JOURNAL OF BACTERIOLOGY, May 1997, p. 2963–2968 Vol. 179, No. 9
0021-9193/97/$04.00+0

Primary Structure and Functional Analysis of the Soluble Transducer Protein HtrXI in the Archaeon *Halobacterium salinarium*

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Received 25 November 1996/Accepted 17 February 1997

Signal transduction in the archaeon *Halobacterium salinarium* is mediated by a family of 13 soluble and membrane-bound transducers. Here, we report the primary structure and functional analysis of one of the smallest halobacterial putative transducers, HtrXI. Hydropathy plot analysis of the primary structure predicts no membrane-spanning segments in HtrXI. The fractionation of the *H. salinarium* proteins confirmed that HtrXI is a soluble protein. Capillary assay with an HtrXI deletion mutant and a complemented strain revealed that this soluble transducer is involved in Asp and Glu taxis. In vivo analysis of the methylesterase activity of the *htrXI-1* deletion mutant suggests that HtrXI plays an important role in the adaptation of the chemotactic responses to His, Asp, and Glu, which are attractants for halobacteria. Stimulation by Asp and Glu causes demethylation of HtrXI and of another putative transducer, HtrVII. But addition of His to halobacterial cells increases HtrXI methylation together with that of other putative transducers. In the absence of HtrXI, stimulation by either Glu or His does not decrease or increase the methylation of any putative transducers. Therefore, the HtrXI transducer appears to have a complex role in chemotaxis signal transduction.

Halobacterium salinarium, a member of the *Archaea* domain, exhibits both phototaxis and chemotaxis (20). Two photoreceptors, SRI and SRII, are responsible for the organism's color vision. Cells swim toward areas with a higher intensity of green light and avoid near-UV and blue light (21). *H. salinarium* is also able to respond to spatial gradients of chemicals. Histidine, glucose, and asparagine are attractants, while phenol is a repellent (15). Methylation and demethylation of halobacterial putative transducers have been shown to be involved in mediation of these responses (1, 18). Unlike for eubacteria, both positive stimulation and negative stimulation of *H. salinarium* cells lead to a transient increase in the rate of methanol production (1). The same type of methanol evolution profile has been shown for the gram-positive bacterium *Bacillus subtilis* (22).

Recently, we and others have identified 13 putative transducers in the archaeon *H. salinarium* (14, 26). On the basis of hydropathy plot analysis and protein fractionation by ultracentrifugation, it was shown that this protein family contains both soluble and membrane-bound putative transducer proteins. There are three distinct subfamilies: (i) eubacterial chemotaxis-type transducers, containing periplasmic and cytoplasmic domains connected by two transmembrane segments; (ii) transducers having two or more transmembrane segments and lacking periplasmic domains, such as the SRI transducer HtrI (25); and (iii) soluble transducer proteins (14, 26). All known eubacterial transducer proteins, except FrzCD in *Myxococcus xanthus* (10–13) and McpA in *Rhodobacter sphaeroides* (24), are integral membrane proteins and contain two transmembrane segments (2, 3). Recently, it was shown that FrzCD, together with the extracellular C-factor, is an essential component for the aggregation and social behavior of *M. xanthus* cells (16). A change in methylation levels of FrzCD is associated with a decrease in reversal frequency and thus facilitates formation of cell aggregates. Until now, functional effects of the archaeal soluble transducers have not been shown.

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MATERIALS AND METHODS

Strains and plasmids. *H. salinarium* Flx15, lacking bacteriorhodopsin and halorhodopsin (17), was used for the isolation of the genomic DNA and for deletion mutant construction. *Escherichia coli* JM109 was used in DNA cloning experiments. Halobacterial DNA fragments were cloned into plasmid vectors pDELTA1 (Gibco BRL, Gaithersburg, Md.) and pBSII SK$^+$ (Stratagene, La Jolla, Calif.). Halobacterial shuttle vector pWL102 (9) was obtained from the American Type Culture Collection.

Media and growth conditions. *H. salinarium* Flx15 was grown aerobically in tryptone medium at 37°C in the dark (17). *E. coli* JM109, containing recombinant plasmids, was grown overnight in Luria-Bertani medium supplemented with the appropriate antibiotic.

Chemicals and electrophoresis reagents. All chemicals were reagent grade. All amino acids for the flow assay experiments were from Sigma, St. Louis, Mo. Mevinolin was a generous gift from B. Alberts (Merck, Rahway, N.J.).

DNA cloning and sequencing. All molecular biology procedures were performed as described previously (26).

Southern hybridization and Western blotting analysis. Two genomic fragments, *Pst*I and *Sac*I, were used for the identification and cloning of the *htrXI* gene. Southern hybridization and Western blotting analysis with HC23 antibody was done as described previously (26).

Preparation of the membrane and soluble protein fractions. The fractionation of the membrane and soluble protein was done as described previously (26). We used HC23 antibody to determine the localization of the transducer proteins in both fractions (26).

Construction of the rescue vector, and selection of the complementary strain. The 1.2-kbp *Eco*RI-*Eco*RI fragment from the 4.5-kbp *Sac*I clone was subcloned into the vector pGEM-7f$^+$ (Promega, Madison, Wis.) to construct plasmid pAB2. A 1.0-kbp *Xba*I-*Pst*I fragment from the plasmid pAB2 was ligated with a 1.9-kbp *Pst*I-*Bam*HI fragment from the 3.6-kbp *Pst*I genomic clone with the fast DNA ligation kit (Boehringer Mannheim, Indianapolis, Ind.) at 25°C for 60 min. Then the *Xba*I-*Bam*HI-digested halobacterial shuttle vector pWL102 (ATCC 77216) was added to the ligation mixture, and incubation was continued for 90 min. The final construction, pAB3, was transformed into Δ*htrXI-1* cells by a standard polyethylene glycol-mediated transformation protocol (9). Transformants were grown on 1.5% agar plates containing mevinolin (16 μg/ml) for 10 to 14 days. Primary screening of the complementary strains was done by Western blotting with HC23 antibody. The positive clones were confirmed by Southern hybridization with *htrXI* gene-specific probes.

Radiolabeling with [*methyl*-^{3}H]methionine and flow assay experiments. Radiolabeling and flow assay experiments for the *H. salinarium* cells were done according to the protocols described previously (1). [*methyl*-^{3}H]methionine (75 to 80 Ci/mmol) was from DuPont, Boston, Mass. Puromycin (Sigma) was added

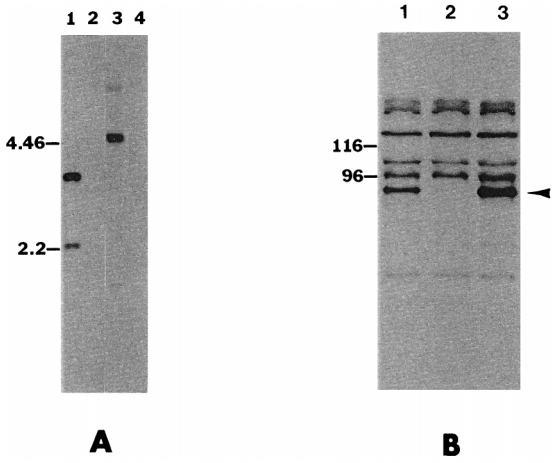

FIG. 1. (A) Southern blotting analysis of the Flx15 and Δ*htrXI-1* genomic DNA digested with *Pst*I and *Sac*I. Lanes: 1, Flx15 with *Pst*I digestion; 2, Δ*htrXI-1* with *Pst*I digestion; 3, Flx15 with *Sac*I digestion; 4, Δ*htrXI-1* with *Sac*I digestion. The bars indicate the positions of the markers (in kilobase pairs). (B) Electrophoretic analysis of *methyl*-^{3}H-labeled transducers of wild-type Flx15 (lane 1), Δ*htrXI-1* (lane 2), and *htrXI*$^{++}$/Δ*htrXI-1* (lane 3) strains. The bars indicate the positions of molecular mass markers (in kilodaltons). The arrowhead indicates the position of HtrXI.

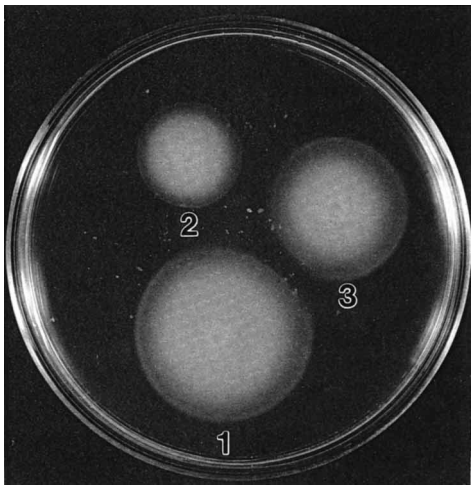

FIG. 3. Chemotaxis mediated by *htrXI*$^+$ parental (1), Δ*htrXI-1* (2), and *htrXI*$^{++}$/Δ*htrXI-1* (3) strains. Motile growing cells were inoculated into semisolid agar plates containing growth medium and were incubated at 37°C for 3 days. The differences between strains in ring size and sharpness were reproducible in assays repeated nine times.

to the chase medium at a concentration of 75 μg/ml. The scintillation cocktail Scinti-Verse BD and polyethylene scintillation vials were from Fisher Scientific, Santa Clara, Calif.

Capillary assay. Capillary assays for chemotaxis migration were performed according to the halobacterial protocol (7). Halobacterial cells were grown to an optical density at 660 nm of 0.6 to 0.7 and washed twice with 4.3 M basal salt solution, and 200 μl of cells was placed in 0.5-ml tubes. Five-microliter capillaries, containing appropriate amino acids (dissolved in basal salt solution), were inserted into the tube containing motile-cell suspensions. Cells were incubated for 1.5 h at 37°C. Following incubation, the contents of the capillaries were transferred into 995 μl of basal salt solution and mixed thoroughly, and 10 μl was plated in triplicate on 2% agar plates. The average from the three individual capillaries was calculated for each amino acid. Each experiment was repeated at least three times.

Agar-in-plug assay. Prewarmed 2% agarose solution (2 to 5 μl), containing appropriate amino acids, was placed in the middle of a microscope slide and immediately covered with a coverslip. Ten to 30 μl of cell suspension was placed around the plug, and the slide was incubated on a thermostable stage (37°C) for 20 min. The distribution of the bacterial cells around the plug surface was video recorded with a BH2 microscope (Olympus, Lake Success, N.Y.) and later analyzed. Details of this optimized agar-in-plug method for halobacteria will be published elsewhere (5).

Nucleotide sequence accession number. The sequence reported in this paper has been deposited in the GenBank database (accession no. U74668).

RESULTS

Cloning and sequencing of the *htrXI* gene. The *htrXI* gene, encoding a 451-amino-acid protein, was identified on overlapping 4.5-kbp *Sac*I and 3.6-kbp *Pst*I genomic fragments. The 4.5-kbp *Sac*I fragment contains an upstream promoter region and the first 120 bp of the open reading frame, while the 3.6-kbp *Pst*I fragment contains the rest of the coding sequence. The calculated molecular mass of the HtrXI protein is 49.5 kDa, and the pI is 4.03. Hydropathy plot analysis of the deduced amino acid sequence of the HtrXI protein indicates a lack of the membrane-spanning segments typical of eubacterial transducers (data not shown).

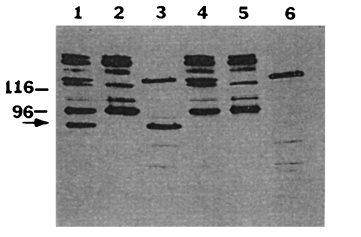

FIG. 2. Western blotting analysis of the membrane and soluble protein fractions of Flx15 and the Δ*htrXI-1* mutant. Lanes: 1, Flx15 total cell lysate; 2, Flx15 membrane fraction; 3, Flx15 soluble fraction; 4, Δ*htrXI-1* total cell lysate; 5, Δ*htrXI-1* membrane fraction; 6, Δ*htrXI-1* soluble fraction. The bars indicate the positions of the molecular mass markers (in kilodaltons). The arrow indicates the position of HtrXI.

Characterization of the *htrXI* deletion and complemented strains. Recently, we have constructed transducer gene deletion mutants by homologous recombination (4). Southern hybridization with the full *htrXI* gene as a probe showed that no

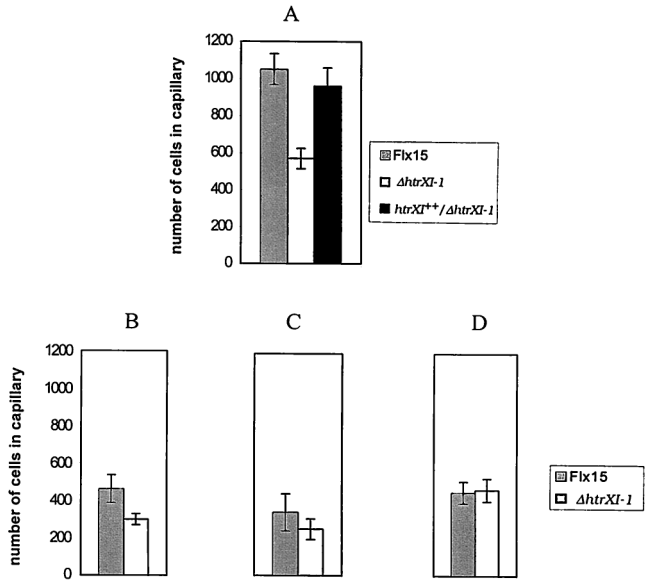

FIG. 4. Capillary assay of the *htrXI*$^+$ parental (Flx15), Δ*htrXI-1*, and *htrXI*$^{++}$/Δ*htrXI-1* strains in growth medium (A), Asp (15 mM) (B), Glu (10 mM) (C), and His (10 mM) (D). Cells were prepared as described in Materials and Methods. The results are the averages for nine experiments (± standard deviations), ×100.

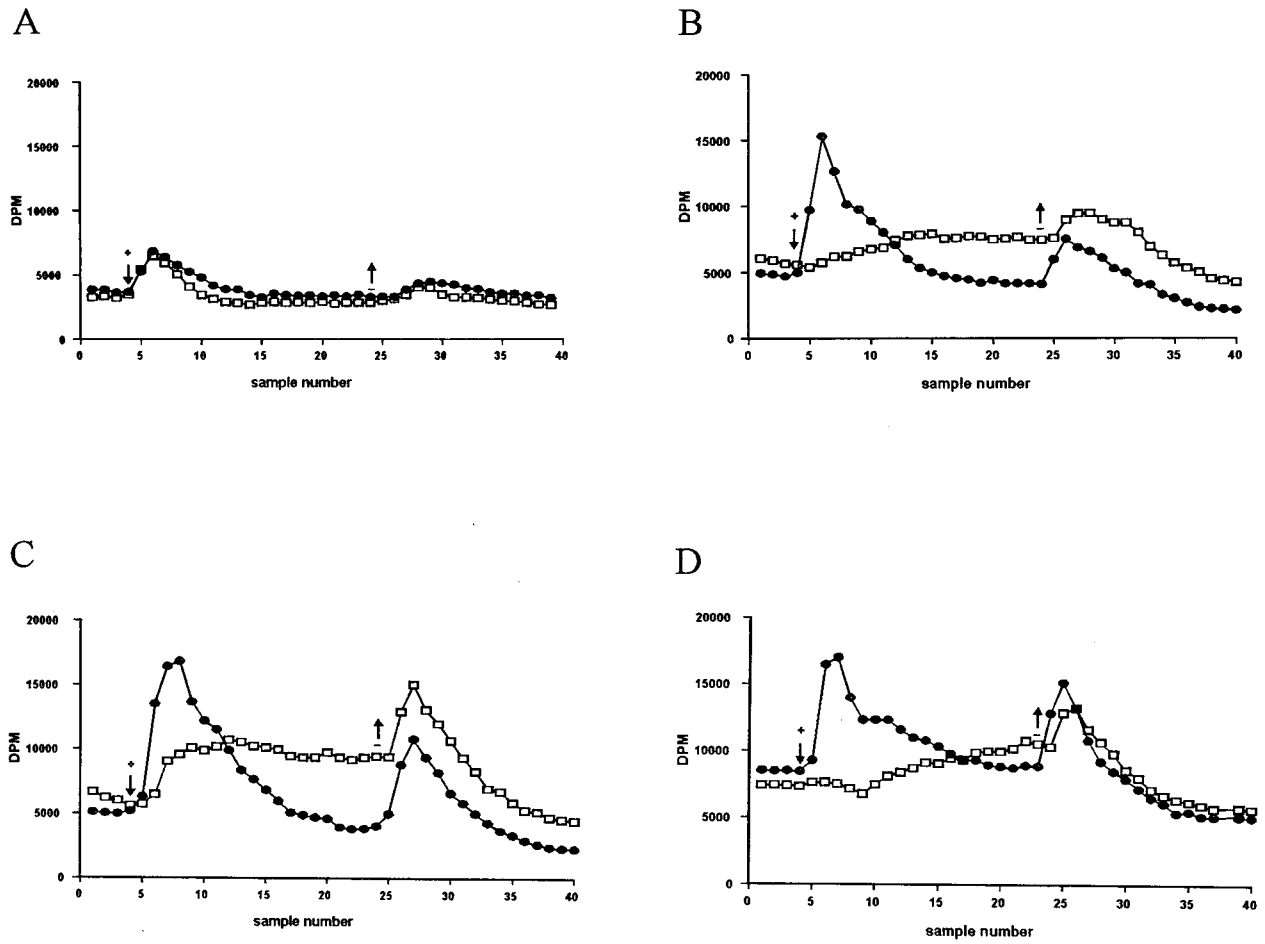

FIG. 5. Chemostimulus-induced changes in rate of release of [^{3}H]methyl groups under conditions of nonradioactive chase of the wild type (Flx15) and *htrXI* deletion mutant (Δ*htrXI-1*). Flx15 and Δ*htrXI-1* cells were radiolabeled as described in Materials and Methods, washed free of unincorporated radiolabeled methionine, placed on a filter-in-flow apparatus, and subjected to a continuous flow (0.5 ml/min) of buffer containing 0.1 mM nonradioactive methionine. Fractions collected every 40 s were analyzed for volatile radioactivity. Chemoeffectors were added (arrows with "+") by changing the contents of the inlet tube from buffer alone to buffer plus 5 mM Leu (A), 5 mM His (B), 5 mM Asp (C), or 5 mM Glu (D). The removal of effectors (arrows with "−") was done by changing the contents of the inlet tube from solution containing an amino acid to buffer alone. DPM, disintegrations per minute.

htrXI-specific fragments were present in *Pst*I- and *Sac*I-digested genomic DNA from Δ*htrXI-1* cells (Fig. 1A). Fluorography of the *methyl*-^{3}H-labeled halobacterial cells confirmed that unlike wild-type strain Flx15, Δ*htrXI-1* cells lack a specific radiolabeled band (Fig. 1B). We constructed a mevinolin-resistant plasmid based on the pWL102 shuttle vector (9), containing the full-length *htrXI* gene in a 2.9-kbp *Eco*RI-*Bam*HI fragment together with its putative promoter and terminator regions. The resultant plasmid, pAB3, was transformed into Δ*htrXI-1* cells, and the mevinolin-resistant *htrXI*$^{++}$/Δ*htrXI-1* strain was picked for the characterization. Southern blotting analysis of DNA from the *htrXI*$^{++}$/Δ*htrXI-1* cells indicated the presence of the *htrXI* gene (data not shown). Fluorography of *methyl*-^{3}H-labeled *htrXI*$^{++}$/Δ*htrXI-1* cells confirmed that the specific radiolabeled band that was missing in the *htrXI-1* deletion mutant is expressed in *htrXI*$^{++}$/Δ*htrXI-1* cells (Fig. 1B). The amount of HtrXI protein produced in the *htrXI*$^{++}$/Δ*htrXI-1* strain is higher than in the wild type due to the expression of *htrXI* from multicopy plasmid pAB3 as assayed by fluorography (Fig. 1B) and immunoblotting (data not shown).

HtrXI is a soluble transducer. The Kyte-Doolittle hydropathy analysis of the primary amino acid sequence of HtrXI (data not shown) indicated that this transducer may be soluble or peripherally membrane bound. To address this issue more thoroughly, fractionation of soluble and membrane-bound proteins from the Δ*htrXI-1* mutant and wild-type Flx15 was carried out. The samples from both of the fractions were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting. The Western blot in Fig. 2 demonstrates that indeed halobacterial transducer protein HtrXI is present in the Flx15 soluble fraction, while the soluble fraction from the Δ*htrXI-1* mutant does not have a cross-reacting band in the same region.

HtrXI is involved in Asp and Glu taxis. Soft agar tryptone "swarm" plates were used to evaluate the chemotaxis ability of the *htrXI*$^+$ parental, Δ*htrXI-1*, and *htrXI*$^{++}$/Δ*htrXI-1* cells. The diameter of the chemotaxis ring of the *htrXI-1* deletion mutant is 50% smaller than that of the wild type, and the wild-type phenotype is partially recovered in the *htrXI*$^{++}$/Δ*htrXI-1* cells (Fig. 3). The quantitative capillary assay confirmed the swarm plate behavior of the *htrXI*$^+$ parental (Flx15), Δ*htrXI-1*, and *htrXI*$^{++}$/Δ*htrXI-1* strains (Fig. 4A). Next, we conducted a detailed study of the *H. salinarium* chemotactic responses to amino acids and sugars by capillary and flow assays. The wild type and deletion mutants were screened for responses to 20

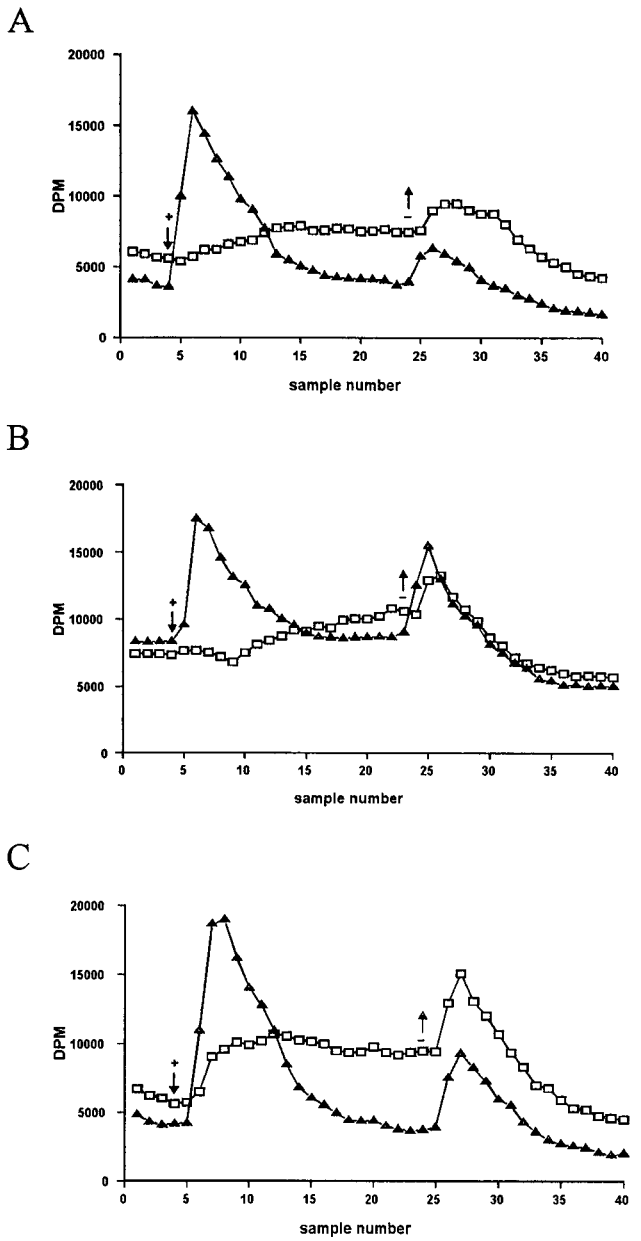

FIG. 6. Chemostimulus-induced changes in rate of release of [^{3}H]methyl groups under conditions of nonradioactive chase of the *htrXI* deletion mutant (Δ*htrXI-1*) and the complementary strain (*htrXI*$^{++}$/Δ*htrXI-1*). Δ*htrXI-1* and *htrXI*$^{++}$/Δ*htrXI-1* cells were prepared as described in the legend to Fig. 5. Chemoeffectors were added (arrows with "+") by switching the contents of the inlet tube from buffer alone to buffer with the appropriate amino acid: 5 mM His (A), 5 mM Glu (B), or 5 mM Asp (C). DPM, disintegrations per minute.

essential amino acids and 11 sugars. We found that the capillary assay responses of the *htrXI-1* deletion mutant to Glu and Asp were weaker than those of the wild-type Flx15 strain (Fig. 4B and C). The partial defect in the Asp and Glu capillary assays was further confirmed by the agar-in-plug method (data not shown). In this method, agar plugs containing these two amino acids were placed under coverslips which were then filled with motile halobacterial cell suspensions. Unlike wild-type Flx15, Δ*htrXI-1* cells do not concentrate around agar plugs containing Asp or Glu.

The *htrXI-1* deletion mutant is defective in methylesterase responses to Asp, Glu, and His. Halobacterial cells continually release methyl groups in volatile chemical form, and the rates of release are altered transiently by chemostimuli and photostimuli (1, 19). We demonstrated that the rates of release of methanol by sensory stimuli also do not exhibit the same symmetry as those of *E. coli*: for halobacteria, both positive and negative stimuli result in an increased rate of methanol release (1). As physiological studies revealed that the Δ*htrXI-1* cells have defects in Asp and Glu taxis, we studied the methylesterase activity in this mutant upon stimulation with various amino acids, including the above-mentioned two. Using the flow assay for methylesterase activity, we found that stimulation of the Δ*htrXI-1* cells with 20 essential amino acids except His, Asp, and Glu produced profiles of methanol release similar to those of the wild type (Fig. 5A). Unlike the wild type, Δ*htrXI-1* cells showed gradual rather than transient increases in methanol production upon addition of His, Asp, and Glu (Fig. 5B to D). The *htrXI*$^{++}$/Δ*htrXI-1* cells fully restored the wild-type profile of methanol release (Fig. 6A to C). It is interesting that for the Δ*htrXI-1* mutant the removal of His, Asp, and Glu stimulation produced a typical transient increase in methanol production, similar to those observed for the wild type (Fig. 5B to D). The gradual rather than transient increase of methanol production was also observed after the second and third additions of Glu (Fig. 7). Unlike that of the wild type, the amplitude of the addition peak is generally lower than the amplitude of the removal peak in the deletion mutant.

Asp and Glu decrease, but His increases, methylation of HtrXI. We have previously demonstrated that stimulation with the attractants His, Leu, and peptone increased the intensity of methylation of specific *methyl*-^{3}H-labeled bands (1). As the methylesterase flow assay data indicate that the *htrXI-1* deletion mutant is partially defective in Asp-, Glu-, and His-induced methanol release, we examined the changes in the intensity of methylation following sensory stimulation and adaptation to these three amino acids. The analytical procedure illustrated in Fig. 1B was used to examine wild-type Flx15 and Δ*htrXI-1* cells. In wild-type cells, Asp and Glu decrease the methylation of HtrXI and another putative transducer, HtrVII (Fig. 8A and B). On the other hand, stimulation with His increases the intensity of the HtrXI together with other radiolabeled transducers (Fig. 8C). In the absence of HtrXI (Δ*htrXI-1*), stimulation with His and Glu does not change the intensity of any *methyl*-^{3}H-labeled transducers (Fig. 8D).

DISCUSSION

HtrXI was originally cloned as a putative transducer based on its high homology in the highly conserved signaling domain with other methyl-accepting proteins (26). Analysis of the DNA sequence revealed that *htrXI* is one of the smallest transducer genes in the archaeon *H. salinarium*. The positions from −32 to −37 upstream of the *htrXI* gene start codon have a very high homology with typical archaeal promoter elements (23). The region upstream of the *htrXI* start codon has an unusually low GC content (45%), while the coding sequence of the gene has a GC content of 60%. A putative ribosomal binding site (GGAGGA) was found at positions −14 to −19 from ATG of the *htrXI* gene (8). As expected from its highly acidic nature, HtrXI migrates more slowly during sodium dodecyl sulfate-polyacrylamide gel electrophoresis than its calculated molecular weight would predict (6).

On the basis of the partial amino acid sequence, we have previously shown that HtrXI has a highly conserved signaling domain and putative K1 and R1 methylation peptides, similar

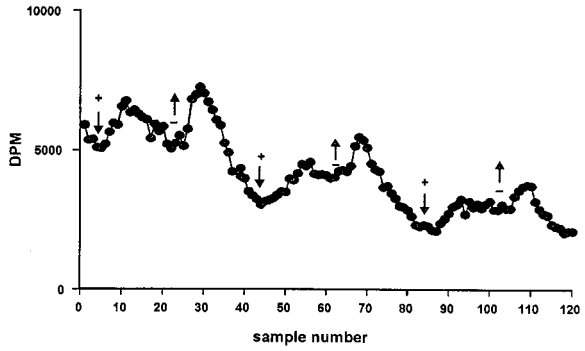

FIG. 7. Repeated stimulation of *methyl*-^{3}H-labeled cells in the flow assay apparatus. Δ*htrXI-1* cells were prepared as described in the legend to Fig. 5. Addition and removal of 5 mM Glu were repeated for three cycles. DPM, disintegrations per minute.

to the known eubacterial transmembrane chemotaxis transducers (26). However, the hydropathy plot indicates that HtrXI is not a transmembrane protein. This was confirmed by a cell fractionation experiment showing that HtrXI is present in the soluble fraction and is not membrane associated.

How can the soluble transducer protein sense the environmental stimulus without the ligand-binding periplasmic domain? If a soluble transducer recognizes some chemoeffector, one must assume that it has to interact with a membrane protein or that the chemoeffector is readily transported into the cell. To confirm that HtrXI is involved in signal transduction, an *htrXI* deletion mutant was constructed. Swarm plate, capillary, and agar-in-plug studies revealed that the Δ*htrXI-1* mutant is defective in the chemotactic responses to Glu and Asp. Analysis of the methylesterase activity of the Δ*htrXI-1* mutant also suggests that HtrXI is involved in the adaptation of the chemotactic responses to these two amino acids. The physiological and biochemical defects in the *htrXI-1* deletion mutant were cured in the *htrXI*$^{++}$/Δ*htrXI-1* cells. We conclude that HtrXI plays a role in adaptation pathways in Asp and Glu taxis.

We have previously demonstrated that stimulation with either of the attractants His and Leu, with a mixture of these two amino acids, or with the mixture of attractants present in peptone resulted in increased methylation of transducer proteins (1). Stimulation of wild-type cells with His confirmed our previous observation of increased methylation of several transducers, including HtrXI. Interestingly, stimulation with Glu and Asp causes the opposite effect, demethylation, which would not be expected since they are attractants. Another interesting observation is that upon stimulation with Asp and Glu, the demethylation of another putative transducer, HtrVII, occurs. Stimulation of the Δ*htrXI-1* cells with these three amino acids does not cause any change in methylation of HtrVII. As the presence of HtrXI is essential for the methylation of HtrVII after stimulation with Asp and Glu, it is possible that the soluble transducer HtrXI and the membrane-bound putative transducer HtrVII interact.

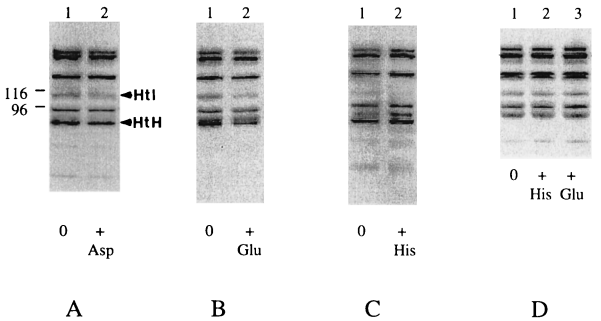

FIG. 8. Changes in methylation following chemostimulation. Flx15 and the Δ*htrXI-1* mutant were labeled with [*methyl*-^{3}H]methionine as described in Materials and Methods and then stimulated with buffer (0) and with 5 mM solution of the indicated amino acid (+). Reactions were terminated 5 min after stimulation. The panels are fluorograms of the segments of 8% acrylamide gels at pH 8.5. The bars indicate the positions of molecular mass markers (in kilodaltons). (A to C) Chemostimulation of wild-type strain Flx15; (D) chemostimulation of the *htrXI-1* deletion mutant. The arrows indicate the positions of HtrXI (HtI) and HtrVII (HtH).

The transient increase in production of volatile methanol after addition of Asp and Glu correlates with the decrease in intensity of radiolabeled methyl groups in the fluorographs. But we cannot explain the His stimulation results, where the increase in production of volatile methanol (as a measure of methylesterase activity) in the flow assay does not correlate with the increase of methylation in the fluorographs. Histidine capillary assay does not show a clear difference between wild-type Flx15 and the *htrXI-1* deletion mutant (Fig. 4D). On the basis of flow assay experiments and in vivo methylation studies, Thoelke et al. suggested that the unusual methanol evolution of *B. subtilis* (similar to that of *H. salinarium*) could be explained by the presence of the methyl-accepting intermediate (22).

In conclusion, we have identified, cloned, and fully sequenced the gene for an archaeal soluble transducer. In this paper we present the first experimental evidence for a functional role for a soluble transducer in *H. salinarium*. Further studies are under way to elucidate molecular events involved in the signaling through this soluble transducer protein, which appears to have complex roles in chemotaxis mechanisms.

ACKNOWLEDGMENTS

We thank G. Hazelbauer, J. Spudich, and P. Patek for critical reading and discussion of the manuscript.

This work was supported by a University of Hawaii Intramural Project Development Award and National Science Foundation Grant MCB-9600860 to M.A.

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