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The methyl-accepting transducer protein HtrI is functionally associated with the photoreceptor sensory rhodopsin I in the archaeon *Halobacterium salinarium*

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We have investigated the functional relationship between two proteins involved in the photosensory system of the archaeon *Halobacterium salinarium*: the photoreceptor sensory rhodopsin I (SRI) and the halobacterial transducer rhodopsin I (HtrI), which has been proposed to be the putative signal transducer of SRI, by genomic DNA analysis of two independent SRI negative mutants, Pho81 and D1. Southern and PCR analyses revealed that both strains bear alterations in the 5′ flanking region of the gene encoding SRI, *sopI*. DNA sequence analysis confirmed the occurrence in this region of *htrI*, the gene encoding the putative transducer protein. PCR and Northern analyses have shown further that *sopI* and *htrI* are expressed as a single transcriptional unit, thus explaining the lack of SRI in mutants with a defective *htrI*. Expression of the cloned *sopI* under the control of a heterologous promoter did not restore the SRI-dependent photoresponse in the strain Pho81. Moreover, the photocycling rate of the expressed pigment was clearly lower than in wild type. HtrI is therefore essential for SRI function and most likely modulates the photochemical properties of the photoreceptor via direct physical interaction. Finally, reintroduction of both *sopI* and *htrI* into Pho81 and D1 restored the SRI photochemistry and its physiological function. Our results provide the first experimental evidence for the functional coupling between SRI and HtrI and corroborate the proposed model in which HtrI acts as the signal transducer of this archaeal seven-helix photoreceptor in a way analogous to the bacterial chemotaxis transducers.

Key words: methylation/operon/phototaxis/sensory rhodopsin I/signal transduction

Introduction

Halobacterium salinarium (formerly called *H.halobium*) is a polarly flagellated archaeon which exhibits both phototaxis and chemotaxis. Its orientation behaviour in response to light or chemical stimuli is achieved by switching the rotational sense of the flagellar motor. Spontaneous switching is suppressed by attractant stimuli and induced by repellent stimuli thus enabling net progress in a favourable direction. The photosensitivity of *H.salinarium* is mediated by two retinal-containing pigments, the sensory rhodopsins (SRs) I and II [for reviews see Spudich and Bogomolni (1988) and Oesterhelt and Marwan (1990)]. SRII elicits a photorepellent response to blue light (λ_{max} = 480 nm). SRI is a photochromic pigment which exists under constant light in two spectrally distinct forms, SR_{587} and SR_{373}, each of which elicits a sensory response: SR_{587} mediates an attractant response to red-orange light and SR_{373} a repellent response to harmful UV light (Spudich and Bogomolni, 1984; Marwan and Oesterhelt, 1990).

SRI is the first prokaryotic photoreceptor that has been purified to homogeneity (Schegk and Oesterhelt, 1988) and its structural gene, *sopI*, has been cloned (Blanck *et al.*, 1989). The primary structure of SRI shares a high degree of sequence similarity with the halobacterial retinal proteins bacteriorhodopsin and halorhodopsin, which function as light-driven ion pumps. The functional counterpart of SRI in eukaryotes is the rhodopsin from vertebrate eyes. Although there is no sequence relatedness between the two photoreceptors, they show the same type of conserved secondary structure consisting of seven membrane spanning hydrophobic α-helices. More significantly, they both detect light via the same fundamental mechanism of retinal isomerization. The SRI-dependent phototaxis therefore provides, in a unicellular organism, a simple model system for the investigation of photosensitive signal transduction processes.

The molecular events that occur in *H.salinarium* after stimulation of its photoreceptors and which result in a response of the flagellar apparatus are the subject of current investigation. Behavioural experiments have demonstrated that photoreceptor excitation causes the catalytic formation of a chemical compound which acts as a switching signal (Marwan and Oesterhelt, 1987). Fumarate has been identified as a switch factor by somatic complementation of a mutant defective in spontaneous and light-induced switching (Marwan *et al.*, 1990) and its cellular concentration has been shown to be controlled by light (Marwan and Oesterhelt, 1991).

Methylation of intrinsic membrane proteins, a fundamental feature of bacterial signalling systems, has also been implicated in the chemo- and phototaxis of *H.salinarium* (Schimz, 1981, 1982; Bibikov *et al.*, 1982). Stimulus-dependent methylesterase activity has been measured *in vivo* (Alam *et al.*, 1989; Spudich *et al.*, 1989). Methyl-accepting membrane proteins with an apparent molecular weight between 90 and 135 kDa have been identified by electrophoresis and fluorography. In addition, these proteins are absent in mutants defective in taxis (Sundberg *et al.*, 1985, 1990). In bacteria, methyl-accepting proteins (MCPs) are central components of the chemotactic system. They act as chemoreceptors, monitoring the environment by binding specific ligands in the periplasm, and as signal transducers by releasing the chemotactic signal to the cytoplasmic sensory pathway. Furthermore, their reversible methylation at several specific glutamyl residues provides the mechanistic basis of sensory adaptation to chemical stimuli (for a review see Hazelbauer *et al.*, 1990). A first indication of structural relatedness between the archaeal and the bacterial methyl-

accepting proteins has come from an immunological study by Alam and Hazelbauer (1991), who showed antigenic cross-reactivity of the archaeal proteins to antisera raised against the chemotaxis transducer proteins of *Escherichia coli*.

Among the methyl-accepting proteins of *H.salinarium*, a 94 kDa species has been shown to be covariant with the SRI photoreceptor in several different mutants. It has therefore been proposed that the 94 kDa protein acts as transducer of the SRI phototactic signal (Spudich and Bogomolni, 1992). Recently, an ORF has been identified in a genomic digest of halobacterial DNA with degenerate oligonucleotide probes derived from peptide sequences of this putative transducer protein (Yao and Spudich, 1992). The derived primary structure of this gene, designated *htrI* (halobacterial transducer rhodopsin I), contains three conserved cytoplasmic regions resembling the two methylation sites and the signalling domain from bacterial MCPs. These sequence similarities corroborate the assumption that the encoded gene product, HtrI, may be involved in sensory signalling. However, no direct experimental evidence for a functional link between *htrI* and *sopI* or their respective gene products has hitherto been presented. In the following work, we have analysed the genetic defects of two SRI negative phototaxis mutants and demonstrate (i) that the lack of SRI phenotype is concomitant with a defective *htrI* and (ii) that their complementation to give the wild type phenotype requires *htrI* expression. These results, together with the analysis of *htrI*-specific transcription, identify *htrI* and its gene product as a molecular component essential to the SRI-dependent signalling pathway.

Results

Transformation of the SRI-deficient H.salinarium strain Pho81 with an SRI expression plasmid

In an attempt to rescue the SRI-dependent photoresponse in the phenotypically SRI-deficient *H.salinarium* strain Pho81 we have transformed this strain with the SRI expression plasmid pEF198. A detailed description of this vector is given in Ferrando-May *et al.* (1993). Its fundamental elements are: (i) an expression cassette in which the coding sequence of *sopI* from the ATG to the stop codon is flanked by the 5′- and 3′-noncoding regions of the bacterio-opsin gene (Betlach *et al.*, 1984) and (ii) the mevinolin resistance gene as a selectable marker for halobacteria (Lam and Doolittle, 1992). Expression of *sopI* in pEF198 is thus driven by the bacterio-opsin promoter.

Transformation of the mutant Pho81 with the vector pEF198 resulted in recombination of the plasmid DNA at the homologous *sopI* locus on the chromosome as is demonstrated by the Southern analysis in Figure 1A. A restriction digest of the isolated vector (pEF198) with *Hin*dIII generates one single fragment of 12.4 kb, which is not visible in the same restriction digest of DNA from the transformant Pho81/pEF198 indicating that the plasmid is not present in these cells as an extrachromosomal element. The smear appearing in the *Hin*dIII restriction digest is due to the low cutting frequency of this enzyme in the halobacterial genome. Digestion of Pho81/pEF198 DNA with the restriction endonuclease *Sac*I yields two fragments, which do not correspond in size to either the plasmid fragment or the *Sac*I genomic fragment of the untransformed recipient strain

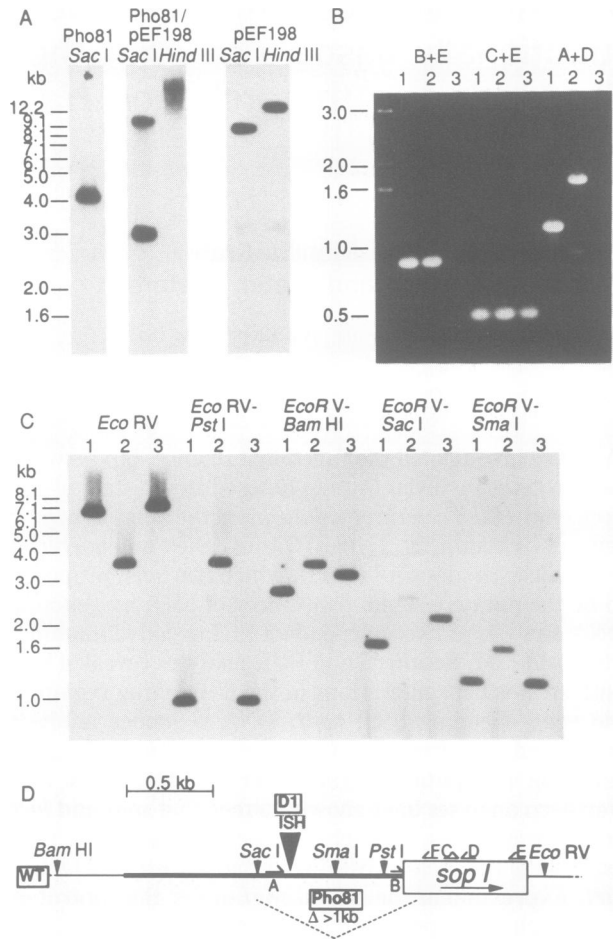

Fig 1. Genetic analysis of *H.salinarium* transformants and mutants. (**A**) Southern blot of total DNA from the mutant Pho81 transformed with the *sopI* expression plasmid pEF198 (= Pho81/pEF198). As a reference, the digested DNA from the untransformed recipient, Pho81, and the isolated plasmid are also shown. A *Pst*I–*Eco*RV fragment from the genomic clone AB2H11 (Blanck *et al.* 1989) was used as *sopI*-specific probe. (**B**) PCR analysis of genomic DNAs from the *H.salinarium* SRI wild type strain L33 (1), and the two SRI negative mutants D1 (3) and Pho81 (2). A, B, C and D are the oligonucleotide primers used. Their location at the *sopI* locus is shown in (D). (**C**) Southern analysis of genomic DNA from the same strains as in *(B)*. The same fragment as above was used as a probe. (**D**) Restriction map of the *sopI* locus on the halobacterial genome. The alterations contained in the SRI negative mutants are: an ~600 bp insertion in D1 and the deletion of at least 1 kb in Pho81.

Pho81. This confirms the occurrence of a homologous recombination event that yields two copies of the *sopI* sequence on the chromosome of the transformant. Site-specific vector integration in *H.salinarium* and the stability of such gene duplications under mevinolin selection have already been demonstrated for a bacterio-opsin expression vector closely related to the plasmid used in this work (Ferrando *et al.*, 1993).

Expression of SRI is still not functional in Pho81 cells

Transformation of Pho81 with the SRI expression plasmid results in the strong expression of a *sopI* transcript with a mobility corresponding to 800 nucleotides (Figure 2A). No endogenous *sopI* mRNA could be detected in the untransformed recipient even after prolonged exposure.

The produced SRI was characterized by measuring the

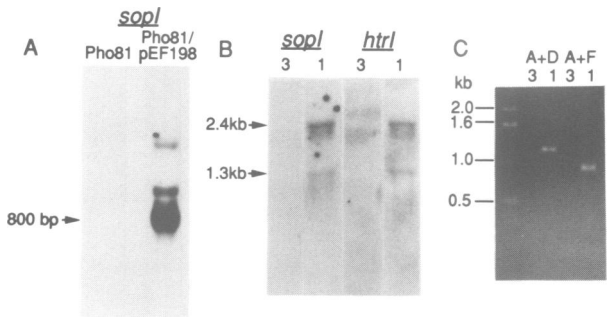

Fig. 2. Analysis of *sopI*-specific transcripts. (**A**) Northern blot of total RNA isolated from the transformed (Pho81-pEF198) and the untransformed mutant Pho81. The *sopI*-specific probe of Fig 1. was used. (**B**) Northern blot of total RNA from the wild type L33 (lane 1) and the mutant Pho81 (lane 3). After hybridization with the *sopI*-specific probe, the blot was stripped off and rehybridized with a DNA fragment comprising the 5′ region of *htrI*. (**C**) PCR products amplified from RNA of the strains L33 (lane 1) and Pho81 (lane 3) using the primer combinations A + D and A + F. The location of the primers is given in Figure 1D.

light-induced absorbance changes in crude membrane preparations (Figure 3). In the light-induced absorption difference spectrum of SRI-containing membranes from wild type cells (Flx3), two main absorbing species of the SRI photocycle were detected: one corresponded to the ground state of the pigment (λ_{max} = 587 nm) and the other to a long-lived intermediate with an absorption maximum at 373 nm (Spudich and Bogomolni, 1984). In the spectrum of membranes from Pho81/pEF198 cells expressing the cloned *sopI*, the 587 nm difference minimum was shifted to shorter wavelengths. This may not exclusively be attributed to an anomalous absorption of the expressed SRI, since the irreversible bleaching of bacterioruberins contained in the membranes of Pho81 interfered with the SRI-specific absorption changes in this spectral range. However, the measurement of the time-dependent recovery of absorbance at 590 nm after bleaching of the membranes with actinic light clearly demonstrates that the gene product expressed in Pho81 cells differs from the wild type pigment (see inserts in Figure 3). In Flx3 membranes the half-time of the recovery of the SR_{587} initial state amounts to ~0.6 s, as has been described by Spudich and Bogomolni (1988), while in the membranes of Pho81/pEF198 the half-time was longer by a factor of 50. In the membranes of the transformed Pho81 the initial state is restored only after almost 1 min, but nevertheless the reaction is completely reversible as established by the repetitive illumination of the same membrane sample (not shown). Further, SRI expressed alone in Pho81 cells was inactive in triggering a detectable photophobic response either to an orange light step down stimulus or to a UV pulse, even under saturating light conditions (Figure 4).

Reintroduction and expression of the *sopI* gene in Pho81 thus generated a pigment with anomalous photochemical properties which was not able to restore the wild type physiological response. However, integrative transformation with the same plasmid DNA fully restores the SRI phenotype if a $sopI^-$ deletion mutant is used as a recipient (Ferrando-May *et al.*, 1993). This confirmed that the lack of SRI phenotype in the transformed Pho81 cells was specific to this strain.

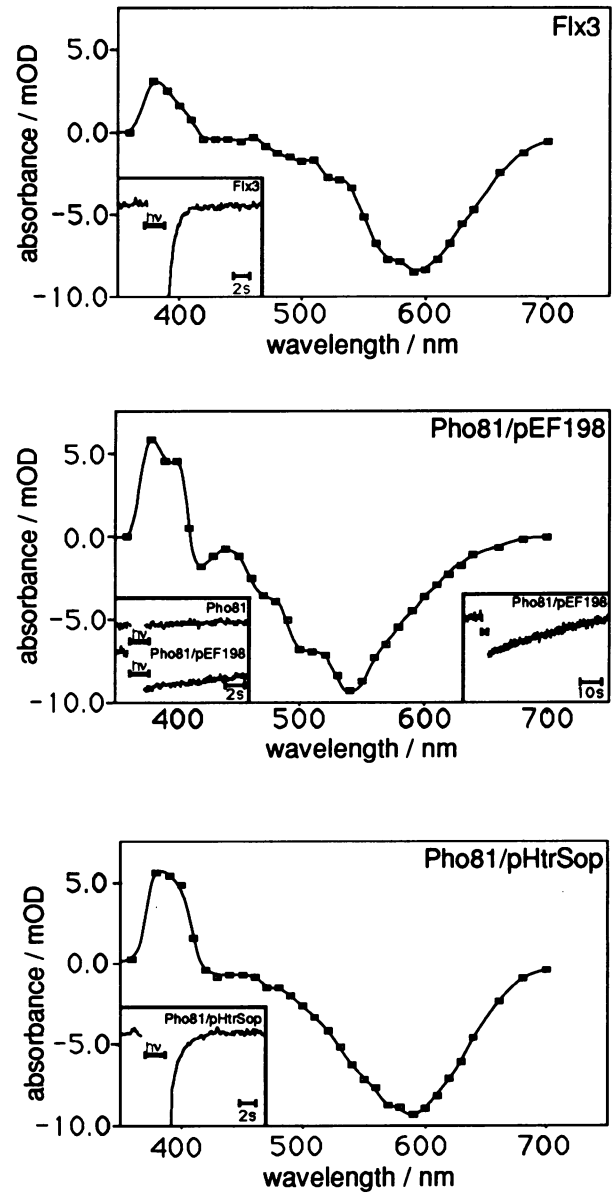

Fig 3. Light-induced absorbance difference spectra of SRI-containing membranes from SRI wild type cells (Flx3), from Pho81 cells transformed with the *sopI* expression vector pEF198 (Pho81/pEF198), and from Pho81 containing both *sopI* and *htrI* (=Pho81/pHtrSop). The spectra were recorded after 2 s of illumination. The inserts show time-dependent absorbance changes at 590 nm after the same period of illumination (indicated by the black bar).

Two independent SRI-deficient H.salinarium strains bear mutations in htrI

The mutant strain Pho81 was obtained by a random mutagenesis procedure and has thus far not been properly characterized at the DNA level. The occurrence of additional, unidentified mutations that affect components closely connected to the function of the photoreceptor is therefore likely. In addition to Pho81, a second *H.salinarium* mutant strain, D1, was available which lacked the photochemically active SRI as well as the SRI-dependent photoresponse but still displayed the physiological response mediated by the blue-light receptor SRII (Scharf *et al.*, 1992). In order to identify mutations resulting in an SRI

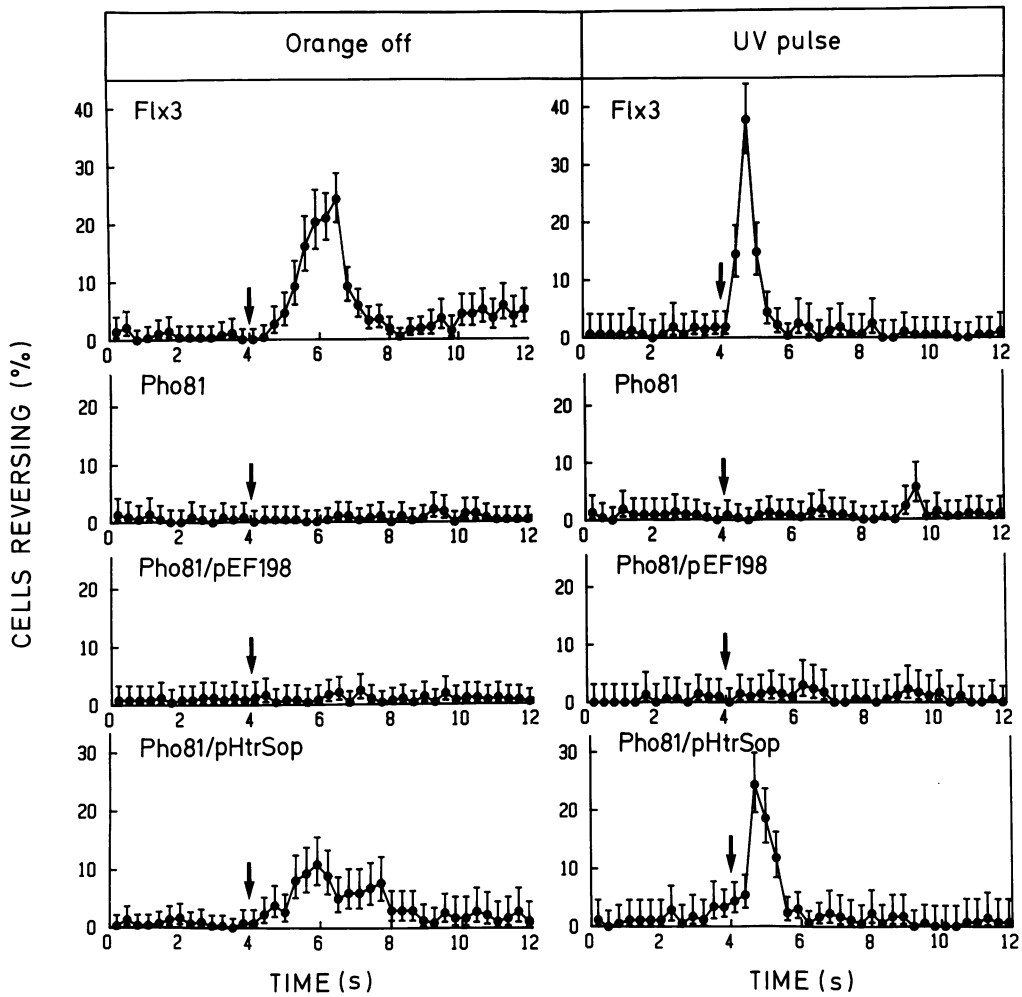

Fig 4. Response of wild type (Flx3), of the mutant Pho81 and of the transformed Pho81 cells (Pho81/pEF198, Pho81/pHtrSop) to orange light step down and UV pulse stimulation. The response of the cells was assayed as described in Materials and methods. Each data point indicates the percentage of cells reversing within a period of 0.3 s. The error bars correspond to 95% confidence. The beginning of stimulus application is marked by an arrow. Control experiments made sure that the applied light stimuli were saturating.

negative phenotype, we performed a genomic analysis of the strains Pho81 and D1. In the Southern blot shown in Figure 1C, double digests of genomic DNA from the wild type strain L33 and the mutants Pho81 and D1 were hybridized to a labelled *sopI* gene fragment. In these digests the restriction endonuclease *Eco*RV, which cuts 106 bp downstream of the *sopI* TGA, was combined with four different enzymes, *Pst*I, *Bam*HI, *Sac*I and *Sma*I. Additionally, the genomic DNA of these strains was analysed by PCR using oligonucleotides complementary to the *sopI* sequence and to sequences upstream of this gene. The result of the PCR is shown in Figure 1B. The data from the Southern blot and the PCR experiment are summarized in Figure 1D which shows a restriction map of the *sopI* locus and the location of the primers used in the PCR (Figure 1D). In the Southern blot of the chromosomal DNA from the SRI negative mutant D1 a *sopI* fragment corresponding to the wild type gene was detected by restriction of this DNA with *Eco*RV+*Pst*I and *Eco*RV+*Sma*I as well as by amplification with PCR primers hybridizing at the 5′ and 3′ ends of *sopI* (B+E). While this suggested that D1 carries a wild type *sopI*, an insertion of ~600 bp, probably due to an insertion element, could be located between the *Sma*I and the *Sac*I restriction site upstream of *sopI*, as demonstrated by restriction digestion of strain D1 DNA with *Eco*RV+*Sac*I and *Eco*RV+*Bam*HI as well as PCR amplification with the primers A and D.

In the mutant Pho81 a complex rearrangement has occurred which consists at minimum in a large deletion of at least 1 kb of DNA sequence including the first 200 bp of *sopI*. Due to this deletion, PCR amplifications using oligonucleotides from the *sopI* 5′ flanking region up to 750 bp upstream of the *sopI* starting codon did not yield DNA products (B+E, A+D). A *sopI*-specific fragment could be amplified in Pho81 chromosomal DNA only if a 5′ oligonucleotide complementary to sequences 200 bp downstream from the start codon was used (C+D). Additionally, the Southern blot clearly demonstrated the absence of the *Pst*I restriction site located 5′ of *sopI*. The size of the restriction fragments obtained with the remaining restriction endonucleases employed in this experiment did not fit into the map of the *sopI* locus and could not be explained in terms of a simple deletion or insertional mutation. We therefore concluded that in Pho81 the deleted sequences had been partially replaced by unknown genomic sequences as a consequence of a radical mutational rearrangement.

Integrative transformation of Pho81 with the SRI

expression plasmid pEF198 in fact introduced an intact copy of *sopI* into the genome, but had no influence on the alterations in the 5′ flanking region. As described above, these cells still lacked the SRI-dependent photoresponse. Consequently, there are two independent strains, the mutant D1 and the transformant Pho81/pEF198, in which the SRI negative phenotype correlates with mutations upstream of *sopI*. DNA sequence analysis of this region confirmed the presence of the 1611 bp ORF reported by Yao and Spudich (1992) encoding the methyl-accepting putative SRI signal transducer HtrI. These results provide the first genetic evidence for a direct connection between the expression of *sopI* and that of *htrI*.

htrI and sopI are transcribed as a single transcriptional unit

A search for regulatory signals for transcription in the regions flanking *htrI* revealed the presence of a consensus promoter upstream of the ATG (Thomm and Wich, 1988; Reiter *et al.*, 1990). However, no close approximation to presumed terminator structures was found within 100 bp downstream of the stop codon (Brown *et al.*, 1989). Additionally, the stop codon of *htrI* overlapped the start codon of *sopI* by one nucleotide (TGATG). In order to determine if *htrI* and *sopI* are cotranscribed, we performed a Northern analysis of total RNA isolated from the SRI wild type strain L33 and the mutant Pho81 (see Figure 2B). Hybridization with the *sopI*-specific probe revealed two transcripts of ~2.4 and 1.3 kb in length which were expressed at a low level in L33 but not in Pho81. Rehybridization of this blot with a probe specific to 5′ sequences of *htrI* labelled the same transcripts as the *sopI*-specific probe. The 2.4 kb transcript most likely represents the full length mRNA which encompasses both genes and is transcribed from the putative promoter upstream of *htrI*. To confirm the result from the Northern blot analysis we synthesized two single-stranded *sopI* cDNAs using the oligonucleotide primers D and F (see Figure 1D) and amplified these cDNAs by PCR using primer A as the 5′ oligonucleotide. As shown in Figure 2C, two fragments corresponding to the entire length of DNA sequence between the primers A and F or A and D could be amplified from RNA of L33 cells, confirming that *sopI* and *htrI* are transcribed as a single transcriptional unit.

The function of SRI is restored by coexpression of sopI and htrI

In order to establish unambiguously the functional link between *sopI* and *htrI*, we constructed an expression plasmid, pHtrSop, for the coexpression of both genes. pHtrSop bears a 5.8 kb genomic fragment encompassing the entire operon together with a novobiocin resistance marker for the selection of positive transformants (Holmes *et al.*, 1991). A PCR analysis of total DNA from Pho81 cells transformed with pHtrSop is shown in Figure 5. Using primers A and D, which hybridize to the *htrI* and *sopI* sequence respectively, a DNA product corresponding to the wild type fragment was obtained, confirming the restoration of the *htrI*−*sopI* operon.

Difference spectroscopy of total membranes prepared from the complemented mutant detected a pigment with the same absorption properties as wild type SRI (Figure 3). Significantly, the recovery of the absorption at 590 nm after actinic illumination displayed the same time-dependence as in the wild type. We therefore conclude that the anomalous

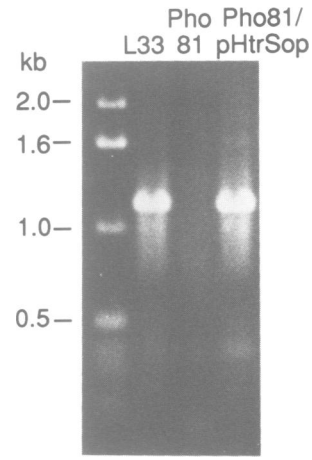

Fig. 5. PCR analysis of total DNA from Pho81 cells after transformation with the plasmid pHtrSop. The oligonucleotide primers A and D (see Figure 1D) yield a DNA product of the same length as in the wild type control (L33) confirming the restoration of the *htrI-sopI* operon.

kinetics of the expressed SRI observed in Pho81/pEF198 cells was due to the lack of *htrI*.

Coexpression of *sopI* and *htrI* finally restored the SRI-dependent photoresponses to orange and UV light in Pho81 cells and also conferred on them the ability to adapt to light stimuli, as demonstrated in the physiological assay shown in Figure 4. The fact that the kinetics of the response of Pho81/pHtrSop are not identical to the wild type flx3 are not identical may be due to a different efficiency in signal amplification (see Marwan and Oesterhelt, 1987).

Restoration of SRI-dependent photoresponses was also obtained when strain D1 was transformed with pHtrSop. Membranes of transformed cells contained photochemically active SRI and 61% (n = 151) of a population displayed stimulus response in the behavioural assay (not shown). Taken together, these data demonstrate that the photochemical behaviour and the phototactic response elicited by stimulation of SRI are functionally linked to HtrI.

Discussion

Our results indicate that the SRI-deficient *H.salinarium* mutants Pho81 and D1 lack at least one additional component besides the photoreceptor which may be required both at the level of *sopI* transcription and SRI function and that the SRI-negative phenotype in these mutants is concomitant to mutations upstream of *sopI*.

DNA sequencing in this 5′ flanking region confirmed the presence of *htrI*, a gene encoding a putatively methylatable protein previously associated with the SRI-dependent phototaxis. It has already been reported that the termination codon of *htrI* overlaps the initiation codon of *sopI* (Yao and Spudich, 1992). This kind of tight physical linkage between two genes has already been observed in *H.salinarium* for the genes encoding ribosomal subunit proteins (Spiridinova *et al.*, 1989) and for the *bat* and *brp* genes in the *bop* gene cluster (Leong *et al.*, 1988) and suggests coordinate expression of these genes. Northern analysis of wild type RNA as well as PCR amplification of *sopI*-specific cDNAs confirmed that *htrI* and *sopI* are organized in a bicistronic operon. This ensures transcriptional coregulation of these

genes and provides a possible explanation for the absence of SRI expression in the *sopI*$^+$, *htrI*$^-$ strain D1.

The 1.3 kb *sopI*-specific transcript which appeared in the Northern blot of Figure 2B did not correspond, as previously assumed (Blanck *et al.*, 1989), to a monocistronic *sopI* mRNA since it hybridized also with a probe spanning the first half of *htrI*. The origin of this transcript remains to be clarified, but it probably represents an inactive species resulting from nucleolytic attack of the functional message at specific processing sites.

In *E.coli* the overlap of the start and stop codons has been demonstrated for polypeptides that are subunits of an enzyme complex, as for example, the products encoded by the tryptophan and by the ribosomal protein operons. In these operons, synthesis of the distal cistron requires translation of the preceding message, thus allowing regulation of both proteins from a single mRNA target site (Oppenheim and Yanofsky, 1980; Sor *et al.*, 1987). This mechanism of translational coupling results in equimolar synthesis rates of the proteins encoded within an operon and may also apply for *sopI* and *htrI*. Therefore, the overlap of their translation signals indirectly suggested the association of the two gene products at a functional level. The conclusive confirmation of this hypothesis is provided by the functional complementation of the mutant strains Pho81 and D1, which requires the presence of both *sopI* and *htrI*. A comparison of the photocycling rates of SRI in the absence (Pho81/pEF198) and in the presence (Pho81/pHtrSop) of *htrI* clearly indicates that *htrI* must act via its gene product in modulating the photochemical activity of the photoreceptor. This indicates a direct physical interaction between SRI and HtrI, as would be expected for a sensory receptor and its dedicated signal transducer.

In the schematic view of Figure 6 we propose a model for HtrI function in halobacterial phototaxis. As already reported by Yao and Spudich (1992), the topography of HtrI closely resembles that of bacterial chemoreceptors. HtrI is probably anchored to the cell membrane by two hydrophobic helices. The portion of the protein protruding outside of the cell is short (about five amino acids) and lacks the large ligand binding domain of bacterial methyl-accepting proteins. This reflects the function of HtrI as a pure signal transducer in contrast to the bacterial proteins which additionally function as receptors for periplasmic ligands. The cytoplasmic part of the molecule bears the conserved methylation regions (1 and 2) and the signalling domain (3).

A unique feature of the sensory system of halobacterial phototaxis, evidenced by the work presented here, is the concomitant occurrence of two structural principles, which are characteristic for bacterial and eukaryotic systems. The association of a rhodopsin-like sensory pigment with a transducer protein bearing domains of bacterial origin raises the possibility of an unknown type of interaction underlying the first molecular steps of the signal relay. In our model we have illustrated two fundamental and hypothetical ways in which the phototactic signal may be transmitted from SRI to HtrI. Interaction may occur through the membrane, where a stimulus-dependent conformational change of the photoreceptor could be sensed by the transducer via the two transmembrane helices and transmitted to the next target of the signal transduction chain. This implies that the two proteins must be tightly associated in the membrane. Alternatively, SRI and HtrI could interact via domains

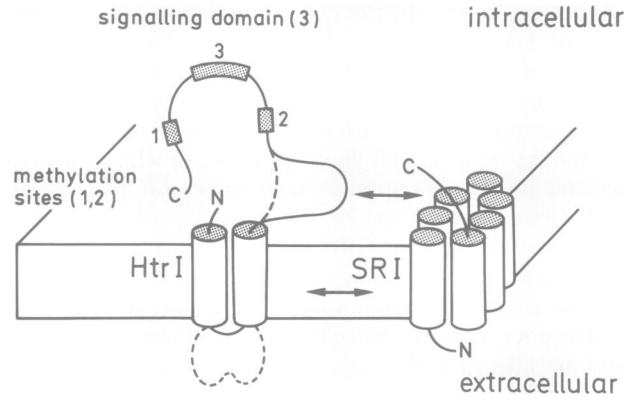

Fig. 6. Model of the structure and the membrane topology of the putative SRI signal transducer HtrI. The hatched lines indicate the length of the corresponding loops in the *E.coli* chemoreceptors. The two possible modes of interaction between SRI and HtrI are indicated by double arrows.

exposed to the cytoplasm. A possible contact site could be the stretch of 229 amino acids between the second hydrophobic helix and methylation site 2 of HtrI which does not bear significant homology to any known sequence and which could sense changes in the conformation of the cytoplasmic surface of the photoreceptor. Further experiments using site-directed mutations of both SRI and the newly identified putative transducer will contribute to the dissection of the individual steps of SRI-dependent phototaxis, which provides the first example at the physiological level for the well-known combination of bacterial and eukaryotic properties in archaeal organisms.

Materials and methods

H.salinarium strains and transformation procedure

The *H.salinarium* strains used are all BR$^-$. Strain L33 (BR$^-$, HR$^+$, SRI$^+$, SRII$^+$) is a bacterio-opsin deficient mutant of S9 (Wagner *et al.*, 1981). Flx3 is an HR$^-$ mutant derived from OD2 by the ion-flux mutant selection, which is wild type in SRI and SRII (Spudich and Spudich, 1982). Pho81 is a mutant derivative of Flx15 that has lost sensitivity to all photostimuli, but retains chemotactic sensitivity (Sundberg *et al.*, 1985). Strain D1 is a BR$^-$, HR$^-$, SRI$^-$ and SRII$^+$ mutant (Scharf *et al.*, 1992). *Halobacterium* cells were grown in peptone medium (Oesterhelt and Stoeckenius, 1973). Transformation of *H.salinarium* was performed following the procedure described by Cline *et al.* (1989).

Plasmid vectors

The *sopI* expression plasmid pEF198 carries the *sopI* coding sequence preceded by the bacterio-opsin promoter together with the mevinolin resistance marker for the selection of resistant transformants (Lam and Doolittle, 1992). Its construction is described in detail in Ferrando-May *et al.* (1993). The vector pHtrSop used for the coexpression of *sopI* and *htrI* is a derivative of the plasmid pSopFS (Ferrando-May *et al.*, 1993). It contains, additionally to the *htrI-sopI* operon from pSopFS, a mutated halobacterial *gyrB* conferring resistance to novobiocin (Holmes *et al.*, 1991).

Southern and Northern analyses

Total halobacterial DNA and RNA were isolated and analysed by Southern and Northern blots as described in Ferrando *et al.* (1993). The *PstI*–*Eco*RV fragment, which was subcloned from a 9.5 kb *Bam*HI genomic fragment (Blanck *et al.*, 1989) and comprises the entire *sopI* sequence, was used as a *sopI*-specific probe. A PCR fragment extending from 777 to 1705 bp upstream of the *sopI* start codon was used as a probe specific for *htrI*.

DNA and RNA amplification

PCRs with total halobacterial DNA were performed according to the protocol supplied with the *Taq* polymerase (Amersham Corp.) using 500 ng of DNA. To amplify RNA the procedure described by Kawasaki (1990) was followed

using 2 μg of total RNA and the oligonucleotides D and F (see Figure 1D) as primers for synthesis of the first strand cDNA and the oligonucleotide A as 5′ primer in the subsequent PCR.

Preparation of halobacterial cell membranes and spectroscopic analysis

H.salinarium cells were grown for 5 days in the dark at 40°C. Cells from a 700 ml culture were harvested by centrifugation at 15 000 *g* at 4°C and the pellet resuspended in 3 ml of basal salts (Oesterhelt and Stoeckenius, 1974). Cell lysis was achieved by the addition of 7 vol of 10 mM Tris–HCl, pH 7.5 with 20 μg/ml DNase I. After a 5 min incubation at room temperature the membrane fraction was collected by centrifugation at 110 000 *g* at 4°C for 1 h. The membranes were then resuspended and homogenized in 1 ml 4 M NaCl, 10 mM Tris–HCl, pH 7.5 and subjected to spectroscopic analysis. The recovery kinetics of the SR_{587} initial state were measured using an Aminco DW2000 spectrophotometer as described in Schegk and Oesterhelt (1988). The samples were bleached by a 2 s illumination with actinic light filtered through a cut-off filter (OG 570 Schott, Mainz, Germany). Time-dependent absorbance changes at selected wavelengths between 360 and 740 nm were simultaneously recorded for 2 s in 100 ms intervals using a laser diode-array spectrophotometer as described in Uhl *et al.* (1985).

Motion analysis

Cells were grown and prepared for behavioural measurements as described by Marwan and Oesterhelt (1990). UV light and orange background light were produced as in Marwan and Oesterhelt (1990), except that a broad band interference filter (570–643 nm) was used. The cells were exposed to a light intensity of 303 W/m^2. At 1 min intervals the light was switched off for a period of 10 s (orange-off stimulation). Alternatively, a pulse of 366 nm light (560 $W/m^{2)}$ was applied on a constant orange light background. The pulse duration was 1 s for Flx3 cells and 5 s for all other strains analysed. The response of the cells was recorded by a computerized cell tracking system (Motion Analysis Corp., Santa Rosa, CA) with a frame rate of 10 frames/s. The experimental set-up and the motion analysis algorithm are described elsewhere (Marwan and Oesterhelt, 1990).

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