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Signal transduction in the Archaeon *Halobacterium salinarium* is processed through three subfamilies of 13 soluble and membrane-bound transducer proteins

(Archaea/multiple antigenic peptide antibody)

WEISHENG ZHANG*†, ALEXEI BROOUN*†, JACQUELINE MCCANDLESS‡, PHILLIP BANDA‡, AND MAQSUDUL ALAM*§

*Department of Microbiology, University of Hawaii, 2538 The Mall, Honolulu, HI 96822; and ‡Applied Biosystem Peptide Synthesis Laboratory, Perkin–Elmer Corporation, 850 Lincoln Centre Drive, Foster City, CA 94404

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ABSTRACT **Eubacterial transducers are transmembrane, methyl-accepting proteins central to chemotaxis systems and share common structural features. We identified a large family of transducer proteins in the Archaeon *Halobacterium salinarium* using a site-specific multiple antigenic peptide antibody raised against 23 amino acids, representing the highest homology region of eubacterial transducers. This immunological observation was confirmed by isolating 13 methyl-accepting taxis genes using a 27-mer oligonucleotide probe, corresponding to conserved regions between the eubacterial and first halobacterial phototaxis transducer gene *htrI*. On the basis of the comparison of the predicted structural domains of these transducers, we propose that at least three distinct subfamilies of transducers exist in the Archaeon *H. salinarium*: (*i*) a eubacterial chemotaxis transducer type with two hydrophobic membrane-spanning segments connecting sizable domains in the periplasm and cytoplasm; (*ii*) a cytoplasmic domain and two or more hydrophobic transmembrane segments without periplasmic domains; and (*iii*) a cytoplasmic domain without hydrophobic transmembrane segments. We fractionated the halobacterial cell lysate into soluble and membrane fractions and localized different halobacterial methyl-accepting taxis proteins in both fractions.**

A basic property of living cells is their ability to respond to specific external chemical signals. In eukaryotic cells, sensory communication is mediated by chemical signals, including hormones, neurotransmitters, and growth factors. Eubacteria, like *Escherichia coli* and *Salmonella typhimurium*, respond to chemical gradients by modulating swimming patterns toward a more favorable chemical environment (1, 2). In eubacteria, the molecular components that mediate chemotaxis have been identified, and some mechanisms have been delineated (1, 2). Central to the response system is a family of transmembrane receptors, called transducers.

Recent progress in phylogenetic research demonstrated that Archaea are more closely related to eukaryotes than to eubacteria (3). *Halobacterium salinarium*, a member of Archaea, responds to changes in the chemical environment and reacts to light. Two membrane proteins, bacteriorhodopsin and halorhodopsin, function as light energy converters that power the energy-driven processes of the halobacterial cell. Moreover, two light sensors, sensory rhodopsins I and II (SRI and SRII), mediate "color vision" (4). Both are retinal-containing proteins distinct from the retinal-based, light-driven ion pumps bacteriorhodopsin and halorhodopsin (5–7). SRI is the photoreceptor for green-red and UV light stimuli, and SRII is the photoreceptor for blue light (7–10). Even in the absence of the dedicated sensory rhodopsins, proton pumping by bacteriorhodopsin generates phototaxis responses, probably because of effects on membrane potential or proton motive force (11, 12).

We and others (13–15) identified a family of methyl-accepting taxis proteins with (apparent) molecular masses between 90 and 135 kDa as components of both the photosensory and chemosensory systems in *H. salinarium*. These proteins are altered in levels of methylation by positive and negative chemostimuli (13), are blocked for methylation in several mutants defective in taxis, are restored to normal methylation in revertants selected for tactic ability (16), and are related to transducer proteins of *E. coli* as determined by immunological crossreaction (17). SRI excitation is transduced by HtrI, a methyl-accepting taxis protein that shares a high sequence homology with the functional domains of eubacterial transducers (18, 19). The HtrI protein lacks a domain that is similar to the periplasmic ligand-binding domain of the enteric transducers. Chromophore cross-linking measurements of SRI (16) and expression studies provided strong evidence that SRI and HtrI form a stable complex in the cell membrane that mediates signal transduction (20, 21). However, except for HtrI, no other transducers have been isolated or characterized.

To identify and isolate other transducers involved in halobacterial signal transduction, we used immunological, genetic, and biochemical approaches. This paper describes for the first time the identification and isolation of all the methyl-accepting taxis genes, and the localization of their products in the Archaeon *H. salinarium*.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions. *H. salinarium* Flx15 is a derivative of S9 that lacks bacteriorhodopsin and halorhodopsin but contains SRI and SRII (6). Strain Flx15 was chosen for its excellent motility. Cells were grown aerobically in peptone medium at 37°C in the dark (6).

Radiolabeling with [*methyl*-^{3}H]Methionine, Membrane Fractionation, Electrophoresis, Immunoblotting, and Fluorography. Radiolabeling experiments were performed according to Alam *et al.* (13). For membrane fractionation, labeled halobacterial cell pellets (OD_{660} = 4) were resuspended in 0.5 ml of the basal salt medium (halobacterial growth medium without tryptone and yeast extracts) and sonicated with 15-s bursts six times with intermittent delays of 30 s. The sonicated material was centrifuged at 100,000 rpm for 1 h in a Beckman tabletop ultracentrifuge TLX (TLC.100.2

Abbreviations: SRI and SRII, sensory rhodopsins I and II; MAP, multiple antigenic peptide.

Data deposition: The nucleotide sequence reported in this paper has been deposited in the GenBank data base (accession no. U53365).

†W.Z. and A.B. contributed equally to this work.

§To whom reprint requests should be addressed.

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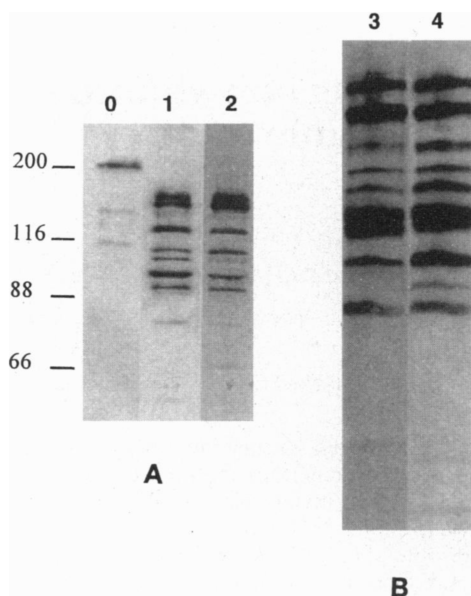

FIG. 1. Identification of a large number of methyl-accepting taxis proteins of the Archaeon *H. salinarium* by site-specific MAP antibody HC23. This antibody was raised against a 23-aa peptide, representing the highest homology region amongst eubacterial and archaeal phototaxis transducer HtrI. (*A*) Lanes 1 and 2 represent two analyses of the same lane of an SDS/10% polyacrylamide gel that has been electroblotted to a nitrocellulose sheet. Lane 1 is a fluorograph of *methyl*-^{3}H-labeled material, and lane 2 is an immunoblot with HC23 antibody. Lane 0 represents the immunoblot with preimmune serum with labeled halobacterial cells that has been processed as in lane 2. (*B*) The gel contained 11% acrylamide (0.073% bisacrylamide) at pH 8.2, thus expanding the spacing between bands in the cluster of high molecular mass. Lanes 3 and 4 represent a fluorograph and an immunoblot, respectively. Each gel lane was loaded with a 5% trichloroacetic acid precipitate from $\approx 10^8$ cells of the tactically wild-type strain Flx15 that had been labeled with [*methyl*-^{3}H]methionine in the absence of protein synthesis. Labeled samples were solubilized by boiling for 2 min in sample buffer. Immunoblotting and fluorography were performed as described (13, 17). The positions of the prestained SDS/PAGE molecular size standards are labeled in kilodaltons.

rotor). Appropriate fractions of the pelleted material and supernatant were processed for SDS/PAGE, which was performed essentially by the procedure of Laemmli (22), with modifications described by Randall and Hardy (23). Immunoblotting and fluorography were performed according to Alam and Hazelbauer (17).

Multiple Antigenic Peptide (MAP) Synthesis and Antibody Production. A MAP of 23 amino acids (VIDIAEQTNMLALNASIEAARAG) was synthesized using the Synergy system (Perkin–Elmer) and was purified on a reverse-phase analytical peptide column. MAP (0.5 mg) was dissolved in 50 μl of 30% ammonium hydroxide and diluted to 500 μl of HPLC-grade water. For the first immunization, we used a 50% peptide solution and Freund's complete adjuvant. After 2 weeks, booster shots containing the same amount of peptide mixed with Freund's incomplete adjuvant were given to the rabbit. The rabbit was bled 2 weeks after the last booster shot. After clotting and centrifugation, the serum was collected and was either further purified by an Affi-Gel protein A antibody purification kit (Bio-Rad) or used without purification. A Bio-Rad Minitransfer apparatus and a 1:10,000 dilution of HC23 antibody were used for Western blotting.

DNA Isolation, Restriction Analysis, and Cloning. Genomic DNA was isolated and purified according to Soppa (24). Standard molecular biological methods were used, if not otherwise indicated, according to Sambrook *et al.* (25). *E. coli* high-efficiency JM109 competent cells were used as the host strain for cloning. The *Pst*I-digested DNA fragments (1.0–23 kbp) from *H. salinarium* strain Flx15 were separated by use of Gene-Clean (Bio 101) and were cloned into the plasmid vectors pDELTA (GIBCO/BRL).

Oligonucleotide Labeling and Southern Hybridization. Total genomic DNA (5–10 μg) was digested with 30–50 units of *Pst*I (Promega) for 3 h at 37°C. DNA samples were loaded on 0.7% agarose gel (SeaKem agarose, FMC) and subjected to electrophoresis for 6 h at 70 V. Southern blotting was performed using Hybond-N$^+$ nylon membrane (Amersham) and Posi-Blot transfer system (Stratagene). Transfer was normally run overnight. After the transfer, membrane was treated with 0.1× standard saline citrate (SSC)/0.5% SDS solution for 1 h at 65°C in a rotary hybridization oven (Hybaid, Middlesex, U.K.) followed by incubation in prehybridization solution (5× SSC/0.02% SDS/0.1% lauryl sarcosine) and 4% blocking regent for 6 h at 55°C. A 27-mer oligonucleotide, 5′-CGC GTT CAG CGC CAG CAT GTT CGT CTG-3′, that represents the highest homology region amongst all known transducers was synthesized and used for hybridization. One hundred picomoles of 27-mer oligonucleotide probe was labeled using the

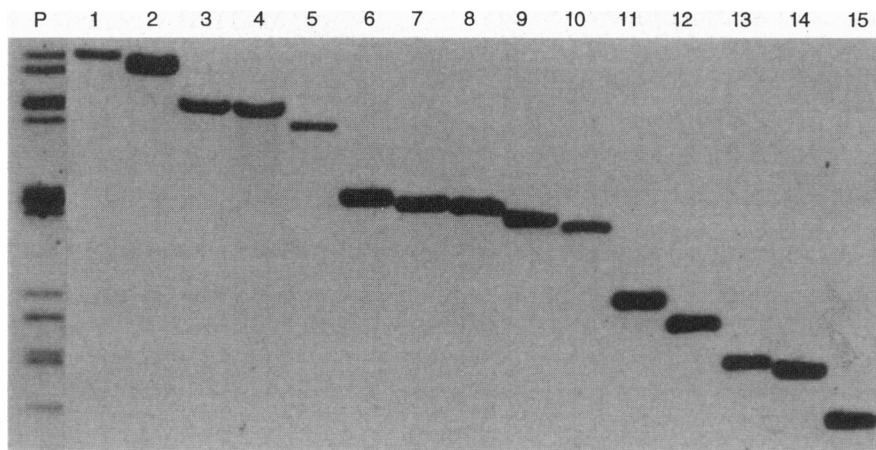

FIG. 2. Southern blot analysis of *Pst*I digestion of DNA from *H. salinarium* strain Flx15 and all possible clones using the 27-mer conserved oligonucleotide probe. Lane P represents *Pst*I digestion of the genomic DNA of the strain Flx15. Lanes 1–15 represent all the clones digested by *Pst*I. Lane 1, HtA (12-kbp fragment); lane 2, HtB (10 kbp); lane 3, HtC (7.8 kbp); lane 4, HtD (7.6 kbp); lane 5, HtE (6.5 kbp); lane 7, HtF (4.2 kbp); lane 8, HtG (4.1 kbp); lane 10, HtH (3.8 kbp); lane 12, HtI (2.2 kbp); lane 13, HtJ (1.9 kbp); lane 14, HtK (1.8 kbp); and lane 15, HtL (1.6 kbp). Lanes 6 (4.4 kbp) and 11 (2.4 kbp) represent DNA fragments hybridized with the 27-mer oligonucleotide probe but do not contain putative transducers. The first phototaxis transducer gene *htrI* (18, 19) was identified in the DNA fragment represented in lane 9 (4.0 kbp).

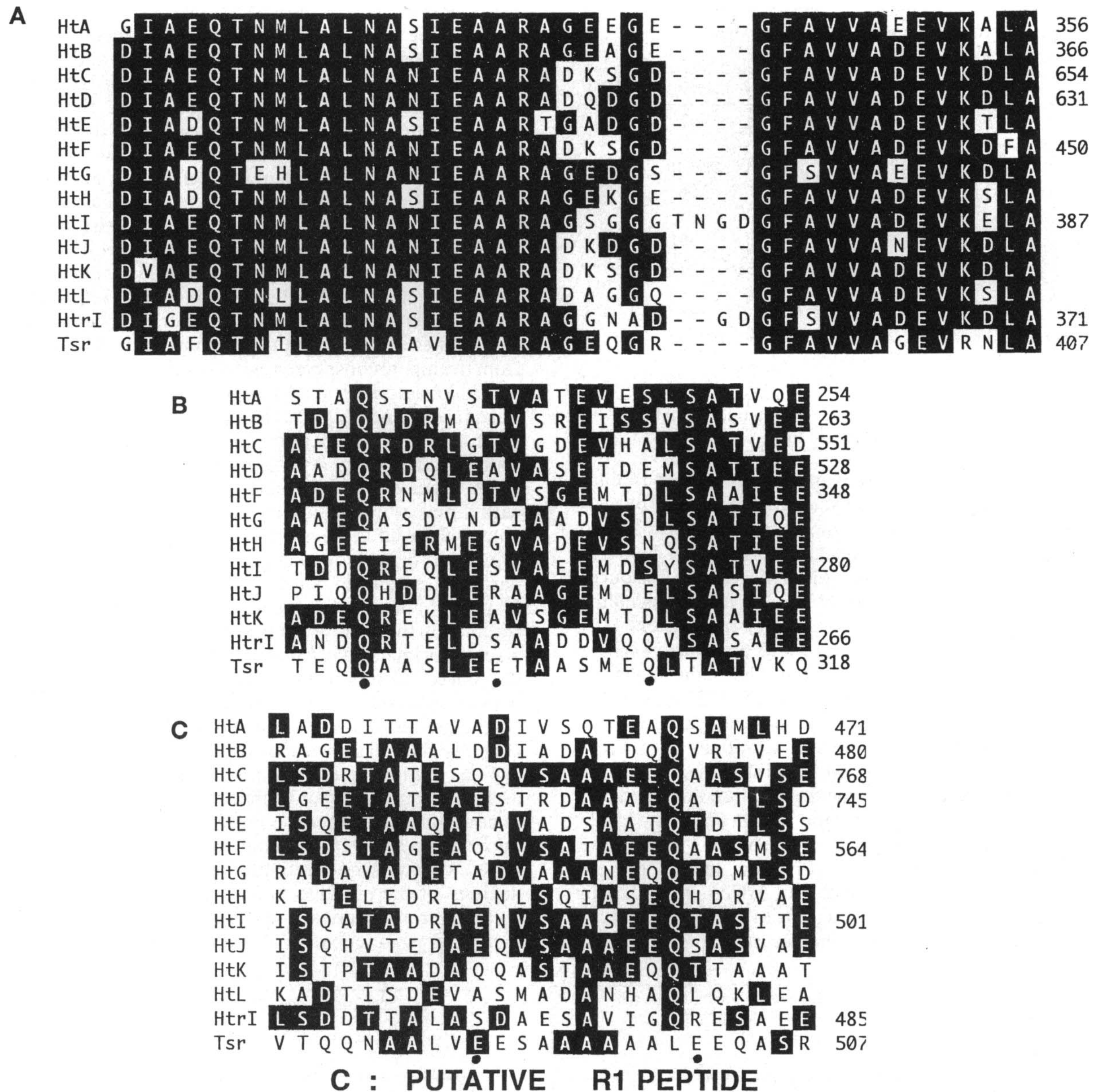

FIG. 3. Comparison of the partial amino acid sequences of the halobacterial transducers HtA through HtL with eubacterial transducer Tsr (28) and HtrI, the first archaeal phototaxis transducer (18, 19). Black boxes indicate positions at which the sequences compared contain identical residues. Sequence alignment was performed using the Clustal method with the default weight table matrix PAM250 from the MEGALIGN program of DNASTAR. As we do not know yet the function of these novel halobacterial transducer proteins, we designated them as HtA through HtL (H, halobacterial; t, transducer; A to L, proteins of unidentified function). (*A*) Highly conserved signaling domain. (*B*) Putative K1 peptide. (*C*) Putative R1 peptide. Filled circles indicate site of methylation in Tsr. Residue numbers are shown only for those transducers in which the full gene has been sequenced.

nonradioactive Genius 3 system (Boehringer Mannheim) with modifications. After terminal transferase reaction, without further purification, the labeled probe was added to a fresh aliquot (15 ml/100 cm^2 of membrane) of prehybridization solution. Hybridization was performed overnight, after which the hybridization membrane was washed twice, first with 2× SSC/0.1% SDS at 25°C and then with 0.5× SSC/0.1% SDS at 45°C. The nylon membrane was soaked with LumoPhos solutions, and chemiluminescent detection was done using a Genius 5′ nucleic acid detection kit (Boehringer Mannheim). Blocking reagent was added to the final concentration of 3% of Genius 2 buffer. X-ray films were exposed to the treated membrane sheet for 1–2 h.

DNA Sequencing and Data Analysis. Double-stranded DNA was sequenced by the chain-termination method using a Sequenase kit (United States Biochemical). We used four sequencing strategies: the bidirectional deletion method using the pDELTA system (GIBCO/BRL), primer walking, the Erase-a-base deletion system (Promega), and automatic DNA sequencing (Applied Biosystems Model 373, Perkin–Elmer). Both strands of the putative transducer genes were sequenced. Protein sequences were aligned using the program MEGALIGN (DNASTAR, Madison, WI), and secondary structure analyses were done using the program PROTEIN (DNASTAR).

RESULTS

Identification of Transducer Proteins by Site-Specific MAP Antibody. On the basis of our previous study (17), using antibody raised to *E. coli* transducers, we estimated that at least five transducer proteins could be expected in the Archaeon *H. salinarium*. But antisera to *E. coli* transducers recognized a few

Table 1. Compositional analysis of putative structural and functional domains of halobacterial transducers HtA through HtD, HtF, and HtI

	HtA	HtB	HtC	HtD	HtF	HtI
Length, aa	482	489	799	777	803	545
Molecular mass, Da	51, 059	52, 820	84, 517	82, 104	84, 540	56, 926
pI	3.63	3.78	3.92	4.10	3.96	3.77
TM residues	—	—	22–49 297–325	24–48 296–323	34–61 320–352	4–29 47–74 80–103
HL residues	—	—	—	—	—	107–171
PD residues	—	—	53–287	51–290	65–319	—
HCR residues	319–356	346–387	617–654	594–631	616–650	346–387

TM, transmembrane segments; PD, periplasmic domain; HCR, highly conserved region; HL, hydrophilic loop.

other nonspecific bands also, thus making it difficult to enumerate the total number of transducer proteins in this Archaeon. We decided to take a more specific approach to identify the total number of transducer proteins in halobacteria. It is well established that all eubacterial transducer proteins show a highly conserved region in the signaling cytoplasmic domain (1). The halobacterial transducers HtrI (18) and HtrII from *Haloarcula vallismortis* and *Natronobacter pharaonis* (26) also show homology in the same signaling domain of the eubacterial transducer (1, 2). A 23-aa peptide representing the highest homology region was synthesized by use of the MAP technique (27) and used as antigen to generate polyclonal antibody HC23. The MAP design maximizes the concentration of antigen for a specific response and produces high-titer antibody. The site-specific MAP antibody HC23 should crossreact with any halobacterial proteins that have the similar conserved region. To identify how many of these immuno-crossreacting proteins with this HC23 antibody will contain typical methyl-accepting regions (a diagnostic feature for the transducer proteins in eubacteria), we radiolabeled halobacterial cells by providing [*methyl*-^{3}H]methionine after blocking the protein synthesis. The radiolabeled halobacterial cells were processed for immunoblotting and analyzed by fluorography. The two films, one with a record of the antibody reaction and the other with the pattern of the [^{3}H]methyl-labeled proteins, were aligned precisely with the nitrocellulose sheet and thus with each other. In Fig. 1, prints of those films are placed next to each other to reflect the alignment of the patterns, illustrating that the crossreacting bands correspond to methyl-accepting species, revealing the existence of a large family of transducer proteins in this Archaeon.

Cloning and Sequencing of Putative Transducer Genes and Homology of Their Predicted Amino Acid Sequences. In the search for transducer genes, a 27-mer oligonucleotide probe was synthesized based on nine amino acids (QTNMLALNA) from the highly conserved region in the signaling domain of the first halobacterial transducer gene, *htrI* (18), and eubacterial transducer genes (28). Halobacterial genomic DNA was digested with *Pst*I restriction endonuclease and used for hybridization and cloning. A total of 15 fragments of halobacterial DNA annealed to the labeled 27-mer oligonucleotide probe with different intensities. By screening >5000 clones, we were able to clone all 15 fragments (Fig. 2). To differentiate putative transducer genes from nonspecific hybridization bands, first we partially sequenced all these positive clones by using the 27-mer oligonucleotide probe, used during Southern hybridization. On the basis of the preliminary sequences, we designed a set of primers that gave us the expected conserved region sequences. Comparison of partial nucleotide and deduced peptide sequences of all clones representing 15 different fragments identified 13 individual putative transducer genes (Fig. 3). They all show extensive identity, from 63% to 95%, to a 42-aa region within the signaling domain among the 13 archaeal transducers and eubacterial transducer Tsr (Fig. 3*A*). Among them was the previously identified phototaxis transducer gene *htrI* (18). Among these 13 putative transducer genes, we have sequenced fully six of them. The compositional analyses of the putative structural and functional domains of these six transducers are summarized in Table 1. The sites of methylation in eubacterial transducer Tsr are the lysine-

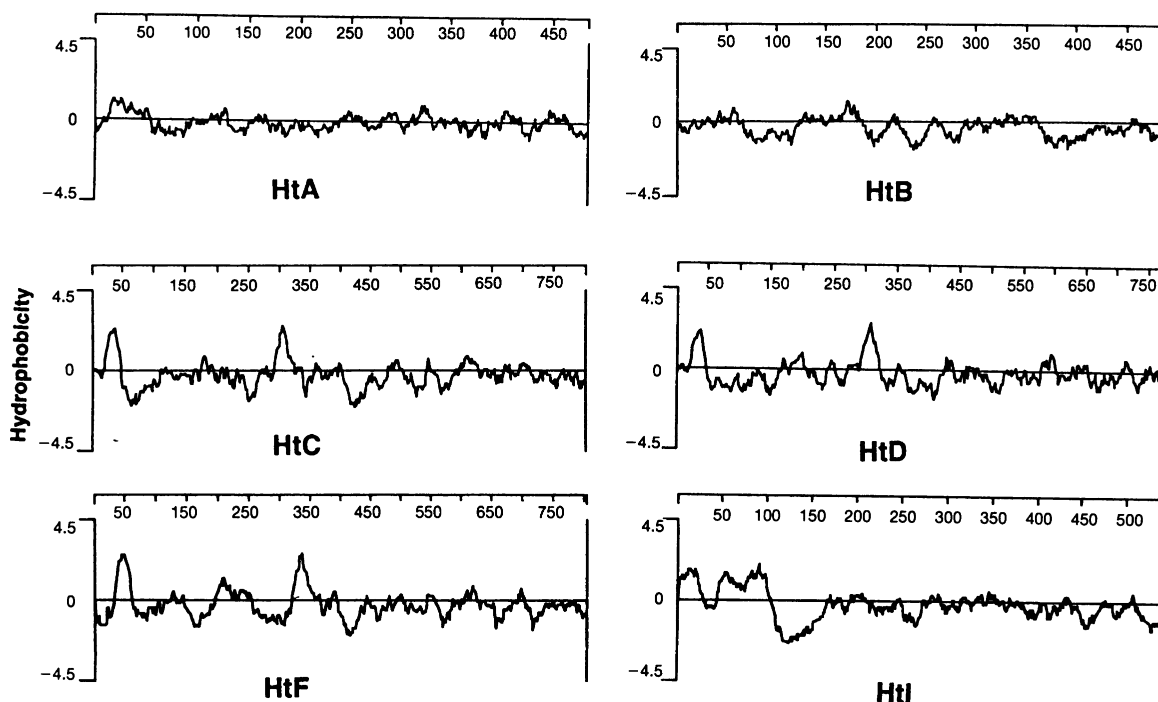

FIG. 4. Kyte–Doolittle hydropathy plot (DNASTAR sequence analysis software package) of the six newly identified halobacterial transducer sequences, HtA, HtB, HtC, HtD, HtF, and HtI.

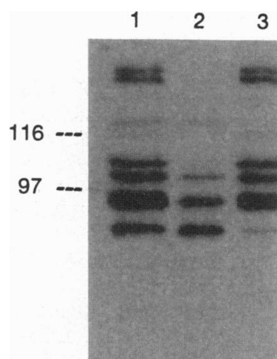

FIG. 5. Distribution pattern of the transducer proteins from the soluble and membrane fractions of *methyl*-^{3}H-labeled halobacterial cells assayed by fluorography. Lane 1, total cell lysate; lane 2, soluble fraction; and lane 3, pellet after 1 h of ultracentrifugation. Bars indicate the position of the molecular weight markers.

containing peptide K1, 295–317, and the arginine-containing peptide R1, 483–507 (28). When we made the homology search for the Tsr K1 and R1 peptides in the new family of the archaeal transducers (partially and fully sequenced), two regions of conserved sequences were visible (Fig. 3 *B* and *C*). The region flanking the methylation domains shows a lesser degree of sequence identity to the archaeal transducer family.

Localization of the Transducer Proteins in the Halobacterial Cell. All the eubacterial transducer proteins, except FrzCD in *Myxococcus xanthus* (29–31), characterized so far are integral membrane proteins and contain two transmembrane segments (1, 2). The Kyte–Doolittle hydropathy analysis of the primary amino acid sequences of HtA or HtB (Fig. 4) indicates that these proteins may be soluble or peripherally membrane bound. To address this issue more thoroughly, halobacterial cells were first labeled with [*methyl*-^{3}H]methionine after inhibition of protein synthesis to identify methyl-accepting taxis proteins (13–15). The cells were then harvested and lysed. The fractionation of soluble and membrane-bound proteins was carried out by ultracentrifugation. The samples from both of the fractions were subjected to SDS/PAGE and fluorography. Fig. 5 shows that halobacterial transducer proteins are localized in both the soluble and the membrane fraction.

DISCUSSION

We have cloned and sequenced all possible DNA fragments that encode the highly conserved signaling domain, and putative K1 and R1 methylation peptides in comparison with the known eubacterial chemotaxis transducers. On the basis of the immunological analysis, fluorography, cloning, and sequencing results, we conclude that the Archaeon *H. salinarium* possesses 13 putative transducers.

Hydropathy plots and comparison of the primary structures of six archaeal transducer proteins reveal very interesting features not observed in eubacteria. When hydropathic indices were plotted against the residue numbers of the six archaeal transducers, HtA through HtD, HtF, and HtI, two possible transmembrane helices with high positive values for HtC, HtD, and HtF are noted (Fig. 4; Table 1). No such patches of hydrophobic amino acids were found in HtA or HtB. It is interesting that HtI has three clearly resolved hydrophobic patches, indicating transmembrane helices. HtI shows the highest homology and sequence identity with HtrI and two other archaeal phototaxis transducers from *H. vallismortis* and *N. pharaonis* (26). On the basis of comparison of the putative functional and structural domains of these six transducers, we propose that at least three structurally distinct subfamilies of transducer exist in the Archaeon *H. salinarium* (Fig. 6). Family A (HtC, HtD, and HtF) contains two transmembrane segments, a periplasmic domain, and a conserved cytoplasmic signaling domain that are similar to eubacterial chemotransducers (1, 2). Family B [HtrI (18) and HtI] has two or more transmembrane segments, no periplasmic domain, but a conserved cytoplasmic domain. Family C (HtA and HtB) possesses neither a periplasmic domain nor transmembrane segments, but does possess a conserved signaling domain. In comparison with eubacterial transducers, the members of family A are relatively large, and the members of the families C are smaller (Table 1). Family B has a unique feature; it possesses a hydrophilic loop structure (residues 107–171 in HtI) just after the last transmembrane segment.

In eubacteria, methylation sites in transducer proteins have been identified as either glutamates or glutamines that are deamidated by the CheB esterase to create methyl-accepting glutamates (32–35). Comparison of the eubacterial K1 and R1 peptides with the archaeal transducers and the surrounding acidic residues allows us to propose methylation sites in these proteins. For example, in the putative K1 peptide, residues Q234, Q253, and E254 in HtA and residues Q508, E513, E527, and E528 in HtD are highly conserved among the archaeal transducers. In the R1 peptide, residues E463 and Q465 are also highly conserved. We cannot rule out that carboxylmethylation sites identified in Tsr may correspond to glutamate and aspartate residues in the archaeal transducer family. The unusual isoaspartate α-methyl ester and aspartate β-methyl ester, whose formation is catalyzed by protein-carboxyl *O*-methyltransferase, are intermediates in the repair process of age-damaged protein in eukaryotes (36, 37).

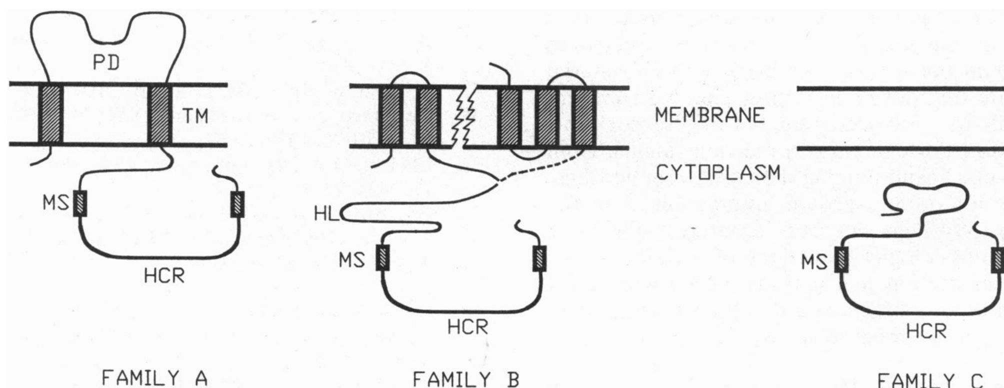

FIG. 6. Schematic models of the three subfamilies of halobacterial transducer proteins. Family A, two transmembrane segments and periplasmic and cytoplasmic domains. Family B, two or more transmembrane segments, a cytoplasmic domain with a specific hydrophilic loop, but no periplasmic ligand-binding domain. Family C, soluble transducer protein. TM, transmembrane segment; PD, periplasmic domain; MS, methylation site; HCR, highly conserved region; HL, hydrophilic loop.

In eubacteria, the transducer serves three functions in chemotaxis: it binds the ligand; it sends an excitatory signal into the cytoplasm; and it is involved in the adaptation to attractants or repellents by being methylated or demethylated, respectively (1, 2). The discovery of the two unique subfamilies B and C of methyl-accepting taxis proteins in *H. salinarium* raises very interesting questions. Why do halobacteria need soluble transducer proteins? Lacking periplasmic ligand-binding domains, how do soluble transducers sense and transduce sensory signals? If a soluble transducer recognizes some chemo-effector, one must assume that it has to interact with a membrane protein or that the chemo-effector is readily transported into the cell. In the case of HtI, three putative transmembrane segments are anchored in the membrane, suggesting that HtI or this type of transducer communicates with other receptor(s) to initiate signal transmission as in the case of the SRI–HtrI interaction (38).

The question of why the Archaeon *H. salinarium* has so many transducer genes in its genome compared with the eubacteria presently cannot be answered. The *dcr* gene family from *Desulfovibrio vulgaris* Hildenborough is the only example of a eubacterium that accommodates a large number of transducer genes (39). Except DcrA, which senses the oxygen concentration or redox potential of the environment, the functions of the other transducers from this family are not known (39). Halobacterial cells are attracted by D-glucose, sodium acetate, sodium benzoate, histidine, asparagine, leucine, methionine, serine, and aspartate and are repelled by sodium phenolate (40), but none of their chemoreceptors are identified. The photoreceptors that are responsible for the phototactic behavior of *H. salinarium* are SRI and SRII (4, 6–10). Interestingly, under intense light, bacteriorhodopsin also mediates a phototactic response by an unknown mechanism based on changes in the proton motive force (11, 12). *H. salinarium* cells are aerotactic (41), and it has been proposed that $\Delta\mu$H-reception is also the mechanism for aerotaxis in *H. salinarium* (42). Aerotaxis is methylation-dependent in the Archaeon *H. salinarium* (43). We have demonstrated that osmotaxis in the Archaeon is a methylation/demethylation-dependent system (data not shown). Thus, in this organism, in addition to chemo- and phototaxis, there must be other transducers that are responsible for aerotaxis, osmotaxis, and $\Delta\mu$H-reception taxis.

The existence of three distinctly different structural classes or subfamilies in the Archaeon raises the question of the evolution of transducer gene families. What was the ancestral transducer? Was it soluble or membrane-bound? Why does the archaeal transducer need more than two transmembrane helices like HtI? Are members of family B an intermediate form? The "transducers" in eukaryotes are generally G proteins that bind to seven-helix membrane proteins. Recent studies indicate that Archaea members are more closely related to eukaryotes, although there are common features between Archaea and eubacteria. The extensive homologies in the signaling domain between the archaeal and eubacterial transducers indicate that during evolution, the "transmitter" part of the molecule was well preserved, but the "sensor" part of the molecule went through various rearrangements, from creating a periplasmic ligand-binding domain to simple transmembrane helices for protein–protein interaction as in the SRI–HtrI complex (38). Thus, of particular interest will be the elucidation of the physiological functions of archaeal transducers and their adaptation mechanisms, which will reveal insights into the molecular organization of the signaling pathways that are not used by eubacteria.

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