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Comparison of the Heme Iron Utilization Systems of Pathogenic Vibrios

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***Vibrio alginolyticus*, *Vibrio fluvialis*, and *Vibrio parahaemolyticus* utilized heme and hemoglobin as iron sources and contained chromosomal DNA similar to several *Vibrio cholerae* heme iron utilization genes. A *V. parahaemolyticus* gene that performed the function of *V. cholerae hutA* was isolated. A portion of the *tonB1* locus of *V. parahaemolyticus* was sequenced and found to encode proteins similar in amino acid sequence to *V. cholerae* HutW, TonB1, and ExbB1. A recombinant plasmid containing the *V. cholerae tonB1* and *exbB1D1* genes complemented a *V. alginolyticus* heme utilization mutant. These data suggest that the heme iron utilization systems of the pathogenic vibrios tested, particularly *V. parahaemolyticus* and *V. alginolyticus*, are similar at the DNA level, the functional level, and, in the case of *V. parahaemolyticus*, the amino acid sequence or protein level to that of *V. cholerae*.**

Vibrios are gram-negative marine bacteria which often cause disease in humans. Infections by *Vibrio cholerae* (7), *Vibrio fluvialis* (4, 15), and *Vibrio parahaemolyticus* (12) are acquired through consumption of contaminated water or seafood, and they lead to excessive watery diarrhea (*V. cholerae* and *V. fluvialis*) or to acute gastroenteritis (*V. parahaemolyticus*). *Vibrio alginolyticus* (5, 20) causes extraintestinal infections, such as wound infections.

Bacterial pathogens must acquire iron inside the host to multiply to numbers sufficient to cause disease (for reviews, see references 14 and 19). *V. cholerae* and *V. parahaemolyticus* acquire iron by at least two methods. Under low-iron conditions, they produce the siderophores vibriobactin (9) and vibrioferrin (25), respectively. These low-molecular-weight compounds bind iron with high affinity and are transported back into the cell. Both *Vibrio* species also acquire iron from heme or hemoglobin (22, 23, 26). *V. fluvialis* and *V. alginolyticus* produce siderophores (2), but neither has been tested for the ability to utilize heme or hemoglobin as an iron source.

V. cholerae heme iron utilization involves the following genes: *hutA*, which encodes the heme receptor (10, 11); *tonB1*, which encodes an inner membrane protein required for the transport of heme into the periplasm; *exbB1D1*, which encodes the inner membrane proteins required for TonB function; and *hutB* and *hutCD*, which encode a periplasmic binding protein and a cytoplasmic membrane permease, respectively, which are involved in the transport of heme to the cytoplasm (18). A second *V. cholerae* TonB system encoded by *tonB2* and *exbB2D2*, which also may be involved in heme transport, recently has been identified (18). To date, no other gram-negative bacterium has been found to contain two TonB systems.

Heme iron utilization systems have been studied for *V. cholerae* and *Vibrio vulnificus* (13), but heme iron utilization systems in other vibrios have not been well characterized. The goals of this study were to (i) identify vibrios that can acquire iron from heme or hemoglobin, (ii) determine if these species have heme utilization and *tonB*-like genes similar to those in *V. cholerae*, and (iii) determine if heme utilization proteins of *V. cholerae* and other heme-utilizing vibrios have similar amino acid sequences and whether they are functionally interchangeable.

Testing *Vibrio* species for heme and hemoglobin iron utilization. The ability of various strains to use several iron-containing compounds was tested. In the assay, cultures were seeded into Luria (L) agar containing the iron chelator ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDA), and 5 μl of each iron-containing compound was spotted onto the media. All of the wild-type vibrios tested, including both clinical and environmental isolates, exhibited substantial zones of growth around the heme and hemoglobin spots (Table 1). *V. cholerae* DHH-11, a heme iron utilization deletion mutant (Table 2), exhibited no detectable growth around the heme or hemoglobin spots, and it served as the negative control (Table 1). All of the strains tested could utilize $FeSO_4$ as an iron source.

Determining if the *Vibrio* species contain DNA similar to *V. cholerae* heme iron utilization genes. Chromosomal preparations (17) were digested with *Hin*dIII, electrophoresed on agarose gels, and subjected to Southern blotting on charged nylon membranes (Boehringer Mannheim, Indianapolis, Ind.) under low-stringency conditions (21). Chromosomal DNA from the noncholera strains was loaded onto the gels at a threefold-higher concentration than that of the *V. cholerae* chromosomal DNA. The Genius DIG DNA labeling and detection system with CDP Star (Boehringer Mannheim) was used to label the probes and detect hybridization. The *hutA* probe contained a 1.8-kb *Eco*RI fragment from pHUT3 (10). The probes for *tonB1*, *exbB1*, and *hutC* and for *tonB2* were generated by PCR from pHUT7 and pOUT11 (18), respectively, and were internal fragments of each gene. All of the strains tested contained DNA sequences similar to *tonB2* (Fig. 1; Table 3), *exbB1*, and *hutC* (Table 3), whereas all the strains except *V. fluvialis* contained DNA sequences similar to *hutA* (Table 3) and *tonB1* (Fig. 1; Table 3). The hybridization signal generated with the *tonB2* probe in *V. fluvialis* DNA (Fig. 1) was barely detectable, but upon prolonged exposure of the blot to X-ray film, a distinct signal at 5.2 kb was detected (Table 3). The *tonB1* probe hybridized to different-sized *Hin*dIII fragments than the

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TABLE 1. Growth of different *Vibrio* species with heme and hemoglobin as iron sources

Bacterial strain	Zone of growth (mm) with:				
	Heme (20 μM)	Hemoglobin (5 μM)	$FeSO_4$ (10 mM)	BE2-542	DTH-1
V. cholerae					
CA401[a]	21	12	21.0	ND[b]	ND
DHH-11[c]	NG[d]	NG	10.0	ND	ND
V. parahaemolyticus[e]					
474801	22.5	22.5	27.5	ND	ND
M47314	25.0	25.0	30.0	ND	ND
115	22.5	20.0	25.0	ND	ND
V. fluvialis[c] BE2-819	32.5	35.0	40.0	ND	ND
V. alginolyticus[e]					
BE2-542	20.0	17.0	26.0	18.0	19.0
DTH-1	NG	NG	26.0	22.0	21.0

[a] 1×10^4 bacteria/ml seeded into L-EDDA agar.
[b] ND, not determined.
[c] 5×10^3 bacteria/ml seeded into L-EDDA agar.
[d] NG, no growth.
[e] 1×10^5 bacteria/ml seeded into L-EDDA agar.

tonB2 probe in *V. alginolyticus* and the three strains of *V. parahaemolyticus*, suggesting that these strains contained two distinct *tonB*-like genes (Fig. 1; Table 3). The *hutA* probe hybridized to two different fragments in all the strains in which a signal was generated (Table 3). This may reflect the presence of an internal *Hin*dIII site in each gene. In all the Southern blots, the hybridization signal of each probe which hybridized to DNA from the *V. cholerae* chromosome was significantly more intense than those obtained with the other *Vibrio* species.

Isolation of a heme iron utilization mutant of *V. alginolyticus*. To isolate a heme iron utilization mutant of *V. alginolyticus* BE2-542, diethylsulfate mutagenesis and nalidixic acid enrichment were performed as previously described (10). Colonies were screened for growth on L-EDDA-hemin agar, and the heme utilization mutant, DTH-1, was isolated. When assayed as described above, DTH-1 exhibited no detectable growth around the spots containing heme or hemoglobin (Table 1). To determine if DTH-1 produced and/or utilized the *V. alginolyticus* siderophore, fully grown cultures of the mutant and its parent strain were spotted onto L-EDDA agar seeded with either bacterial strain. Significant zones of growth occurred around the spots of the mutant and the wild type on both plates, indicating that DTH-1 both produces and utilizes its siderophore. These data suggest that the heme utilization defect did not affect the siderophore synthesis or transport system.

Complementation of *V. alginolyticus* DTH-1 with the *V. cholerae* TonB1 system. To determine if the defect in DTH-1 was in a TonB system, the mutant was transformed by electroporation (18) with pHUT11, which contains *V. cholerae tonB1* and *exbB1D1*, or with the vector pACYC184. DTH-1/pHUT11 grew as well as the parent strain, BE2-542, in L-EDDA-hemin broth (Table 4), suggesting that DTH-1 has a defective TonB, ExbB, or ExbD protein and that the comparable *V. cholerae* protein is functionally interchangeable. Combined with the data in Table 1 showing that the mutation in DTH-1 had no effect on siderophore production or transport, these data indicate that the *V. alginolyticus* siderophore transport system may use a second TonB system. Occhino et al. (18) recently determined that mutations in either the TonB1 system or the TonB2 system in *V. cholerae* had no impact on heme iron or vibriobactin uptake in *V. cholerae*. However, when both TonB systems in *V. cholerae* were defective, both heme iron and vibriobactin utilization were disrupted. Thus, either TonB system in *V. cholerae* can function in both siderophore and heme uptake. This does not appear to be the case in *V. alginolyticus*, where apparent disruption of one of the TonB systems leads to the loss of only heme iron utilization, not siderophore uptake. Thus, *V. alginolyticus* may contain one TonB system that plays a role in heme uptake and another that plays a role in siderophore uptake.

Additional work was performed to determine if the defect in one of the DTH-1 TonB systems was in a *tonB* gene. DTH-1 was transformed with pTONB1 (containing *V. cholerae tonB1*) or with pTEE1 (containing *V. cholerae tonB1* and *exbB1D1*). DTH-1/pTONB1 failed to grow in L-EDDA-hemin broth, whereas DTH-1/pTEE1 grew as well as DTH-1/pHUT11 (Table 4). These data suggest that while the mutation in DTH-1 is in a *tonB* locus, it is not in a *tonB* gene. It is not clear whether the defect in DTH-1 is in an *exbBD* gene(s) or a promoter that controls expression of all three genes or is a polar mutation in *tonB*.

Isolation of a *V. parahaemolyticus* gene that is functionally interchangeable with *V. cholerae hutA*. A cosmid library of *V. parahaemolyticus* 474801 DNA was constructed (10) and transferred by triparental mating to *Escherichia coli* 1017 containing pHUT10 (10), which contains all the *V. cholerae* heme iron utilization genes except *hutA*. Bacteria were plated on L-EDDA-hemin agar, and a heme utilization-positive isolate was identified. The cosmid was named pPHU1 (parahaemolyticus heme utilization), and *E. coli* 1017/pHUT10/pPHU1 grew to a density similar to that of *E. coli* 1017/pHUT10/pHUT2 (pHUT2 contains the *V. cholerae hutA* gene) when tested for growth in L-EDDA-hemin broth (Table 4). These data suggested that pPHU1 contains the *V. parahaemolyticus hutA* equivalent and that it is functionally interchangeable with *V. cholerae hutA*. *E. coli* 1017 transformed with pPHU1 alone grew poorly in L-EDDA-hemin medium, indicating that it needs *tonB1* and accessory genes present on pHUT10. To confirm that pPHU1 contained the *V. parahaemolyticus hutA* equivalent, pPHU1 was digested with *Hin*dIII, electrophoresed on a gel, and probed with the *hutA* probe described above. The probe annealed to cosmid clone fragments of 2.9 and 0.6 kb, which are the same size as the fragments observed in Southern blots of genomic DNA (Table 3 and data not shown).

Cloning and sequencing of a portion of the *tonB1* locus from *V. parahaemolyticus*. Additional work was performed to confirm that *V. parahaemolyticus* has a TonB1 system similar to that in *V. cholerae*. As indicated in Table 3, both the *tonB1* and *exbB1* probes hybridized to a 2.8-kb *Hin*dIII fragment from *V. parahaemolyticus*, suggesting that these two genes are linked, as they are in *V. cholerae* (18). To clone the *tonB1* and *exbB1* genes, 2.5- to 3.4-kb *Hin*dIII fragments from *V. parahaemolyticus* 474801 chromosomal preparations were isolated, ligated into pACYC184, and transformed into *E. coli* DH5α. Tetracycline-sensitive colonies were pooled into groups of 25, and plasmids were screened by Southern hybridization with the *V. cholerae tonB1* probe. A clone was identified (pPHU2) to which the *V. cholerae tonB1* and *exbB1* probes, but not the *tonB2* or *hutC* probes, hybridized. This indicated that pPHU2 contained *tonB1* and *exbB1*, but not *hutC* or *tonB2*.

The DNA sequence of both strands of the insert in pPHU2 was determined with a ABI Prism 377 DNA sequencer from Applied Biosystems and was analyzed with the DNA Strider program (16). The BLAST program of the National Center for Biotechnology Information (1) was used to determine homol-

TABLE 2. Bacterial strains and plasmids

Strain or plasmid	Description or relevant phenotype	Source or reference
Strains		
V. parahaemolyticus		
474801	Clinical isolate	Texas Department of Health
M47314	Clinical isolate	Texas Department of Health
115	Environmental isolate	P. Baumann
V. alginolyticus		
BE2-542	Clinical isolate; Amp^r	Texas Department of Health
DTH-1	Heme utilization mutant of BE2-542	This study
V. fluvialis BE2-819	Clinical isolate	Texas Department of Health
V. cholerae		
DHH-11	$TonB^-$ Vib^- mutant of CA401	10
CA401	Classical strain	8
E. coli 1017	Ent::Tn*5* mutant of HB101	S. M. Payne
Plasmids		
pHUT2	Tet^r; 16-kb *Sau*3A fragment of *V. cholerae* CA401 DNA cloned into pLAFR3; encodes outer membrane receptor protein HutA	10
pHUT3	Amp^r; 3-kb *Hin*dIII-*Sal*I fragment of pHUT2 cloned into pAT153; encodes outer membrane receptor protein HutA	10
pHUT10	Cm^r; 10.3-kb *Hin*dIII fragment of *V. cholerae* CA401 DNA cloned into pACYC184; contains *tonB1*, *exbB1D1*, and *hutBCD*	10
pHUT7	Cm^r; 6.7-kb *Sal*I-*Hin*dIII fragment from pHUT10::Tn*5*f (10) cloned into pACYC184; contains *tonB1*, *exbB1D1*, and *hutBCD*	18
pHUT11	Cm^r; 3.1-kb *Hpa*I fragment from pHUT7 cloned into the *Eco*RV site of pACYC184; contains *tonB1* and *exbB1D1*	This study
pOUT11	Amp^r; 10.5-kb *Hin*dIII fragment of *V. cholerae* CA401 cloned into pWSK29; encodes TonB2 system	18
pTONB1	Cm^r; 1.1-kb PCR fragment containing *V. cholerae tonB1* cloned into pACYC184	18
pTEE1	Cm^r; 2.1-kb PCR fragment containing *V. cholerae tonB1* and *exbB1D1* cloned into pACYC184	18
pPHU1	Tet^r; 7-kb *Sau*3A fragment of *V. parahaemolyticus* 474801 DNA cloned into pLAFR3; contains *hutA*-like gene	This study
pPHU2	Cm^r; 2.8-kb *Hin*dIII fragment of *V. parahaemolyticus* cloned into pACYC184; contains *tonB1* and part of *exbB1* and *phuW*	This study

ogies of the deduced amino acid sequences, and MacVector Clustal W was used to determine protein identity and similarity. Our analyses of pPHU2 indicated that the cloned DNA contained three open reading frames (ORFs) (Fig. 2). ORFs 1 and 3 are missing the region encoding the carboxy termini of the respective proteins, as no stop codon was identified in either ORF. ORFs 1 to 3 encoded proteins that are homologous to the *V. cholerae* HutW (18a), TonB1, and ExbB1 proteins, respectively (18) (Fig. 2; Table 5).

V. parahaemolyticus TonB1 (predicted molecular weight [MW], 27,100; 247 amino acids; pI 9.20) has 66% amino acid similarity with *V. cholerae* TonB1 (18), and it has weaker homology to numerous TonB proteins in other organisms, most notably to that in *Pseudomonas putida* (3) (Table 5). The similarity between *V. cholerae* TonB1 and *V. parahaemolyticus* TonB1 is greatest in the 103 amino acids at the carboxy terminus, where the similarity is 87%.

V. parahaemolyticus ExbB1 (predicted MW, 25,000; 231 amino acids; pI 8.32) is 81% similar to *V. cholerae* ExbB1 (18), and it exhibits weak homology to other ExbB proteins, such as that from *Haemophilus ducreyi* (Table 5). The incomplete ExbB1 protein contains three more amino acids than *V. cholerae* ExbB1 (228 amino acids).

V. cholerae HutW is a protein that has weak homology with a number of putative coproporphyrinogen oxidases in other organisms (18a). PhuW (predicted MW, 40,400; 420 amino acids; pI 6.62), the HutW homologue in *V. parahaemolyticus*, has 82% similarity to HutW and has weak homology with a

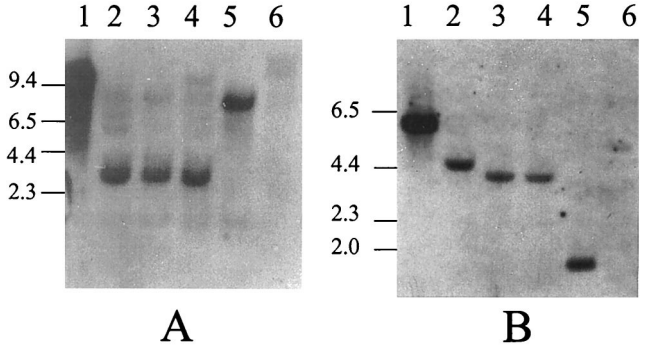

FIG. 1. Autoradiograms of Southern blots of *Vibrio* strains probed with *V. cholerae tonB1* (A) and *tonB2* (B). The following strains were tested: *V. cholerae* CA401 (lane 1); *V. parahaemolyticus* 474801, M47314, and 115 (lanes 2 to 4, respectively); *V. alginolyticus* BE2-542 (lane 5); and *V. fluvialis* BE2-819 (lane 6). Size markers are indicated on the left.

TABLE 3. Presence of heme iron utilization genes in *Vibrio* species

V. cholerae DNA probe	Fragment size(s) (kb) obtained in hybridization to chromosomal DNA from[a]:				
	V. parahaemolyticus			*V. alginolyticus* BE2-542	*V. fluvialis* BE2-819
	474801	M47314	115		
hutA	2.9, 0.6	2.9, 0.6	2.9, 0.6	6.0, 2.0	NDH[b]
hutC	3.5	4.4	3.5	9.0	2.9
tonB1	2.8	2.8	2.8	9.0	NDH
exbB1	2.8	2.8	2.8	9.0	6.1
tonB2	4.4	3.8	3.8	1.6	5.2[c]

[a] Values are approximate sizes of the *Hin*dIII fragments to which the probes hybridized.
[b] NDH, no detectable hybridization.
[c] Hybridization signal was weaker than that observed with other species.

number of putative coproporphyrinogen oxidases, such as HemN in *Bacillus subtilis* (Table 5).

The arrangement of genes in pPHU2 (Fig. 2) is similar to that in the *V. cholerae tonB1* locus, which contains *hutW*, *tonB1*, and *exbB1*, in that order (18, 18a), with *hutW* being transcribed in the opposite direction of *tonB1* and *exbB1*. The proposed promoters for *V. parahaemolyticus phuW* and for *tonB1* and *exbB1* contain a sequence similar to the *E. coli* consensus Fur box sequence (Fig. 2) (6). This sequence overlaps the predicted −35 region of the *phuW* promoter and the predicted −10 region of the promoter for *tonB1* and *exbB1*, suggesting that expression of the genes is iron regulated.

We have shown that the heme iron utilization systems of *V. parahaemolyticus*, *V. alginolyticus*, and *V. fluvialis* are similar at the DNA level to that of *V. cholerae* and that some of the heme utilization proteins of *V. cholerae*, *V. parahaemolyticus*, and *V. alginolyticus* are functionally interchangeable. *V. fluvialis* can use heme and hemoglobin as iron sources, but our data suggest that its heme utilization system has diverged from that of *V. cholerae*. The gene encoding the *V. fluvialis* heme receptor is not sufficiently similar to *V. cholerae hutA* to be detected by Southern hybridization. Since a *tonB2*-like gene, but not a *tonB1*-like gene, was detected in *V. fluvialis*, its heme receptor may function with a TonB2-like protein. Or *V. fluvialis* may have a TonB1-like protein, but the gene may not be similar enough to the *V. cholerae tonB1* gene to be detected by Southern hybridization.

TABLE 4. Growth of *V. alginolyticus* DTH-1 and *E. coli* 1017 transformed with various recombinant heme iron utilization plasmids

Bacterial strain	Absorbance[a] of cultures grown in:		
	L broth	L-EDDA broth	L-EDDA-hemin broth
V. alginolyticus			
BE2-542	1.40	0.12	1.27
DTH-1/pHUT11	1.40	0.08	1.20
DTH-1/pTEE1	1.53	0.04	1.30
DTH-1/pTONB1	1.30	0.03	0.12
DTH-1/pACYC184	1.50	0.12	0.12
E. coli			
1017/pHUT10/pPHU1	1.88	0.14	1.56
1017/pHUT10/pHUT2	2.40	0.13	1.42
1017/pPHU1	2.47	0.46	0.29
1017/pHUT10	2.32	0.24	0.29

[a] Absorbance at 600 nm after 7 h of growth for *V. alginolyticus* and 18 h of growth for *E. coli* 1017.

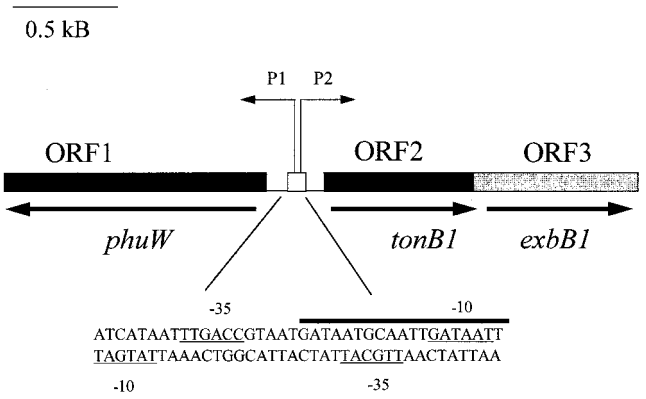

FIG. 2. Genetic map of cloned DNA from pPHU2. The putative Fur box is indicated by an open box. The arrows labeled P1 and P2 indicate the locations of the proposed promoters for *phuW* and for *tonB1* and *exbB1*, respectively. The arrows beneath the filled boxes denote the direction of transcription. Below the map is the DNA sequence containing the predicted divergent promoters and the proposed Fur box, which is marked with a thick black line above the DNA.

This study indicates that *V. parahaemolyticus* and *V. alginolyticus* contain two *tonB*-like genes similar in DNA sequence to *V. cholerae tonB1* and *tonB2*. Our data suggest that heme utilization in *V. alginolyticus* requires one TonB system and that the other TonB system can function in siderophore uptake but not in heme iron uptake. This is contrary to what occurs in *V. cholerae*, where the TonB systems appear to be redundant in that either system can support both heme and siderophore uptake (18). Future work will center on confirming that *V. alginolyticus* uses each TonB system to support a different function and on determining if *V. parahaemolyticus* is more similar to *V. cholerae* or *V. alginolyticus* in this regard.

Our sequencing data for pPHU2 supported our Southern blotting data concerning the presence of *tonB1*- and *exbB1*-like genes in *V. parahaemolyticus*. In addition, the sequencing data indicated that *V. parahaemolyticus*, like *V. cholerae*, contains a coproporphyrinogen oxidase-like gene linked to the TonB1 system genes. It is not clear at this time if HutW and PhuW are involved in heme iron utilization in their respective organisms. *Shigella dysenteriae* and *E. coli* O157:H7 also contain coprophorphyrinogen oxidase-like genes linked to heme iron utilization genes (24). Future work will be done to construct a *V. parahaemolyticus phuW* mutant that can be tested for heme iron utilization.

TABLE 5. Proteins with highest homology to products of *V. parahaemolyticus phuW*, *tonB1*, and *exbB1*

V. parahaemolyticus protein	Homologue	Amino acid identity (%)	Amino acid similarity (%)
PhuW	*V. cholerae* HutW	67	82
	B. subtilis HemN[a]	24	38
TonB1	*V. cholerae* TonB1	52	66
	P. putida TonB[b]	23	38
ExbB1	*V. cholerae* ExbB1	67	81
	H. ducreyi ExbB[c]	29	44

[a] Accession no. Z99117 (2634996).
[b] Data are from reference 3.
[c] Accession no. AF001034.

Nucleotide sequence accession number. The nucleotide and amino acid sequences corresponding to this region can be found under GenBank/EMBL accession no. AF119047.

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