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Car: a cytoplasmic sensor responsible for arginine chemotaxis in the archaeon *Halobacterium salinarum*

Kai-Florian Storch, Johannes Rudolph[1] and Dieter Oesterhelt[2]

Max-Planck Institut für Biochemie, D-82152 Martinsried, Germany

[1]Present address: Mitotix, Inc., One Kendall Square, Cambridge, MA 02139, USA

[2]Corresponding author
e-mail: oesterhe@biochem.mpg.de

A new metabolic signaling pathway for arginine, both a chemoeffector and a fermentative energy source, is described for *Halobacterium salinarum*. Systematic screening of 80+ potentially chemotactic compounds with two behavioral assays identified leucine, isoleucine, valine, methionine, cysteine, arginine and several peptides as strong chemoattractants. Deletion analysis of a number of potential halobacterial transducer genes led to the identification of Car, a specific cytoplasmic arginine transducer which lacks transmembrane helices and was biochemically shown to be localized in the cytoplasm. Flow assays were used to show specific adaptive responses to arginine and ornithine in wild-type but not Δ*car* cells, demonstrating the role of Car in sensing arginine. The signaling pathway from external arginine to the flagellar motor of the cell involves an arginine:ornithine antiporter which was quantitatively characterized for its transport kinetics and inhibitors. By compiling the chemotactic behavior, the adaptive responses and the characteristics of the arginine:ornithine antiporter to arginine and its analogs, we now understand how the combination of arginine uptake and its metabolic conversion is required to build an effective sensing system. In both bacteria and the archaea this is the first chemoeffector molecule of a soluble methylatable transducer to be identified.

Keywords: antiporter/arginine deiminase/methyl-accepting chemotaxis protein/signal transduction/transducer

Introduction

Halophilic archaea live in extreme habitats such as solar evaporation ponds which are characterized by high salinity, intense illumination and oxygen limitation. For optimal survival in their stressful environment halobacteria have evolved the capacity of fermentation, respiration under aerobic and anaerobic conditions and photosynthesis (for review see Oren, 1994). Orientation in their environment is guaranteed by the flagellar motor of the cells, allowing migration toward favorable attractant stimuli and away from repellent stimuli. Halobacteria respond to physical as well as chemical stimuli, including photons, oxygen, ion motive forces and amino acids (Oesterhelt and Marwan, 1993).

Halobacterium salinarum uses a dual system for sensing tactic stimuli. The first is the well known two-component system of eubacterial signal transduction, which consists of a transducer-mediated histidine kinase (CheA) activation and its response regulator (CheY), a substrate of CheA, which becomes phosphorylated if a repellent stimulus interacts with the transducer (Rudolph and Oesterhelt, 1995, 1996; Rudolph *et al*., 1995). The phosphorylated response regulator causes the flagellar motor to switch and the cell thereby moves away from the source of the repellent. The positive correlate involves the deactivation of the histidine kinase following stimulation with attractant and results in low levels of phosphorylated response regulator and prolonged swimming periods of cells towards an attractant source. The second sensing system triggered by the activated transducer involves the metabolite fumarate, which plays the role of a second messenger in the cell and acts together with the response regulator at the flagellar motor (Marwan *et al*., 1990; Montrone *et al*., 1993). This principle, first shown in archaea, has now also been verified for eubacteria, demonstrating an interesting link between metabolic signaling (by fumarate) and external stimulation (Barak *et al*., 1996; Montrone *et al*., 1998).

In *H.salinarum*, 13 genes have been identified so far, which encode homologs of eubacterial methyl-accepting chemotaxis proteins, MCPs (Rudolph *et al*., 1996; Zhang *et al*., 1996a). Their gene products, denoted as halobacterial transducer proteins (Htps, see Rudolph *et al*., 1996), share highly conserved regions for signaling to the histidine kinase and for adaptive methylation at the C-terminus. The N-termini define the specific function of the various transducers by their modular architecture with domains for ligand binding and for membrane anchoring using two or more transmembrane helices. For example, the transducer for green and UV light stimuli, HtrI, has no ligand-binding domain but forms a complex with the photoreceptor sensory rhodopsin I (SRI) (Olson and Spudich, 1993; Krah *et al*., 1994). Photostimulation of the complex then results in the same conformational change in HtrI to activate the histidine kinase CheA, as does ligand binding in chemoreceptors. At least two of the Htps generally lack transmembrane domains and consequently occur as soluble proteins in the cytoplasm of the cell (Rudolph *et al*., 1996; Brooun *et al*., 1997). The following members of this transducer family have been functionally assigned. HtpI and -II have been identified as the transducers for phototaxis HtrI and -II (Yao and Spudich, 1992; Zhang *et al*., 1996b). HtrXI is involved in taxis towards the amino acids Glu, Asp and His (Brooun *et al*., 1997) and HtrVIII, which exhibits homology to the heme-binding sites of eukaryotic cytochrome *c* oxidase,

mediates the aerotactic response (Brooun *et al*., 1998). Furthermore, the phototactic transducer HtrII additionally functions as a chemotactic transducer for serine (Hou *et al*., 1998). For most other cellular responses to various stimuli, however, no causal link between the phenotype and genotype has yet been established.

In search of such links, we systematically screened a large body of chemical compounds which could eventually serve as external stimuli for unicellular organisms and present a preliminary list of tested compounds and those which were found to be active. Of special interest seemed to be arginine since it is an essential amino acid (Helgerson *et al*., 1992) and at the same time the only fermentative substrate of halobacteria (Hartmann *et al*., 1980). Fermentation of arginine via the arginine deiminase (ADI) pathway is not unique to archaea but also occurs in eubacteria (for review see Cunin *et al*., 1986). It comprises the active uptake of the substrate arginine, conversion first to citrulline and then to ornithine, which is secreted, and to carbamoylphosphate from which ATP is produced (see inset, Figure 9). In *H.salinarum*, the gene cluster encoding the enzymes of the ADI pathway has been isolated, sequenced and characterized for regulation of expression (Ruepp and Soppa, 1996). We therefore considered this an attractive system to study the interaction between bioenergetics and signal transduction. To link genotypes and phenotypes, we systematically deleted *htp* genes of which sufficient sequence information was available and screened for a phenotype not responding to arginine. The search resulted in the identification of a soluble transducer protein for arginine. We describe here its primary structure, intracellular distribution and the pathway by which it receives its signal from the outside world.

Results

Screen for chemotactic active compounds

We screened the chemotactic competence of *H.salinarum* cells with the established capillary assay (Adler, 1973) and confirmed the results with the chemical-in-plug assay (Tso and Adler, 1974). The >80 compounds of our initial screen, subgrouped as shown in Table I, were selected with the following rationale: halobacteria live on amino acids, peptides and proteins in their environment and therefore naturally occurring amino acids, their derivatives and dipeptides are good candidates for chemoattractants. Since several halophilic archaea are able to metabolize carbohydrates or sugars, selected examples were also tested (Oren, 1994). A third group of compounds was comprised of carboxylic acids often found as the central metabolites of all living cells. Additionally, the halophilic nature of the cells led us to test specific inorganic ions. Finally, a few esoteric compounds were tested. The results of this screening are shown in Table I with the active compounds underlined. Essentially, a group of six amino acids and seven peptides were identified as attractants and three compounds known from the eubacterial world as typical repellents were shown to induce phobic responses. The active di- and tri-peptides always contain at least one strongly hydrophobic amino acid. Among the amino acids which are attractants all but cysteine are essential for cell growth. Thus, only two amino acids which are essential do not cause chemotaxis, namely lysine and tyrosine (Helgerson *et al*., 1992).

We used the capillary assay as a semiquantitative measurement of chemotactic activity. Figure 1 shows the concentration dependence of chemotaxis for arginine in S9 wild-type cells (Figure 1A) and Δ*htpV-3* cells (Figure 1B), an arginine-negative mutant described in more detail below. Cells grown aerobically and anaerobically showed the same response to arginine, which is maximal at ~500 μM and drops to the level of basal salt at ~10 mM, whereas the threshold response is <5 μM (data not shown). The amino acids leucine, isoleucine, valine and methionine are approximately equally potent as attractants. Cysteine has an intermediate potency, whereas threonine, alanine, phenylalanine and tryptophan show only a slight chemoattractant effect above that of the negative control (CBS, chemotaxis buffer) and are therefore not marked in Table I. Interestingly, no response was observed to cystine, and cysteine taxis was significantly decreased when the cysteine solution used in the assay was exposed to air for 12 h, indicating that cysteine is only active in its reduced form.

The results obtained with the capillary assay were confirmed by the rapid chemical-in-plug method (Tso and Adler, 1974). Typical results using the strongest attractants among the amino acids are shown in Figure 2. The cells included in soft agar migrate up an attractant gradient towards the plug that contains the active compound. This results in the formation of chemotactic rings which represent zones depleted of cells (Figures 2 and 6). The diameter of the depletion rings depends on the amount of cells in the soft agar and on the attractant concentration in the hardplugs (Figure 6). An increase of the cell number results in smaller rings (not shown), whereas higher concentrations of the chemoeffector lead to larger rings (Figure 6). These findings suggest that the attractant gradient causing the tactic response is generated by diffusion as well as the active uptake of the compound by the cells. Therefore, the reduced ring around the 50 mM arginine plug (Figure 2) is presumably due to a higher uptake efficiency compared with that of the other chemotactic solutes. The dark-colored disk around the cysteine plug is an artifact most likely caused by precipitated oxidation products of cysteine. The depletion zone, which is not visible in this photograph, is directly adjacent to the dark area. In another experiment with freshly prepared cysteine and a shorter incubation time (4 h), a chemotactic ring similar to that of the other active amino acids was observed (not shown).

Amino acid taxis involves methylatable transducers

We anticipated that halobacterial chemotaxis is mediated by members of the methylatable transducer family (Rudolph *et al*., 1996; Zhang *et al*., 1996a). Therefore, we tested the adaptive response to amino acids in the so-called flow assay (Kehry *et al*., 1984). This well-established method measures the changes in turnover of methyl groups caused by reversible methylation–demethylation of MCPs at specific carboxyl groups during adaptation. For the assay, cells are incubated with L-[methyl-^{3}H]methionine to equilibrate the transducer proteins with ^{3}H-methyl groups (Alam *et al*., 1989). The

Table I. Test compounds for chemotactic activity in *H.salinarum*

Amino acids I	Amino acids II	Dipeptides	Tripeptides	Sugars and analogs	Carboxylic acids	Inorganic ions	Others
Ala	cystine	Gly–Gly	Gly–Leu–Tyr	D-glucose	acetate	NH^{4+}	glycerol
Asn	homoarginine	Gly–Ile	Leu–Gly–Gly	D-mannose	lactate	K^+	mannitol
Asp	canavanine	Gly–Leu	Leu–Gly–Pro	D-galactose	propionate	Mg^{2+}	TMAO
Gln	ornithine	Gly–Met	Val–Gly–Gly	D-fructose	oxalate	Mn^{2+}	uracil
Glu	citrulline	Gly–Tyr	Leu–Gly–Leu[a]	D-ribose	succinate	Co^{2+}	phenolate[b]
Gly	D-arginine	Gly–Pro		D-maltose	fumarate	Ni^{2+}	indole[b]
His		Leu–Tyr		D-sucrose	malate	NO_3^-	
Lys		Ile–Trp		D-glucosamine	ketoglutarate	HCO_3^-	
Phe		Val–Gly			nicotinate	SO_4^{2-}	
Pro		Val–Val			benzoate[b]	HPO_4^{2-}	
Ser		Val–Met					
Thr		Met–Ala					
Trp		Met–Lys					
Tyr		Lys–Tyr					
Leu[a]		Pro–Gly					
Ile[a]		Tyr–Arg					
Val[a]		Leu–Ala[a]					
Met[a]		Leu–Gly[a]					
Cys[a]		Leu–Leu[a]					
Arg[a]		Met–Ile[a]					
		Met–Val[a]					
		Met–Arg[a]					

Active compounds are underlined. [a]Attractants; [b]repellents. All compounds were tested at 1 mM with the capillary assay in four replicates, except: Tyr at 0.5 mM due to its weak solubility in basal salt; K^+ at 3 and 30 mM; Mg^{2+} and SO_4^{2-} at 8 and 80 mM; HCO_3^- at 10 and 100 mM; NH^{4+}, NO_3^- and HPO_4^{2-} at 10 mM. All amino acids, di- and tripeptides tested were L-isomers if not otherwise indicated. Phobic response towards benzoate was detected at pH 6.0.

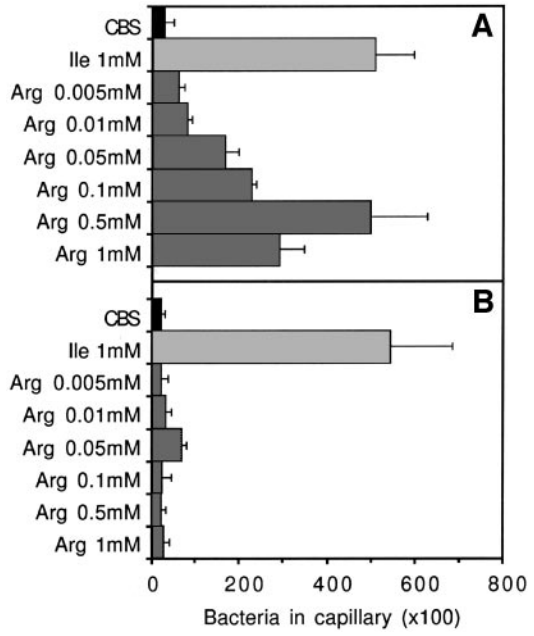


Fig. 1. Capillary assay of cells from *H.salinarum* strains S9 (**A**) and Δ*htpV-3* (**B**) at various concentrations of arginine. Chemotaxis buffer (CBS) with and without isoleucine served as positive and negative controls, respectively. Cells were prepared as described in Materials and methods. Capillaries were filled with the active compound in CBS, dipped into the cell suspension and incubated for 2 h at 37°C in the dark. The bars correspond to the average number of cells which migrated into the capillaries in four independent experiments. The error bars indicate the standard deviation.

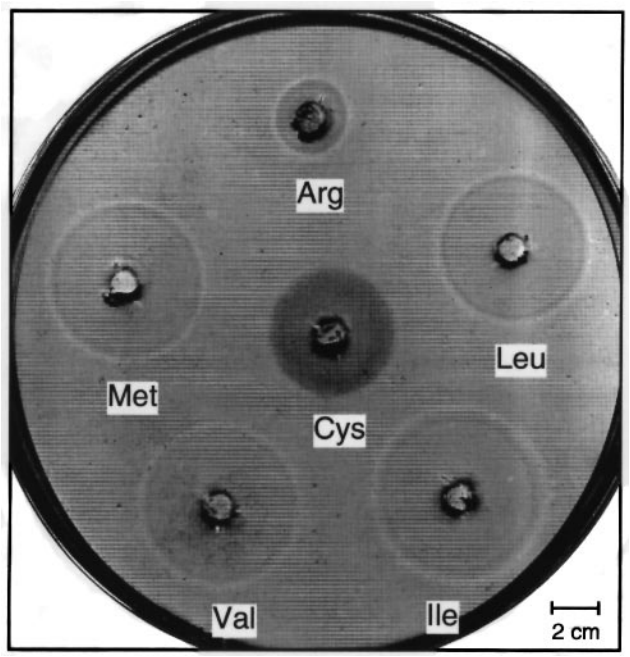


Fig. 2. Chemotactic response of S9 cells to amino acid attractants using the chemical-in-plug method. Amino acid concentration in the hard plugs (2% agar) was 50 mM. Cells were included in 0.25% soft agar and the plate was incubated at 40°C for 12 h in the dark (see Figure 6 for a negative control).

cells are then subjected to a chase medium with unlabeled methionine. Supplementation of the chase medium with a stimulating amino acid causes an adaptive response and release of ^{3}H-methanol. The onset and offset of stimulation by the chemoattractants leucine, isoleucine, valine, cysteine and arginine are accompanied by a burst of methanol release (Figure 3). Interestingly, the addition of arginine causes a much stronger signal compared with the other attractants, whereas the 'off' response is only slightly above background. As a negative control, glutamic acid, not a chemoattractant, is shown at the bottom of Figure 3. As reported previously (Alam *et al*., 1989), and in contrast

to *Escherichia coli* (Kehry *et al*., 1984), halobacteria always produce a burst of methanol release with both the onset and offset of a chemotactic stimulus, with the onset signal being stronger than the offset signal.

Deletion analysis of transducer genes

Given the chemotactic response to various amino acids in halobacteria, the existence of more than twelve putative transducer genes and the observed adaptive response, we set out to screen for phenotypic defects after deletion of known transducer genes, thus establishing a direct link between a specific chemoattractant and its transducer.

Since *htpI* and *II* code for the known transducers HtrI and HtrII involved in the halobacterial phototaxis we began by deleting *htpIII*, *IV, V* and *VI* (Rudolph *et al*., 1996) from the genome of strain *H.salinarum* S9. Deletion of *htpIII*, *IV* or *VI* gave no phenotype in the capillary assay using the active compounds listed in Table I. Although two out of three clones carrying the deletion of *htpV* (Δ*htpV-1* and Δ*htpV-2*) were wild-type when tested for arginine taxis, one clone, Δ*htpV-3*, was found to be deficient (Figure 1B). However, complementation of

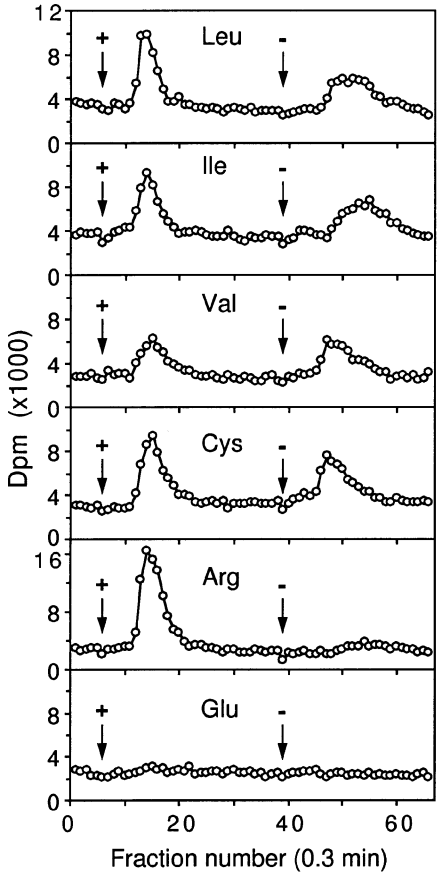


Fig. 3. Amino acid-induced changes in the rate of release of volatile ^{3}H-labeled methyl groups. S9 cells were radiolabeled as described in Materials and methods, washed free of L-[methyl-^{3}H]methionine, placed on a filter in a flow apparatus and subjected at room temperature to a continuous flow (1.5 ml/min) of chase medium containing CBS plus 0.1 mM unlabeled methionine. Fractions collected for periods of 18 s were analysed for volatile radioactivity. Amino acids were added (arrows '+') by switching the inlet tube of the filter from chase medium to chase medium plus 5 mM amino acid and removed by transferring the inlet tube back to chase medium alone (arrows '–').

Δ*htpV-3* with the intact *htpV* gene did not restore wild-type behavior. We therefore performed a genomic analysis of Δ*htpV-3* using a probe to the signaling domains of the *htps (*see Materials and methods). The Southern blot for clone Δ*htpV-3* lacked a band at 2.9 kb (arrow a) in the *Pst*I and at 2.0 kb (arrow b) in the *Bam*HI digest which are present in the S9 wild-type and the arginine-positive clone Δ*htpV-2* (Figure 4A). The blot also demonstrates that Δ*htpV-3* is indeed deleted in the *htpV* gene: both Δ*htpV-2* and Δ*htpV-3* lack the bands corresponding to *htpV* at 2.2 kb (arrow c) and 7.0 kb (arrow d) in the *Pst*I and *Bam*HI digests, respectively (Figure 4A). We concluded, therefore, that Δ*htpV-3* carries a spontaneous mutation in addition to the constructed deletion of *htpV* probably caused by transposition of halobacterial insertion sequences (Pfeifer, 1988). This mutation presumably inactivated an as yet unidentified *htp* gene required for arginine taxis.

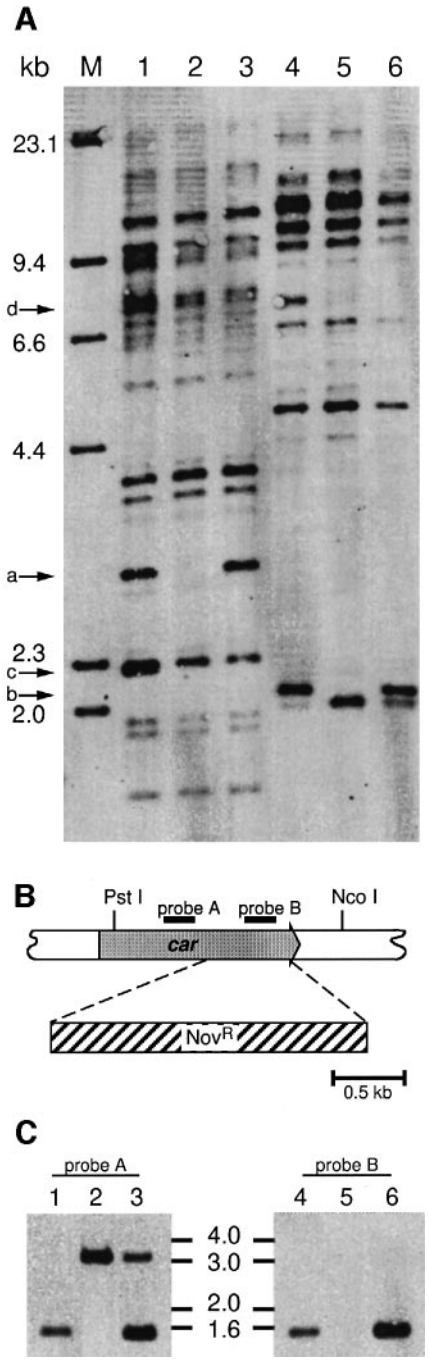
Identification and specific deletion of the transducer gene car

We isolated the DNA fragment corresponding to the 2.9 kb band of the fluorograph (Figure 4A) from a genomic *Pst*I digest from strain S9. Positive clones were identified by probing with the *htp* signaling domain. The nucleotide sequence of this fragment revealed an incomplete *orf* with significant homologies to the known *htp*s. To complete the coding region, a 5′-overlapping *Sal*I-fragment (3.1 kb) was cloned. The sequence of the entire gene (DDBJ/EMBL/GenBank accession No. AJ132321) confirmed that the encoded protein is a new member of the halobacterial transducer family and shares its modular composition. Thus, we named the new gene *car* for cytoplasmic arginine transducer. Car is 452 amino acids long and is expected to be cytoplasmic because it contains the conserved domains for adaptation and signaling, but lacks transmembrane domains based on hydropathy analysis, i.e. Car resembles HtpIII, another soluble transducer protein with an as yet unknown function (Rudolph *et al*., 1996). A sequence (AGTTTATAACT) with significant similarities to the consensus sequence for a strong halobacterial promotor (Danner and Soppa, 1996) was found upstream from the start codon of *car* at nucleotides –40 to –30 and a putative ribosomal binding site (AGGAGG) was found at nucleotides –21 to –16 (Kagramanova *et al*., 1982). A search in the DDBJ/EMBL/GenBank database revealed that a very similar gene exists in halobacteria which also encodes a soluble transducer and was reported as being involved in the tactic behavior against histidine, glutamic acid and aspartic acid (Brooun *et al*., 1997).

A specific *car* deletion mutant of the wild-type strain S9 was constructed using the gene replacement technique (Figure 4B). In strain Δ*car* the C-terminal section of the *car* gene comprising the signaling and one of the adaptation domains (R1 peptide) is replaced by a novobiocin resistance cassette. Thus, Southern analysis of *Pst*I–*Nco*I digested genomic DNA reveals a band at 3.3 kb for Δ*car* with probe A or no band if a probe against the deleted part is used (Figure 4C, lanes 2 and 5). Correspondingly, the complementation strain with the intact *car* gene reintroduced into the genome of Δ*car*, reveals both the wild-type 1.55 kb and the Δ*car* 3.3 kb band (lane 3) or only the wild-type band (lane 6).

Localization of the transducer protein Car

To prove the soluble nature of Car, antibodies against the protein as expressed in *Escherichia coli* were raised in rabbits and used to detect the transducer protein in Western blots. Figure 5A compares a staining pattern of membrane and cytoplasmic fractions (lanes 3 and 4) with total protein extracts from wild-type S9 cells and cells of the deletion strain as positive and negative controls, respectively (lanes 1 and 2). The protein migrating at ~85 kDa is found almost exclusively in the cytoplasmic fraction (Figure 5A, lane 4). The substantial difference between the calculated (49 kDa) and the apparent (85 kDa) molecular mass of Car determined by SDS–PAGE is not uncommon for halophilic proteins and attributed to their acidic nature (Monstadt and Holldorf, 1991).



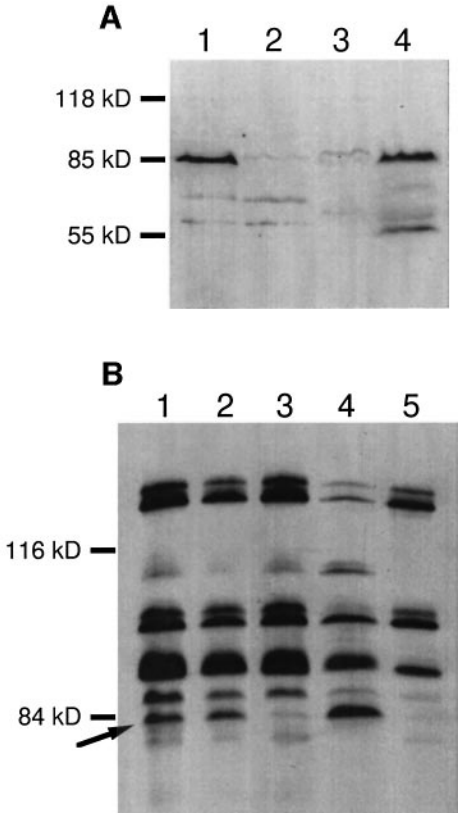


Fig. 5. (**A**) Immunochemical detection and localization of Car in *H.salinarum* using anti-Car antiserum. Lanes: 1, S9 total protein preparation; 2, Δ*car* total protein preparation; 3, S9 membrane fraction; 4, S9 soluble fraction. (**B**) Patterns of methyl-^{3}H-labeled proteins in *H.salinarum* strains S9, Δ*car* and Δ*car/car*$^+$. Represented is the fluorogram of a SDS–PAGE gel (10% acrylamide and 0.066% bisacrylamide). Lanes: 1, S9 total protein preparation; 2, Δ*car/car*$^+$ total protein preparation; 3, Δ*car* total protein preparation; 4, S9 soluble fraction; 5, S9 membrane fraction. The arrow indicates the position of Car. Prior to extraction of the proteins cells were radiolabeled with L-[methyl-^{3}H]methionine as described in Materials and methods. Each lane was loaded with protein fractions corresponding to 50 μg of total cellular protein.

Fig. 4. (**A**) Southern blot analysis of *H.salinarum* strains S9 (lanes 1 and 4), Δ*htpV-3* (lanes 2 and 5) and Δ*htpV-2* (lanes 3 and 6) with a probe to the highly conserved signaling domain of halobacterial transducer proteins (Htps). Genomic DNA was digested with *Pst*I (lanes 1–3) and *Bam*HI (lanes 4–6). Arrows 'a' and 'b' indicate the absence of a band at 2.9 kb (lane 2) and 2.0 kb (lane 5) in Δ*htpV-3*. The band at 2.2 kb (arrow 'c', lane 1), which is superimposed by a slightly slower migrating band at 2.3 kb and the band at 7.0 kb (arrow 'd', lane 4) represent the *htpV* gene and are consequently absent in the deletion strains Δ*htpV-2* and Δ*htpV-3*. Markers (M) are a λ-*Hin*dII digest end-labeled with digoxigenin. (**B**) Schematic representation of the *car* deletion. The 0.55 kb of the C-terminus of *car* were replaced by a Nov^R cassette (2.3 kb). (**C**) Southern blot indicating the absence of the C-terminal portion of *car* (amino acids 266–447 containing the signaling domain and the R1 peptide) from strain Δ*car* (lanes 2 and 5) in comparison with the wild-type strain S9 (lanes 1 and 4) and the complementation strain Δ*car/car*$^+$ (lanes 3 and 6). All samples were subjected to *Pst*I–*Nco*I digestion. Lanes 1–3 were stained with a probe to the N-terminal region of car (amino acids 147–191, probe A). Lanes 4–6 were stained with a probe to the deleted part of car (amino acids 349–384, probe B). Each of the lanes contains 10 μg of genomic DNA. The bars indicate the positions of the markers (in kilobase pairs).

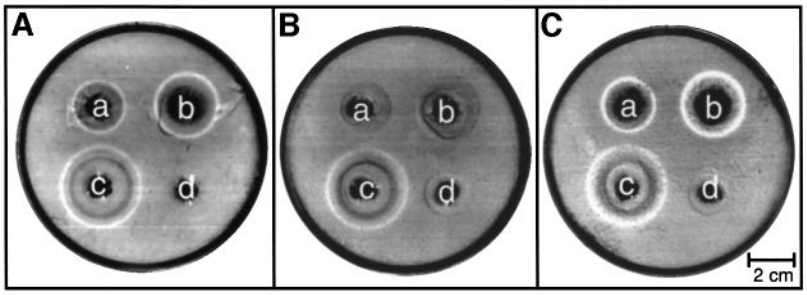


Fig. 6. Chemotactic response in plug plates of *H.salinarum* strains S9 (**A**), Δ*car* (**B**) and Δ*car/car*$^+$ (**C**). Plates were prepared as in Figure 2. Hard plugs contained 50 mM arginine (a), 100 mM arginine (b), 10 mM leucine (c) or no test compound (d). The plates were incubated at 40°C for 12 h in the dark. Bright rings around the plugs represent depletion zones and are therefore indicative of chemotactically responding cells.

The cytoplasmic localization of the transducer was confirmed in a second approach. Following radiolabeling with L-[methyl-^{3}H]methionine as described for the flow experiments, protein extracts were prepared and subjected to SDS–PAGE under conditions of high resolution in the upper molecular weight range (Alam *et al*., 1989). The corresponding fluorograph of the gel is represented in Figure 5B. Total extract from S9 cells (lane 1) and from cells of the complementation strain (lane 2) show the appearance of a labeled band at ~85 kDa which is present in the cytoplasmic fraction (lane 4) but not in the membrane fraction of wild-type extracts (lane 5). The *car*-deleted strain (lane 3) shows only a faint band whereas the intensity is fully restored when this strain is complemented with an intact *car* (lane 2). This clearly indicates that the missing band represents Car and that this transducer is a member of the methyl-accepting protein family. In line with this experimental finding is the identification of three putative methylation sites in the peptide sequence of Car, Glu225, Glu232 and Gln407. The adjacent sequences of Glu225 and Glu232 are in good homology to, whereas Gln407 perfectly matches the consensus sequence derived from the most rapidly methylated sites of the enteric transducers. According to Le Moual and Koshland (1996), the consensus sequence is defined as X_1-X_1-X-X_2-X_2-X_2-X-Z-Z*-X-X_2-A-S/T-X-X_1-X_1 (X = any residue; X_1 = E, Q, D, N, T or S; X_2 = A, S or T; Z = Glx; Z* = methylatable Glx).

Functional analysis of Car

Wild-type (A), the *car* deletion strain (B) and its complementation strain (C) were compared for their tactic behavior in the presence of 50 mM arginine (a) and 100 mM arginine (b) (Figure 6). Plugs containing 10 mM leucine (c) or buffer alone (d) served as positive and negative controls, respectively. Cells of the wild-type and complementation strain form significantly larger rings around the 100 mM arginine plugs than around 50 mM plugs (for explanation see above). Clearly, the deletion strain, while retaining normal taxis towards leucine, has lost the capacity to respond to arginine. This result was confirmed by the capillary assay at arginine concentrations of 0.1 and 1 mM (data not shown). The deletion strain showed no response in agreement with the results for the mutant strain (Figure 1B), whereas the complemented strain reached response levels of the wild-type. When the deletion strain was tested against the remaining amino acid attractants listed in Table I, it showed the same strong responses as wild-type, indicating an apparently absolute specificity of the transducer for arginine among these compounds.

To summarize so far, Car is a cytoplasmic and methylatable transducer of *H.salinarum* which mediates specifically the internal chemotactic signal of arginine through the two-component system to the flagellar motor. Apparently, the signaling pathway involves internalization of the chemoeffector. To elucidate the underlying mechanism further, the mode of arginine uptake and metabolism was characterized and their link to chemotaxis established.

Arginine transport and metabolism

In contrast to the ADI operon of *Pseudomonas aeruginosa* which includes the *arcD* gene encoding the arginine: ornithine antiporter, no corresponding *orf* could be assigned in the recently identified ADI gene cluster of *H.salinarum* (Ruepp and Soppa, 1996). However, the observation that halobacterial cell suspensions secrete an equivalent amount of ornithine when taking up arginine from the medium strongly suggested the existence of such an exchange protein (Hartmann *et al*., 1980).

For a detailed characterization of the exchange reaction we performed transport experiments with membrane vesicles generated from S9 cells. Vesicles were loaded with 500 μM ornithine and diluted by a factor of 100 into a buffer containing 19 μM [^{14}C]arginine. At room temperature, the rate of arginine uptake was stimulated ~40-fold when ornithine was present inside the vesicles, indicating the existence of an Arg:Orn antiporter (data not shown). Since the saturating accumulation of arginine occurs within 5 s at room temperature, the stoichiometry of the exchange and all kinetic parameters were measured at 0°C. Vesicles were loaded with 200 μM [^{14}C]ornithine and diluted into buffer containing 5.8 μM [^{3}H]arginine. Rapid uptake of arginine was accompanied by efflux of ornithine (Figure 7A). In a parallel experiment, [^{14}C]ornithine loaded vesicles were diluted into arginine-free buffer to assess the slow arginine-independent rate of ornithine efflux (Figure 7A, filled circles). Ornithine-independent uptake of arginine was measured by diluting unloaded vesicles into a buffer containing [^{3}H]arginine (Figure 7A, filled squares). From Figure 7A the stoichiometry of the arginine:ornithine exchange is estimated to be ~1:1. This is in agreement with the ratio found for Arg:Orn exchange in *Lactococcus lactis* (Driessen *et al*., 1987) and *P.aeruginosa* (Verhoogt *et al*., 1992).

The kinetic constants of the exchange were estimated

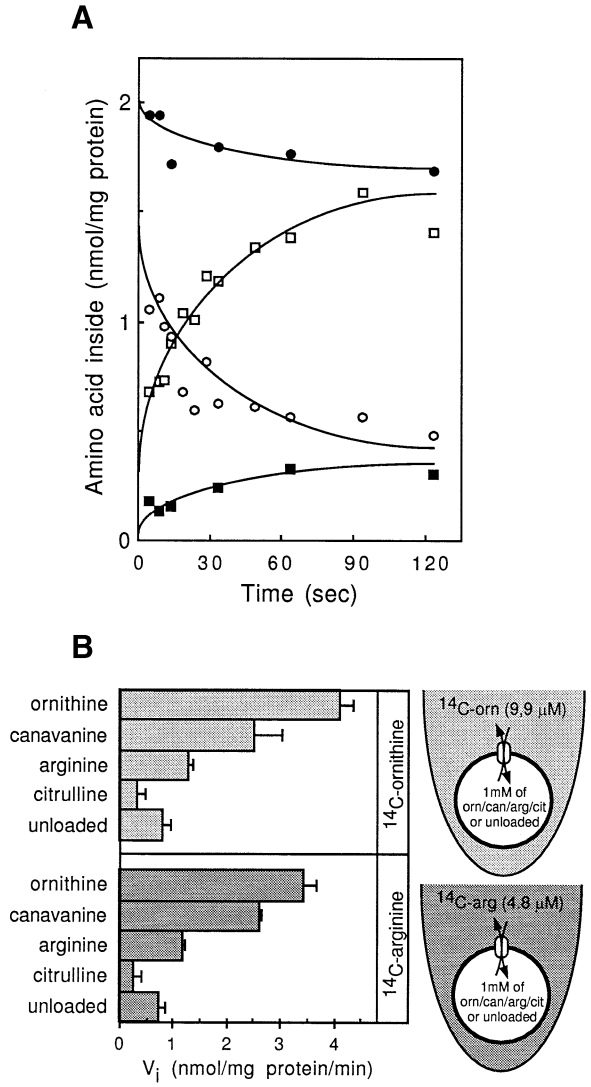


Fig. 7. (**A**) Arginine:ornithine exchange in membrane vesicles of *H.salinarum* S9. Membrane vesicles were either loaded or not loaded with 200 µM [^{14}C]ornithine and diluted 100-fold in transport buffer (TBS). [^{14}C]Ornithine efflux in the presence (○) or absence (●) of 5.8 µM [^{3}H]arginine. [^{3}H]Arginine uptake by membrane vesicles loaded (□) or unloaded (■) with [^{14}C]ornithine. (**B**) Initial uptake rates (15 s) of [^{14}C]arginine or [^{14}C]ornithine into membrane vesicles loaded with 1 mM of the solute indicated. The drawings on the right illustrate the uptake assay with a loaded vesicle suspended in the solution containing the radiolabel. Uptakes were performed at 0°C. Membrane vesicles were used at a final protein concentration of 0.3–0.4 mg/ml.

from the initial rates of arginine uptake at 0°C by varying the arginine concentrations from 0.2 to 10 µM with 200 µM unlabeled ornithine inside the vesicles. A Michaelis constant for arginine entry transport (K_t^{entry}) of 1.1 (±0.1) µM and a maximal velocity (V_{max}) of 6.8 (±0.5) nmol arginine/min·mg of protein was found. The substrate specificity for the external binding site of the Arg:Orn antiporter was analyzed under the same conditions as for the determination of K_t^{entry} except that the arginine concentration was varied in the presence of fixed concentrations of analogs. L-ornithine, L-homoarginine and L-canavanine, an oxo-analog of arginine (see Figure 8B, inset), are strong competitive inhibitors of arginine uptake with apparent inhibition constants (K_i) of 1.5 (±0.2), 1.3 (±0.3) and 3.0 (±0.5) µM, respectively. In contrast, L-citrulline, the intermediate metabolite of the ADI pathway did not affect the K_t^{entry} for arginine, indicating that citrulline is not competing for the Arg:Orn antiporter.

The substrate specificity for the internal binding site of the antiporter was determined by preloading membrane vesicles with 1 mM of the solute to be tested. Loaded vesicles were diluted by a factor of 100 into buffer containing 4.8 µM [^{14}C]arginine or 9.9 µM [^{14}C]ornithine and initial uptake rates were measured for 15 s. The uptake dependency on the preloaded solute was very similar for both cases, indicating that the substrate properties of ornithine for uptake equals that of arginine (Figure 7B). As expected for a cosubstrate of the antiporter, ornithine was found to be the best substrate at the inside. Surprisingly, the accumulation of radiolabel into vesicles loaded with citrulline was significantly lower than into unloaded vesicles. This could be explained by an inhibitory effect of citrulline at the cytoplasmic binding site of the exchanger which blocks uptake of external arginine. The strong decrease of radiolabel accumulation detected in vesicles preloaded with L-arginine compared with L-canavanine-loaded vesicles is presumably due to residual ADI activity in the vesicle preparations converting arginine into the inhibitory citrulline (data not shown, see below).

The transport experiments clearly show that *H.salinarum* contains an Arg:Orn antiporter which exchanges both solutes in a 1:1 ratio. The exchanger's affinities to canavanine and ornithine are comparable to that of arginine at both faces, outside and inside. Accumulation of reasonable amounts of labeled ornithine with different solutes present at the inside of the vesicles indicate that the antiporter is capable of reverse and homologous exchange.

In order to test whether the arginine analogs, D-arginine, L-canavanine and L-homoarginine, interfere with the arginine metabolism, the substrate specificity of the ADI was tested. Crude cell extracts were incubated with 2 mM L-arginine at 37°C in the presence of 10 mM D-arginine, L-homoarginine and L-canavanine, respectively. When initial rates of citrulline formation were measured using a colorimetric assay specific for ureido-compounds (Oginsky, 1957), only L-canavanine inhibited arginine conversion (95% inhibition, data not shown). Moreover, when crude extracts were incubated with 10 mM of each of the three analogs alone, no colored product could be detected. L-homocitrulline, the presumed product of the deimination of L-homoarginine, as well as urea form products with absorption properties and extinction coefficients, which are comparable to that of the citrulline product (data not shown). Thus, none of these analogs is apparently a substrate for the ADI.

Analysis of the signaling pathway of arginine taxis

All of these results prompted us to undertake a more detailed flow assay analysis of the Car-mediated methanol release in intact cells. Figure 8A compares wild-type and deletion strain behavior in an experiment with sequential stimulation by ornithine and arginine. Cells were incubated with 10 mM ornithine for 1 h, washed free from ornithine and pumped onto the filter of the flow apparatus. After 10 min flow with chase medium neither the addition nor the later removal of ornithine caused any effect. In contrast, addition of arginine results in a burst of methanol release

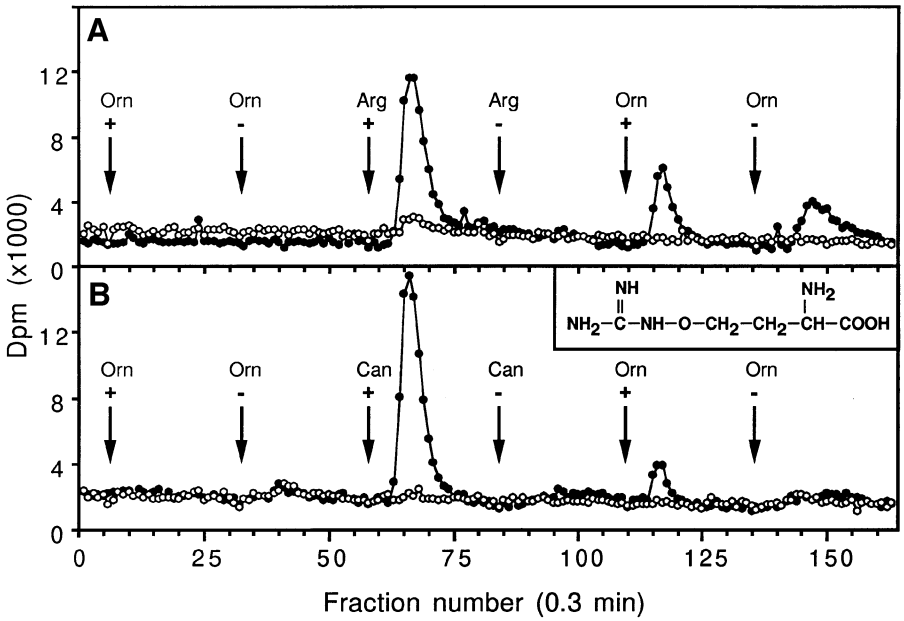


Fig. 8. Volatile [^{3}H]methyl group release of *H.salinarum* strains S9 (●) and Δ*car* (○) upon sequential stimulation with ornithine and arginine (**A**) or its oxo-analog canavanine (**B**). Assay conditions were as in Figure 3, except that the cells were radiolabeled in the presence of 10 mM ornithine. Cells were stimulated with ornithine, arginine and canavanine (Orn/Arg/Can arrows) at concentrations of 10 mM. Inset in B: structural formula of canavanine.

in wild-type cells, but not in the deletion strain (Figure 8A, open circles). The small signal seen in the *car*-deleted cells might be caused by unspecific arginine stimulation of other transducer proteins in the cell. A second stimulation with ornithine following the arginine treatment, however, gives a significant response of wild-type cells. The discrepancy between the two ornithine stimuli can be explained by a reverse action of the Arg:Orn antiporter in the following way. First, during preincubation with ornithine, the reverse action of the antiporter depletes the cells of internal arginine. Therefore, the first ornithine stimulation causes only a homologous exchange of external and internal ornithine via the antiporter, i.e. no net change occurs. The stimulation with arginine reloads the cells with arginine and now, different from the first ornithine treatment, the subsequent addition of ornithine causes the efflux of this internal arginine by reversed action of the Arg:Orn antiporter. This concentration drop in return lowers the occupancy of the taxis-specific arginine receptor and thus results in a phobic response of the cells. The removal of ornithine stops the arginine efflux and therefore causes a detectable 'off' response.

The same experiment carried out with canavanine instead of arginine is shown in Figure 8B. Canavanine acts nearly identically to arginine with a slightly stronger 'on' response. The effect of the second ornithine stimulus, however, is evidently weaker with no visible 'off' response. This result demonstrates that canavanine enters the cell, presumably via the Arg:Orn exchanger like arginine, and is able to excite specifically the Car-mediated adaptive signaling pathway, since Δ*car* does not respond in this assay (Figure 8B, open circles). As shown by the ADI activity assay, canavanine is not degraded by the ADI pathway, indicating that canavanine by itself is the effector, which triggers the methanol release. This in turn suggests that arginine and not a metabolite is the native chemoeffector of arginine taxis, since canavanine is structurally closer to arginine than to any of its metabolic products. The finding that canavanine does not cause an attractant response in the behavioral assays (data not shown) but does show an adaptive response fits well with the hypothesis that the cytoplasmic concentration of an internally monitored chemoeffector must correspond to its external concentration to enable cells to orient in an attractant gradient. Canavanine, which is not metabolized and therefore rapidly reaches an internal concentration which is saturating, causes the cell to adapt and to become unresponsive to external changes. On the other hand, canavanine and ornithine abolish arginine taxis completely if admixed (25 mM) to cells in plug plates with 50 mM arginine in the plugs (data not shown). Considering the results of the transport studies, this might be simply due to the inability of the cells to actively generate an arginine gradient in the plates when the Arg:Orn exchanger is inhibited by canavanine or ornithine.

Discussion

We optimized two behavioral methods, the capillary and chemical-in-plug assay, for halobacteria and used them to identify halobacterial chemoattractants and repellents. In contrast to *E.coli* (Adler, 1975), the attractants of *H.salinarum* identified are restricted to amino acids and peptides. This finding is consistent with the metabolic properties of this archaeon. First, all of the active amino acids except cysteine are essential for cell growth (Helgerson *et al*., 1992) and all active peptides contain at least one of these amino acids. Secondly, unlike in the case of *E.coli*, there is no evidence that *H.salinarum* utilizes carbohydrates as an energy source. Consequently this species has not evolved any sugar-specific chemoreceptors, as seen in its unresponsiveness towards the carbohydrates tested.

Our results further demonstrate that taxis towards argin-

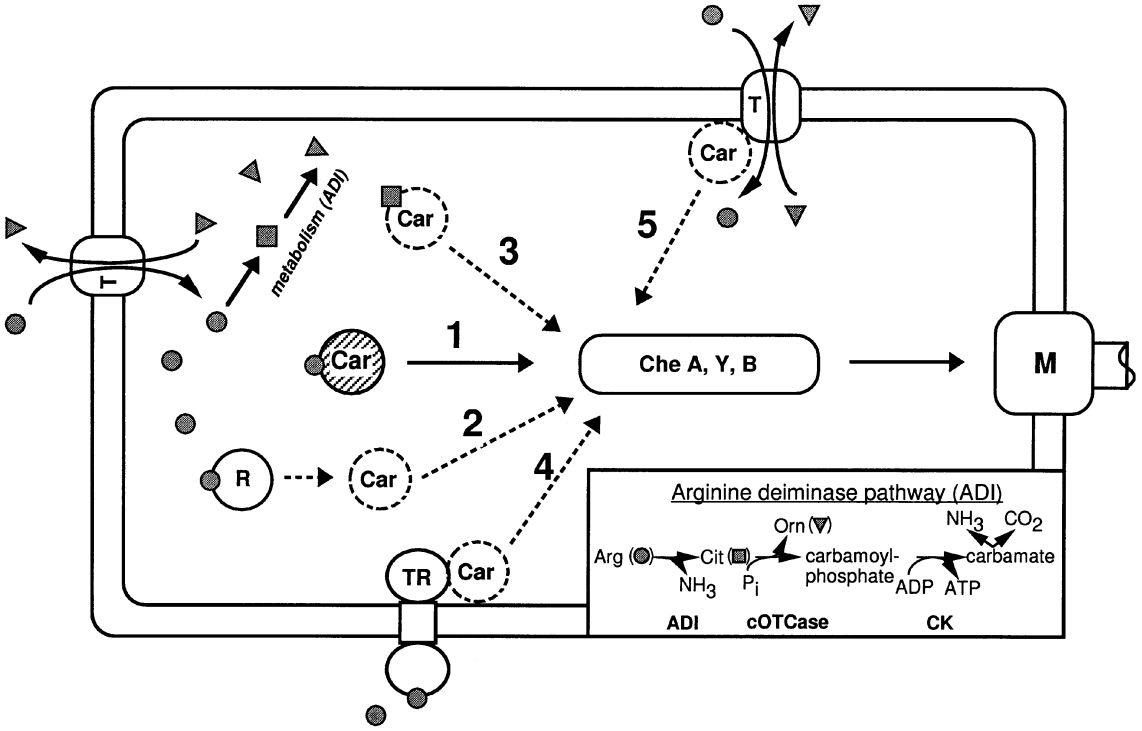


Fig. 9. Proposed signaling pathway for arginine taxis in *H.salinarum* (1, 2) and alternative routes (3–5). Arginine is transported into the cell by action of an Arg:Orn antiporter, which thereby releases one molecule ornithine per molecule of arginine. Internalized arginine is continuously degraded by the enzymes of the ADI pathway. Arginine is converted to citrulline and further to ornithine, which is secreted. (1) Car measures cytoplasmic arginine levels via direct binding and relays the sensory signal into the Che pathway, i.e. Car acts as an arginine receptor. (2) Alternatively, Car is a transducer, which interacts with an as yet unknown cytoplasmic receptor component, before the signal is mediated to CheA, CheY and the flagellar motor. (3) A metabolite of the ADI pathway, e.g. citrulline, is the chemoeffector which binds to Car. (4) Car acts as a transducer of an as yet unknown transmembrane receptor for arginine. (5) Car monitors the arginine occupancy of the antiporter. Inset: scheme of the ADI pathway. M, flagellar motor; T, Arg:Orn antiporter; R, unknown cytoplasmic receptor; TR, unknown transmembrane receptor; ●, arginine; ■, citrulline; ▲, ornithine; ADI, arginine deiminase; cOTCase, catabolic ornithine transcarbamoylase; CK, carbamate kinase.

ine is mediated by Car in *H.salinarum*. In both bacteria and the archaea this is the first chemoeffector molecule of a soluble methylatable transducer to be identified. Based on our data, we propose the following pathway for halobacterial arginine taxis (Figure 9). (i) Arginine is internalized before eliciting a chemotactic signal. This can be deduced from the flow assays of Figure 8, since a plausible explanation of the ornithine effect seen here requires that arginine signals inside the cells. We assume that ornithine acts indirectly by modulating the cytoplasmic arginine concentration and thereby the occupancy of the cytoplasmic arginine receptor. This modulation is caused by the reversed action of the Arg:Orn exchanger upon addition of ornithine to arginine-loaded cells and by feedback inhibition of fermentative arginine degradation (ornithine is a product of the ADI pathway). Consistent with this interpretation is the finding that there is no adaptive response to ornithine if the cells are arginine depleted. Also, the plug plate experiments, showing that ornithine by itself is not an attractant, instead inhibiting arginine taxis, supports this conclusion. (ii) Arginine, rather than one of its metabolites, is the chemoeffector in arginine taxis. Canavanine, the oxo-analog of arginine, competes with arginine uptake and gives a strong and Car-specific response in the flow assay (Figure 8B) even though it is not degraded by the ADI pathway. We therefore assume that canavanine triggers the adaptive response seen in the flow assay by binding to the receptor component of the signaling pathway.

Interestingly, canavanine is not an attractant, but rather inhibits the arginine response as shown using plug plates. At first sight, the lack of behavioral activity of this compound appears to contradict the results of the flow assay. This paradox is best explained by the metabolic persistence of this analog in contrast to arginine. Consider that arginine is a chemoeffector specifically acting in the cytoplasm. Coordinated taxis is only possible, therefore, if the attractant's internal concentration reflects its external concentration. A prerequisite for this type of taxis is the metabolism of the chemoeffector. A cytoplasmic receptor monitors a steady-state level of arginine. If the ligand is not degraded, its cellular concentration rises due to continuous solute uptake. The chemoreceptor and/or solute uptake systems become saturated and the cytoplasmic solute levels do not drop below these saturating concentrations, even if the cell is swimming down the chemoeffector's gradient. Consequently, internal and external concentrations are decoupled and the cells are unable to orient in the chemical gradient, even when an interaction with a specific chemoreceptor is possible.

In summary, the data presented provide substantial evidence that arginine metabolism is a prerequisite for an appropriate tactic response, but it is the interaction of arginine and not of a metabolic product with Car, which specifically triggers the signal transduction machinery in the cytoplasm. Thus, in addition to a chemotactic transducer and the Che proteins, a transport protein and a degradation pathway are both required for this new type of metabolic signaling.

Whether arginine interacts directly with the N-terminal domain of Car, which has no significant homology to any of the peptide sequences deposited in the PIR databases as of November 1, 1998, or binds to an as yet unknown upstream component (Figure 9, route 2) of the arginine-specific signaling pathway, remains to be determined. In this context it is interesting that the aspartate receptor from *E.coli* is able to signal from the ligand binding to the effector domain even after the transmembrane domains have been deleted (Ottemann and Koshland, 1997).

Figure 9 describes five pathways theoretically possible for arginine taxis (routes 1–5). Our results are clear evidence for routes 1 or 2 and make it very unlikely that an intermediate of the arginine metabolism, like citrulline, is the chemoeffector compound (route 3), because the non-metabolizable oxo-analog of arginine, canavanine, carries the same positively charged guanidino group as arginine and further shows an even stronger adaptive response in the flow assay than arginine. However, a mechanism of sensory detection whereby a metabolite of an internalized attractant triggers the chemotactic response has been suggested as the predominant mode for chemotaxis in *Rhodobacter sphaeroides*. Responses to glutamate and sugars depend on uptake and metabolism of the attractant molecules in this species (Jacobs *et al*., 1995; Jezioresassoon *et al*., 1998), but a specific receptor protein has not yet been identified. Route 4 proposes that external arginine levels are measured directly via a membrane-bound receptor component which relays the stimulus to the Car protein. This metabolism-independent sensing mode is characteristic of the enteric MCPs, which monitor changes of chemoeffector concentration in the medium by their periplasmic binding domain (reviewed in Stock and Surette, 1996). In this case, Car would functionally resemble the Htr proteins which interact with the halobacterial receptors for light stimuli, SRI and SRII (Hoff *et al*., 1997). However, as discussed above, the differential effect of ornithine demonstrated in the flow assay (Figure 8) is only explainable if arginine signals in the cytoplasm. Alternatively, Car directly interacts with the Arg:Orn antiporter, thereby monitoring the arginine occupancy of the exchange protein (route 5). A comparable mode of signaling has been reported for carbohydrates transported by the phosphotransferase system (PTS) in *E.coli*. There, the transport of these sugars across the cellular membrane triggers the chemotactic response (Lux *et al*., 1995). The results of our transport studies have shown that ornithine competes strongly with arginine for the external binding site of the exchange protein. However, ornithine elicits an adaptive response only if arginine is present inside the cells (Figure 8). Therefore, binding of a substrate to the antiporter, or even its transport, is not sufficient to stimulate the Car-specific signaling pathway. Taken together, our results strongly favor routes 1 or 2 of signaling and rule out routes 4 and 5. Only a deletion of the ADI, however, will provide the final argument for the exclusion of route 3.

Additional evidence that the Arg:Orn antiporter might act as a link between the outside world and the chemotactic machinery comes from the comparison of aspartate taxis in *E.coli* and the halobacterial arginine taxis. Not only the affinities of the membrane-bound receptor Tar towards aspartate, K_d = 1 μM and of the halobacterial exchanger towards arginine, K_t^{entry} = 1.1 μM are nearly identical, also the peak responses in the capillary assay are in the same range of 1 and 0.5 mM, respectively (Reader *et al*., 1979; Biemann and Koshland, 1994). Even the threshold responses might be comparable, although not quantitated for *H.salinarum*, with $<5\times10^{-6}$ M for arginine and 6×10^{-8} M for aspartate (Mesibov and Adler, 1972).

Soluble MCP homologs have been found recently in several eubacterial species, but little or nothing is known about the sensory mechanisms in which they are involved or the ligands with which they interact (Armitage and Schmitt, 1997; Ward and Zusman, 1997). The putative soluble proteins TlpA and B of *Sinorhizobium meliloti*, which both exhibit MCP-like motifs, are involved in the tactic response to proline and the corresponding deletion mutants show reduced swimming speed (Armitage and Schmitt, 1997). The cytoplasmic MCP-like TlpA of *R.sphaeroides* is proposed to mediate the response to a metabolic intermediate produced primarily by aerobic metabolism, since *tlpA* inactivation results in a reduced chemotaxis to various carbon sources under aerobic conditions (Ward *et al*., 1995; Armitage and Schmitt, 1997). In *Myxococcus xanthus*, response to all known repellents and attractants requires the Frz signal-transduction system, including the soluble receptor component FrzCD, which is homologous to the enterobacteric MCPs. In contrast to repellent responses, which are likely to follow the enteric paradigm, the attractant responses seem to be more complex in this gliding bacterium. For the latter, it is suggested that the respective chemoeffector is an autoattractant rather than a specific nutrient (Ward and Zusman, 1997). Recently, discrete phospholipids have been identified as chemoattractants for this species. Since null mutants of *frzCD* and *frzE*, a CheA/CheY homolog, failed to adapt to dilauroyl phosphatidylethanolamine, it was suggested that the *frz* system is involved in the sensory adaptation process towards this autoattractant (Kearns and Lawrence, 1998).

Considering that the mechanism for arginine taxis presented here is tightly coupled to the presence of the ADI pathway, it is likely that other organisms which exhibit this metabolic feature might mediate their chemotactic arginine response in a similar manner. *Pseudomonas aeruginosa*, however, does not appear to use this mechanism. This species is strongly attracted by all commonly occurring amino acids, including arginine, and is equipped with the enzymes of the ADI pathway, yet it mediates its response to arginine and all other amino acids by membrane-bound MCP homologs, much like Tar and Tsr from *E.coli* (Taguchi *et al*., 1997). Furthermore, the ADI activity in *P.aeruginosa* is substantially lower under the aerobic conditions of the behavioral assays, whereas the halobacterial ADI cluster seems to be differentially regulated with a high ADI and Arg:Orn exchanger activity even under aerobiosis (this work; Gamper *et al*., 1991; Monstadt and Holldorf, 1991). Final proof that metabolism via the ADI pathway is an absolute requirement for a proper chemotactic response towards arginine in *H.salinarum* will be provided by a mutant strain with an inactivated ADI cluster.

Recently, the term energy taxis has been introduced as metabolism-dependent behavior, which requires the metabolism of the signaling molecule in order to produce a signal (Taylor and Zhulin, 1998). This was contrasted

with the metabolism-independent chemotaxis mediated by transmembrane receptors, e.g. *E.coli* MCPs, which are monitoring changes of chemoeffector concentration in the medium. Since the tactic response towards arginine is essentially linked to fermentative arginine degradation which comes along with ATP production, the halobacterial arginine taxis could also be referred to as energy taxis. It should be noted, however, that all behavioral assays employed in this work were carried out under aerobic conditions in the dark. Under these conditions the majority of intracellular ATP is provided by respiration and it is questionable whether ATP production by the ADI pathway contributes to the overall energy status of the cell.

Materials and methods

Strains and culture conditions

All strains were grown under standard conditions in the absence of light (Oesterhelt and Krippahl, 1983). A highly motile single colony isolate of *H.salinarum* strain S9 (Wagner *et al*., 1981) collected from expanding rings on semisolid agar plates was used in behavioral assays, for generation of the *htpV* and *car* deletion strains, and for isolation of membrane vesicles and crude lysate.

General molecular biological techniques

General molecular biological techniques were performed according to Sambrook *et al*. (1989). Standard PCR reactions with *Taq* (Perkin Elmer) or VENT (New England Biolabs) polymerases were performed in the buffers provided by the supplier. To samples in a volume of 100 µl, 50 ng template, 5 pmol of each primer and 50 µM of each dNTP were added. All PCR cycling reactions (30 cycles of the sequence, 30 s at 95°C, 30 s at the annealing temperature and variable extension times at 72°C) were performed in a Perkin-Elmer DNA Thermal Cycler. Oligonucleotides were synthesized on an Applied Biosystems DNA Synthesizer, Model 394. DNA sequencing was performed using the chain termination method (Sanger *et al*., 1977) and cycle sequencing (PRISM Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit, Applied Biosystems). An Applied Biosystems Sequencer, Model 377A, was used for detection of sequence data and the program Sequence Navigator and Sequencing Analysis (Applied Biosystems) for their interpretation.

Identification and cloning of car

A 100 bp degenerate probe against the halobacterial signaling domains was synthesized by PCR to identify *car*. The primer sequences were deduced from an alignment of HtpIII–VI and HtrI (Rudolph *et al*., 1996). The upstream primer corresponding to the signaling domains was 5′-GACATCGCSGAVCAGACS-3′ and the downstream primer was 5′-GCSACSACSGCGAAVCC-3′. The PCR reaction was performed at an annealing temperature of 55°C (1 min) and an extension time of 1 min using these two degenerate primers with the addition of 0.5 µg digoxigenin-dUTP (DIG–dUTP, Boehringer Mannheim) and genomic DNA of strain S9. The probe was used without further purification in Southern blots of *Pst*I genomic digests of halobacterial DNA using the DIG detection kit from Boehringer Mannheim. According to the Boehringer protocol (Boehringer Mannheim, 1995) blots (Hybond-N^+ nylon membranes, Amersham) were hybridized in standard buffer with 50% formamide for 12 h, followed by a stringency wash with 0.1× SSC/0.1% SDS solution at 48°C. The 2.9 kb fragment present in strain S9 and Δ*htpV-2*, but missing in Δ*htpV-3* was chosen for cloning. Preparative digestion of genomic DNA from strain S9 with *Pst*I was followed by 1% agarose gel electrophoresis and isolation of the correct-sized fragment using the Prep-A-Gene kit (Bio-Rad). The fragments were ligated into pBKS+ (Stratagene) which had been digested with *Pst*I and dephosphorylated with Shrimp Alkaline Phosphatase (Amersham). Clones into XL1-Blue cells (Stratagene) were screened by standard colony blot hybridization using the same DIG-labeled probe described above. The plasmid DNA of positive clones (pFS40) was isolated and sequenced on both strands.

The upstream overlapping fragment needed to complete the cloning and sequencing of *car* was obtained as follows. A specific DIG-labeled probe (130 bp) for the *car* gene was prepared under standard PCR conditions with the *Pst*I clone (pFS40) as the template, the primers 5′-TCGGCAGCTTTGGAGTGG-3′ and 5′-ACCTCGTCGATGGATTCG-3′, an annealing temperature of 52°C and 30 s extension time. A genomic digest of strain S9 with *Sal*I, followed by 1% agarose gel electrophoresis and Southern blotting with this probe showed a single signal at 3.1 kb. The 3.1 kb *Sal*I fragment was ligated into the *Sal*I site of pBKS+. Clones into XL1-Blue cells were screened as above and the plasmid DNA of positive clones (pFS41) was isolated and sequenced on both strands from the vector as well as the known overlapping regions.

Construction of a deletion strain (Δcar)

A 1.7 kb *Hin*cII–*Hin*dIII fragment (comprising nucleotide 1341 of the *car* gene and the following downstream sequence) from pFS40 was ligated into the *Hin*dIII- and *Sma*I-digested vector pBN1 (Nowosad, 1991) to form pFS401. The vector pBN1 was generated by ligating a 2.3 kb PCR fragment, amplified from pMDS1 (Holmes *et al*., 1991) and containing the novobiocin resistance cassette, into a *Xba*I–*Bam*HI digested pBSK– (Stratagene). Then, a 0.7 kb *Eag*I fragment (comprising nucleotides 109–791 of the *car* gene) from pFS40 was ligated into pFS401 which had been digested with *Not*I and checked for proper orientation to generate pFS402. Transformation of pFS402 into halobacterial strain S9 was performed as described by Cline *et al*. (1989). Novobiocin resistant clones were initially screened by colony hybridization using a DIG-labeled PCR probe (comprising amino acids 349–384 of *car*) to the deleted region. Colonies with weak signals compared with wild-type were further screened by Southern blotting following a *Pst*I–*Nco*I digest of genomic DNA using DIG-labeled PCR probes to both the deleted region and the upstream region which was retained (see Figure 4B).

Construction of a complementary strain (Δcar/car$^+$)

A 1.6 kb *Sac*II fragment from pFS40 was ligated into the *Sac*II-digested vector pFS41 (with removal of a 0.9 kb fragment) and checked for proper orientation to generate pFS42 and an intact *car*. A 2.8 kb *Bam*HI fragment containing the *mev*^{R} cassette from the vector pJR3557 (Rudolph and Oesterhelt, 1995) which confers mevinolin resistance (Lam and Doolittle, 1992) was ligated into the vector pBKS+, which had been digested with the same enzyme, to generate pFSMev+. A 3.8 kb *Apa*I fragment (comprising *car* and 2.0 kb of the upstream and 0.5 kb of the downstream flanking region) of pFS42 was then ligated into the *Apa*I-digested vector pFSMev+, resulting in pFS8c.

Transformation of pFS8c into strain Δ*car* was performed as described (Cline *et al*., 1989). Mevinolin-resistant clones were screened by Southern blotting following a *Pst*I–*Nco*I digest of genomic DNA using the DIG-labeled probe hybridizing upstream of the deleted region (see Figure 4B).

Heterologous expression and immunodetection of Car

The Car protein-coding region was amplified from plasmid pFS42 by standard PCR with VENT polymerase with the primers 5′-AATAAGAAACATATGGATCCAGCATCG-3′ and 5′-AGCAACGACGAAGCTTTAGCGGCG-3′, an annealing temperature of 50°C and 2 min extension time. The first primer introduces an *Nde*I site at the start codon of the gene, the second primer inserts a *Hin*dIII site 10 nucleotides downstream of the *car* stop codon. The resulting PCR fragment of 1395 bp was cloned into *Nde*I- and *Hin*dIII-restricted plasmid pT7-7 (Tabor and Richardson, 1985) to generate the expression vector pFS4000. The amplified region was resequenced to ensure that no errors had been incorporated. Transformation of pFS4000 into *E.coli* strain BL21(DE3) (Stratagene) yielded the isopropyl-β-D-galactopyranoside (IPTG)-inducible expression system for Car, denoted HSR.

Induction of Car: strain HSR was grown in Luria Broth containing 50 µg/ml ampicillin at 37°C until the OD_{600} was 0.7. Following the addition of IPTG to a final concentration of 0.4 mM, the cells were shaken at 37°C for an additional 2.5 h. Induction led to the appearance of a protein band migrating at 85 kDa. In order to confirm the expression of the correct protein, the first four residues were identified by N-terminal sequencing and found to be M-D-P-A, in agreement with the sequence expected from the nucleotide sequence.

Antibodies to Car were generated by subcutaneous injection of SDS–PAGE slices containing 50 µg of protein mixed with adjuvant (GERBU Adjuvant 100, Gerbu Biotechnik) into a Chinchilla bastard rabbit (Johnstone and Thorpe, 1987). Booster shots were given weekly containing the same amount of protein mixed with the same adjuvant. The rabbit was bled 7 days after the fourth booster shot. After clotting and centrifugation the serum was collected and used without further purification.

Preparation of halobacterial protein extracts of strains S9, Δ*car* and Δ*car/car*$^+$, fractionation of cell lysates and Western blot analysis were

performed as described (Rudolph *et al*., 1996). The anti-Car polyclonal antiserum was used at a dilution of 1:1000 for Western blots.

Capillary assay

Halobacterial cells were grown under standard conditions to a density of ~ 5×10^8 cells/ml washed three times (20 000 *g*, 3 min) with chemotaxis buffer, CBS [halobacterial complete medium (Oesterhelt and Stoeckenius, 1974) without peptone, buffered with 20 mM HEPES pH 7.0] and diluted with CBS to a density of 1×10^8 cells/ml. Aliquots of 200 µl cell suspension were placed in a microtiter plate (96 holes, Falcon) and the compartments of a second microtiter plate (washing plate) were filled with aliquots of 300 µl CBS.

Capillaries (10 µl micropipettes, Brandt) were closed at one end in a flame and pulled through the flame 10 times before they were placed in 2 ml reaction vessels (Eppendorf), which contained 200 µl CBS or the test substances dissolved in CBS. The capillaries and the microtiter plates were incubated at 37°C for 30 min before the capillaries were pushed through holes of a special cover to dip into the buffer solution of the washing plate. Then the cover with the capillaries was placed onto the cell plate and incubated for 2 h at 37°C in the dark. Finally, the cover with the capillaries was placed again onto the washing plate, the capillaries were removed, cut below the closed end and the liquid was blown into 1 ml CBS (1.5 ml reaction vessels, Eppendorf). After dilution 1:10 in CBS, aliquots of 50 µl were streaked onto 1.5% agar plates with complete medium and incubated for 5 days at 40°C. The number of cells which migrated into the capillary during the chemotactic assay was calculated from the numbers of colonies on the plates. Each compound was tested in four parallel experiments.

Chemical-in-plug assay

Cell cultures grown to a density of ~7×10^8 cells/ml were pelleted for 20 min at 3000 *g* and resuspended in CBS to a density of 1×10^9 cells/ml. The suspension was warmed to 40°C and mixed with the 0.5% (w/v) agar solution in CBS of the same temperature and filled into Petri dishes. Agar plugs, made from 2% agar (w/v) in CBS, containing either CBS alone or the compounds to be tested, were pushed into the soft agar at room temperature and the Petri dishes were incubated for at least 4 h at 40°C in the dark.

In vivo labeling of halobacterial transducer proteins with [methyl-^{3}H]methionine, analysis of adaptive release of volatile methyl groups and fluorographic detection of the methylated proteins

Labeling of the cells with [methyl-^{3}H]methionine and analysis by a flow assay of volatile products released from labeled cells were essentially performed as described in Alam *et al*. (1989). Briefly, cells were grown to a density of ~5×10^8 cells/ml, washed in CBS and suspended at the same density in CBS supplemented with 130 µg/ml of puromycin. After incubation for 30 min at 37°C, L-[methyl-^{3}H]methionine (Amersham) with a specific activity of 80 Ci/mmol was added to a final concentration of 0.25 µM and incubation was continued for another 45 min. Cells were washed free from radiolabel with CBS, pumped onto a sterile filter (0.22 µm pore size) and subjected to a continuous flow (1.5 ml/min) of 0.1 mM unlabeled methionine in CBS (chase medium). Fractions collected for periods of 18 s (500 µl) were analyzed for volatile radioactivity. For detection of methylated proteins, cells were radiolabeled as described above and proteins were fractionated and extracted as described (Rudolph *et al*., 1996). SDS–PAGE of the protein preparations and fluorography of the gels was carried out in accordance with Alam *et al*. (1989).

Isolation of membrane vesicles and transport assays

Halobacterium salinarum S9 cells grown to a density of ~7×10^8 cells/ml were pelleted for 45 min at 6000 *g* and resuspended in transport buffer, TBS [halobacterial complete medium (Oesterhelt and Stoeckenius, 1974) without citrate and peptone, buffered with 50 mM HEPES pH 7.0] to a density of 0.25×10^9 cells/ml. The suspension was frozen in liquid nitrogen, thawed to room temperature and stirred for 1 h upon addition of DNase I (final concentration 0.1 mg/ml) (Oesterhelt and Stoeckenius, 1974). After low speed centrifugation (6000 *g* for 20 min, 4°C) membrane vesicles were collected (160 000 *g* for 1 h, 4°C) and suspended in TBS to a final protein concentration of 15 mg/ml. The supernatant of the ultracentrifugation step (crude lysate) was used for assaying ADI activity. In order to confirm that the vesicles were intact and not leaky, bacteriorhodopsin (BR)-mediated proton pumping upon illumination was examined and the specific activity was found to be 70 H^+/min·BR at pH 7.0.

For exchange experiments, membrane vesicles were washed and equilibrated for at least 12 h at 4°C in TBS supplemented with 0.2–1.0 mM ornithine or other solutes in a final volume of 1.5–5.0 ml (0.5–3.0 mg protein). Vesicles were diluted with TBS to 20 ml and pelleted by centrifugation (200 000 *g* for 30 min, 4°C). The membrane vesicles were washed once with 1 ml TBS and concentrated to 30 mg/ml of protein. Then, small aliquots of the suspension (4 µl) were rapidly diluted into TBS (400 µl) supplemented with labeled substrate. At the time intervals indicated, samples were rapidly diluted with 1 ml TBS, filtered (cellulose nitrate membranes, 0.45 µm pore size, 2.5 cm diameter, Schleicher & Schuell) and washed twice with 1 ml TBS. Radioactivity retained on the filters was determined by liquid scintillation spectroscopy. All transport studies were performed at 0°C. Kinetic parameters as well as competitive inhibitor constants were estimated from Eadie–Hofstee and Hanes–Woolf plots (Dixon and Webb, 1979). All initial rate measurements were performed in triplicate and normalized on the basis of protein content. L-[4,5-^{3}H]arginine (3.9 TBq/mmol) and L-[U-^{14}C]ornithine (9.5 GBq/mmol) were obtained from ICN Pharmaceuticals. L-[Guanido-^{14}C]arginine (1.9 GBq/mmol) was purchased from DuPont NEN.

ADI activity assay and protein determination

Crude lysate from S9 cells obtained by the freeze–thaw method as described above was diluted with TBS buffer to a final protein concentration of 0.25 mg/ml. The reaction was started upon addition of arginine and ADI activity was measured as the amount of citrulline formed by using a colorimetric assay essentially as described by Oginsky (1957). Initial rates were calculated from citrulline formed in the time interval 30–60 min after starting the assay. Protein concentrations of crude lysates and membrane vesicles were determined with the BCA-Protein Assay Reagent (Pierce). Solutions of bovine serum albumin in TBS were used as standard.

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