ELSEVIER

Biochimica et Biophysica Acta 1505 (2001) 131–143

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.bba-direct.com

Review

Towards the molecular mechanism of Na^+/solute symport in prokaryotes

Heinrich Jung *

Universität Osnabrück, Fachbereich Biologie/Chemie, Abteilung Mikrobiologie, D-49069 Osnabrück, Germany

Received 28 July 2000; received in revised form 19 October 2000; accepted 14 November 2000

Abstract

The Na^+/solute symporter family (SSF, TC No. 2.A.21) contains more than 40 members of pro- and eukaryotic origin. Besides their sequence similarity, the transporters share the capability to utilize the free energy stored in electrochemical Na^+ gradients for the accumulation of solutes. As part of catabolic pathways most of the transporters are most probably involved in the acquisition of nutrients. Some transporters play a role in osmoadaptation. With a high resolution structure still missing, a combination of genetic, protein chemical and spectroscopic methods has been used to gain new insights into the structure and molecular mechanism of action of the transport proteins. The studies suggest a common 13-helix motif for all members of the SSF according to which the N-terminus is located in the periplasm and the C-terminus is directed into the cytoplasm (except for proteins containing a N- or C-terminal extension). Furthermore, an amino acid substitution analysis of the Na^+/proline transporter (PutP) of *Escherichia coli*, a member of the SSF, has identified regions of particular functional importance. For example, amino acids of TM II of PutP proved to be critical for high affinity binding of Na^+ and proline. In addition, it was shown that ligand binding induces widespread conformational alterations in the transport protein. Taken together, the studies substantiate the common idea that Na^+/solute symport is the result of a series of ligand-induced structural changes.

Keywords: Secondary transport; Sodium/solute symport; Sodium/proline transporter PutP

1. Introduction

Due to the function of (Na^+/K^+)-ATPase, Na^+ is the predominant coupling ion in secondary transport in animal cells. In prokaryotes, Na^+ is employed as coupling ion particularly by cells living in extreme environments like elevated temperatures, high pH or high external Na^+ concentrations [1,2]. These cells use primary Na^+ pumps (Na^+-dependent electron transport complexes, decarboxylases, ATPase) and Na^+/H^+ antiporter(s) to generate Na^+-electrochemical gradients [3]. Although in neutrophilic bacteria solute uptake by secondary transport systems is mainly coupled to H^+, accumulation of some solutes is energized by electrochemical Na^+ gradients. In *Escherichia coli*, for example, transport of melibiose, proline, pantothenate and glutamate are coupled to Na^+. The necessary Na^+-electrochemical gradient is established by the action of Na^+/H^+ antiporter(s) [4] (Fig. 1).

Abbreviations: AZT, L-acetidine-2-carboxylate; cL, putative cytoplasmic loop; DAACS, dicarboxylate/amino acid-cation symporter family (TC No. 2.A.23); DHP, 3,4-dehydro-D,L-proline; GHP, glycoside-pentose-hexuronide family (TC No. 2.A.2); NSS, Na^+/neurotransmitter symporter family (TC No. 2.A.22); pL, putative periplasmic loop; SSF, sodium/solute symporter family (TC No. 2.A.21); TM, putative transmembrane domain

* Fax: +49-541-969-2870;
E-mail: jung_h@biologie.uni-osnabrueck.de

0005-2728/01/$ – see front matter
PII: S0005-2728(00)00283-8

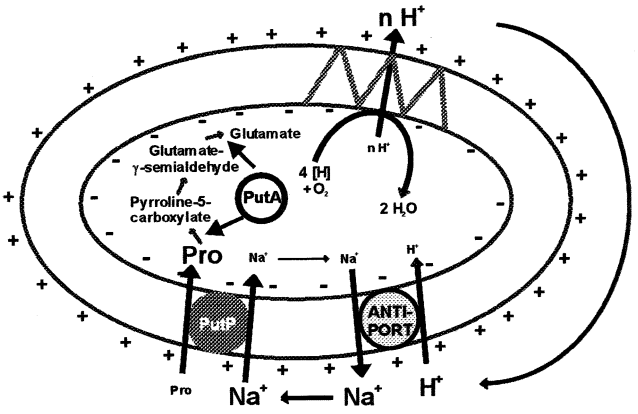

Fig. 1. Role of PutP in *E. coli*. PutP catalyzes the Na^+ coupled uptake of proline with a stoichiometry of 1:1 [57–60]. The electrochemical Na^+ gradient required to energize proline transport is generated by the action of Na^+/H^+ antiporter(s). Subsequent to the uptake process proline is oxidized via pyrroline 5-carboxylate to L-glutamate [14]. The two oxidation steps are catalyzed by a single protein, the product of the *putA* gene, which also acts as a repressor of *put* operon transcription in the absence of L-proline [111].

The ever growing number of known transport proteins including Na^+-dependent cotransporters is classified in different families based on their sequence similarities. There are mixed families of cotransporters that despite their sequence similarity accept either Na^+ or H^+ or both as coupling ion. Examples are the glycoside-pentose-hexuronide (GPH) family (identification number according to the transporter classification (TC) system: No. 2.A.2) and the dicarboxylate/amino acid-cation symporter (DAACS) family (TC No. 2.A.23) [5–8]. Other transporters fall into distinct families which either contain only Na^+ or H^+ cotransporters [9]. The Na^+/solute symporter (SSF) family (or SGLT/PutP family, TC No. 2.A.21) and the Na^+/neurotransmitter symporter (NSS) family (TC No. 2.A.22) are examples for families of Na^+-dependent cotransporters [5,8–10].

Lots of efforts are currently made to elucidate the structure and molecular mechanism(s) of function of these and other transport proteins. Unfortunately, structural information at the atomic level is not available yet for any secondary transporter. Besides intensive attempts at crystallization, genetic, protein chemical, spectroscopic and kinetic methods are employed to obtain new insights into the architecture and way(s) of energy transduction of the transporters.

This article summarizes recent findings on the structure and function of members of the SSF, thereby focusing on the prokaryotic part of the family. The results are discussed with respect to achievements and models for other secondary transporters.

2. Prokaryotic members of the SSF

The SSF currently comprises more than 40 similar proteins from archaea, bacteria, yeast, insects, and mammals [10]. The proteins catalyze the following general transport process:

$$nNa^+_{(out)} + solute_{(out)} \leftrightarrows nNa^+_{(in)} + solute_{(in)}$$

Under physiological conditions usually an inwardly directed electrochemical Na^+ gradient ($\Delta\tilde{\mu}_{Na^+} = RT\ln([Na^+_{(out)}]/[Na^+_{(in)}]) - F\Delta\Psi$, where F is the Faraday constant, $\Delta\Psi$ is the membrane electrical potential difference, R is the gas constant and T is the absolute temperature) is used to drive the uphill transport of a solute [11,12]. Among the eukaryotic members of the SSF are the human transporters for glucose (SGLT1), nucleosides (SNST1), *myo*-inositol (SMIT1) and iodide (NIS) [10]. The known prokaryotic members of the SSF use $\Delta\tilde{\mu}_{Na^+}$ to specifically accumulate proline, galactose (glucose), pantothenate or phenyl acetate in cells (Table 1). PanF, the Na^+/pantothenate transporter of *E. coli* and other prokaryotes, scavenges extracellular pantothenate for coenzyme A biosynthesis in the cells [13]. Most of the other characterized transporters are part of catabolic pathways and are used for the acquisition of the corresponding solute as carbon source [14–17]. For example, the *putP* gene encoding the Na^+/proline transporter of *E. coli* and *Salmonella typhimurium* is part of the *put* operon which also encodes a proline dehydrogenase (product of *putA* gene) allowing the use of proline as a source of nitrogen and/or carbon [14,18] (Fig. 1). Expression of the transporter genes is usually induced by the specific solute or some product thereof.

In this context, OpuE, a Na^+/proline transporter of *Bacillus subtilis*, is an exception since it serves osmoprotective purposes and is apparently not involved in a catabolic pathway [19,20]. While the activity of the catabolic Na^+/proline transporter PutP

Table 1
Prokaryotic members of the SSF (TC No. 2.A.21)

Transported solute	Organisms	Gene name	Accession No.	Reference
proline	*E. coli*	*putP*	P07117	[21,37]
	S. typhimurium	*putP*	P10502	[59,97]
	Pseudomonas putida	*putP*	Q9R9T6	[16]
	Pseudomonas fluorescens	*putP*	Q57023	[98]
	K. pneumoniae	*putP* (?)[a]	P23723	[112]
	Helicobacter pylori	*putP* (?)	O24896	[99]
	Campylobacter jejuni	*putP* (?)	Q9PMG1	[113]
	Neisseria meningitidis	*putP* (?)	Q9JR87 (preliminary)	[114]
	Vibrio cholerae	*putP* (?)	–[b]	[115]
	Haemophilus influenzae	*putP* (?)	P45174	[100]
	Rickettsia typhi	*putP* (?)	Q53255	[96]
	S. aureus	*putP*	O30986	[26,101]
	C. glutamicum	*putP*	O32355	[15]
	B. subtilis	*opuE*	O06493	[20,24]
		ycgO (?)	P94392	[20,24]
	Chlamydia pneumoniae	*putP* (?)	Q9Z865	[102]
	Archaeoglobus fulgidus	*putP*-1 (?)	O30220	[103]
		putP-2 (?)	O29293	
		putP-3 (?)	O29046	
	Pyrococcus horikoshii	*putP* (?)	O59086	[104]
	Methanobacterium thermoautotrophicum	*putP* (?)	O27884	[105]
pantothenate	*E. coli*	*panF*	P16256	[13]
	H. influenzae	*panF* (?)	P44963	[100]
	Rickettsia prowazekii	*panF* (?)	Q9ZCY0	[106]
	Borrelia burgdorferi	*panF* (?)	O51754	[107]
	A. fulgidus	*panF*-1 (?)	O29193	[103]
		panF-2 (?)	O29063	
		panF-3 (?)	O30002	
phenyl acetate	*P. putida*	*ppa*	O50471	[17,108]
galactose (glucose)	*Vibrio parahaemolyticus*	vSGLT	P96169	[109]
?	*Synechocystis* sp.	Na^+/solute symporter (?)	P74252	[110]

[a]The functional assignment is based on sequence similarities.
[b]*V. cholerae El Tor* N16961 gene locus VCA1071 [116].

of *E. coli* and *S. typhimurium* is actually reduced when the cells are grown under high osmolarity conditions, OpuE activity is strongly enhanced in hypertonic medium, thereby highlighting the distinct physiological functions of the PutP and OpuE proteins [20,21]. The osmoregulated proline transport activity of OpuE is entirely dependent on de novo protein synthesis and is controlled at the transcriptional level [19,20]. Osmosensory properties as observed for the osmoprotectant uptake systems ProP of *E. coli* or BetP of *Corynebacterium glutamicum* could not be detected for OpuE [20,22,23]. The genome sequencing project of *B. subtilis* revealed that the bacterium probably contains a second proline transporter, YcgO, which is highly similar to OpuE and probably involved in proline catabolism [24] (E. Bremer, personal communication). Thus, two closely related proteins are obviously used for different physiological functions by putting their structural genes under the control of different regulatory schemes [20]. Beside OpuE of *B. subtilis*, PutP-1 of the hyperthermophilic archaeon *Pyrococcus abyssi* (28% identical amino acids with OpuE of *B. subtilis*) is postulated to be osmoregulated [25].

Interestingly, some of the prokaryotic members of the SSF may represent new targets for the development of new drugs against pathogens. Thus, the high affinity Na^+/proline transporter PutP of *Staphylococcus aureus* contributes to in vivo survival of this causative organism of a wide variety of human infections

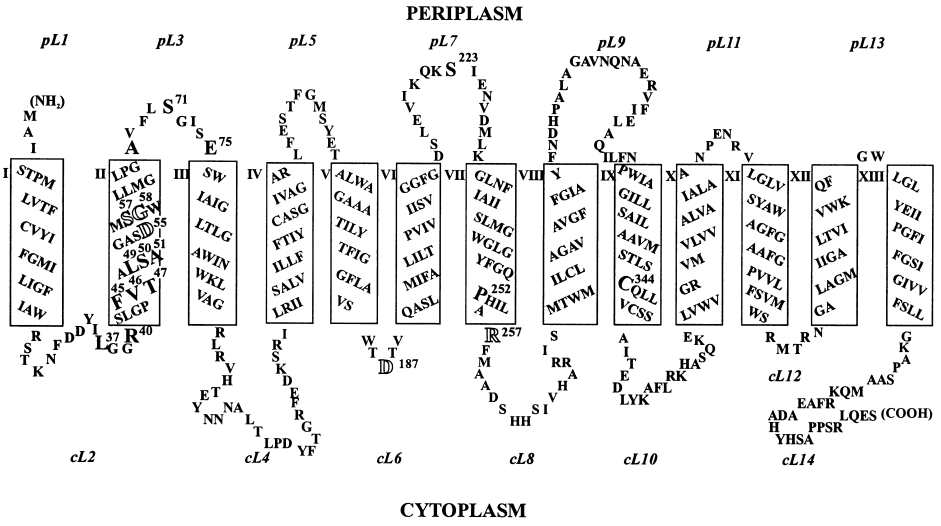

Fig. 2. Secondary structure model of PutP of *E. coli* (according to [39,42]). Putative transmembrane domains are represented as rectangles and numbered with Roman numerals; loops are numbered with Arabic numerals starting from the N-terminus. Amino acid residues shown to be critical for Na^+ and/or proline binding are indicated by open letters. Residues proposed to participate in conformational alterations are shown as bold letters.

[26]. It could be shown that insertional inactivation of the *putP* gene of *S. aureus* leads to significantly reduced virulence in experimental endocarditis [27].

3. Transporter structure

Understanding the molecular mechanism of function of the transport proteins requires detailed structural information. Indeed, X-ray crystallography has yielded major advances in the understanding of channel-type proteins and both light- and electron flow-driven ion pumps [28–31]. However, as stated above a major problem in the field of secondary transport is the difficulty inherent in obtaining structural information on the proteins involved at a relevant level of resolution. The first direct glimpse into the structure of a secondary transporter was provided by two-dimensional crystallization and electron cryo-microscopy of the Na^+/H^+ antiporter NhaA of *E. coli* [32]. Based on these studies a three-dimensional map of NhaA was produced that reveals 12 tilted, bilayer spanning helices. A roughly linear arrangement of six helices is adjacent to a compact bundle of six helices, with the density for one helix in the bundle not continuous through the membrane [33]. Besides intensive trials to crystallize the transporters, cysteine cross-linking studies and site-directed labeling analyses in combination with spectroscopic methods are used to obtain information on helix packing in secondary transport proteins like the lactose permease of *E. coli* [34–36]. Information on tertiary interactions within SSF proteins is not available yet. However, knowledge on the secondary structure of the polypeptide chains and their topological arrangement in the membrane has significantly improved in the last couple of years.

The average hydropathy plot for SSF proteins predicts 11–15 transmembrane domains (TMs) in α-helical conformation [9]. Most experimental evidence on the topological arrangement comes from studies on the Na^+/proline transporter PutP of *E. coli* (Fig. 2). PutP has originally been proposed to consist of a short hydrophilic N-terminal region, 12 transmembrane domains in α-helical conformation that traverse the membrane in zigzag fashion connected by hydrophilic loops, and a hydrophilic C-terminal tail [37]. According to the model 60% of the 502 amino acids of PutP are found in hydrophobic domains with a mean length of 24 amino acids. Circular dichroic measurements of purified and reconstituted PutP indicate that the protein is about 63% α-helical in conformation thereby approaching the predicted value (T. Pirch, J. Greie and H. Jung, unpublished).

The 12-helix model of PutP has further been tested by applying a gene fusion approach, immunological

and site-directed labeling techniques. Analysis of a series of *putP-phoA* and *putP-lacZ* fusions yields a reciprocal activity pattern of the reporter proteins alkaline phosphatase (PhoA) and β-galactosidase (LacZ) that is in agreement with the topology of TMs III–XII of the 12-helix model [38,39]. However, differing from the topology prediction, the fusion analysis suggests a shift of the boarders of putative TM II by eight amino acids towards the C-terminus, thereby creating a large preceding periplasmic loop. Placement of PutP-PhoA junction points into this loop does not yield conclusive results. Therefore, a cysteine accessibility analysis was performed to determine the topology of the N-terminal part of PutP. Surprisingly, cysteine residues placed close to the N-terminus of PutP (Ile3→Cys, Thr5→Cys) are highly accessible to membrane permeant and impermeant thiol reagents from the periplasmic space. In contrast, cysteine at the C-terminus is accessible only from the cytosolic side of the membrane as indicated also by immunological studies [39,40]. These results contradict the 12-helix motif and indicate a periplasmic location of the N-terminus while the C-terminus faces the cytoplasm. In addition, the accessibility pattern of cysteine residues placed into the periplasmic loop (pL) preceding TM II (pL 2) suggests the formation of an additional TM formed by amino acids of this loop (Fig. 2). The results are in good agreement with site-specific proteolysis studies performed with unidirectionally reconstituted PutP [39,41].

Finally, site-directed spin labeling and electron paramagnetic resonance (EPR) spectroscopy were used to test the altered structural arrangement of PutP [42]. Information on spin label topography was obtained by analyzing the residual mobility of site-specifically attached nitroxide side chains and determination of collision frequencies of the nitroxide with non-polar oxygen and polar CROX. The method is well established with a variety of membrane proteins [43–47]. For PutP, the measurements indicate that TM II is composed of amino acids Ser41 to Gly66 while TM III (TM II in the 12-helix model) comprises residues Ser76 to Gly95 [42] (Fig. 2).

Based on these studies a new secondary structure model of PutP was proposed according to which the protein consists of 13 TMs with the N-terminus on the periplasmic side of the membrane and the C-terminus facing the cytoplasm [39,42] (Fig. 2). An additional TM is formed by amino acid residues of former pL 2 placing the functionally important residues Asp55 and Ser57 (see below) from a periplasmic loop region into a TM (now TM II). The new secondary structure model of PutP is in agreement with the recently proposed topological arrangement of TMs I to XIII of SGLT1 which is based on a *N*-glycosylation study [48]. The 13-helix feature was also verified for the mammalian Na^+/I^- transporter [49]. Taken together, the results support the idea of a common topological motif for members of the SSF according to which, for example, the bacterial transporters for proline (PutP) and pantothenate (PanF) and the mammalian Na^+/I^- transporter are composed of 13 TMs. Transporters with a C-terminal extension (for example, the human SGLT1 and the Na^+/*myo*-inositol transporter (SMIT1)) are proposed to have an additional 14th TM [10,48].

The periplasmic location of the N-terminus is an unexpected feature of the 13-helix motif implying that, beginning from the N-terminus, the transporter starts with a stop transfer sequence and not a signal anchor sequence. The mechanism of insertion of the polypeptide chain is not known. For PutP of *E. coli* it can be stated that despite the N-terminus itself the stretch of the first 27 amino acids of PutP is highly hydrophobic and does not contain any charged residues. The N-terminal primary structures of PutP and PanF of other bacteria and of vSGLT are similar. So, it can be speculated that the energy required to translocate the short N-terminal tail of PutP and other members of the SSF across the membrane is gained from the transition of hydrophobic TM I from the aqueous phase into the apolar phase of the bilayer. Indeed, analysis of hybrid proteins generated from *E. coli* leader peptidase and the phage Pf3 coat protein reveals that short tails which do not contain positively charged residues are efficiently translocated independent of the Sec machinery [50]. The cytoplasmic loop that follows the inwardly directed TM I according to the 13-helix model contains three positively and two negatively charged residues. Altogether, the new topological arrangement fits very well the positive inside rule [51].

As far as investigated, the 13-helix motif appears to be a special feature of the SSF. The 12-helix arrangement is most common among secondary trans-

porters including Na^+-dependent systems [5]. The melibiose permease of *E. coli*, a member of GPH family (TC No. 2.A.2), is a well studied example for a 12-helix transporter able to catalyze Na^+/solute cotransport [52,53]. However, different TM numbers are not exceptional [5,7]. For example, a specific topological model in which seven α-helical TMs are followed by a reentrant loop-pore structure followed by one final TM is proposed for members of the DAACS family (TC No. 2.A.23) [7,54], and an 11 TM topology has been experimentally documented for the *Klebsiella pneumoniae* Na^+/citrate transporter CitS, a member of the cation/citrate symporter (CCS) family (TC No. 2.A.24) [55,56].

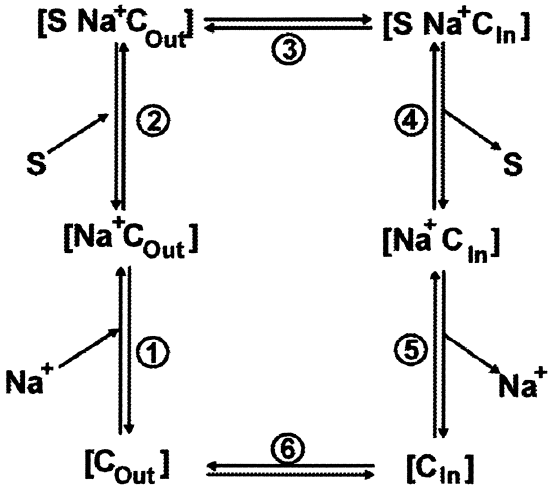

Fig. 3. Ordered binding model of Na^+/solute symport (based on [71–74]; see text for details).

4. Functional properties of PutP

PutP of *E. coli* is the best characterized prokaryotic member of the SSF, and its main functional properties are therefore briefly summarized here. The gene product is an integral protein of the cytoplasmic membrane and catalyzes the coupled translocation of proline and Na^+ with a stoichiometry of 1:1 [57–60]. PutP is highly specific for proline and has to be discriminated from ProP and ProU which transport proline but also glycine betaine and other betaines in response to osmotic stress [61–65]. ProP is a secondary, H^+-linked transport system and belongs to the major facilitator superfamily (TC No. 2.A.1) [66]. The isolated protein has recently been shown to act as an osmosensor and an osmoregulator [22]. ProU is a binding protein-dependent ABC transporter which preferentially transports glycine betaine and shows a relatively low affinity and uptake rate for proline compared to glycine betaine [61].

The *putP* gene was cloned and sequenced [37,67,68], and the gene product has been solubilized from the cytoplasmic membrane, purified, reconstituted into proteoliposomes, and shown to be solely responsible for Na^+/proline transport [41,69,70]. Li^+ can substitute for Na^+; however, despite earlier assumptions H^+ driven proline uptake by PutP could not be demonstrated [57,58]. In the cell, Na^+/proline symport is thermodynamically coupled to Na^+/H^+ antiport, and in this way PutP is part of the Na^+ circuit of *E. coli* (Fig. 1).

The K_m values for Na^+ and proline uptake by PutP were determined with 30 and 2 μM, respectively [18]. Kinetic analyses of Na^+/solute symport catalyzed by different members of the SSF including PutP suggest that transport occurs according to an ordered binding mechanism [71–74] (Fig. 3). In this scheme, Na^+ binds to the empty transporter first thereby inducing a conformational alteration which increases the affinity of the transporter for the solute. The formation of the ternary complex induces another structural change that exposes Na^+ and solute to the other site of the membrane. Solute and Na^+ are released and the empty transporter reorientates in the membrane allowing the cycle to start again.

5. Sites important for Na^+ and/or solute binding

Labeling experiments, random and site-directed mutagenesis have been employed to identify functionally important sites in members of the SSF. Most comprehensive studies are available for PutP of *E. coli* and *S. typhimurium*.

To identify residues involved in cation binding, *putP* mutants that confer resistance to Li^+ during growth on proline were isolated. The location of each mutation was determined by deletion mapping: the mutations cluster in two small deletion intervals at the 5′ and 3′ termini of the *putP* gene [75]. Recent site-directed mutagenesis studies have established that amino acids of TM II of PutP (Fig. 2) are of particular functional importance, thereby strongly

supporting the idea that the N-terminal part of the transporter is responsible for Na^+ binding. Thus, out of five acidic residues in the N-terminal domain analyzed, the carboxylate of Asp55 (TM II) proved to be essential for transport while Asp33, Asp34 (both cytoplasmic loop (cL) 2), and Glu75 (pL 3) were dispensable for function [76]. Significant albeit highly reduced activity was detected with Glu in place of Asp55 (PutP-D55E). Kinetic analysis of active transport catalyzed by the latter PutP derivative revealed a 50-fold decrease of the apparent affinity of the protein for sodium ions compared to the wild-type transporter. On the other hand, only a relatively small alteration of the apparent affinity for proline was observed [76]. Furthermore, Na^+ binding could not been detected for PutP missing a carboxylate at position 55. These results suggest that Asp55 is located at or close to a binding site of the coupling ion.

In addition, individual replacement of Met56, Ser57 or Gly58 following Asp55 in TM II led to significantly altered transport kinetics. Thus, substitution of Ser57 with Ala, Cys, Gly, or Thr increased the apparent K_m values for Na^+ (and Li^+) AND proline by up to two orders of magnitude with little influence on V_{max} values [77]. Similarly, Cys in place of Met56 or Gly58 caused an increase in the apparent K_m values for Na^+ AND proline although the extent of the effect was less dramatic as in case of Ser57 (M. Nietschke, S. Landmeier, M. Quick and H. Jung, unpublished). These results support the idea that the sites of Na^+ and proline binding are overlapping. The latter idea is in agreement with findings on melibiose permease of *E. coli* implicating Asp55 of the sugar transporter in the binding of both ion and substrate [78]. However, in the case of PutP, Cys accessibility analyses challenge the idea of Ser57 and Gly58 being part of a Na^+ binding site. Thus, the reaction rate of a Cys placed at the positions of Ser57 and Gly58 of PutP with sulfhydryl-specific reagents was not blocked by Na^+ as expected for residues being directly involved in binding of the ligand but significantly stimulated by the ion (T. Pirch and H. Jung, unpublished). Obviously, ion binding to a site different from Ser57 and Gly58 induced a conformational alteration that increased the accessibility of the amino acids at positions 57 and 58. These observations make a direct involvement of Ser57 and Gly58 in Na^+ binding unlikely. The accessibility of Cys at the position of Met56 was not significantly influenced by Na^+.

In the *Propionigenium modestum* F_1F_0 ATP synthase Gln32, Glu65 and Ser66 of subunit c were shown to be Na^+ binding ligands [79]. In view of these findings, Ser54 which precedes Asp55 in TM II of PutP was another candidate for a Na^+ liganding group of the transporter. Replacement of Ser54 with Cys yielded an about 8-fold decrease of the apparent affinity of PutP for Na^+ with no significant effect on the proline affinity. Na^+ did not significantly affect the Cys accessibility at position 54 similar as observed for Cys in place of Met56 [39] (T. Pirch, M. Nietschke and H. Jung, unpublished). Clearly, further substitution analyses are necessary to clarify the role of Ser54 and Met56 in Na^+ binding.

Arg40 (cytoplasmic end of TM II) of PutP which is conserved within the SSF was also subjected to amino acid substitution analysis [80]. Removal of the positive charge at position 40 (PutP-R40C, Q, E) led to a dramatic decrease of the turnover numbers for Na^+-coupled proline uptake (1–10% of PutP-wild-type). The reduced transport rates were accompanied by decreased apparent affinities of the transporter for Na^+ and Li^+ while the apparent affinity for proline was only slightly altered. Furthermore, single Cys PutP-R40C reacted with *N*-ethylmaleimide (NEM), and this reaction was partially inhibited by proline and more efficiently by sodium ions. Remarkably, NEM modification of Cys40 inhibited Na^+-driven proline uptake almost completely while facilitated influx of proline into de-energized cells was stimulated by this reaction indicating an at least partially uncoupled phenotype under these conditions [80]. These results suggest that Arg40 is located close to the site of ion binding and is important for the coupling of ion and proline transport.

Besides the discussed role of amino acids of TM II in Na^+ liganding, some residues in this segment proved to be important for proline binding. As already described above, even conservative replacements for Ser57 and Gly58 caused a dramatic decrease of the apparent affinity of PutP for proline. In addition, substitution of Ser57 reduced the sensitivity of *E. coli* cells to the toxic proline analogues L-acetidine-2-carboxylate (AZT) and 3,4-dehydro-D,L-proline (DHP) [77]. Remarkably, while Na^+ in-

creased the accessibility of Cys in place of Ser57 or Gly58, proline inhibited the reaction of the residue with sulfhydryl-specific reagents at both positions, thereby reversing the Na^+ effect almost completely (T. Pirch and H. Jung, unpublished). Taken together the findings propose a location of Ser57 and Gly58 at or close to the proline binding site of PutP. Clearly, it cannot be excluded that protection of Cys at positions 57 and 58 is achieved by a proline-induced conformational alteration and not by direct steric hindering. In any case, it is very likely that Ser57 and Gly58 participate in the transmission of a Na^+-induced conformational alteration leading to high affinity proline binding to a site that is at least close to these positions in the tertiary structure of the transporter. The observations confirm the functional importance of TM II described above.

The proposed participation of TM II in proline binding apparently contradicts results obtained with SGLT1, suggesting that the C-terminal domain is responsible for substrate binding and translocation. Thus, analysis of chimeras consisting of sequences of the high affinity transporter SGLT1 and the low affinity transporter SGLT2 suggest that recognition/transport of organic substrate is mediated by interactions distal to amino acid 380 [81]. In addition it was shown that a SGLT1 fragment comprising only the last five TMs of the transporter is able to catalyze Na^+-independent facilitated diffusion of sugar [82,83]. However, it has to be taken into account that the sugar affinity of the C-terminal fragments of SGLT1 tested was highly reduced, thereby leaving the possibility that amino acid residues of the N-terminal part of the protein contribute to high affinity binding of the organic substrate.

The idea that multiple regions of PutP contribute to solute binding is supported by the analysis of *putP* mutants with an altered specificity for proline, AZT and DHP [84]. Deletion mapping of the mutants revealed that the mutations cluster in three distinct regions of the *putP* gene. One set of mutants was mapped at the distal end of the *putP* gene, thereby implicating the C-terminal region of PutP in substrate recognition.

Besides amino acid residues of TM II, Asp187 (cL 6) and Arg257 (cL 8) of PutP were shown to be of functional importance [85,86] (Fig. 2). The residues are conserved within members of the SSF. Substitution of Asp187 yielded highly reduced transport rates which in case of PutP-D187C could be stimulated by acetylation or amidoacetylation of the engineered Cys [85]. In contrast to the wild-type, proline binding to PutP-D187C was apparently independent from Na^+. In addition, reaction of PutP containing a single Cys at position 187 with NEM was inhibited by Na^+ but not by Li^+ or proline. The results indicate that electrostatic interactions of the amino acid side chain at position 187 in PutP with other parts of the transporter and/or the coupling ion are crucial for active proline transport. It was suggested that Asp187 is located close to the pathway of the coupling ion through the membrane and may be involved in the release of Na^+ on the cytoplasmic side of the membrane [85]. A reduced Na^+ dependence of proline binding was also observed upon replacement of Arg257 with Cys [86]. The authors speculate that removal of the positive charge leads to an enhanced affinity of PutP for Na^+. However, the effect of the substitution of Arg257 on proline uptake was much less dramatic than in case of Asp187.

Originally, the native Cys residues at positions 281 (TM VIII) and 344 (TM IX) of PutP were identified as substrate-protectable residues and suggested to reside close to the binding site for the coupling ion and/or proline [87,88]. However, replacement of these Cys residues neither affected proline uptake nor altered the sensitivity of *E. coli* cells to the toxic proline analogue AZT significantly, thereby indicating that the two Cys residues may not be directly involved in binding [87,88]. Finally, a fully functional PutP molecule devoid of all five native Cys residues was constructed, demonstrating that Cys residues are not important for the function of PutP similar as shown for other secondary transporters [39,89,90].

Finally, alignment of amino acid sequences of members of the SSF led to the proposal of a sodium binding (SOB) motif which has the following consensus sequence: $GX_{35-37}AX_3(EQ)LX_3GR$ [9]. In PutP of *E. coli* the conserved amino acids are Gly328 (TM IX), Ala366, Glu370 (cL 10), Leu371, Gly375 and Arg376 (TM X). However, a substitution analysis of Arg376 reveals that the residue does not reside at the Na^+ binding site, thereby suggesting that the proposed motif is not essential for function [91].

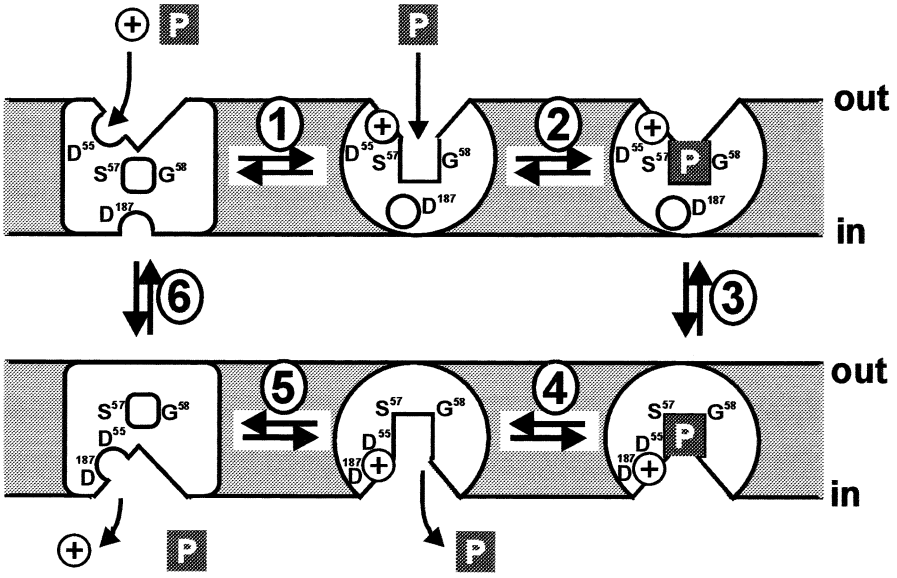

Fig. 4. Model of Na^+/proline symport by PutP of *E. coli*. Transport is proposed to proceed according to the following consecutive steps. (1) Binding of Na^+ to the empty transporter at or close to Asp55 in TM II of PutP. Binding of the ion induces a conformational change that involves at least TM II and the adjoining loops. (2) High affinity proline binding to the transporter at or close to Ser57 and Gly58 of TM II. (3) Conformational alterations of the ternary complex involving at least TM II, VII, IX and adjoining loops expose the ligand binding sites to the other site of the membrane. (4) Release of proline and (5) Na^+ whereby Asp187 in cL 6 plays a critical role. (6) Conformational alteration of the empty transporter to allow the cycle to start again (see text for further information).

6. Ligand-induced conformational alterations

The ordered binding model implies that Na^+/solute transport is the result of a series of ligand-induced conformational changes in the protein. To explore the nature of these changes protein labeling and spectroscopic methods were used. For PutP, these changes were analyzed by site-directed spin labeling and EPR spectroscopy [42]. The study utilized the sensitivity of nitroxide mobility to tertiary interactions as means of detecting conformational movements in PutP. Analysis of EPR lineshapes revealed ligand-induced mobility changes of nitroxide attached to positions 37 (cL 2) and 45 (TM II) of the transporter. In the case of single Cys PutP-L37C, binding of Na^+ and/or proline to the transporter led to a mobilization of the spin label while the nitroxide at position 45 became less mobile upon addition of proline, and Na^+ alone had no effect. From these findings it was concluded that proline binding induces a conformational alteration of PutP that involves at least parts of TM II and the preceding cytoplasmic loop. Na^+ could only be shown to affect the structure of cL 2 [42].

The EPR studies were confirmed and extended by Cys accessibility studies in the presence and absence of ligands. Thus, Na^+ was shown to increase the accessibility of Cys placed at the position of Ser57, Gly58 (TM II), Ser71 or Glu75 (pL 3). In contrast to Ser57 and Gly58 (see above), proline did not affect the accessibility of Cys at positions 71 and 75. Furthermore, proline inhibited the reaction of sulfhydryl-specific reagents with Cys at the positions of Phe45, Val46, Thr47, Leu49, Ser50 and Ala51 (all TM II) (T. Pirch, M. Langkamp and H. Jung, unpublished). The substrate protected sites are not clustered on one phase of the putative α-helix but distributed around it. Furthermore, substitution of these residues had little or no effect on the proline uptake kinetics, suggesting that in these cases protection was achieved by proline-induced conformational alterations and not by direct steric hindering as discussed for Ser57 and Gly58. Similarly, proline reduced the accessibility of Cys at the positions of Ser223 (pL 7), Pro252 (TM VII), Arg257 (cL8) and Cys344 (TM IX) ([38,39,86–88]; T. Pirch and H. Jung, unpublished). Na^+ alone had no significant effect. Replacement of these amino acids had little

or no effect on the apparent proline affinity of the transporter despite reduced turnover numbers. In addition, analysis of 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid (MIANS)-labeled PutP-S223C revealed significant changes in the fluorescence emission spectrum upon addition of proline if Na^+ was present. The studies suggest that at least parts of TM VII and adjoining loops are involved in structural alterations probably occurring after formation of the ternary complex between transporter, Na^+ and proline (Fig. 4).

For SGLT1 it was shown by similar labeling experiments that the reaction of Cys in place of Gln457 with a sulfhydryl-specific probe requires Na^+, and the reaction is blocked by external sugar [92]. The study implicates Gln457 in conformational alterations that are responsible for the coupling of Na^+ and sugar transport.

The precise nature of the structural alterations in the transport proteins is still unclear. It can be speculated that binding of Na^+ and solute induces changes in helix tilt or helix rotation similar as it has been proposed for ligand-induced helix movements in the lactose permease of *E. coli* [93–95].

7. Conclusions

The SSF is a large family of Na^+/solute cotransporters of pro- and eukaryotic origin. Most of the transporters are most probably required for the acquisition of nutrients. Analyses of secondary structure and membrane topology propose a 13-helix arrangement as a common structural motif for all members of the SSF. Kinetic analyses in combination with amino acid substitution, site-directed labeling and spectroscopic studies provide first glimpses of the sites of ligand binding. Furthermore, the results indicate that the Na^+ and solute binding sites communicate with each other via conformational alterations. For Na^+/proline symport by PutP of *E. coli* the studies support the following model (Fig. 4). In a first step Na^+ binds to the empty transporter at or close to Asp55 in TM II of PutP. Binding of the ion induces a conformational change that involves at least TM II and the adjoining loops. As part of this change Ser57 and Gly58 move from a buried into a highly accessible position (step 1). The structural rearrangement results in high affinity proline binding to the transporter whereby Ser57 and Gly58 either participate directly in proline binding or are located close to the binding site in the tertiary structure of the protein (step 2). The formed ternary complex undergoes another structural alteration that exposes the ligand binding sites to the other site of the membrane. This rearrangement involves at least TM II, VII, IX and adjoining loops (step 3). Subsequently, proline and Na^+ are released whereby Asp187 in cL 6 plays a critical role (steps 4 and 5). In the last step of the cycle, the empty transporter reorientates in the membrane to allow the cycle to start again (step 6). Clearly, additional analyses are required to obtain a complete picture of the sites of Na^+ and proline binding and of the residues forming the translocation pathway. Furthermore, since conformational changes are apparently essential for the transport process more information on the precise nature and the extent of the changes must be obtained, i.e., by double spin labeling techniques or FTIR spectroscopy. Finally, notwithstanding the power of molecular biological, protein chemical and biophysical techniques it is obvious that without high resolution structural information, interpretation of the results of part of the manipulations, particularly those related to the molecular mechanism of energy coupling, will remain speculative. Clearly, new insights into the structure and function of prokaryotic members of the SSF will contribute to a better understanding of the basic biochemical mechanism of energy transduction. Moreover, since some of the transporters proved to be important for bacterial virulence, the studies may also lead to new strategies for the development of drugs against pathogens.

Acknowledgements

This work was financially supported by the Deutsche Forschungsgemeinschaft (SFB 431-D4).

References

[1] B. Tolner, M.E. van der Rest, G. Speelmans, W.N. Konings, in: E. Quagliariello, Palmieri (Eds.), Molecular Mechanisms of Transport, Elsevier Science Publishers, Amsterdam, 1992, pp. 43–50.

[2] P. Dimroth, Antonie Van Leeuwenhoek 65 (1994) 381–395.
[3] P. Dimroth, Biochim. Biophys. Acta 1318 (1997) 11–51.
[4] E. Padan, S. Schuldiner, in: E.P. Bakker (Ed.), Alkali Cation Systems in Prokaryotes, CRC Press, Boca Raton, FL, 1993, pp. 3–24.
[5] M.H. Saier, http://www-biology.ucsd.edu/~msaier/transport/, 2000.
[6] B. Poolman, J. Knol, C. van der Does, P.J. Henderson, W.J. Liang, G. Leblanc, T. Pourcher, I. Mus-Veteau, Mol. Microbiol. 19 (1996) 911–922.
[7] D.J. Slotboom, W.N. Konings, J.S. Lolkema, Microbiol. Mol. Biol. Rev. 63 (1999) 293–307.
[8] M.H.J. Saier, Microbiol. Mol. Biol. Rev. 64 (2000) 354–411.
[9] J. Reizer, A. Reizer, M.H.J. Saier, Biochim. Biophys. Acta 1197 (1994) 133–166.
[10] E. Turk, E.M. Wright, J. Membr. Biol. 159 (1997) 1–20.
[11] G.A. Gerencser, B.R. Stevens, J. Exp. Biol. 196 (1994) 59–75.
[12] B. Poolman, W.N. Konings, Biochim. Biophys. Acta 1183 (1993) 5–39.
[13] S. Jackowski, J.H. Alix, J. Bacteriol. 172 (1990) 3842–3848.
[14] E. McFall, E.B. Newman, in: F.C. Neidhardt (Ed.), *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology, ASM Press, Washington, DC, 1996, pp. 358–379.
[15] H. Peter, A. Bader, A. Burkovski, C. Lambert, R. Krämer, Arch. Microbiol. 168 (1997) 143–151.
[16] S. Vilchez, L. Molina, C. Ramos, J.L. Ramos, J. Bacteriol. 182 (2000) 91–99.
[17] E.R. Olivera, B. Minambres, B. Garcia, C. Muniz, M.A. Moreno, A. Ferrandez, E. Diaz, J.L. Garcia, J.M. Luengo, Proc. Natl. Acad. Sci. USA 95 (1998) 6419–6424.
[18] I. Yamato, Y. Anraku, in: E.P. Bakker (Ed.), Alkali Cation Systems in Prokaryotes, CRC Press, Boca Raton, FL, 1993, pp. 53–76.
[19] F. Spiegelhalter, E. Bremer, Mol. Microbiol. 29 (1998) 285–296.
[20] C. von Blohm, B. Kempf, R.M. Kappes, E. Bremer, Mol. Microbiol. 25 (1997) 175–187.
[21] J.M. Wood, J. Membr. Biol. 106 (1988) 183–202.
[22] K.I. Racher, R.T. Voegele, E.V. Marshall, D.E. Culham, J.M. Wood, H. Jung, M. Bacon, M.T. Cairns, S.M. Ferguson, W.J. Liang, P.J. Henderson, G. White, F.R. Hallett, Biochemistry 38 (1999) 1676–1684.
[23] R. Rubenhagen, H. Ronsch, H. Jung, R. Krämer, S. Morbach, J. Biol. Chem. 275 (2000) 735–741.
[24] F. Kunst, N. Ogasawara, I. Moszer, A.M. Albertini, G. Alloni, V. Azevedo, M.G. Bertero, P. Bessieres, A. Bolotin, S. Borchert, R. Borriss, L. Boursier, A. Brans, M. Braun, S.C. Brignell, S. Bron, S. Brouillet, C.V. Bruschi, B. Caldwell, V. Capuano, N.M. Carter, S.K. Choi, J.J. Codani, I.F. Connerton, A. Danchin, Nature 390 (1997) 249–256.
[25] R. Heilig, EMBL accession No. Q9V083, 1999.
[26] W.R. Schwan, S.N. Coulter, E.Y. Ng, M.H. Langhorne, H.D. Ritchie, L.L. Brody, S. Westbrock-Wadman, A.S. Bayer, K.R. Folger, C.K. Stover, Infect. Immun. 66 (1998) 567–572.
[27] A.S. Bayer, S.N. Coulter, C.K. Stover, W.R. Schwan, Infect. Immun. 67 (1999) 740–744.
[28] H. Michel, J. Behr, A. Harrenga, A. Kannt, Annu. Rev. Biophys. Biomol. Struct. 27 (1998) 329–356.
[29] H. Luecke, B. Schobert, H.T. Richter, J.P. Cartailler, J.K. Lanyi, J. Mol. Biol. 291 (1999) 899–911.
[30] M. Kolbe, H. Besir, L.O. Essen, D. Oesterhelt, Science 288 (2000) 1390–1396.
[31] D.A. Doyle, C.J. Morais, R.A. Pfuetzner, A. Kuo, J.M. Gulbis, S.L. Cohen, B.T. Chait, R. MacKinnon, Science 280 (1998) 69–77.
[32] K.A. Williams, U. Geldmacher-Kaufer, E. Padan, S. Schuldiner, W. Kühlbrandt, EMBO J. 18 (1999) 3558–3563.
[33] K.A. Williams, Nature 403 (2000) 112–115.
[34] K. Jung, H. Jung, J. Wu, G.G. Prive, H.R. Kaback, Biochemistry 32 (1993) 12273–12278.
[35] J. Sun, J. Voss, W.L. Hubbell, H.R. Kaback, Biochemistry 38 (1999) 3100–3105.
[36] C.D. Wolin, H.R. Kaback, Biochemistry 39 (2000) 6130–6135.
[37] T. Nakao, I. Yamato, Y. Anraku, Mol. Gen. Genet. 208 (1987) 70–75.
[38] H. Jung, Biochim. Biophys. Acta 1365 (1998) 60–64.
[39] H. Jung, R. Rübenhagen, S. Tebbe, K. Leifker, N. Tholema, M. Quick, R. Schmid, J. Biol. Chem. 273 (1998) 26400–26407.
[40] Y. Komeiji, K. Hanada, I. Yamato, Y. Anraku, FEBS Lett. 256 (1989) 135–138.
[41] H. Jung, S. Tebbe, R. Schmid, K. Jung, Biochemistry 37 (1998) 11083–11088.
[42] C. Wegener, S. Tebbe, H.J. Steinhoff, H. Jung, Biochemistry 39 (2000) 4831–4837.
[43] G.L. Millhauser, Trends Biochem. Sci. 17 (1992) 448–452.
[44] W.L. Hubbell, H.S. Mchaourab, C. Altenbach, M.A. Lietzow, Structure 4 (1996) 779–783.
[45] W.L. Hubbell, A. Gross, R. Langen, M.A. Lietzow, Curr. Opin. Struct. Biol. 8 (1998) 649–656.
[46] M. Pfeiffer, T. Rink, K. Gerwert, D. Oesterhelt, H.J. Steinhoff, J. Mol. Biol. 287 (1999) 163–171.
[47] B. Tiebel, N. Radzwill, L.M. Aung-Hilbrich, V. Helbl, H.J. Steinhoff, W. Hillen, J. Mol. Biol. 290 (1999) 229–240.
[48] E. Turk, C.J. Kerner, M.P. Lostao, E.M. Wright, J. Biol. Chem. 271 (1996) 1925–1934.
[49] O. Levy, A. De la Vieja, C.S. Ginter, C. Riedel, G. Dai, N. Carrasco, J. Biol. Chem. 273 (1998) 22657–22663.
[50] G. Cao, S. Cheng, P. Whitley, G. von Heijne, A. Kuhn, R.E. Dalbey, J. Biol. Chem. 269 (1994) 26898–26903.
[51] G. von Heijne, Nature 341 (1989) 456–458.
[52] T. Pourcher, E. Bibi, H.R. Kaback, G. Leblanc, Biochemistry 35 (1996) 4161–4168.
[53] C. Gwizdek, G. Leblanc, M. Bassilana, Biochemistry 36 (1997) 8522–8529.
[54] M. Grunewald, A. Bendahan, B.I. Kanner, Neuron 21 (1998) 623–632.
[55] M. van Geest, J.S. Lolkema, J. Biol. Chem. 274 (1999) 29705–29711.

[56] M. van Geest, J.S. Lolkema, Biochim. Biophys. Acta 1466 (2000) 328–338.
[57] C.C. Chen, T. Tsuchiya, Y. Yamane, J.M. Wood, T.H. Wilson, J. Membr. Biol. 84 (1985) 157–164.
[58] T. Tsuchiya, Y. Yamane, S. Shiota, T. Kawasaki, FEBS Lett. 168 (1984) 327–330.
[59] J. Cairney, C.F. Higgins, I.R. Booth, J. Bacteriol. 160 (1984) 22–27.
[60] L.M.D. Stewart, I.R. Booth, FEMS Microbiol. Lett. 19 (1983) 161–164.
[61] L.N. Csonka, W. Epstein, in: F.C. Neidhardt (Ed.), *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology, ASM Press, Washington, DC, 1996, pp. 1210–1223.
[62] J.M. Wood, Microbiol. Mol. Biol. Rev. 63 (1999) 230–262.
[63] J. Cairney, I.R. Booth, C.F. Higgins, J. Bacteriol. 164 (1985) 1218–1223.
[64] S. Grothe, R.L. Krogsrud, D.J. McClellan, J.L. Milner, J.M. Wood, J. Bacteriol. 166 (1986) 253–259.
[65] D.E. Culham, B. Lasby, A.G. Marangoni, J.L. Milner, B.A. Steer, R.W. van Nues, J.M. Wood, J. Mol. Biol. 229 (1993) 268–276.
[66] S.S. Pao, I.T. Paulsen, M.H.J. Saier, Microbiol. Mol. Biol. Rev. 62 (1998) 1–34.
[67] R.R. Anderson, R. Menzel, J.M. Wood, J. Bacteriol. 141 (1980) 1071–1076.
[68] J.M. Wood, D. Zadworny, Can. J. Biochem. 58 (1980) 787–796.
[69] C.C. Chen, T.H. Wilson, J. Biol. Chem. 261 (1986) 2599–2604.
[70] K. Hanada, I. Yamato, Y. Anraku, J. Biol. Chem. 263 (1988) 7181–7185.
[71] I. Yamato, FEBS Lett. 298 (1992) 1–5.
[72] I. Yamato, Y. Anraku, J. Membr. Biol. 114 (1990) 143–151.
[73] S. Eskandari, D.D. Loo, G. Dai, O. Levy, E.M. Wright, N. Carrasco, J. Biol. Chem. 272 (1997) 27230–27238.
[74] E.M. Wright, D.D. Loo, M. Panayotova-Heiermann, B.A. Hirayama, E. Turk, S. Eskandari, J.T. Lam, Acta Physiol. Scand. Suppl. 643 (1998) 257–264.
[75] R.S. Myers, S.R. Maloy, Mol. Microbiol. 2 (1988) 749–755.
[76] M. Quick, H. Jung, Biochemistry 36 (1997) 4631–4636.
[77] M. Quick, S. Tebbe, H. Jung, Eur. J. Biochem. 239 (1996) 732–736.
[78] T. Pourcher, M. Deckert, M. Bassilana, G. Leblanc, Biochem. Biophys. Res. Commun. 178 (1991) 1176–1181.
[79] G. Kaim, F. Wehrle, U. Gerike, P. Dimroth, Biochemistry 36 (1997) 9185–9194.
[80] M. Quick, S. Stölting, H. Jung, Biochemistry 38 (1999) 13523–13529.
[81] M. Panayotova-Heiermann, D.D. Loo, C.T. Kong, J.E. Lever, E.M. Wright, J. Biol. Chem. 271 (1996) 10029–10034.
[82] M. Panayotova-Heiermann, S. Eskandari, E. Turk, G.A. Zampighi, E.M. Wright, J. Biol. Chem. 272 (1997) 20324–20327.
[83] M. Panayotova-Heiermann, D.W. Leung, B.A. Hirayama, E.M. Wright, FEBS Lett. 459 (1999) 386–390.
[84] D.K. Dila, S.R. Maloy, J. Bacteriol. 168 (1986) 590–594.
[85] M. Quick, H. Jung, Biochemistry 37 (1998) 13800–13806.
[86] M. Ohsawa, T. Mogi, H. Yamamoto, I. Yamato, Y. Anraku, J. Bacteriol. 170 (1988) 5185–5191.
[87] K. Hanada, T. Yoshida, I. Yamato, Y. Anraku, Biochim. Biophys. Acta 1105 (1992) 61–66.
[88] I. Yamato, Y. Anraku, J. Biol. Chem. 263 (1988) 16055–16057.
[89] P.R. van Iwaarden, J.C. Pastore, W.N. Konings, H.R. Kaback, Biochemistry 30 (1991) 9595–9600.
[90] A.C. Weissborn, M.C. Botfield, M. Kuroda, T. Tsuchiya, T.H. Wilson, Biochim. Biophys. Acta 1329 (1997) 237–244.
[91] I. Yamato, M. Kotani, Y. Oka, Y. Anraku, J. Biol. Chem. 269 (1994) 5720–5724.
[92] D.D. Loo, B.A. Hirayama, E.M. Gallardo, J.T. Lam, E. Turk, E.M. Wright, Proc. Natl. Acad. Sci. USA 95 (1998) 7789–7794.
[93] S. Frillingos, M. Sahin-Toth, J. Wu, H.R. Kaback, FASEB J. 12 (1998) 1281–1299.
[94] J. Wu, D. Hardy, H.R. Kaback, Biochemistry 37 (1998) 15785–15790.
[95] J. Wu, D. Hardy, H.R. Kaback, J. Mol. Biol. 282 (1998) 959–967.
[96] K. Nelson, R.K. Selander, J. Bacteriol. 174 (1992) 6886–6895.
[97] D.R. Hahn, R.S. Myers, C.R. Kent, S.R. Maloy, Mol. Gen. Genet. 213 (1988) 125–133.
[98] H. Hosoya, K. Nakamura, Biosci. Biotechnol. Biochem. 58 (1994) 2099–2101.
[99] J.F. Tomb, O. White, A.R. Kerlavage, R.A. Clayton, G.G. Sutton, R.D. Fleischmann, K.A. Ketchum, H.P. Klenk, S. Gill, B.A. Dougherty, K. Nelson, J. Quackenbush, L. Zhou, E.F. Kirkness, S. Peterson, B. Loftus, D. Richardson, R. Dodson, H.G. Khalak, A. Glodek, K. McKenney, L.M. Fitzegerald, N. Lee, M.D. Adams, J.C. Venter, Nature 388 (1997) 539–547.
[100] R.D. Fleischmann, M.D. Adams, O. White, R.A. Clayton, E.F. Kirkness, A.R. Kerlavage, C.J. Bult, J.F. Tomb, B.A. Dougherty, J.M. Merrick, Science 269 (1995) 496–512.
[101] P.A. Wengender, K.J. Miller, Appl. Environ. Microbiol. 61 (1995) 252–259.
[102] S. Kalman, W. Mitchell, R. Marathe, C. Lammel, J. Fan, R.W. Hyman, L. Olinger, J. Grimwood, R.W. Davis, R.S. Stephens, Nat. Genet. 21 (1999) 385–389.
[103] H.P. Klenk, R.A. Clayton, J.F. Tomb, O. White, K.E. Nelson, K.A. Ketchum, R.J. Dodson, M. Gwinn, E.K. Hickey, J.D. Peterson, D.L. Richardson, A.R. Kerlavage, D.E. Graham, N.C. Kyrpides, R.D. Fleischmann, J. Quackenbush, N.H. Lee, G.G. Sutton, S. Gill, E.F. Kirkness, B.A. Dougherty, K. McKenney, M.D. Adams, B. Loftus, J.C. Venter, Nature 390 (1997) 364–370.
[104] Y. Kawarabayasi, M. Sawada, H. Horikawa, Y. Haikawa, Y. Hino, S. Yamamoto, M. Sekine, S. Baba, H. Kosugi, A. Hosoyama, Y. Nagai, M. Sakai, K. Ogura, R. Otsuka, H. Nakazawa, M. Takamiya, Y. Ohfuku, T. Funahashi, T. Tanaka, Y. Kudoh, J. Yamazaki, N. Kushida, A. Oguchi, K. Aoki, H. Kikuchi, DNA Res. 5 (1998) 55–76.

[105] D.R. Smith, L.A. Doucette-Stamm, C. Deloughery, H. Lee, J. Dubois, T. Aldredge, R. Bashirzadeh, D. Blakely, R. Cook, K. Gilbert, D. Harrison, L. Hoang, P. Keagle, W. Lumm, B. Pothier, D. Qiu, R. Spadafora, R. Vicaire, Y. Wang, J. Wierzbowski, R. Gibson, N. Jiwani, A. Caruso, D. Bush, J.N. Reeve, J. Bacteriol. 179 (1997) 7135–7155.
[106] S.G. Andersson, A. Zomorodipour, J.O. Andersson, T. Sicheritz-Ponten, U.C. Alsmark, R.M. Podowski, A.K. Naslund, A.S. Eriksson, H.H. Winkler, C.G. Kurland, Nature 396 (1998) 133–140.
[107] C.M. Fraser, S. Casjens, W.M. Huang, G.G. Sutton, R. Clayton, R. Lathigra, O. White, K.A. Ketchum, R. Dodson, E.K. Hickey, M. Gwinn, B. Dougherty, J.F. Tomb, R.D. Fleischmann, D. Richardson, J. Peterson, A.R. Kerlavage, J. Quackenbush, S. Salzberg, M. Hanson, R. van Vugt, N. Palmer, M.D. Adams, J. Gocayne, J.C. Venter, Nature 390 (1997) 580–586.
[108] C. Schleissner, E.R. Olivera, M. Fernandez-Valverde, J.M. Luengo, J. Bacteriol. 176 (1994) 7667–7676.
[109] R.I. Sarker, W. Ogawa, M. Tsuda, S. Tanaka, T. Tsuchiya, Biochim. Biophys. Acta 1279 (1996) 149–156.
[110] T. Kaneko, S. Sato, H. Kotani, A. Tanaka, E. Asamizu, Y. Nakamura, N. Miyajima, M. Hirosawa, M. Sugiura, S. Sasamoto, T. Kimura, T. Hosouchi, A. Matsuno, A. Muraki, N. Nakazaki, K. Naruo, S. Okumura, S. Shimpo, C. Takeuchi, T. Wada, A. Watanabe, M. Yamada, M. Yasuda, S. Tabata, DNA Res. 3 (1996) 109–136.
[111] R. Menzel, J. Roth, J. Biol. Chem. 256 (1981) 9762–9766.
[112] L.M. Chen, S. Maloy, J. Bacteriol. 173 (1991) 783–790.
[113] J. Parkhill, B.W. Wren, K. Mungall, J.M. Ketley, C. Churcher, D. Basham, T. Chillingworth, R.M. Davies, T. Feltwell, S. Holroyd, K. Jagels, A.V. Karlyshev, S. Moule, M.J. Pallen, C.W. Penn, M.A. Quail, M.A. Rajandream, K.M. Rutherford, A.H. van Vliet, S. Whitehead, B.G. Barrell, Nature 403 (2000) 665–668.
[114] H. Tettelin, N.J. Saunders, J. Heidelberg, A.C. Jeffries, K.E. Nelson, J.A. Eisen, K.A. Ketchum, D.W. Hood, J.F. Peden, R.J. Dodson, W.C. Nelson, M.L. Gwinn, R. DeBoy, J.D. Peterson, E.K. Hickey, D.H. Haft, S.L. Salzberg, O. White, R.D. Fleischmann, B.A. Dougherty, T. Mason, A. Ciecko, D.S. Parksey, E. Blair, H. Cittone, E.B. Clark, M.D. Cotton, T.R. Utterback, H. Khouri, H. Qin, J. Vamathevan, J. Gill, V. Scarlato, V. Masignani, M. Pizza, G. Grandi, L. Sun, H.O. Smith, C.M. Fraser, E.R. Moxon, R. Rappuoli, J.C. Venter, Science 287 (2000) 1809–1815.
[115] J.F. Heidelberg, J.A. Eisen, W.C. Nelson, R.A. Clayton, M.L. Gwinn, R.J. Dodson, D.H. Haft, E.K. Hickey, J.D. Peterson, L. Umayam, S.R. Gill, K.E. Nelson, T.D. Read, H. Tettelin, D. Richardson, M.D. Ermolaeva, J. Vamathevan, S. Bass, H. Qin, I. Dragoi, P. Sellers, L. McDonald, T. Utterback, R.D. Fleishmann, W.C. Nierman, O. White, Nature 406 (2000) 477–483.
[116] http://www.tigr.org/tigr-scripts/CMR/report.spl?db=gvc and orf=VCA1071, 1999.