. strain 60.** Although we assayed the ADP-ribosyltransferase activity from the supernatants of *V. fischeri* cultures, the lack of a convincing signal peptide (15, 22) in the deduced amino acid sequence of *V. fischeri* ADP-r led us to formally test the possibility that the activity we measured was derived from a cytoplasmic enzyme and merely released by cell lysis. Therefore, we made use of a well-described halophilic wild-type marine bacterium, *Vibrio* sp. strain 60 (10), that can secrete heterologous exotoxins from related organisms, including the pore-forming exotoxin aerolysin (27) and the B subunit of *E. coli* heat-labile enterotoxin (14). Pleiotropic secretory mutants of *Vibrio* sp. strain 60 have previously been constructed (11); these strains are unable to secrete aerolysin or the B subunit of *E. coli* heat-labile enterotoxin (14, 27). We cloned the *V. fischeri* ADP-r *EcoRI* fragment into a broad-host-range expression vector (pMMB208) (21), transferred this recombinant expression vector into *Vibrio* sp. strain 60 by conjugation, and tested these clones for the ability to secrete *V. fischeri* ADP-r activity into the supernatant, while the secretory mutant MVT1203 does not. Negative control experiments with cultures of *Vibrio* sp. strain 60/pMMB208 and *Vibrio* sp. strain 60 alone are ADP-r negative. Cell sonicates made from MVT1203/pMMB208/*EcoRI* had significant ADP-r activity (data not shown), proving that the mutant is defective in secreting *V. fischeri* ADP-r but not in synthesizing the enzyme.

Characterization of *V. fischeri* ADP-r: reducing agent requirement and pH activity profile. The lack of amino acid homology between *V. fischeri* ADP-r and CTX led us to re-evaluate assumptions about the enzymatic activity of *V. fischeri*

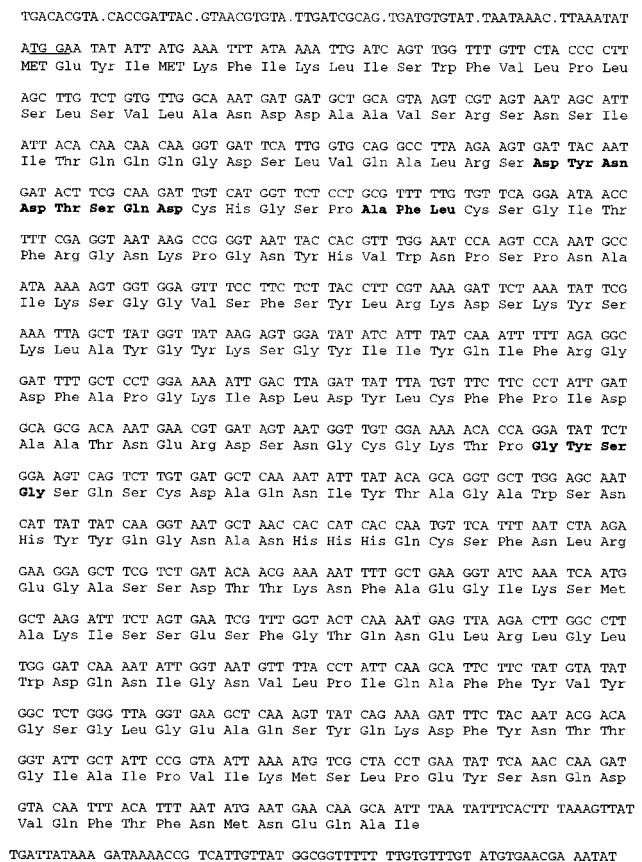


FIG. 5. DNA and deduced amino acid sequences of *V. fischeri* ADP-r. Possible initiation methionine residues are capitalized, the putative ribosome binding site is underlined, and amino acid sequences derived from sequenced tryptic fragments of the purified protein are in bold.

ADP-r and, in particular, if the conditions that activate CTX are also required to activate *V. fischeri* ADP-r. Therefore, we tested the purified enzyme for activation with mild proteolysis, SDS treatment, and a reducing agent, the conditions that ac-

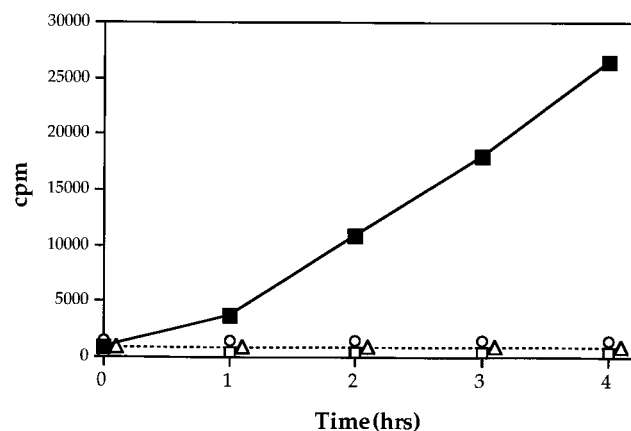


FIG. 6. Recombinant *Vibrio* sp. strain 60 secretes *V. fischeri* ADP-r. ADP-ribosyltransferase activities were measured from growing cultures of *Vibrio* sp. strain 60. The supernatant was harvested at the indicated times and assayed for *V. fischeri* ADP-r activity. ■, MVT606/pMMB208/*EcoRI*; □, MVT1203/pMMB208/*EcoRI*; ○, MVT606/pMMB208; △, MVT606. See the text for details.

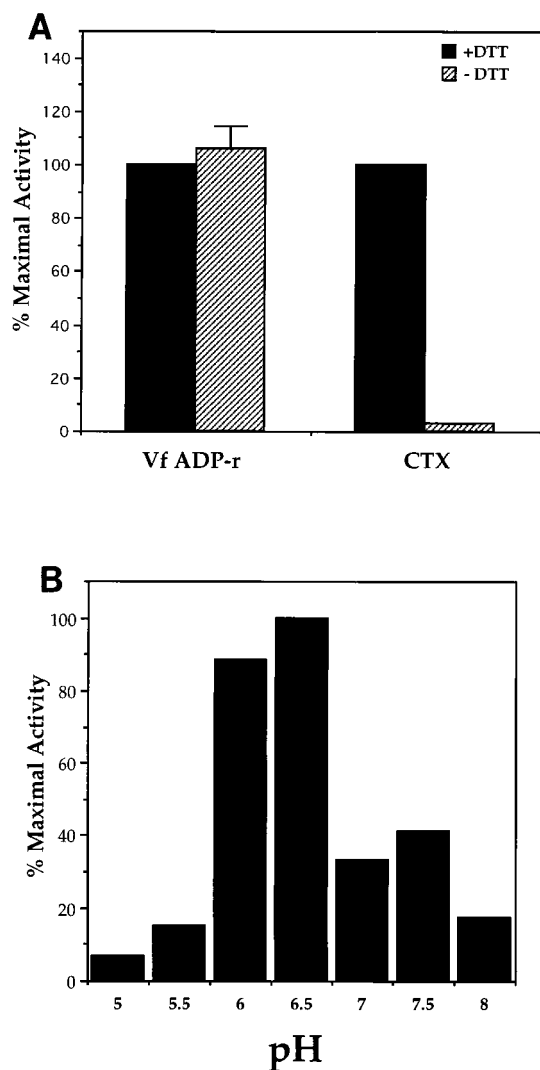


FIG. 7. Reducing agent requirement and pH activity profile of halovibrin. (A) Activities of halovibrin and CTX in the presence (+) or absence (-) of 20 mM DTT. (B) *V. fischeri* ADP-r activities measured at various pHs. When visible, an error bar is shown.

tivate CTX. No increase in enzymatic activity was observed after treatment with trypsin, chymotrypsin, or SDS (data not shown). Purified *V. fischeri* ADP-r (Fig. 7A) was added to ADP-r reaction mixes containing 20 mM DTT or lacking this reducing agent. With authentic CTX as the control, the results show that *V. fischeri* ADP-r is insensitive to the presence or absence of this reducing agent, but as expected, CTX required this reducing agent for enzymatic activity (18).

The pH activity profile of *V. fischeri* ADP-r was assayed in reaction mixes over a range of pHs (Fig. 7B). Purified *V. fischeri* ADP-r was added to these solutions, and ADP-ribosyltransferase activities were measured as described in Materials and Methods. The enzyme exhibited maximal activity at slightly acidic pHs (pH 6 to 6.5), which is suggestive of a pH titration of a single amino acid side chain required for enzymatic activity.

DISCUSSION

In this report, we have described a new member of the family of ADP-ribosyltransferases and the first example of an

ADP-ribosyltransferase secreted from a nonpathogenic bacterium. We believe that this enzyme is representative of a new class of ADP-ribosyltransferases. To distinguish this enzyme from other ADP-ribosyltransferases and in anticipation of the discovery of additional related enzymes in other marine bacteria, we propose the name halovibrin (gene designation, *hvn*) for this new enzyme. Our discovery of halovibrin presents a number of interesting facets, including the biochemistry of this enzyme, the lack of sequence homology with other ADP-ribosyltransferases, the mechanism of secretion, the distribution of similar enzymes in marine *Vibrio* species, and of course the potential function of this enzyme in the symbiotic association of *V. fischeri* with its squid host.

V. fischeri secretes halovibrin in an enzymically active form that requires no processing; many members of the ADP-ribosyltransferase family require either proteolytic cleavage or reduction or both for enzymatic activity. Halovibrin requires neither and is active when expressed from a heterologous host, either *E. coli* or *Vibrio* sp. (Table 2 and Fig. 6). Molecular weight determinations, conducted under nonreducing conditions, of ADP-r activity from *V. fischeri* spent culture media (data not shown) do not provide evidence for a tightly associated B-like receptor-binding protein, and there is no homology with other receptor binding domains or subunits in the other open reading frames on the *EcoRI* fragment that contains *hvn*. Halovibrin is a stable, soluble protein; no detectable loss of activity was noted after 6 months at 4°C (data not shown). The sharp pH activity profile curve is possibly due to the titration of a single amino acid side chain (most likely a histidine) required for enzymatic activity. A histidine residue has been postulated to be involved in the binding of NAD for other ADP-ribosyltransferase (6) and proven for diphtheria toxin fragment A (2). In experiments in progress, we are trying to identify active-site residues in halovibrin by UV-mediated NAD cross-linking (4) and sequencing of the photolabeled peptide, an experimental approach that has been informative for other bacterial ADP-ribosyltransferases.

We initially hypothesized that *V. fischeri* ADP-r is homologous to the ADP-ribosylating A subunit of CTX because in previously reported work from our laboratory, we had found that *V. fischeri* has a homolog of *V. cholerae* *toxR* (23). However, this prediction was not borne out by amino acid sequence analysis, since the deduced amino acid sequence of halovibrin has no homology with those of CTX, other ADP-ribosyltransferase enzymes, or any other protein in the data bank. The supposition that *V. fischeri* *toxR* would regulate *V. fischeri* ADP-r, again in analogy to the biology of *V. cholerae*, also has not stood up to experimental testing, since a *V. fischeri* *toxR* null mutant strain is unaffected in *V. fischeri* ADP-r activity (data not shown). Taken together, these data do not provide evidence for structural, biochemical, or sequence similarity between CTX and halovibrin, thus pointing to an independent evolutionary origin for *V. fischeri* ADP-r. We have searched the deduced amino acid sequence of halovibrin for the limited amino acid homologies and motifs that do exist among selected members of the ADP-ribosyltransferase enzyme family and can find no evidence for these signatures in our enzyme. These motifs include RPP--VST--IYIY (among CTX and pertussis and mosquitocidal toxins [6]), EXXXXW (between diphtheria toxin and exotoxin A [6]), and the more recently described EVXGHT (12). We have recently screened a large number of marine bacterial species for ADP-ribosyltransferase activities and *hvn*-like genes, and preliminary experiments show a small family of related sequences and enzymatic activities from other marine *Vibrio* species.

Inspection of the amino acid sequence reveals two possible

translation starts, with a possible ribosome binding sequence located 8 bp upstream of the second ATG (Fig. 5). According to the deduced amino acid sequence, halovibrin is rich in tyrosine and serine and possesses four tryptophan and six cysteine residues, which, if oxidized, could form three intrachain disulfide bonds. We have used molecular weight determination by FPLC gel permeation chromatography in the absence of a reducing agent to test for the presence of intermolecular disulfide bonds in halovibrin and found no dimer or higher multimer formation.

Halovibrin is secreted directly into the medium from *V. fischeri*. *E. coli* clones harboring *hvn* produce but do not secrete this protein into the culture supernatant. Inspection of the halovibrin amino acid sequence reveals no obvious signal peptide. Therefore, we wished to test if the mechanism of secretion of halovibrin was specific for this protein or if a general secretory mechanism was responsible for its export. A wild-type marine bacterium (*Vibrio* sp. strain 60) harboring cloned *hvn* secretes halovibrin into the culture medium, while a secretory mutant of *Vibrio* sp. strain 60 cannot; this observation provides confirmatory evidence that halovibrin is actively secreted and that our ability to measure ADP-ribosyltransferase activity in spent culture media is not the result of cytoplasmic ADP-ribosyltransferase activity being released by cell lysis. As a secreted protein, halovibrin could interact with the host cells lining the light organ.

ADP-ribosyltransferase exotoxins are widely distributed in the bacterial world, and the majority have a clear role in pathogenesis. In this report, we have described the purification and biochemical characterization of a novel member of this enzyme family. All of the other family members have eucaryotic target proteins that, when modified, profoundly affect the biology of the host organism. *V. fischeri* participates in a symbiotic relationship with *Euprymna scolopes*, in part by triggering the morphological development of its light organ. While little is known about the bacterial signals, functions, or genes required for initiating or maintaining this symbiosis, the specificity of this interaction leaves little doubt of the extensive communication between the two partners. By virtue of its enzymatic activity and its secretion to the extracellular milieu, halovibrin is an obvious candidate for a bacterial signalling molecule. We are actively testing the hypotheses that *V. fischeri* ADP-r is a symbiotic determinant and that this enzyme is required for the initiation or maintenance of symbiosis and/or for triggering the developmental program of the host.

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