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An Archaeal Aerotaxis Transducer Combines Subunit I Core Structures of Eukaryotic Cytochrome *c* Oxidase and Eubacterial Methyl-Accepting Chemotaxis Proteins

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Signal transduction in the archaeon *Halobacterium salinarum* is mediated by three distinct subfamilies of transducer proteins. Here we report the complete *htrVIII* gene sequence and present analysis of the encoded primary structure and its functional features. HtrVIII is a 642-amino-acid protein and belongs to halobacterial transducer subfamily B. At the N terminus, the protein contains six transmembrane segments that exhibit homology to the heme-binding sites of the eukaryotic cytochrome *c* oxidase. The C-terminal domain has high homology with the eubacterial methyl-accepting chemotaxis protein. The HtrVIII protein mediates aerotaxis: a strain with a deletion of the *htrVIII* gene loses aerotaxis, while an overproducing strain exhibits stronger aerotaxis. We also demonstrate that HtrVIII is a methyl-accepting protein and demethylates during the aerotaxis response.

An aerotactic response in the archaeon *Halobacterium salinarum* has previously been characterized as an accumulation of motile cells around an air bubble (4, 30). In *H. salinarum*, the aerotactic response was shown to be methylation dependent (21). Inhibition of methylation in *H. salinarum* by depletion of the methyl donor *S*-adenosylmethionine resulted in defective aerotaxis, providing further evidence that methylation is involved in aerotaxis in *H. salinarum* (21). Recently, a transducer for aerotaxis and other energy-dependent responses was identified in *Escherichia coli* (3, 24). Membranes with overexpressed Aer protein contained high levels of noncovalently associated flavin adenine dinucleotide (FAD) (3).

Signal transduction in the archaeon *H. salinarum* is mediated by a family of 13 putative transducers (28, 35). On the basis of hydropathy plot analysis and protein fractionation by ultracentrifugation, it was shown that this family contains both soluble and membrane-bound putative transducer proteins. There are three distinct subfamilies of these proteins: subfamily A consists of eubacterial chemotaxis-type transducers, containing periplasmic and cytoplasmic domains connected by two transmembrane segments; subfamily B contains transducers with two or more transmembrane segments and lacking a periplasmic domain, such as the SRI transducer HtrI (34); and subfamily C contains soluble transducer proteins (7, 35). Here, we report the full primary structure of HtrVIII, a member of subfamily B, and present experimental evidence that it is an aerotaxis transducer in *H. salinarum.*

MATERIALS AND METHODS

Strains and plasmids. Halobacterial strain Flx15 (29), which lacks bacteriorhodopsin and halorhodopsin, was used for the identification of the *htrVIII* gene. After appropriate restriction enzyme digestion, halobacterial DNA fragments were cloned into the commercial vector pDELTA1 (Gibco BRL, Gaithersburg, Md.). *H. salinarum* Pho81, which lacks bacteriorhodopsin, halorhodopsin, SRI, and SRII (31), was used for the deletion mutant construction. *E. coli* JM109 was used in routine cloning experiments. Halobacterial shuttle vector pMDS20 was a generous gift of M. Dyall-Smith (University of Melbourne, Melbourne, Australia).

Media and growth conditions. *H. salinarum* Flx15 and Pho81 were grown aerobically in tryptone medium at 37°C in the dark. *E. coli* JM109 cells, containing recombinant plasmids, were grown overnight in Luria-Bertani medium supplemented with the appropriate antibiotic.

Chemicals and electrophoresis reagents. All chemicals were reagent grade. Novobiocin was purchased from Sigma, St. Louis, Mo.

DNA isolation, restriction analysis, and cloning. The 1.8-kbp *Pst*I and 6.4-kbp *Bam*HI genomic fragments were used for the identification and cloning of the *htrVIII* gene. Southern hybridization and Western blotting analysis with antibody HC23 were performed as described previously (35). Standard molecular biological methods were followed, if not otherwise indicated, as described in reference 35.

DNA sequencing and data analysis. Double-stranded DNA was sequenced by the chain termination method with a Sequenase kit (United States Biochemicals). We used four sequencing strategies: the bidirectional deletion factory method with the pDELTA system (Gibco BRL), primer walking, the Erase-a-Base deletion system (Promega, Madison, Wis.), and automatic DNA sequencing (Applied Biosystems model 373; Perkin-Elmer).

Secondary-structure analysis. A prediction of the secondary structures of the halobacterial transducers was generated by a consensus among computer prediction algorithms PHD sec (25–27), Predator (9, 10), and four other prediction methods courtesy of SOPMA (8, 12–14, 20) on the Internet. The Kyte-Doolittle hydrophobicity plot was used to determine the approximate borders of the transmembrane regions. Generation of the prediction consensus was done with Microsoft Excel and was based on the individual predictions with the highest frequencies. All diagrams illustrating the predicted secondary structures were created with Microsoft PowerPoint.

Isolation of the deletion mutant *htrVIII* gene. The 6.4-kbp *Bam*HI-*Bam*HI fragment containing the full-length *htrVIII* gene was subcloned into vector pGEM-7Zf$^+$ (Promega) to yield recombinant plasmid phtrVIIIG. The halobacterial shuttle vector pMDS20 (17) was digested with *Pst*I and *Sma*I restriction endonucleases to produce a 2.9-kbp fragment containing the *gyrB* gene, conferring resistance to novobiocin. The resultant 2.9-kbp fragment was cloned into *Pst*I sites of phtrVIIIG, replacing most of the coding sequence of the *htrVIII* gene (from bp 56 to 1864) with a *Sma*I-*Pst*I adapter. The final plasmid (phtrVIIIGnov) was transformed into halobacterial strain Pho81 according to a standard polyethylene glycol-mediated protocol (19). Transformants were grown on 2% agar plates containing novobiocin (2 μg/ml). Primary screening of the deletion mutants was performed by two independent methods: Southern hybridization with a 27-mer conserved oligonucleotide probe and immunoblot detection with the antibody HC23 (35). This polyclonal antibody was raised against a 23-amino-acid peptide representing the most highly conserved region of all the transducers. We used a synergy system to synthesize a 23-amino-acid multiply antigenic peptide as an antigen to generate polyclonal antibody HC23, described in detail by Zhang et al. (35). For additional control of the deletion construction, we performed

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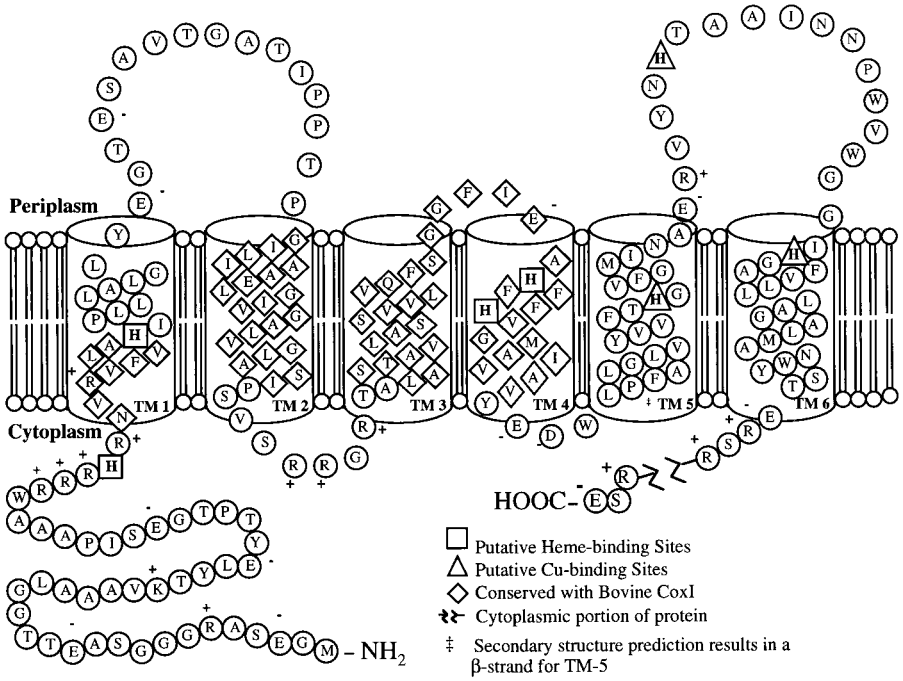

FIG. 1. Schematic representation of the transmembrane portion of HtrVIII. The putative transmembrane segments predicted as α-helices lie within the cylinders. Diamond-shaped residues have high homology with bovine COXI. The extensive cytoplasmic portion of the protein has been left out and is designated by the jagged break in the chain.

PCR with a set of specific primers to detect the presence of the *gyrB* cassette in the genome.

Construction of an HtrVIII overexpression strain. The 6.4-kbp *Bam*HI-*Bam*HI fragment containing the full-length *htrVIII* gene was subcloned into the multicopy halobacterial shuttle vector pMDS20. The resultant expression vector, phtrVIIIGwt2, which contained the *htrVIII* gene under the control of its own promoter, was transformed into strain Pho81. Transformants were grown on 2% agar plates containing novobiocin (2 μg/ml). The *htrVIII*$^{++}$/*htrVIII*$^{+}$ overexpression strains were screened by immunoblotting with our HC23 polyclonal antibody. The *htrVIII*$^{++}$/*htrVIII-1* strain was chosen for the high-level expression of *htrVIII*.

Radiolabeling with [*methyl*-^{3}H]methionine, electrophoresis, immunoblotting, and fluorography. Radiolabeling experiments were performed according to the method of Alam and Hazelbauer (1). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed essentially by the procedure of Laemmli (18), with modifications described by Randall and Hardy (23). Immunoblotting and fluorography were performed according to the method of Alam and Hazelbauer (1). The demethylation in vivo flow assay was performed according to the method of Lindbeck et al. (21). We used the scintillation cocktail Scinti-Verse BD and polyethylene scintillation vials (Fisher Scientific, Santa Clara, Calif.).

Aerotaxis assay. Highly motile halobacterial cells were grown to an optical density at 660 nm of 0.6 to 0.7. Cells were washed and resuspended in basal salt medium (25). Microslide capillaries (VitroCom, Mountain Lakes, N.J.) were filled halfway with washed cells. Both ends of the capillary were sealed and the capillary was placed for 5 to 6 h on a microscope stage that had been prewarmed to 37°C. The distribution of halobacterial cells close to the surface of an air bubble was recorded by time-lapse dark-field microscopy.

Nucleotide sequence accession number. The sequence reported in this paper has been deposited in the GenBank database (accession no. AF031641).

RESULTS

Cloning, sequencing of the *htrVIII* gene, and analysis of the deduced amino acid sequence. The *htrVIII* gene encodes a 642-amino-acid protein. A portion of the gene was identified in the 1.8-kbp *Pst*I fragment, and the full-length gene was identified in the 6.4-kbp *Bam*HI genomic fragment. The calculated molecular mass of the HtrVIII protein is 67.1 kDa, and it has a pI of 4.1. The newly identified transducer is a member of subfamily B (6, 35). It has six putative transmembrane segments (residues 1 to 209) (Fig. 1) that have homology with eukaryotic mitochondrial cytochrome *c* oxidase subunit I (COXI). Transmembrane 4 (TM4) of HtrVIII has 50% identity with helix X of bovine COXI, and TM5 and TM6 have 36% identity with helices VII and VIII (33). HtrVIII residues 386 to 550 are about 31% identical and residues 459 to 492 are 71% identical to the cytoplasmic signaling domains of eubacterial methyl-accepting chemotaxis proteins (12).

Secondary-structure prediction for the HtrVIII protein. Generation of the secondary structure from the primary sequence was accomplished with alignments and computer prediction algorithms as described in the Materials and Methods section. The algorithms predicted the presence of four α-helices in the cytoplasmic domain of HtrVIII (Fig. 2) instead of the predicted six helices in the eubacterial chemotransducers (16).

Isolation of the *htrVIII* deletion strain gene. The *htrVIII* deletion mutant was isolated by a standard gene knockout technique using the *gyrB* cassette. Southern hybridization with a 27-mer oligonucleotide probe showed that the 1.8-kbp *Pst*I *htrVIII*-specific fragment is missing in the Δ*htrVIII-24* strain (data not shown). An immunoblot with HC23 antibody of total cell lysate of Pho81 and the Δ*htrVIII-24* strain indicated that one of the cross-reacting bands is absent in the deletion strain (Fig. 3A). In sodium dodecyl sulfate-polyacrylamide gel electrophoresis, recombinant HtrVIII expressed in *E. coli* showed a mobility similar to that of the native protein from the halobacterial strain Pho81 (5).

HtrVIII is a methyl-accepting protein. The expression level of HtrVIII in strain Pho81 is relatively low compared to that in the other halobacterial transducers (Fig. 3A). To demonstrate that HtrVIII is indeed a methyl-accepting protein, we needed to express enough of HtrVIII for it to be distinctly visible in fluorography. Thus, we constructed an overexpression *htrVIII*$^{+}$/*htrVIII*$^{++}$*-1* strain based on the multicopy shuttle vector pMDS20 (13). The immunoblot clearly shows that the expres-

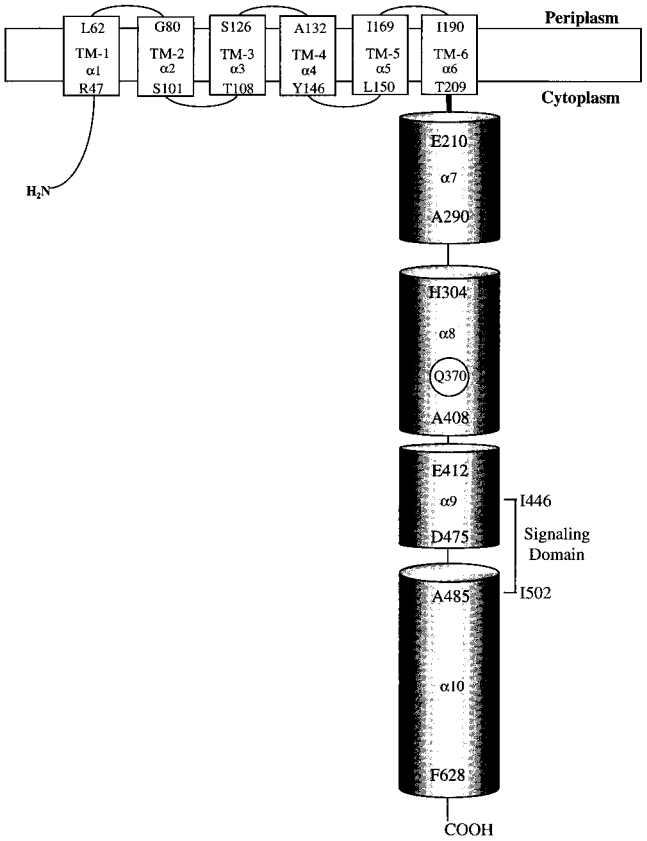

FIG. 2. Schematic representation of the secondary-structure prediction of the cytoplasmic domain of HtrVIII. The six transmembrane portions are represented as boxes, the cytoplasmic α-helices are represented as cylinders, and the linker regions are represented as broad lines.

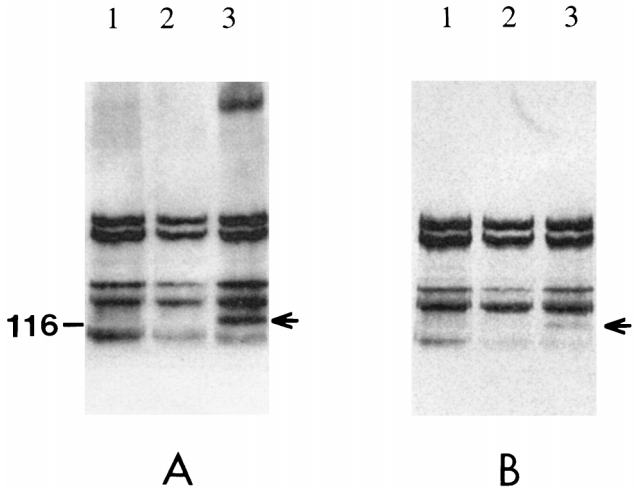

FIG. 3. Immunoblot and fluorograph of Pho81, the *htrVIII*$^{++}$/*htrVIII*$^{+}$*-1* strain, and the Δ*htrVIII-24* strain. (A) Western blotting analysis with HC23 antibody. Lanes: 1, Pho81; 2, the Δ*htrVIII-24* strain; 3, the *htrVIII*$^{++}$/*htrVIII*$^{+}$*-1* strain. (B) Electrophoretic analysis of *methyl*-^{3}H-labeled transducers. Lanes: 1, Pho81; 2, the Δ*htrVIII-24* strain; 3, the *htrVIII*$^{++}$/*htrVIII*$^{+}$*-1* strain. The molecular mass is in kilodaltons. Arrows indicate the position of HtrVIII.

sion level of HtrVIII in the *htrVIII*$^{+}$/*htrVIII*$^{++}$*-1* strain is higher than in the Pho81 strain (Fig. 3A). Fluorography of radiolabeled *htrVIII*$^{+}$ (strain Pho81), Δ*htrVIII-24*, and overexpression strains demonstrates that the specific radiolabeled band is missing in the deletion strain. The same band is present in wild-type and overexpression strains, and its intensity corresponds to the expression levels of the HtrVIII protein in those strains (Fig. 3B).

HtrVIII is involved in aerotaxis. The ability of the cells to sense an oxygen gradient and thus to concentrate around the trapped air in the flat microcapillary was analyzed by time-lapse dark-field microscopy. Wild-type halobacterial cells congregated around the interface between air and cell suspensions (Fig. 4A). In contrast, cells of the Δ*htrVIII-24* strain failed to gather around the air boundary (Fig. 4C). The aerotaxis band of the overexpression strain after 5 to 6 h at 37°C is much broader than that of the wild-type strain (Fig. 4A and B). We postulate that this difference in aerotaxis response is due to the multicopy plasmid bearing the wild-type *htrVIII* gene. The swimming speeds of the deletion and overexpression strains are comparable with that of the wild-type strain.

HtrVIII is involved in demethylation during the adaptation to the aerotaxis response. Halobacterial cells transiently release methanol, which is an indication of carboxymethyl group turnover by chemostimuli and photostimuli (2, 30). The effects of sensory stimuli on the rate of release of methanol in *H. salinarum* do not exhibit the same symmetry as in *E. coli*; both positive and negative stimuli result in an increased rate of methanol release. Lindbeck et al. showed transient increases in the methanol production by *H. salinarum* cells in response to a step up or down in the oxygen concentration (21). Because fluorography showed that HtrVIII is indeed a methyl-accepting protein, transient increases in methanol release should not be seen in the deletion strain (the Δ*htrVIII-24* strain) in response to the addition or removal of oxygen in a flow assay. To test our hypothesis, we studied the methylesterase activity in the deletion and overexpression strains. Indeed, unlike the overexpression strain, the Δ*htrVIII-24* strain showed no transient changes in the methanol production following a step up or down in the oxygen concentration (Fig. 5). These results further confirmed that HtrVIII is a methyl-accepting protein and that covalent modifications of putative methylation residues are involved in aerotaxis.

DISCUSSION

HtrVIII was originally cloned as a putative transducer protein based on its homology in the highly conserved signaling domain with other methyl-accepting proteins (35). The position −22 to −28 bp from the start codon of *htrVIII* has a strong homology with the putative archaeal promoter element (32).

The BLAST sequence homology search of the six transmembrane segments in HtrVIII revealed that this region has high homology with eukaryotic COXI (Fig. 1). The C-terminal domain has high homology with the eubacterial methyl-accepting chemotaxis protein. Cytochrome *c* oxidase is a heme/copper protein that catalyzes the four-electron reduction of dioxygen to water. Therefore, we suspected that HtrVIII functions as an oxygen-sensing transducer protein.

All three methylation-dependent taxis responses (chemo-, photo-, and aerotaxis) described so far for *H. salinarum* exhibit similar profiles of methanol release. Methylation is not involved in *E. coli* aerotaxis (22). The putative oxygen sensor DcrH from *Desulfovibrio vulgaris* was shown to be methylated, but no physiological data regarding its involvement in aerotaxis has been reported so far (11).

These experimental results provide the answer to the first key question, i.e., whether HtrVIII is an aerotaxis transducer in

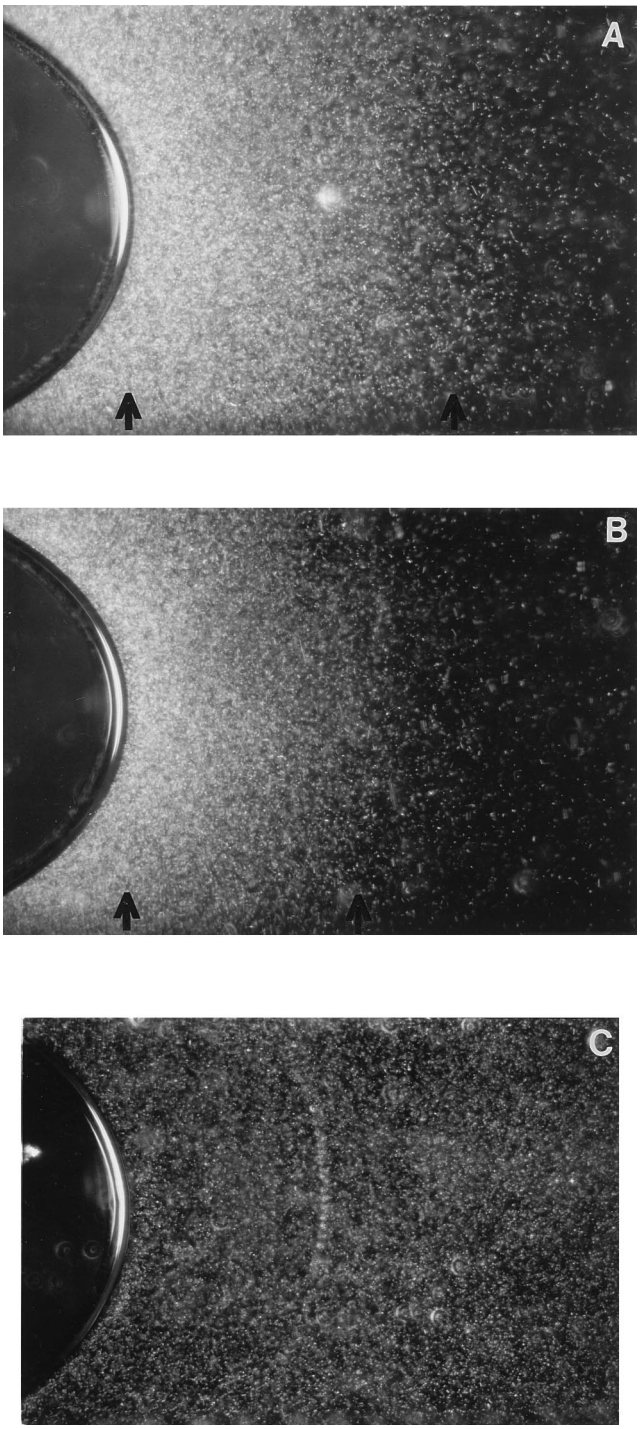

FIG. 4. Aerotaxis response (seen as white ring designated by two arrows in the dark background) in the air bubble assay. (A) *htrVIII*$^{++}$*/htrVIII*$^{+}$*-1* overexpression strain; (B) wild-type Pho81; (C) Δ*htrVIII-24* deletion strain.

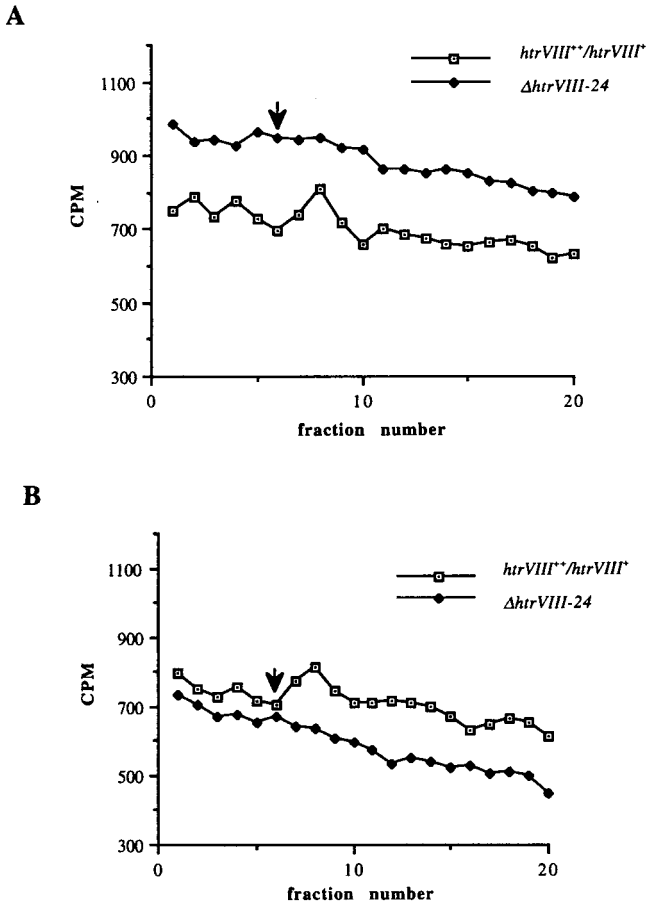

FIG. 5. Aerotaxis-induced changes in the rate of release of [^{3}H]*methyl* groups under conditions of nonradioactive chase of the *htrVIII*$^{++}$*/htrVIII*$^{+}$*-1* overexpression strain and the Δ*htrVIII-24* deletion mutant. Arrows indicate switch from N_2-equilibrated buffer to O_2-equilibrated buffer (A) and from O_2 to N_2 buffer (B).

H. salinarum. The next logical question is: what is the active site(s) that acts as the oxygen-sensing center? Several types of oxygen-sensing proteins are now known, including FixL (*Rhizobium meliloti*) and the recently discovered aerotaxis protein Aer in *E. coli*. A functional distinction between these two proteins is the nature of their oxygen-sensing sites. In the case of FixL, the active site is a heme chromophore, while in Aer it is a putative FAD. In the case of Aer, the oxygen sensing is believed to be indirect, with electron transfer to the FAD occurring via the respiratory chain, while FixL directly binds dioxygen to the heme iron (15).

Given the primary sequence of HtrVIII, we cannot rule out the possibility of any sort of prosthetic group, but the presence of histidine residues in HtrVIII (Fig. 1) is consistent with metal binding. Cytochrome *c* oxidase binds oxygen via a five-coordinate heme *a* chromophore located in subunit I. The conserved histidine residues are diagnostic for the oxidase family. The corresponding residues His-376 and His-378 of bovine COXI are conserved in HtrVIII; they are His-133 and His-135. The corresponding position of His-290 of COXI is His-163 in HtrVIII, and His-291 is replaced by Gly-164 in HtrVIII. It is tempting to speculate that HtrVIII is a heme protein (based upon the homology between HtrVIII TM4 and subunit I helix X of COXI). An alternative hypothesis is that HtrVIII contains a multinuclear copper center similar to the binuclear copper centers of hemocyanins (also oxygen-binding proteins) or a FAD as in the aerotaxis transducer of *E. coli* (3, 24). In the case of the hemocyanin binuclear copper sites, six histidines are required.

This report describes the first example of a methyl-accepting aerotaxis protein in the archaea. Further studies are under way to determine the nature of the oxygen-binding chro-

mophore and the molecular mechanism of signal transduction by HtrVIII in the archaeon *H. salinarum.*

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