**Vibrio fischeri** Genes hvnA and hvnB Encode Secreted NAD⁺-Glycohydrolases

ERIC V. STABB,†,* KARL A. REICH,‡ AND EDWARD G. RUBY

Pacific Biomedical Research Center, University of Hawaii, Honolulu, Hawaii 96813, United States of America, and Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, California 94305

Received 22 June 2000/Accepted 27 September 2000

HvnA and HvnB are proteins secreted by Vibrio fischeri ES114, an extracellular light organ symbiont of the squid Euprymna scolopes, that catalyze the transfer of ADP-ribose from NAD⁺ to polyarginine. Based on this activity, HvnA and HvnB were presumptively designated mono-ADP-ribosyltransferases (ARTases), and it was hypothesized that they mediate bacterium-host signaling. We have cloned hvnA and hvnB from strain ES114. hvnA appears to be expressed as part of a four-gene operon, whereas hvnB is monocistronic. The predicted HvnA and HvnB amino acid sequences are 46% identical to one another and share 44% and 34% identity, respectively, with an open reading frame present in the Pseudomonas aeruginosa genome. Four lines of evidence indicate that HvnA and HvnB mediate polyarginine ADP-ribosylation not by ARTase activity, but indirectly through mechanisms that parallel ARTase cholera toxin. ARTases directly ribosylate protein targets, whereas ARTases rather than NADases in bacterium-host interactions is unknown beyond the observations that clinical streptococcal isolates and Vibrio cholerae secrete NADases (42, 52). We present here (i) the characterization of hvnA and hvnB from an E. scolopes light organ isolate, (ii) the disruption of these genes in this wild-type V. fischeri strain, (iii) biochemical data supporting a reclassification of HvnA and HvnB as NADases rather than as ARTases, and (iv) evidence that HvnA and HvnB are not required to initiate the V. fischeri-E. scolopes symbiosis.

**MATERIALS AND METHODS**

**Bacteria, media, and reagents.** Wild-type V. fischeri ES114, isolated from E. scolopes (3), was the parent strain for mutant construction and was the source of DNA for the cloning of hvnA and hvnB. Escherichia coli strains DH5α (16) and BW25474 (14) were used as hosts for plasmids with ColE1 or R6K replication origins, respectively, with the exception of plasmid pUTminiTn5-Sm3p, which was maintained in strain CCI18Oqar (19). E. coli was grown in Luria-Bertani (LB) medium (36), and V. fischeri was grown in either SWT medium (3) or LBS medium, which contained, per liter of water, 10 g of tryptone, 5 g of yeast extract, 20 g of NaCl, and 20 mM Tris-hydrochloride (Tris) (pH 7.5).

All chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo), except [α-32P]NAD⁺ (1 Ci/μmol), which was obtained from Amersham Pharamacia and Department of Biology and Department of Biology, Stanford University School of Medicine, Stanford, California 94305

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* Corresponding author. Mailing address: Pacific Biomedical Research Center, University of Hawaii, Honolulu, HI 96813, Phone: (808) 539-7311. Fax: (808) 599-4817. E-mail: stabb@hawaii.edu.
† Present address: Abbott Laboratories, Abbott Park, IL 60064.
Biotecnology (Piscataway, N.J.). Restriction enzymes and DNA ligase were obtained from New England Biolabs (Beverly, Mass.). AmpliTaq DNA polymerase was obtained from Perkin-Elmer (Branchburg, N.J.). Oligonucleotides were synthesized by Operon Technologies, Inc. (Alameda, Calif.). When added to LB medium for the selection of E. coli, ampicillin, trimethoprim, chloramphenicol, streptomycin, and kanamycin were used at concentrations of 100, 20, 20, 100, and 40 μg/ml, respectively. When added to LBS medium for selection in V. fisheri, trimethoprim, chloramphenicol, streptomycin, and kanamycin were used at concentrations of 5, 5, 20, and 40 μg/ml, respectively.

Cloning, sequence analysis, and disruption of hvnA and hvnB. hvnB was cloned using hybridization techniques and a DNA probe based on a partial peptide sequence of HvnB. HvnB was purified as described previously (46) and subjected to in-gel trypsin digestion, and peptides were separated by reversed-phase high-pressure liquid chromatography, prior to peptide sequencing (Beckman Center Protein and Nucleic Acid Facility, Stanford, Calif.). Based on this partial HvnB amino acid sequence, an oligonucleotide (5′-GGT GGA GTT TTC TTC TAC GTT CAA GAT ACT AAA TCG AAG TTA GCT TAT GG-3′) was designed, end labeled with digoxigenin (DIG Oligonucleotide Tailing Kit; Boehringer Mannheim), and used in Southern and dot blotting experiments (DIG DNA Labeling and Detection Kit; Boehringer Mannheim) to identify hvnB-containing DNA fragments. Blots were hybridized with the probe overnight at 32°C. Membranes were washed under low-stringency conditions (two 30-min washes in 30 mM sodium citrate-300 mM NaCl-0.1% sodium dodecyl sulfate [SDS] [pH 7.0] at 42°C) prior to development, according to the manufacturer’s instructions. A 3.9-kb Xbal fragment containing hvnB was identified and purified and cloned into the XbaI site of pKNG101. A 0.8-kb SalI fragment from the XbaI-XbaI fragment was cloned into the mobilizable suicide vector, pEVS54, generating pEVS57. pEVS54 is a derivative of pKNG101 (23) with a unique SalI site inserted in each direction, was cloned into the mobilizable suicide vector, pEVS54, generating pEVS57. pEVS54 was subjected to in-gel trypsin digestion, and peptides were separated by reversed-phase high-pressure liquid chromatography, prior to peptide sequencing (Beckman Center Protein and Nucleic Acid Facility, Stanford, Calif.). Based on the unique SalI fragment, we isolated plasmid pEVS30, which carries the hvnA gene in hvnA-null ES114 by marker exchange (see below). The rate of HvnA-mediated NAD+ degradation (see below). The rate of HvnA-mediated NAD+ hydrolysis was determined (see below) using 25 ng of purified HvnA and 400, 200, 133, 100, 67, or 50 μM NAD+.

To assess HvnA and HvnB activity in the presence of other secreted proteins, filtrates were concentrated and then separated from small solutes (<10 kDa) using Centricon YM-10 (Amicon, Beverly, Mass.). Ten milliliters of culture filtrate was concentrated to 300 μl and then subjected to three size filters in SP buffer. Cholera toxin and NADase enzymes were maintained as 1 mg/ml stock solutions and diluted in reaction buffer immediately prior to addition.

Assays of ADP transfer from NAD+ were performed as described elsewhere (47), except that 100-μl reactions were spotted onto 2.5-mm filter disks soaked in 10% trichloroacetic acid (TCA) and washed four times with 5 ml of 5% TCA using a vacuum manifold. Polyarginine, polylysine, and polyhistidine were added to a final concentration of 1 mg/ml. Hydroydrolamine-HCl was neutralized with NaOH prior to use. NAD+ concentrations were assayed by the addition of KCN (667 mM, final concentration), measurement of absorbance at 340 nm and comparison to a standard curve (2, 5). e-NAD+ degradation was monitored by measuring the increase in fluorescence at 465 nm of samples excited with a 340-nm-wavelength light. Measurements of NAD+ and e-NAD+ degradation were performed on a Perkin-Elmer HTS7000 fluorometer (24). This layer chromatography (TLC) was performed after 1 h incubations of samples with [32P]NAD+. A 5-μl portion of each reaction was spotted onto TLC plates and developed in one of three solvent systems: (i) Cellulose 300 plates (Selecto Scientific) and isobutyrate-H2O-NH4OH (96:19:4, vol/vol/vol) running buffer (13), (ii) silica plates (PE SIL G/UV; Whatman, Ltd., Maidstone, Kent, England) containing 2 M NaOH and 10% water-ethanol-NaCl (30%:70%:0.2 M) solvent, or (iii) silica plates with H2O-ethanol-NH4HCO3 (30%:70%:0.2 M) solvent (20). Plates were dried, developed with autoradiography. ADP was added as a standard and visualized under UV light. ADP cyclase from Aplysia californica was incubated with [32P]NAD+ to generate a cADPr standard. In each TLC system, NAD+ was detected by autoradiography. ADP, cADPr, and hADP displayed relative mobilities to those previously reported (13, 20).

Plasmids were purified using the PerfectPrep plasmid DNA kit (5 Prime-3 Prime, Inc., Boulder, Colo.). Between restriction and ligation reactions DNA was recovered with the Wizard DNA Cleanup System (Promega Corp., Madison, Wis.). DNA sequencing was conducted on an ABI automated DNA sequencer at the University of Hawaii Biotechnology/Molecular Biology Instrumentation and Training Facility. Both strands of each insert were sequenced. Sequence analysis (e.g., the identification of ORFs and putative stem-loops) was performed using DNA Strider 1.2, and comparisons of ORF and protein sequences were conducted with either the CLUSTAL W (53) or the BLASTP (1 algorithms), using the BLOSUM62 scoring matrix (17).
between strains ES114 and MJ1 in ORF3 and ORF4, amounting to 1.3 and 0.1% nonidentity, respectively. The ORF immediately downstream of hvnB displayed 30% identity and 50% similarity to chitinase A of Vibrio harveyi. ORF5 and ORF6, upstream of hvnB, were similar to YKGJ and YGJP, genetically unlinked ORFs of unknown function in E. coli.

The Hvna and Hvnb sequences derived from their respective genes are 46% identical to one another, share 44% and 34% identity, respectively, with an ORF present in the Pseudomonas aeruginosa genome, and contain regions that are similar to a 47-amino-acid stretch in human nicotinamide nucleotide transhydrogenase (Fig. 2). No other significant similarities were observed in protein database searches, suggesting that Hvna, Hvnb, and the ORF in P. aeruginosa comprise a new protein family.

Absence of secreted ADP-ribosylating activity in an hvna and hvnb double mutant. Null mutant derivatives of hvna and hvnb were constructed singly and in tandem by marker exchange in wild-type V. fischeri ES114. The chloramphenicol acetyltransferase gene replaced a deleted portion of hvna in strains EVS498 and EVS500, and a miniTn-5-Sm/Sp insertion was used to interrupt hvnb in strains EVS499 and EVS500 (Fig. 1). Construction of these mutants was confirmed by Southern blotting (data not shown). To test whether a third secreted Hvna-like activity was present in V. fischeri, culture supernatants of ES114, EVS498, EVS499, and EVS500 were tested for their ability to catalyze ribosylation of polyarginine. The single mutants EVS498 and EVS499 showed activity levels similar to that of ES114 (Fig. 3). Although we initially expected intermediate activities from the single mutants, the data in Fig. 3 are consistent with a model (see below) wherein Hvna and Hvnb have high NADase activity, quickly hydrolyzing the $[^{32}\text{P}]\text{NAD}^+$ to $[^{32}\text{P}]\text{ADPr}$, with subsequent nonenzymatic incorporation of label onto the polyarginine substrate. The double mutant EVS500 showed no detectable activity above background (Fig. 3), and supernatant-mixing experiments revealed no anti-Hvna activity secreted by EVS500 (data not shown). Based on these data, there does not appear to be a third Hvna-like protein expressed and secreted by V. fischeri ES114 in culture, and the ribosylating activities of EVS498 and EVS499 are presumably solely due to Hvnb and Hvna, respectively.

**RESULTS**

The goals of this study were (i) to clone hvna and hvnb from an E. scolopes isolate of V. fischeri, (ii) to determine whether an hvna hvnb double mutant possesses any other extracellular Hvna-like activity, (iii) to test whether Hvna- and Hvnb-catalyzed ribosylation of polyarginine is mediated directly by an ARTase activity or indirectly by NADase activity, and (iv) to determine whether an hvna hvnb double mutant is capable of colonizing and triggering development of the E. scolopes light organ.

**Cloning of hvna and hvnb.** hvna and hvnb were cloned from the squid light organ isolate ES114. The hvna gene from ES114 was identical to hvna previously cloned from the fish light organ isolate, V. fischeri MJ1, although there were minor differences in nearby ORFs (see below). Because the amino acid sequence derived from the hvnb gene matched peptide sequences within the Hvnb protein and because the derived molecular mass (35 kDa) approximated the 32 kDa deduced from Hvnb by gel electrophoresis (46), we concluded that this cloned gene encoded Hvnb. Based on the location and orientation of the ORFs, the gaps between ORFs, and the location of putative transcriptional terminators, hvna appears to be part of a four-gene operon, whereas hvnb is monocistronic (Fig. 1). None of the ORFs upstream of, and putatively co-transcribed with, hvna showed convincing similarity to known proteins, although short (50 to 179 amino acid) stretches of ORF3 and ORF4 were 43 to 60% similar to certain bacterial outer membrane proteins, including the enteric flagellar hook protein FlgE (ORF3) and the Haemophilus influenzae filamentous adhesin Hmw2A (ORF4). There were minor variations
amine insensitivity of cholera toxin activity demonstrates that, under these conditions, hydroxylamine did not significantly disrupt ADPr-arginine bonds formed by ARTase activity. ADPr transfer catalyzed by HvnA and HvnB was consistent with a reaction mediated by a free ADPr intermediate, because ribosylation of polyarginine was hydroxylamine sensitive, and ribosylation of polylysine and polyhistidine was also catalyzed (Fig. 4). Therefore, in this assay, HvnA and HvnB catalyzed reactions consistent with NADase activity and inconsistent with ARTase activity. We considered the possibility that other secreted proteins might be required for ARTase activity by HvnA or HvnB, but that does not seem to be the case because these experiments were performed with preparations that included other proteins secreted by *V. fischeri*. Similar results were obtained with purified HvnA, which ribosylated polyarginine, polylysine, and polyhistidine, each in a hydroxylamine-sensitive manner (data not shown).

If the first step in HvnA- and HvnB-mediated ADP-ribosylation of polyarginine is the breakdown of NAD$^+$; then these enzymes should degrade NAD$^+$ in the absence of a polypeptide target. Several lines of evidence suggest that this degradation occurs. First, HvnA and HvnB each catalyzed the degradation of ε-NAD$^+$, an NAD$^+$ analog whose fluorescence increases when the ADP moiety is released from the fluorescence-quenching nicotinamide (Fig. 5A). These data are consistent with HvnA and HvnB possessing NADase activity; however, the reaction of ε-NAD$^+$ with phosphodiesterase and NAD$^+$ pyrophosphatase activities also results in an increase in fluorescence (13). In a second assay, the breakdown of NAD$^+$ was measured by the addition of cyanide, which forms a light-absorbing complex with N-substituted nicotinamide compounds (5), allowing detection of nicotinamide-ADPr bond cleavage. We found that NAD$^+$ is degraded by HvnA and HvnB, indicating that these enzymes cleave the nicotinamide-ribose bond of NAD$^+$ (Fig. 5B).

The data presented in Fig. 4 and 5 suggested that the cleavage of NAD$^+$ by HvnA and HvnB produced free ADPr; how-

![FIG. 2. Sequence comparison of HvnA and HvnB. Amino acid alignment of HvnA, HvnB, an ORF present in the *P. aeruginosa* genome, indicated by PaORF, and residues 67 to 113 of the human nicotinamide nucleotide transhydrogenase, indicated by NNTH. Identical aligned residues are indicated by white letters on black background. Dashes indicate gaps introduced into the sequence to facilitate alignment. Shaded boxes are aligned above segments with similarity to regions I, II, and III, which are conserved among ARTases and NADases (see Discussion) (43).](image)

![FIG. 3. Transfer of ADPr to polyarginine catalyzed by *V. fischeri* halovibrin mutants. Culture supernatants from strain ES114 (wild type), the EVS498 hvnA, EVS499 hvnB, and EVS500 hvnA hvnB mutant strains, and an LBS medium control were filtered and incubated with $^{32}$P-NAD$^+$ and polyarginine. Polyarginine was precipitated on TCA-soaked filters, which were washed and assayed for ADPr incorporation. ADPr incorporation was calculated from the counts per minute, assuming that the ADPr moiety from $^{32}$P-NAD$^+$ corresponds to recovery of the ADPr moiety from [32P]NAD$^+$ (see Discussion) (43).](image)
ever, these results were also consistent with a different hydrolysis product, such as cADPr. Using TLC with three different mobile-phase–stationary-phase combinations, we determined that ADPr was the major product of HvnA- and HvnB-mediated NAD$_1$ breakdown (Fig. 6 and data not shown), confirming that HvnA and HvnB catalyze an NADase reaction. Most ARTases possess minor NADase activity; however, NADases typically possess specific activities higher than the low residual NADase activity inherent in ARTases. To test whether halovibrin NADase activity resembled that of genuine NADases or the residual NADase activity of an ARTase, we purified HvnA and assessed the kinetics of its NADase activity. HvnA had a $K_m$ for NAD$_1$ of 10$^{-4}$ M and a $V_{max}$ of 400 mol of NAD$_1$ consumed/min/mol HvnA (Fig. 7). This $V_{max}$ is 10$^2$- to 10$^4$-fold higher than the minor NADase activity reported in certain other bacterial ARTases (15, 39–41). It is also a higher activity than that reported for NADases purified from rabbit erythrocytes and Bungarus fasciatus venom, while lower than the activity of NADases purified from N. crassa and streptococci (12, 25, 35, 58). Thus, the specific NADase activity of HvnA lies within a range reported for NADases and would be exceptionally high for the background NADase activity of an ARTase.

Symbiosis proficiency of an $hvnA$ and $hvnB$ double mutant. In order to test possible roles of HvnA and/or HvnB in the $E$. scolopes light organ symbiosis, symbiont-free hatchling squids were inoculated with ES114 and $hvn$ mutant strains, and the initiation of symbiosis was monitored. Strains EVS499, EVS498, and EVS500, each successfully infected juvenile $E$. scolopes. The extent of colonization, as measured by luminescence, is equivalent in ES114 and $hvn$ mutant-infected animals over 48 h (Fig. 8). In addition, the number of CFUs per light organ is equivalent for ES114 and the $hvnA$ $hvnB$ mutant EVS500 (data not shown). Furthermore, when juvenile animals were exposed to low $V$. fischeri inoculum densities, such that only a fraction of inoculated squid became infected, the percentage of animals infected by $hvn$ mutants was not significantly different from the percentage infected by wild type (data not shown). SEM analysis revealed that EVS500 triggered regression of the light organ ciliated field in a time frame similar to the regression induced in a wild-type infection. TEM analysis of $E$. scolopes juveniles infected with EVS500 or ES114 similarly revealed no qualitative difference in the morphological effect of infection on host epithelial cells or the spatial pattern of light organ infection by the bacteria.

**DISCUSSION**

HvnA and HvnB were previously identified as potential signaling molecules in the $V$. fischeri-$E$. scolopes light organ symbiosis, based on the ability of purified HvnA and HvnB to catalyze polyarginine ribosylation using NAD$^+$ as a substrate, a feature presumptively described as ARTase activity (46, 47). We have demonstrated that the NADase activity of HvnA and HvnB (Fig. 5, 6, and 7) and the subsequent nonenzymatic reaction of free ADPr and polyarginine accounts for the observed HvnA- and HvnB-mediated ADP-ribosylation of polyarginine (Fig. 4). The NADase activity of HvnA displayed a
$V_{\text{max}}$ (Fig. 7) that was $10^2$- to $10^4$-fold higher than the minor NADase activity reported in bacterial ARTases such as cholera toxin, pertussis toxin, iota toxin, and exotoxin A (15, 39–41). Consistent with this, the inherent NADase activity of cholera toxin was undetectable in our assays (Fig. 5 and 6), even though cholera toxin catalyzed greater ADP-ribosylation of polyarginine than either HvnA or HvnB (Fig. 4). It remains possible that HvnA and HvnB are ARTases, targeting a specific, as-yet-unknown, host protein, or that some host-derived factor is required to stimulate Hvn ARTase activity. However, neither HvnA nor HvnB have demonstrated bona fide ARTase activity, and the specific activity of NAD$^+$ hydrolysis by HvnA lies within the range of enzymes categorized as NADases (25, 58). Therefore, based on the available data, we propose that HvnA and HvnB be reclassified as NADases and not as ARTases.

Although ARTases and NADases encompass several distinct protein lineages, three conserved structural motifs have been identified among these families that may be involved in NAD$^+$ binding and ADPr transfer (43). The new family described here and comprised of HvnA, HvnB, and a putative $P.\ aeruginosa$ protein has conserved regions similar to these motifs that are arranged in the same order (Fig. 2). Region I is comprised of an R or an H residue, often preceded by an aromatic residue and usually followed by a G or A residue (7), a pattern most closely matched by position R113 in the alignment shown in Fig. 2. Region II is a stretch of 13 to 19 amino acids, 20 to 40% of which are aromatic residues, criteria met by positions 147 to 164. Region III is comprised of an active site Q/E-X-E motif, of which the latter E is implicated in NAD$^+$ hydrolysis in various proteins by cross-linking, site-directed mutagenesis, and crystallographic studies (4, 7, 43, 45). A conserved Q-N-E from positions 276 to 278 in the alignment shown in Fig. 2 could represent this active site motif. It is interesting that although a Q-N-E sequence is found within the alignment of the NADH-utilizing domain of the human nicotinamide nucleotide transhydrogenase with HvnA and HvnB, this conserved E278 residue is not believed to function in

**FIG. 5.** HvnA and HvnB hydrolysis of e-NAD$^+$ and NAD$^+$. Catalysis of e-NAD$^+$ and NAD$^+$ hydrolysis was measured for cholera toxin (L), HvnA (C), and HvnB (N). e-NAD$^+$ or NAD$^+$ were added to a 100 μM final concentration. HvnA and HvnB were added as dialyzed preparations of proteins secreted by strains EVS499 and EVS498, respectively. Secreted proteins from strain EVS500 were a negative control (L). Amounts of HvnA, HvnB, and control preparations corresponded to 150 μl of original culture supernatant. A total of 750 ng of cholera toxin was added. Error bars (often too small to visualize) indicate standard errors ($n = 3$). In panel A, the cleavage of e-NAD$^+$ is indicated by an increase in the relative fluorescence at 465 nm, when samples were excited at 340 nm. In panel B, NAD$^+$ was assayed by the addition KCN and measurement of absorbance at 340 nm.

**FIG. 6.** Product analysis of NAD$^+$ degradation by TLC. Enzymes were incubated with [$^{32}$P]NAD$^+$ in 100-μl reaction volumes, and 5 μl was spotted onto silica TLC plates. After the solvent front had progressed approximately 10 cm, the plates were dried and scored by autoradiography. Lanes 1, 2, and 3 indicate reactions with cholera toxin (75 ng), no enzyme, and $N.\ crassa$ NADase (300 ng), respectively. Lanes 4, 5, and 6 correspond to crude HvnB (secreted proteins from strain EVS498), crude HvnA (secreted proteins from strain EVS499), and a negative control (secreted proteins from strain EVS500), respectively. The mobile phases were H$_2$O-ethanol-NaCl (A) and H$_2$O-ethanol-NH$_4$HCO$_3$ (B). Arrows mark the mobilities of ADPr or cADPr standards. In panel B, the lanes have been digitally shuffled to match the order in panel A.
NAD was made over a range of data that fit a linear model \((r > 0.9)\). Each velocity measurement was made over a range of data that fit a linear model \((r > 0.9)\). The NAD concentration was assayed by the addition of KCN, measurement of the absorbance at 340 nm, and comparison to a standard curve. (Inset) SDS-PAGE of (from left): lane 1, 97-, 66-, 45-, 31-, and 21-kDa protein standards; lanes 2 through 5, 6

Little is known about secreted bacterial NADases. Clinical streptococcal isolates often secrete NADases (24), and an NADase activity distinct from cholera toxin has been described as an extracellular product of \(V. cholerae\) (52). Also, an ORF resembling HvnA and HvnB occurs in the \(P. aeruginosa\) genome, suggesting that this organism may also secrete an NADase. Each of these bacterial species, as well as \(V. fischeri\), colonizes the extracellular surface of animal cells, leading us and others (24) to speculate that secretion of NADases may play a role in host colonization or bacterium-animal signaling. NADases could function in animal-bacterium interactions by any of a number of mechanisms. Secreted bacterial NADases might enable bacteria to use NAD\(^+\) as a nutrient in animal tissue; however, using NAD\(^+\) as a sole carbon source, strain EVS500 (\(hvnA\) \(hvnB\) mutant) grew at a similar rate and to a similar final density as the wild type (data not shown). NADases could also act essentially like ARTases, mediating a host response via the indirect ribosylation and altered function of a particular host protein (21). Alternatively, if bacterial NADases gained access to host cytoplasm, they could potentially interfere with a number of NAD\(^+\)-dependent intracellular processes, for example, by inhibiting a respiratory burst response, inducing necrosis, or interfering with signaling by endogenous ARTases. ADPr cyclases, or poly(ADPr) polymerase (18, 28, 56). However, preliminary experiments using immunocytochemistry to localize HvnA in the light organ crypt environment suggest that this NADase does not accumulate inside host cells (A. Small and M. McFall-Ngai, personal communication), arguing against intracellular NAD\(^+\) as a primary target.

Although NAD\(^+\) has traditionally been thought of as an intracellular metabolite, the discoveries of NAD(H)-metabolizing ectoenzymes anchored to the outer membrane of eukaryotic cells suggest that extracellular NAD\(^+\) is important as well. These ectoenzymes include CD38, an ADPr cyclase-cADPr hydrolase (30); PC-1, a nucleotide phosphodiesterase (6); ART-1, an ARTase (29); and a lipoxigenase (42). Both CD38 and ART-1 mediate changes in lymphocyte physiology, and Detter et al. (6) speculate that NAD\(^+\) released from lysing cells may initiate this effect. Secreted bacterial NADases could readily subvert such a signaling system by rapidly degrading extracellular NAD\(^+\). Future studies of eukaryotic NAD\(^+\)-utilizing ectoenzymes and the NADases secreted by animal-associated bacteria will likely be complementary and lead to a better understanding of the role of extracellular NAD\(^+\) in animals.

Whatever effect, if any, HvnA and HvnB have on colonization of host tissue may be subtle, considering that mutants defective in one or both \(hvn\) genes were apparently unaffected in the ability to initiate colonization of the \(E. scolopes\) light organ and to stimulate apoptotic regression of the light organ ciliated field. The observation that an \(hvn\) homolog appears in \(P. aeruginosa\), which like \(V. fischeri\) establishes long-term chronic colonization in hosts, could intimate that these proteins are involved in persistence, rather than initiation, of infection. Also, although an \(hvnA\) \(hvnB\) mutant triggered observable morphological changes in the light organ in a manner similar to the wild type, these anatomical bacterium-triggered developments may represent only a fraction of the total changes in host cell metabolism and gene expression triggered by \(V. fischeri\). A deeper understanding of the interspecies signaling in this symbiosis may help elucidate a symbiotic function for HvnA and HvnB.

![Graph](image)

**FIG. 7.** Kinetics of HvnA NADase activity. Rate of NAD\(^+\) degradation was measured in 100-\(\mu\)l reactions with 25 ng of purified HvnA and different concentrations of NAD\(^+\). Each velocity measurement was made over a range of data that fit a linear model \((r > 0.9)\). The NAD\(^+\) concentration was assayed by the addition of KCN, measurement of the absorbance at 340 nm, and comparison to a standard curve. (Inset) SDS-PAGE of (from left): lane 1, 97-, 66-, 45-, 31-, and 21-kDa protein standards; lanes 2 through 5, 6

![Graph](image)

**FIG. 8.** Colonization of juvenile squid by \(V. fischeri\) halovibrin mutants. For each treatment, 20 hatching \(E. scolopes\) were exposed to 5,000 CFU of \(V. fischeri\) ml\(^{-1}\) or no \(V. fischeri\) as a control for 3 h and then rinsed with \(V. fischeri\)-free seawater. Luminescence was measured with a luminometer at 24 and 48 h after inoculation. Error bars indicate the standard errors.
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