Published in final edited form as:
Biochim Biophys Acta. 2012 March ; 1818(3): 703–717. doi:10.1016/j.bbamem.2011.12.005.

Bioinformatic Characterization of the 4-Toluene Sulfonate Uptake Permease (TSUP) Family of Transmembrane Proteins

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Abstract

The ubiquitous sequence diverse 4-Toluene Sulfonate Uptake Permease (TSUP) family contains few characterized members and is believed to catalyze the transport of several sulfur-based compounds. Prokaryotic members of the TSUP family outnumber the eukaryotic members substantially, and in prokaryotes, but not eukaryotes, extensive lateral gene transfer occurred during family evolution. Despite unequal representation, homologues from the three taxonomic domains of life share well-conserved motifs. We show that the prototypical eight TMS topology arose from an intragenic duplication of a four TMS unit. Possibly, a two TMS α-helical hairpin structure was the precursor of the 4 TMS repeat unit. Genome context analyses confirmed the proposal of a sulfur-based compound transport role for many TSUP homologues, but functional outliers appear to be prevalent as well. Preliminary results suggest that the TSUP family is a member of a large novel superfamily that includes rhodopsins, integral membrane chaperone proteins, transmembrane electron flow carriers and several transporter families. All of these proteins probably arose via the same pathway: 2 → 4 → 8 TMSs followed by loss of a TMS either at the N- or C-terminus, depending on the family, to give the more frequent 7 TMS topology.

Keywords

Transport proteins; secondary active transport; TSUP; uptake/efflux; evolution; superfamily

1. Introduction

Using functional, phylogenetic, and membrane topological information derived from over 10,000 publications on transport systems, we have classified most recognized transport systems into over 600 families. Our work is summarized in the IUBMB-approved Transporter Classification (TC) Database (TCDB; http://www.tcdb.org; [1, 2]), a carefully curated database presenting the TC system [3], which is analogous to the function-only-based Enzyme Commission (EC) system (http://www.chem.qmul.ac.uk/iubmb/enzyme/). The current study focuses on the putative 4-Toluene Sulfonate Uptake Permease (TSUP) family (TC# 2.A.102; previously TC# 9.A.29).

Transport systems play crucial roles in all processes associated with life. They catalyze nutrient uptake, metabolite excretion, the establishment of electrochemical gradients,

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macromolecular export, drug and toxin efflux and intercellular communication by transporting signaling molecules [3]. However, their effectiveness can be utilized in ways that are detrimental to humans and other organisms. This is exemplified by multi-drug resistant (MDR) pathogenic microbial strains, arising partially because of the excessive use of antibiotics in meat production and medicine. Characterizing transporters can pave the way for computational modes of drug discovery, which would allow us to more effectively target various MDR pathogens and diseases [4, 5]. The importance of transport proteins, constituting roughly 10% of the proteome of an organism, on the average, cannot be overstated.

The TSUP family includes thousands of currently sequenced members spanning the bacterial, eukaryotic and archaeal domains. Within the prokaryotes, we have found these proteins in virtually all well studied phyla (see below). The occurrence of multiple organismal sources within single phylogenetic clusters implies extensive horizontal transfer of genes encoding the homologues [6]. A majority of prokaryotic protein members are shown to range in size from 240 amino acids (aas) to 280 aas with few exceptions. The archaeal members, on average, are slightly smaller than the bacterial members, but the eukaryotic members are typically 40–50% larger and range from 400–500 amino acyl residues in size [7]. Eukaryotic members often possess N- and C-terminal extensions, which may play regulatory roles [8, 9]. When only the bacterial members were analyzed, an 8 transmembrane segment (TMS) topology proved to predominate [6], although eukaryotic and archaeal homologues may exhibit greater topological variation. We show that some of the prokaryotic members have undergone intragenic duplication of a 4 TMS unit yielding 8 TMS proteins.

Functions for most TSUP family members have not been assigned and cannot be assumed due to the great sequence divergence among homologues. In fact, the few analyses that have been performed with TSUP homologues suggest differing functionalities. One TSUP member, TsaS in *Comamonas testosteroni* (TC# 2.A.102.1.1), was proposed to catalyze 4-toluene sulfonate uptake via a proton symport mechanism [10, 11]. A cotranscribed protein, TsaT, was proposed to be membrane anion porin [12], but we found that it is homologous to periplasmic binding proteins and therefore propose that, as for TRAP-T family members [13, 14], it is an extracytoplasmic binding receptor that feeds substrates to the TsaS carrier. TsaT is only expressed in the presence of 4-toluene sulfonate, whereas TsaS is expressed following growth in the presence of this and other compounds [12]. TsaS has 7 putative TMSs, and the tight expressional control implied that TsaT might confer specificity to TsaS.

TsaS homologue, TauE (TC# 2.A.102.2.1), was predicted to be a sulfite exporter, but its mechanism of action was not investigated [15]. A putative sulfate uptake porter termed CysZ (TC# 2.A.102.6.1) was also shown to belong to the TSUP family. As for TauE, its mechanism of action is unknown [16]. Yet another homologue, SafE1 (TC# 2.A.102.2.2), was proposed to be a sulfoacetate exporter [17]. A recent study has identified PmpC (TC# 2.A.102.4.2), a TSUP family member, to be part of the PigP regulon in *Serratia* sp. strain ATCC 39006. It was predicted to be inner-membrane localized along with the DUF395 family proteins PmpA and PmpB. PmpA, B and C were all predicted to transport sulfur-containing compounds [18].

It is not known how TSUP family members arose, and conserved motifs have not been identified. Additionally, structural characteristics such as sidedness and rigorous determination of TMS numbers have not been performed. In this report, we use bioinformatic approaches to correct these deficiencies.

2. Methods

2.1 Obtaining homologues and removing redundancies

Query sequences used to identify TSUP family members were (1) Orf of *Pyrococcus abyssi* (gi# 74545625; TC# 2.A.102.4.1), (2) YfcA of *Escherichia coli* (gi# 82592533; TC# 2.A. 102.3.1) and (3) Orf of *Oryza sativa* (gi# 75252893; TC# 2.A.102.5.1). NCBI PSI-BLAST with two iterations were performed with these 3 proteins using default settings, with the output set to 1000 sequences, and with a cutoff of e^{-4} and e^{-6} for the first and second iterations, respectively [19, 20]. Due to program restrictions, the corresponding TinySeq XML files were inputted into the MakeTable5 program separately, and a 70% cutoff was used to remove fragments, redundancies, and sequences having greater than 70% identity with a retained sequence [6]. CD-HIT was then used with a 45% cutoff after combining the three files [21]. 216 sequences remained, and MakeTable5 then created a FASTA file for the sequences as well as a table, which included the corresponding abbreviation, sequence description, organismal source, size, gi number, organismal group or phylum, and organismal domain for each protein. The methods used here to establish homology have been shown to exceed other available methods in terms of reliability and sensitivity as documented in [22, 23].

2.2 Multiple alignment and phylogenetic/16S/18S rRNA trees

The ClustalX program was used to create multiple alignments of homologous proteins and a neighbor-joining phylogenetic tree, visualized using the FigTree program [24, 25]. Based on the multiple alignment, 27 fragmented sequences were removed, bringing the total number to 189. 16S/18S rRNA sequences were obtained using the Silva rRNA database.

2.3 Topological analyses

For topological analyses of single protein sequences, the WHAT, TMHMM 2.0, and HMMTOP programs were used [26–30]. The TMHMM 2.0 program was used in TMS count determinations while the HMMTOP program was used for determining protein sidedness [31, 32]. In cases where the TMS count was in agreement, but protein sidedness differed between the two programs, the positive inside rule was used to make educated guesses concerning protein sidedness [33]. Inputting the multiple alignment file generated by ClustalX into the Average Hydropathy, Amphipathicity and Similarity (AveHAS) program facilitated topological assessments of multiple proteins or entire subfamilies [34].

2.4 Establishing internal repeats

Based on visual analysis of AveHAS plots, potential internal repeats were examined using the IntraCompare (IC) program [35]. The best comparison scores, reported in standard deviations (S.D.), were confirmed and analyzed further using the GSAT/GAP program [36]. The GSAT/GAP program was set at default settings with a gap creation penalty of 8 and a gap extension penalty of 2 with 500 random shuffles. A length of 60 amino acyl residues, the average size of a typical protein domain, and 10 S.D., corresponding to a probability of 10^{-24} that the level of similarity arose by chance, is considered sufficient to prove homology between two proteins or internal repeat units [2, 6, 37, 38]. Optimization of the GSAT/GAP alignment was performed on sequences by maximizing the number of identities, minimizing gaps, and removing non-aligned sequences at the ends. Optimization yields a higher comparison score that better represents the level of similarity between two shorter internal sequences.

2.5 Functional domains and motifs

A search for recognized functional domains in TSUP family members was performed using the conserved domain database (CDD) of NCBI [39]. Protein sequence motifs were identified using the MEME and MAST programs in 2 separate runs due to program restrictions [29, 30]. The most conserved motifs across the 2 runs were analyzed and blended into a single conserved motif based on individual amino acid conservation. The appearance of duplicates of conserved motifs was noted as further evidence of internal repeat elements.

2.6 Genome and operon context analyses

To propose possible related functions, genome context analyses were performed using The SEED-Viewer, which allows the exploration of over 1,500 curated genomes in order to find homologous genes, their operon context, and consequently their known or putative roles in other organisms [40]. This was done alongside RegPrecise and RegPredict, which allow for the identification of transcription factor binding sites [41, 42].

3. Results

3.1 Phylogenetic analysis

The 189 TSUP family members included in this study fell into 15 phylogenetic clusters as shown in Figures 1 and S1, while the phylogenetic tree for the corresponding 16S/18S rRNAs, representing the genuses from which the proteins derived, is shown in Figures 2 and S2. In Table 1, the proteins with their properties are tabulated according to their positions in the phylogenetic tree in Figure 1 in a clockwise fashion. The Conserved Domain Database (CDD) [39] referred to TSUP family members as DUF81, TauE and COG0730 domains [15, 17]. These proteins will be discussed according to cluster.

Cluster 1 (43 proteins) includes proteins derived from plants, apicomplexa, ciliates and other unicellular eukaryotes (See Figure 1 and Table 1). Considering the organismal diversity of cluster 1 proteins, it is not surprising that these sequences are so diverse. The average size of these proteins is 572 ± 312 amino acids (aas), but five of them (Tth4, Cre1, Gla2, Cre2 and Tgo1) are much larger than the others. Excluding these five proteins, the average size is 476 ± 71 aas. Extra hydrophilic domains were found at the N-termini and between TMSs, but not at the C-termini. However, these regions did not represent domains recognized by CDD. Apicomplexa proteins occur in five distinct positions, plant homologues occur in three positions, and the Bacillariophyta, Codonosigidae and Oligohymenophorea phyla are each present in two positions in cluster 1, suggesting a lack of orthology. Explanations for this diversity include (1) the occurrence of pseudogenes, (2) early arising paralogues and (3) horizontal gene transfer.

Cluster 2 consists of 40 homologues, all much smaller than those in cluster 1 (average size is 264 ± 21 aas). The largest of these (366 aas) is from *Franscisella tularensis*, a γ-proteobacterium, with a unique N-terminal 110 aa hydrophilic extension. Although individual proteins are predicted to have from 5 to 8 TMSs, based on the TMHMM 2.0 program [31, 32], an AveHAS plot [35] suggested the presence of 8 TMSs.

Cluster 2 proteins derive from (five sub-phyla) Proteobacteria, Actinobacteria, Verrucomicrobia, Fusobacteria, Firmicutes, Spirochetes and Thermotogae plus one plant, *Ricinus communis*. This last mentioned protein could be a chloroplast protein, explaining its clustering with bacterial homologues. Extensive horizontal gene transfer has evidently occurred during the evolution of this cluster.

The five **cluster 3** proteins derive from δ-Proteobacteria, Chloroflexi and a Euryarchaeon. The average size of these proteins is 358 ± 174 aas. Dde1 (379 aas) from *Desulfovibrio desulfuricans* contains an insertion of unknown function between the first and second TMSs

The larger Orf6 protein contains an N-terminal 160 aa N-terminal degP_htrA_DO domain [43] with probable serine protease and/or chaperone activity [44, 45]. An N-terminal TMS preceding this domain may target it to the cell surface. Members of this serine protease family usually reside in the periplasm of Gram-negative bacteria. It is possible that Orf6 hydrolyzes peptides and transports the products. Although a phylum assignment for Orf6 has not been made, the corresponding 16S rRNA (Fig. 2) clusters with the Verrucomicrobia.

The average protein size of **cluster 4** (6 proteins) is 432 ± 214 aas, but when the large Bsp1, Cgl1 and Gla1 proteins were excluded, it was 260 ± 18 aas. Members possess 7, 8 or 10 predicted TMSs. Bsp1 was found to have an extra C-terminal domain with two tandem copies of the Universal Stress Protein (USP_like) domain. USP proteins are upregulated in response to stress-causing agents [46] and possess an ATP-binding alpha/beta fold motif. The presence of a potential ATP-binding or hydrolyzing domain introduces the possibility that this transporter may either be regulated by ATP or be energized by ATP hydrolysis and thus function as a primary active transporter rather than a secondary carrier (see [47, 48]). Bacterial arsenite and ECF transporters provide examples of carriers with superimposed ATPases that allow secondary carriers to function as primary active transporters (see [47, 49] and references cited therein).

The nine proteins comprising **cluster 5** derive from all three domains of life. The bacterial proteins are from Actinobacteria, Cyanobacteria and δ-Proteobacteria, while the single eukaryotic and archaeal proteins are from Bacillariophyta and Crenarchaeota, respectively. When the large (2798 aa) eukaryotic Ptr3 homologue was excluded, an average value of 282 ± 25 aas was calculated. Ptr3 was predicted to have 8 TMSs by the WHAT program and 10 TMSs based on visual inspection of the hydropathy profile together with comparison with its 8 TMS homologues. Ptr3 has an N-terminal hydrophobic domain followed by a >2000 aa hydrophilic domain that shows sequence similarity with a hydrophilic protein of 2409 aas from *Thalassiosira pseudonana*. Based on the 16S rRNA tree, the three Actinobacterial homologues and the three Cyanobacterial homologues may all be orthologous.

The fourteen **cluster 6** proteins derive from a diversity of bacterial and archaeal phyla. This fact, plus the observation that the bacterial and archaeal homologues are interspersed, clearly suggests that extensive lateral gene transfer has occurred. The sizes of these proteins range from 250 aas to 333 aas (average, 277 ± 20 aas with 7 or 8 putative TMSs). Pto1 from *Picrophilus torridus*, a Euryarchaeota, is the largest protein with 333 aas, reflecting a unique 60 aa hydrophilic insert between TMSs 5 and 6.

Cluster 7 contains only 2 proteins derived from Actinobacteria. Bad1 from *Bifidobacterium adolescentis* and Gva1 from *Gardnerella vaginalis* are 292 and 267 aas in size, respectively, both with 8 putative TMSs.

Cluster 8 (30 proteins) is the third largest cluster and includes proteins solely from bacteria. These proteins cluster closely together in two sub-clusters. The first sub-cluster derives from α- and γ-Proteobacteria while the second, primarily from β- and γ-Proteobacteria, but including one protein each from Bacteroidetes and Cyanobacteria, are interspersed, suggestive of lateral gene transfer. Very little size variation is observed with the average size being 275 ± 11 aas and the majority possessing 8 TMSs. Each of **clusters 9** and **10** consists of a single protein from a γ-Proteobacterium with sizes, and topologies similar to those in cluster 8.

Cluster 11 (3 proteins) and **cluster 12** (4 proteins) derive from archaea. These proteins possess 8 putative TMSs with an average size of 266 ± 7 aas. Comparison with the 16S rRNA tree revealed that these proteins cannot be orthologous.

Cluster 13 (10 proteins) derives primarily from Firmicutes, and most of these proteins may be orthologues. However, homologues from a single Crenarchaeota and a bacterium of unknown phylum are also present. These proteins are of uniform size (273 ± 13 aas) and topology (7 to 8 putative TMSs). The bacterial Tte1 homologue 16S rRNA clusters with the Chloroflexi.

Cluster 14 (7 proteins) derives from α-Proteobacteria, Firmicutes, Spirochetes, and Euryarchaeota. Considerable size variation is observed with the Bja1, Dau1 and Dre4 proteins being the largest. The average size for this cluster is 340 ± 61 aas. Despite the size variation and large phylogenetic distances, the topologies are fairly uniform with members possessing 7 to 9 putative TMSs.

Cluster 15 (14 proteins) derives from eight different archaeal and bacterial phyla. Three Firmicute homologues are sandwiched in between those derived from other phyla. The size and sequence divergence of these homologues is tremendous (246 aas to 569 aas), but their predicted topologies range from 7 to 9 TMSs with the majority exhibiting 8 TMSs. The average size for the proteins in this cluster is 316 ± 86 aas.

3.2 Topological analyses

The average hydropathy (top, dark lines), amphipathicity (top, light lines) and similarity (bottom) plots for the 189 TSUP family members included in this study are presented in Figures 3 and S3. Eight major peaks of hydrophobicity correlate with 8 major peaks of similarity. The peaks of amphipathicity observed between the TMSs exceed the amphipathicities of the transmembrane domains as is frequently the case for transmembrane permeases. TMSs 1 to 4 cluster together separated from TMSs 5 to 8 which also cluster together, suggestive of intragenic duplication as demonstrated below. Additionally, TMSs 1 and 2 cluster closely together, as do TMSs 3 and 4, consistent with duplication of a primordial 2 TMS hairpin peptide. TMSs 5 to 8 are more distant from each other than are TMSs 1 to 4.

The first four TMSs are separated from the last four by a large hydrophilic loop not recognized as a conserved domain by CDD. Two peaks of hydrophobicity and two corresponding peaks of similarity are observed within the loop region prior to TMS 5. These peaks may be due to the 10 TMS proteins in eukaryotes.

3.3 Establishing internal repeats

Most TSUP family members possess 8 predicted TMSs, while those that deviate from this topology may have 7 or 9. To establish the evolutionary origin of this family, the IC and GSAT/GAP programs were used to compare putative repeat elements [2, 6]. Comparing TMSs 1–4 of all TSUP proteins with TMSs 5–8 of the same homologues yielded many comparison scores in excess of 10 S.D. For example, comparing TMSs 1 – 4 with TMSs 5–8 of Tko1 resulted in a comparison score of 26 S.D. (Fig. 4A), while the same comparison for Mch1 resulted in a comparison score of 17 S.D. (Fig. 4B). Thus, an intragenic duplication event occurred during the evolution of the TSUP family. The large comparison scores indicate that the duplication event occurred fairly recently in evolutionary time. When comparing 2 TMS hairpin structures with the adjacent hairpins [50], comparison scores were insufficient to establish homology. However, we have demonstrated homology for these 2 TMS repeats in proteins of other families homologous to TSUP proteins (see Discussion).

3.4 Conserved motifs

The ClustalX program did not identify fully conserved amino acyl residues [24, 25], but the MEME program [29, 30] revealed two well conserved motifs (Figure 5A–B) derived from a 46 residue stretch that spans the first and second TMSs. Motif 1, spanning the second half of TMS 1, is 21 residues in length (Fig. 5A). Glycines (Gs) at positions 1, 5, 9, 12, and 14–16 are the best conserved. I, V and P at positions 13, 20 and 21, respectively, are also well conserved. The large number of Gs in TMS 1 suggests a high degree of conformational flexibility, possibly important for function. A "reverse" Rossmann fold (GxGxxG) sub-motif (GxxGxG) is observed in motif 1 which resides in TMS 1 and may play a role in regulating transport given its potential for binding nucleotides [49, 51, 52].

Motif 2, the second best conserved motif, is 25 residues in length (Fig. 5B). The sub-motif A(VI)(AG)TSL(AF)(TM) (positions 2–9) is highly conserved. Residues 1–20 of this motif mainly comprise TMS 2 within which I, T, and three Ss are well conserved at positions 10, 13, 14, 16 and 17. Residue 21 (H or Y) marks the beginning of the loop region connecting TMSs 2 and 3, with a conserved G at position 25. Motifs 1 and 2, spanning TMSs 1 and 2, respectively, repeat in TMSs 5 and 6, as expected since the second half of these proteins arose by an intragenic duplication event.

3.5 Genome context analyses for functional predictions

Small gene size, high gene density, intronless coding regions and simple operon organization in bacterial genomes render functional predictions feasible [53]. Our previous molecular genetics studies based on operon context have proven to be successful in identifying the substrates of transporters of unknown function [54–56]. Operon context analyses and identification of transcription factor-controlled regulons were facilitated by the use of the SEED database [40] along with RegPrecise and RegPredict [41, 42]. SEED identifies close homologues using the PSI-BLAST algorithm [19, 20]. Our findings are summarized in Table 2. For detailed analyses by cluster, see Supplementary Material designated "Functional Predictions."

3.5.1 Functional predictions—Cluster 1 homologues in *Mycoplasma penetrans*, *M. gallisepticum*, *Staphylococcus aureus* and *Holdemania filiformis* were designated iron-sulfur (FeS) cluster assembly proteins, SufB. The SUF system, encoded by the *sufABCDSE* operon is one of the three FeS cluster assembly systems with the other two being the iron-sulfur cluster (ISC) and nitrogen fixation (NIF) systems [57]. FeS clusters serve as cofactors mediating electron transfer [58, 59]. SufA may be an iron chaperone and is essential for FeS cluster assembly under aerobic, but not anaerobic conditions [60]. SufS is a cysteine desulfurase, and SufE is a scaffold protein. SufB and the paralogous SufD, both of which are homologous to Ath3 from *A. thaliana*, function as parts of a cytoplasmic complex along with SufC [61]. It seems likely that Ath3 is chloroplastic, and its bacterial homologues mediate the uptake of sulfur-based compounds (see also cluster 5 analyses).

In **cluster 2**, the *E. coli* YfcA protein (TC# 2.A.102.3.1) is one of eight presumed orthologues. One homologue is encoded in an operon with a gene encoding a phosphoserine phosphatase in *Silibacter* sp. TM1040. Genes encoding phosphoserine aminotransferase, D-3-phosphoglycerate dehydrogenase, serine/threonine phosphatase and L-threonine 3-dehydrogenase are near this operon. These genes encode the serine biosynthetic pathway starting with 3-phosphoglycerate. Glycerate is a known carbon source for *E. coli* and *S. typhimurium* although the transporter gene has not been identified [62, 63]. Therefore YfcA could be the long sought glycerate uptake porter.

Pas1 from *Photorhabdus asymbiotica* and its homologue from *Neisseria meningitidis*, are encoded in monocistronic operons, but surrounding genes are involved in the methycitrate cycle, acetyl-CoA generation and the propionate-CoA to succinate module. Dno1 is in an operon along with EngB, a GTP-binding protein, and is next to L-asparaginase and a putative protease in *Dichelobacter nodosus*. In *Marinomonas* sp. MWYL1, genes involved in glycine and serine utilization and glycerolipid metabolism surround the Dno1 homologue. Par1 from *Psychrobacter* sp. 273-4 is encoded near a gene for a putative glutamate symporter of the Dicarboxylate/Amino Acid:Cation (Na^+ or H^+) Symporter (DAACS) family (TC# 2.A.23) and a butyryl-CoA dehydrogenase involved in Ile/Val degradation and Lys fermentation. Its homologue in *Mannheimia succiniciproducens* is part of an operon along with a murein endopeptidase, in an arrangement similar to that observed for the Dno1 homologue in *P. multocida*. A function involving peptide/amino acid transport is suggested.

Mka1 from *M. kandleri* in **cluster 3** is part of an operon along with a predicted nucleotide-binding protein related to the universal stress protein, UspA, which is upregulated by metabolic, oxidative and temperature stresses [64]. Genes involved with oxidative stress also surround the Mka1 homologue from *Thermococcus kodakarensis*. The Mka1 homologues in *Pyrococcus furiosus* and *P. horikoshii* are found in operons that appear to function in protein degradation, possibly suggesting an amino acid transport role, and/or representing a part of the stress response. The large Dde1-like proteins of *Desulfovibrio desulfurican*, *D. vulgaris* and *D. baculatum* are encoded adjacently and transcribed divergently from a sigma-54 (σ^{54})-dependent transcriptional regulator. σ^{54} or σ^{N} often plays a global regulatory role for genes encoding proteins involved in nitrogen metabolism [65]. RegPrecise identified RpoN as the regulator of Dde1 transcription.

Homologues of the *Roseiflexus castenholzi* Rca1 protein are encoded in operons that are divergently transcribed from a gene encoding a putative efflux pump of the Arsenical Resistance-3 (ACR3) family (TC# 2.A.59). Upstream of this operon is a gene of the putative permease Duf318 family (TC# 9.B.28). Duf318 homologues have been implicated in arsenate/arsenite resistance [66]. Consistent with this finding, closely upstream of the Duf318 genes, genes encoding redox-active disulfide proteins and an ArsR transcriptional regulator are found. ArsR homologues regulate many transporters including Duf318 transporters, and it may also regulate Rca1 [49]. The two transporters may transport arsenate/arsenite, and/or other stress-related substrates.

Cluster 4 orthologues in two *Thermus thermophilus* strains are located in operons along with genes encoding a ferredoxin-sulfite reductase, a sulfate adenylyltransferase involved in inorganic sulfur assimilation and a (phospho)adenylyl-sulfate reductase involved in cysteine biosynthesis. Several genes homologous to those involved in heme and siroheme biosynthesis are also part of the operon. The siroheme prosthetic group is known to be essential for the function of sulfite-reductases, which convert sulfite (derived from sulfate) to sulfide [67]. Thus, cluster 4 proteins may be involved in the uptake of sulfate or other sulfur-based compounds, a suggestion supported by sequence similarity between cluster 4 proteins and CysZ of *Corynebacterium glutamicum*, a putative sulfate transporter [16].

Proteins of **cluster 5** appear to exhibit functional diversity. One of two *Syntrophobacter fumaroxidans* paralogues is located adjacent to 3 genes involved in cobalt/zinc/cadmium resistance with sequence similarity to members of the RND superfamily (TC# 2.A.6). It is possible that the second Sfu1 paralogue extrudes anions or mediates the uptake of cations during $Co^{2+}/Zn^{2+}/Cd^{2+}$-induced stress. The homologue in *Meiothermus ruber* is surrounded on one side by an operon encoding an acyltransferase and peptidase M19, and on the other side by an ATP-dependent protease, suggestive of a possible amino acid or peptide transport role. However, the homologue in *Meiothermus silvanus* is located near a gene encoding a scaffold protein for (4Fe-4S) cluster assembly, which makes transport of sulfur-based compounds a reasonable possibility. A *Trichodesmium erythraeum*-derived Ter2 homologue in *Nostoc punctiforme* is in an operon also coding for a cysteine desulfurase and the sulfur oxidation molybdopterin C protein. Thus, cluster 5 proteins may transport sulfur-based compounds or amino acids/peptides in response to stress.

Cluster 6 includes Sus1 and its homologues, which all appear in operons along with transcriptional regulators of the GntR superfamily [68–71]. Members of the GntR family usually respond to sugar metabolite effector molecules and control genes involved in carbon metabolism [72, 73]. Unlike its homologue in *Chitinophaga pinensis*, Sus1 from *Solibacter usitatus* is not part of an operon with a GntR transcription regulator, but is instead in an operon with two hypothetical proteins and rhodanase (thiosulfate sulfurtransferase; see cluster 15). Roles in carbon or sulfur-based compound transport are implied.

The only protein from **cluster 7** to be in SEED was Bad1 from *Bifidobacterium adolescentis*. Bad1 is found in a monocistronic operon adjacent to operons encoding heat stress and pathogenicity genes. Thus, Bad1 and other cluster 7 proteins could play roles in stress responses and virulence.

In the mainly proteobacterial **cluster 8**, Rru1 from *Rhodospirillum rubrum*, Rsp1 and its homologues from *Silibacter* sp. TM1040 and *Magnetococcus* sp. MC-1, are found in operons with or nearby genes encoding a γ-glutamyltranspeptidase involved in glutathione and poly-gamma-glutamate biosynthesis as well as enzymes involved in the utilization of glutathione as a sulfur source. Rsp1 and its homologues may transport glutathione or another compound to supply sulfur to supplant the glutathione utilization pathway. TsaS (TC# 2.A. 102.1.1) is a cluster 8 protein predicted to be a 4-toluenesulfonate uptake permease, further supporting a role in transport of sulfur-containing compounds [10, 12, 14].

The 2 proteins comprising **clusters 9** and **10** are from organisms not included in the SEED database. In **cluster 11**, Mma2 from *Methanococcus maripaludis* is in an operon with genes encoding an FMN adenylyltransferase and a putative membrane protein with sequence similarity to members of the Autoinducer-2 Exporter (AI-2E) family (TC# 2.A.86). Tko1 of *T. kodakarensis* (**cluster 12**) is encoded within an operon along with a gene encoding glycyl-tRNA synthetase. Therefore, Tko1 may function as a glycine uptake protein. It is likely that Cbe1 from *Clostridium beijerincki* in **cluster 13** functions in sulfur-based compound uptake. Although not present in a polycistronic operon, it is near genes encoding an iron-sulfur-binding protein, the dissimilatory sulfite reductase (desulfoviridin) and the CoA-disulfide reductase. The *nma1* gene from *Nitrosopumilus maritimus* is in a dense gene cluster containing the iron-dependent repressor IdeR of the DtxR family. Deregulation of iron metabolism or superoxide dismutase deficiency can favor the Fenton reaction, which contributes to oxidative stress, DNA damage, mutagenesis and sensitivity to H_2O_2 [74, 75]. Nma1 may therefore be regulated by IdeR and function in iron uptake. The Nma1 homologue in *Thermococcus onnurineus*, localized to an operon coding for an aminopeptidase and a dehydrogenase, may be an amino acid/peptide uptake permease.

Pth1 of **cluster 14** from *Pelotomaculum thermopropionicum* appears in an operon with 2 hypothetical proteins and is accompanied by an adjacent transporter, not part of the same operon, with greatest similarity to Sulfate/Tungstate Uptake Transporters (SulT; TC# 3.A. 1.6) within the ABC superfamily. Accordingly, Pth1 may transport a sulfur-containing compound.

Homologues in *Magnetospirillum magnetotacticum* and *M. magneticum* are located in operons along with genes encoding UspA stress homologues, and next to an operon encoding the NifU protein. The NifU scaffold protein is part of the N_2 fixation system [57] (see cluster 1) of FeS cluster assembly and is known to colocalize with the Fe-S center-containing rubrerythrin, a peroxidase involved in hydroperoxide detoxification [76, 77]. Homologues of Pth1 may serve as a means for the uptake of inorganic sulfide [78, 79].

Cluster 15 includes a homologue in *Cupriavidus* (*Ralstonia*) *metallidurans* encoded in a monocistronic operon. It may be co-regulated with the two nearby operons, the first, a large operon encoding enzymes involved in cytochrome c biogenesis and acting on sulfur-based compounds. These proteins include thioredoxin, the thiol:disulfide interchange protein, and a protein-disulfide reductase [80]. Rme1 and its homologues are likely to transport sulfur-based compounds and play a role in metabolic pathways. *Sulfolobus solfataricus*-derived Sso1, also in a monocistronic operon, is orthologous to a protein in *S. acidocaldarius*, which is located closely upstream of an operon encoding the various subunits of the CoB-CoM heterodisulfide reductase, further supporting a possible sulfur-based compound transport role.

Aau1 from *Arthrobacter aurescens* is encoded within an operon with a gene encoding a rhodanase domain-containing protein. It is surrounded by several other small genes encoding a thioredoxin and proteins that also contain rhodanase-like domains. Rhodanase catalyzes the transfer of a sulfur atom from sulfane sulfur-containing compounds (sulfur atoms at the 0 or -1 oxidation state) to sulfur acceptors like cyanide and thiols in order to generate molecules that are less toxic to the cell [81]. An example of a reaction which rhodanase catalyzes is:

$$\text{Thiosulfate}+\text{Cyanide}\xrightarrow{\text{Rhodanase}}\text{Sulfite}+\text{Thiocyanate}$$

Aau1 and its homologues in *Corynebacterium glutamicum*, *C. efficiens*, *Salinispora tropica* and *Mycobacterium* sp. JLS are all present in operons containing genes encoding hydroxyacylglutathione hydrolase, which may also serve as a polysulfide binding protein. In *C. efficiens* and *S. tropica*, hydroxyacylglutathione hydrolase may be encoded in an operon with the Aau1 homologue.

Bsp2 from *Bacillus* B-14905 and its homologues in *B. cereus*, *Geobacillus kaustophilus* and *Exiguobacterium sibiricum* have essentially the same gene arrangement as Aau1 and its respective homologues, with various rhodanase-like domain proteins and the hydroxyacylglutathione hydrolase joining them in operons. In addition, Bsp2 and its homologues are in an operon with, or are surrounded by a putative sulfide reductase, protein disulfide isomerase (S-S rearrangase) and/or a putative pyridine nucleotide-disulfide oxidoreductase. Aau1 and Bsp2, as well as their respective homologues, may therefore function in sulfur-based compound uptake, or equally likely, sulfite export, the assigned function of TauE (TC# 2.A.102.2.1) [15], a member of cluster 15. Biochemical and genome context analyses suggest that SafE1 is a putative exporter of sulfoacetate (TC# 2.A.102.2.2), while PmpC (TC# 2.A.102.4.2) may be an organo-sulfur compound transporter. Both belong to cluster 15, providing further evidence for a sulfur-based compound transport role for members of this cluster [17, 18].

3.6 The novel Rhodopsin Superfamily

Comparing the TSUP family to TC subclasses 2.A, 3.E, 4.B, 5.A and 9.A proved to be fruitful and led to the identification of a novel superfamily including at least ten previously recognized families of transporters. This novel superfamily includes members mostly of 7 or 8 TMSs (Table 3). Surprisingly, in addition to families of transporters, the superfamily includes vertebrate and invertebrate rhodopsins, G-protein coupled receptors (GPCRs), a variety of hormone receptors, and invertebrate odorant receptors among others (MA Shlykov, DC Yee, VS Reddy, S Aurora, JS Chen, EI Sun and MH Saier Jr., manuscript in preparation). A summary of the comparisons performed is presented in Figure 6A, while specific comparisons between the TSUP, LCT and NiCoT families are presented in Figures 6B and 6C. These comparisons also demonstrate the primordial 4 TMS repeat unit found in the members of all of these families. More details, including phylogenetic and topological analyses of each family as well as proposed evolutionary pathways for the appearance of these proteins will be presented in a forthcoming publication (Shlykov et al., in preparation).

3.6.1 The LCT and MR families—The evolutionary pathway of the 7 TMS LCT family has been partially elucidated (Zhai et al., 2001), and LCT family members were found to be homologous to members of the Microbial Rhodopsin (MR) family which includes fungal chaperone proteins (see TC entries under 3.E.1). All of the transporters are light-driven ion pumps or light-activated ion channels. Because MR family members are the best characterized from structural, functional and mechanistic standpoints, we have designated this new superfamily the Rhodopsin Superfamily.

LCT family members range in size from 300 to 400 amino acyl residues (aas) and are generally larger than MR proteins which have ~220 to 300 residues. Eukaryotic homologues within a single family tend to be ~40% larger than their bacterial homologues [7]. Whereas the LCT family is found exclusively in the eukaryotic domain, the MR family is ubiquitous. Despite these differences, both families possess a uniform 7 TMS topology.

TMSs 1–3 in LCT family members duplicated to give rise to TMSs 5–7, with TMS 4 showing insignificant sequence similarity to any one of the other six TMSs [82]. The precursor could have been an 8 TMS protein which generated the present-day 7 TMS proteins by loss of TMS 1 or 8, and strong evidence for this possibility is presented here.

The 8 TMS Axy3 protein of the TSUP family is homologous to the 7 TMS Asu1 protein of the LCT family (Fig. 6B). The repeat regions shown in Fig. 6B include TMSs 1–3 of Axy3 and 4–6 of Asu1 (comparison score = 11.2 S.D.). This demonstrates that TSUP TMSs 1–3 and 5–7 are homologous to LCT/MR TMSs 4–6. TMS 1 of most TSUP members was lost to generate the 7 TMS topology of the LCT/MR families.

3.6.2 The NiCoT family—Members of sub-family 1 within the ubiquitous NiCoT family are typically 300 to 380 aas in size and possess 6–8 putative TMSs [83]. NiCoT sub-family 2 is comprised of distant homologues of great size, sequence and topological variation. NiCoT transporters catalyze the uptake of Ni^{2+} and Co^{2+} using a pmf-dependent mechanism; however, a Ni^{2+} and Co^{2+} resistance protein that is believed to export the two metals to the external environment has been reported [84, 85].

Comparing TMSs 1–3 of TSUP Bja1 (8 TMSs) with TMSs 4–6 of NiCoT Pla1 (6 TMSs) yielded a comparison score of 12.8 S.D. (Fig. 6C). This comparison establishes homology between members of these two families. Based on this and other alignments, it is likely that the 6 TMS NiCoT proteins arose from the loss of TMSs 1 and 8 after the 4 TMS intragenic duplication event that gave an 8 TMS topology.

4. Discussion

Members of the ubiquitous TSUP family appear to function as secondary carriers primarily for sulfur-based compounds. The vast majority of homologues within the TSUP family possess eight putative TMSs, with some predicted to have seven or nine TMSs, possibly as a result of N- or C-terminal deletions or extensions. Conserved motifs were identified, and their presence in multiple copies within TSUP homologues supports a two-fold symmetry within the proteins (Figs. 5A–B).

The greatest size and topological variation of all phylogenetic TSUP clusters was observed in the diverse cluster 1, which consists solely of eukaryotic members that are in general, 40–50% larger than their prokaryotic counterparts [7], although large homologues were also identified in prokaryotic clusters. These may have been the products of gene fusion events where hydrophilic domains were introduced during their evolutionary histories. Most hydrophilic domains proved to be unrecognizable by CDD, but the degP_htrA_DO domain of Orf6 and the USP_like domain of Bsp1 were identified. Their presence suggests a group-translocation-like function for Orf6 and a stress response role for Bsp1. The known functions of these domains correlate nicely with the predicted functions of the phylogenetic clusters in which they reside.

Comparative analyses of the phylogenetic protein and 16S/18S rRNA trees revealed that lateral gene transfer was common within the bacterial and archaeal domains but much less common within the eukaryotic domain. Lateral gene transfer between bacteria and archaea must have been frequent but rare between prokaryotes and eukaryotes. As a result, orthology was generally not observed within the bacterial domain with the notable exception of the Actinobacterial and Cyanobacterial homologues in cluster 5.

We were able to demonstrate a 4 TMS repeat in homologues from bacteria, eukaryotes and archaea. 2 TMS repeat units have been found in several families of transport proteins, including the Oligopeptide Transporter (OPT; TC# 2.A.67) and CRAC channel/CDF carrier superfamily [23, 50]. However, our methods were unable to detect a 2 TMS repeat unit within TSUP homologues. Sequence divergence may have accounted for this failure.

Comparisons between the TSUP family and other transport systems have revealed superfamily relationships with the Ion-Translocating Microbial Rhodopsins, Sweet sugar porters, Branched Chain Amino Acid Exporters, Nicotinamide Ribonucleotide Uptake Permeases, Ni^{2+}-Co^{2+} Transporters, Organic Solute Transporters, Disulfide Bond Oxidoreductase D 2-electron carriers, Phosphate:Na^{+} Symporters and Lysosomal Cystine Transporters (See Table 3). These ten families comprise the novel Rhodopsin Superfamily. Of these proteins, high resolution 3-d structures are available only for the microbial rhodopsins [86]. We predict that all members of these ten families will prove to have similar structures. The functional diversification of this superfamily is unprecedented among transmembrane protein superfamilies (see Superfamily link in TCDB). However, it is worth noting that in some of these families it was possible to demonstrate that duplication of a 2 TMS hairpin structure gave rise to the 4 TMS precursor that duplicated internally in all of the proteins of these families to give the current 8 or 7 TMS proteins. Using the Superfamily Principle, we therefore conclude that the same must have been true for the TSUP family.

Genome context analyses supported the few biochemical assays that have been performed using TSUP homologues. Results for clusters 1, 5, 6, 8 and 12–15 substantiate a transport role for sulfur-based compounds (Table 2). Given the apparent functional diversity of our predictions as well as the sequence diversity inherent in the TSUP family, it may be that members transport a wide range of compounds. These may include (1) amino acids/peptides, (2) nucleotides/nucleosides/nucleobases and (3) carbohydrates. Some TSUP proteins may function as parts of stress responses and/or play roles in cofactor precursor transport. At least some TSUP members may function with auxiliary cytoplasmic and periplasmic proteins. The elucidation of these functions, using the predictions presented here as guides, are likely to open up new fields of study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The work reported in this paper was supporter by NIH Grant 2 RO1 GM077402-05A1.

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Highlights

- The 4-toluene sulfonate uptake permease (TSUP) family is ubiquitous in nature.
- Uptake of sulfur-containing compounds is mediated by members of the TSUP family.
- No bioinformatic characterization of this family of porters has yet been described.
- We here demonstrate that these proteins arose by intragenic duplication of 4 TMSs.
- Phylogeny, motifs, topologies and functional predictions are described herein.

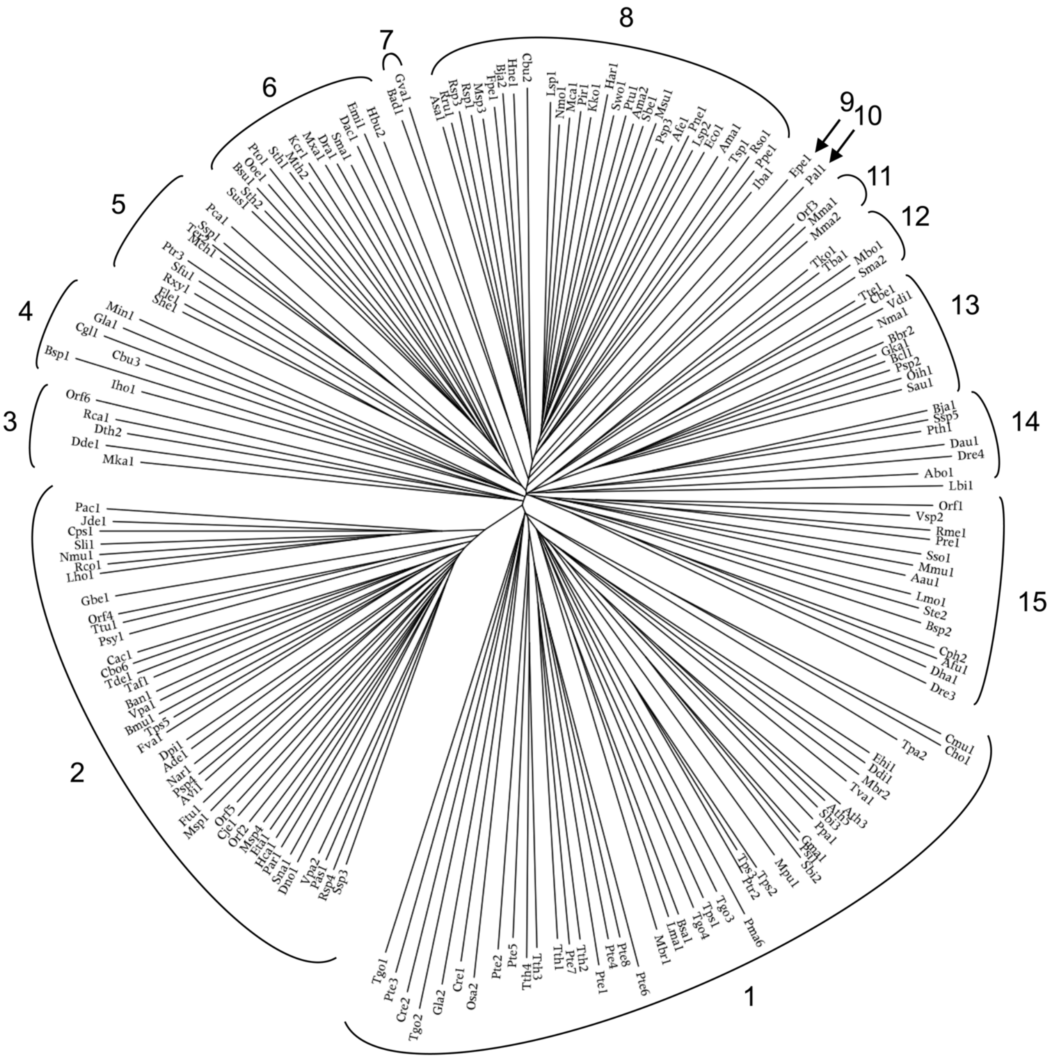


Figure 1.

Phylogenetic tree of the 189 TSUP family proteins included in this study. The tree was generated using the ClustalX multiple alignment and FigTree programs. Protein abbreviations and their descriptions are listed in Table 1 in a clockwise fashion starting from cluster 1. The positions of individual proteins within the phylogenetic tree are revealed in Fig. S1.

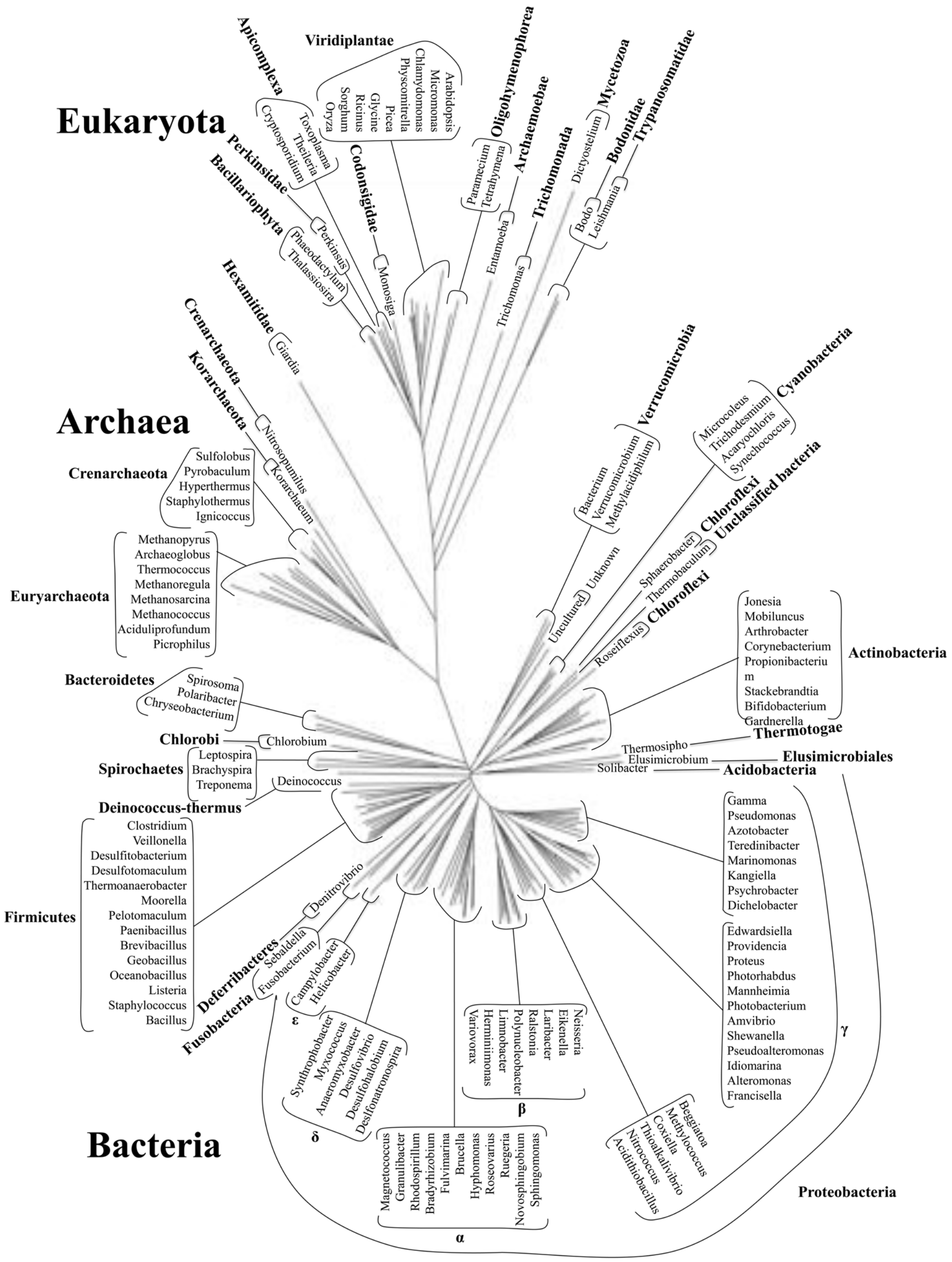


Figure 2.

16S/18S rRNA phylogenetic tree of genuses represented in this study. The *Cloacamonas*, *Symbiobacterium*, *Oenococcus*, *Endoriftia* and *Desulforudis* genera were excluded due to unreliable sequence data.

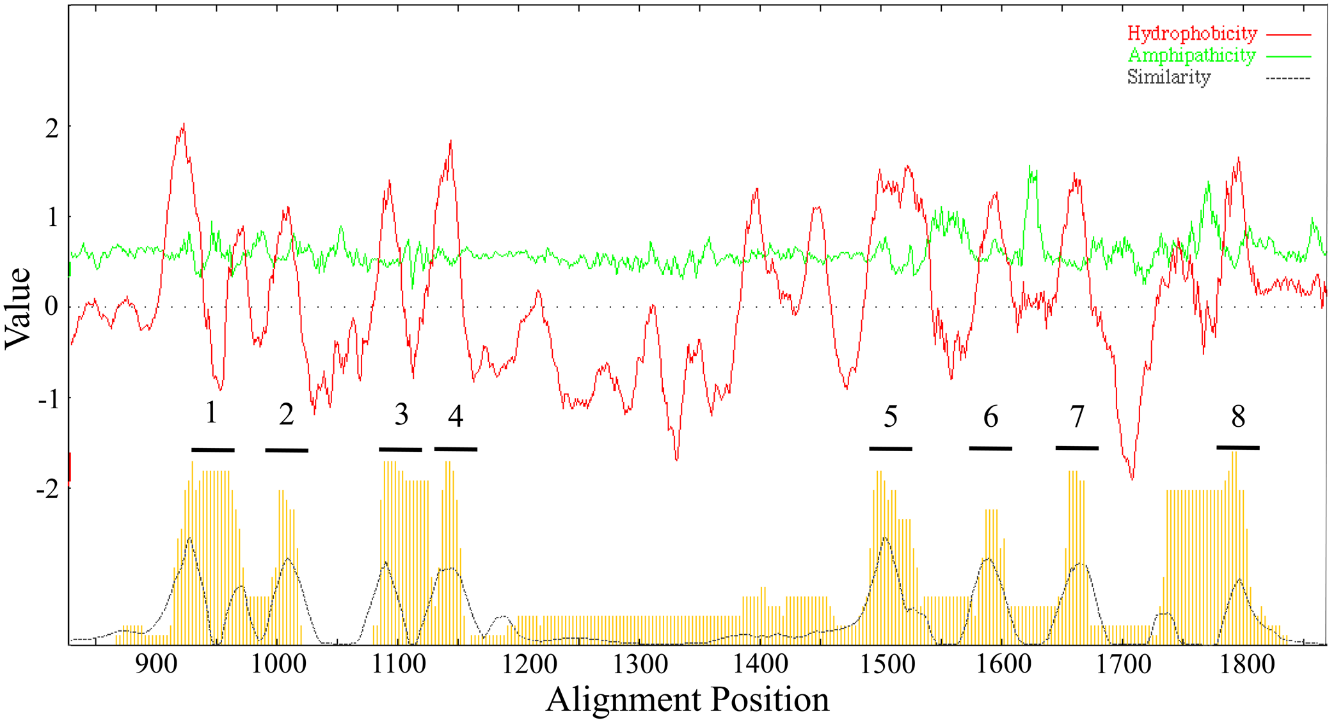


Figure 3.

Portion of average hydropathy, amphipathicity, and similarity (AveHAS) plots for the 189 TSUP family proteins included in this study. A magnification of the TMS-containing region is presented due to the large size of the plot which reveals 8 clear TMSs. However, as many as 12 poorly conserved peaks of hydrophobicity can be seen, suggesting that some homologues have additional TMSs.

A

```
                  1                            2
Tko1  11 GIVIGILAAMFGLGGGFLIVPTLNFLGVEIHHAVGTSSAAVVFTSLSSAI 60
         | : |: . : |:||| : || | ::|. ||:|| ||| |:|||. | ||
Tko1 141 GFIAGVASGLLGIGGGAINVPFLTYMGLPIHYAVATSSFAIVFTATSGAI 190
                 5                              6
                            3                     4
Tko1  61 AYHRQRRIHYKAGLLLASTAVIGAYIGAWATSYISAAQLKVIFGVVLFLV 110
          ::    :  .  .||    :||| :||       |.||   | ||:  .
Tko1 191 KHYTLGNVEVEWLVLLVPGLIIGAQLGAKIAKRTKASQLTKAFAVVMAFL 240
                              7                        8
            4
Tko1 111 AIRLYRK 117
         |||:  |
Tko1 241 AIRMILK 247
            8
```

B

```
                      1                            2
Mch1  11 SAGLFAGILAGFLGIGGGTVLVPL.LVTLGYDYQQAVATSTLSIVITAIS 59
         | |  ||:|||  |:||| :|||| :. ||   . |: ||   |||||||
Mch1 152 STGSTAGLLAGVFGVGGGVILVPLQILLLGESIKTAIQTSLGVIVITAIS 201
                           5                       6
                                 3                  4
Mch1  60 GTVQNWRLGNIDFKRIIAIGFPAIITAPIGAYLTELFADYWLKAAFGLLL 109
           | .   ||: .   : :|   ::   |         |  .  ||  ||
Mch1 202 ACVGHAVQGNVLWIEGLLLGTGGLLGVQISTRFLPKLPDQVVSLAFSALL 251
                            7                        8
Mch1 110 LI 101
          |
Mch1 252 AI 102
```

Figure 4.

Demonstration that 8 TMS TSUP family members arose by intragenic duplication of a primordial 4 TMS encoding genetic element. **(A)** GAP alignment of TMSs 1–4 of TSUP Tko1 (*Thermococcus kodakarensis*; gi# 57640914) with TMSs 5–8 of the same protein. Initial identification of repeat units was done using the IC program. The GAP program was run with default settings and 500 random shuffles. Residue identities are signified by vertical lines, while close and more distant similarities are signified by colons or periods, respectively. The numbers at both ends of each line signify the positions of the residues in the proteins. TMS positions were predicted using the TMHMM 2.0 program for this and subsequent comparisons. A comparison score of 26 S.D. was obtained. **(B)** GAP alignment of TMSs 1–4 of TSUP Mch1 (*Microcoleus chthonoplastes*; gi# 224407624) with TMSs 5–8 of Mch1. A comparison score of 17 S.D. was obtained.

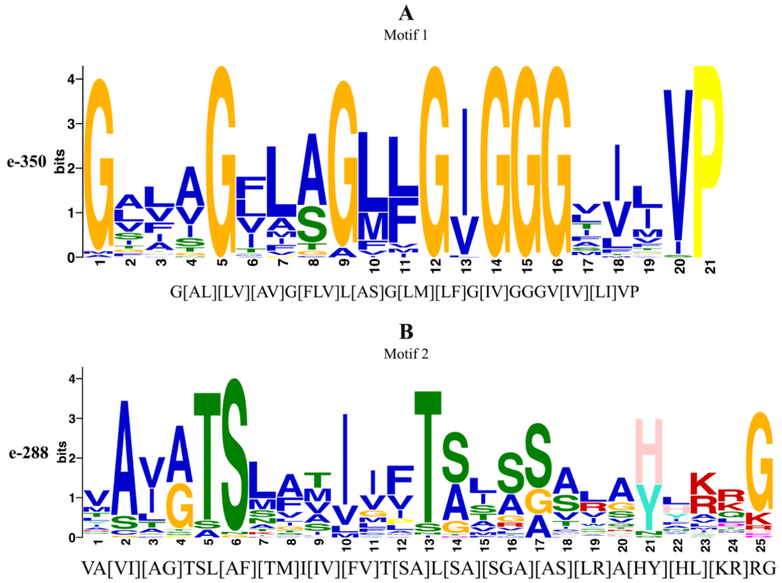
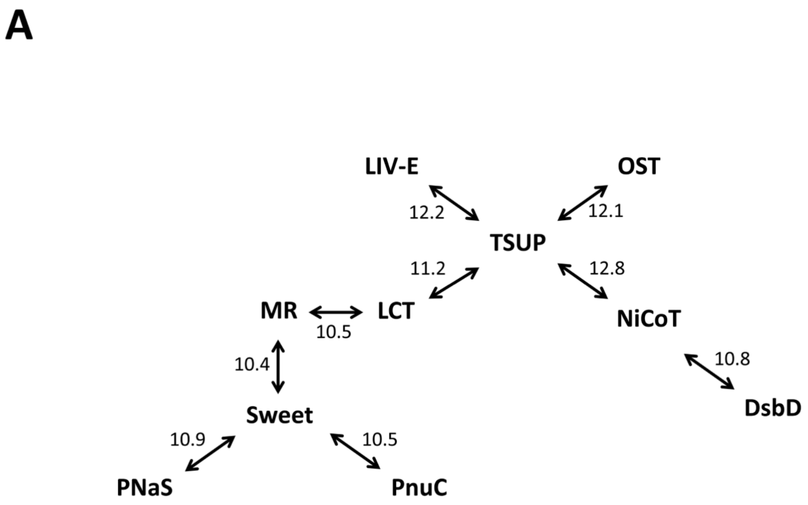


Figure 5.

(A–B) The two best conserved motifs found within the TSUP family as predicted by MEME. Corresponding statistical scores are presented on the y-axis. MAST predictions of motifs based on the MEME results are presented on the x-axis below each motif graphic.



B

```
              1                             2
Axy3  21 TMTGFAFGLVLLGL-SGVFQLASVSEVANVVSVLSLVNAAVTLARAKPQV 69
         |  | :   :|  | | |  : :|      :: || :   ||| :  ||
Asu1 251 TCAGISSAFILFALVSFVLTMVNVINALQFITFLSYIKMGVTLCKYFPQA 300
                     4                     5
                         3                 4
Axy3  70 NWSLMRPAMASSLVGVGAGVAALAWISGSMSVLLQLLLG 108
          ::  |     | ||   |   | :: ||| :   :| |
Asu1 301 FFNFKR----KSTVGWSIGNVLLDFLGGSMDICQMILQG 334
                              6
```

C

```
Bja1  25 EGAHLHCRNDHAQSHHTPHTHSPRPKFLIRSTANSAIERRGNRSVSPVRG 74
         |  | |   ||   |   | | | |  :       |  |:|      |||
Pla1 179 EHGHVHHDHDH.DTHEHDHAHIPTPADI......RAAKRKG......VRG 215
                               1                   2
Bja1  75 SMQLYLPIADLPVNVFLVLAMGAAVGFVSGMFGIGGGFLMTPLLIFIG.. 122
            : | :   |    :   : |      | | ||    |  :   :|
Pla1 216 MAAMILSVGLRPCTGAILVLLFAV...TQGAFSIG...VMSAIVMSVGTA 259
                     4                               5
                2                                   3
Bja1 123 IT.PAVAVASVASHIAASSFSGAI.SYWRR...RAIDPALASVLLCGGVT 167
         ||  | |   | |   |  | | : | | |   | :  |  ||:|
Pla1 260 ITVSALALMTVFSKRLALRFAGGVDSPWARRVERGLKIAGGSVIL...LF 306
               5                                    6
              3
Bja1 168 GTALGVWTFTQ 178
         |  | |  |||
Pla1 307 GMMLLVASFTQ 317
              6
```

Figure 6.

(A) Rhodopsin superfamily homology established through the use of GSAT/GAP and the Superfamily Principle. Established Rhodopsin superfamily proteins from TCDB and their homologues were used to establish homology between all members of the ten families.

GSAT/GAP scores, adjacent to the arrows, are expressed in terms of standard deviations (S.D.). (**B)** Homology between members of the TSUP and LCT families. GAP alignment of TMSs 1–3 of TSUP Axy3 (*Achromobacter xylosoxidans*; gi 311107599) with TMSs 4–6 of LCT Asu1 (*Ascaris suum*; gi 324511247). A comparison score of 11.2 S.D. was obtained with 43.8% similarity and 28.1% identity. **(C)** Homology between members of the TSUP and NiCoT families. GAP alignment of TMSs 1–3 of TSUP Bja1 (*Bradyrhizobium japonicum*; gi 27376265) with TMSs 4–6 of NiCoT Pla1 (*Parvibaculum lavamentivorans*; gi 154252649). A comparison score of 12.8 S.D. was obtained with 44.7% similarity and 36.4% identity.

Table 1

The 189 TSUP proteins included in this study. Proteins are listed clockwise starting from Cluster 1 in Fig. 1. Protein abbreviations, taxonomic origins, protein sizes (aas), gi numbers, organismal phyla, organismal domains, putative numbers of TMSs, and N-terminal orientation are included (see key below). N-terminal orientation results lacking asterisks signify agreement between programs. Average size and standard deviation values are provided for all clusters with two or more members. Additional average size and SD values are provided after removing the specified outliers.

Abbreviation	**Organism**	**Protein Size**	**GenBank No.**	**Phylum**	**Domain**	**No. of TMSs**
Cluster 1						
Cmu1	*Cryptosporidium muris* RN66	525	209881434	Apicomplexa	Eukaryota	11
Cho1	*Cryptosporidium hominis* TU502	518	67601741	Apicomplexa	Eukaryota	10
Tpa2	*Theileria parva* strain Muguga	409	71033393	Apicomplexa	Eukaryota	9
Ehi1	*Entamoeba histolytica*	460	67466247	none	Eukaryota	10
Ddi1	*Dictyostelium discoideum* AX4	549	66825573	none	Eukaryota	10
Mbr2	*Monosiga brevicollis* MX1	499	167525260	Codonosigidae	Eukaryota	8
Tva1	*Trichomonas vaginalis* G3	448	123437805	Trichomonada	Eukaryota	9
Ath3	*Arabidopsis thaliana*	491	6554197	Viridiplantae	Eukaryota	9
Ath5	*Arabidopsis thaliana*	431	2911082	Viridiplantae	Eukaryota	9
Sbi3	*Sorghum bicolor*	473	242058941	Viridiplantae	Eukaryota	9
Ppa1	*Physcomitrella patens* subsp. *patens*	405	168065030	Viridiplantae	Eukaryota	9
Gma1	*Glycine max*	469	83853809	Viridiplantae	Eukaryota	9
Psi1	*Picea sitchensis*	505	148906357	Viridiplantae	Eukaryota	10
Sbi2	*Sorghum bicolor*	383	242044420	Viridiplantae	Eukaryota	7
Mpu1	*Micromonas pusilla* CCMP1545	461	226458924	Viridiplantae	Eukaryota	8
Tps2	*Thalassiosira pseudonana* CCMP1335	564	223992571	Bacillariophyta	Eukaryota	11
Tps3	*Thalassiosira pseudonana* CCMP1335	385	223998204	Bacillariophyta	Eukaryota	7
Ptr2	*Phaeodactylum tricornutum* CCAP 1055/1	644	219112381	Bacillariophyta	Eukaryota	11
Pma6	*Perkinsus marinus* ATCC 50983	385	239878631	Perkinsidae	Eukaryota	6
Tgo3	*Toxoplasma gondii* GT1	482	221485444	Apicomplexa	Eukaryota	10
Tps1	*Thalassiosira pseudonana* CCMP1335	522	224014684	Bacillariophyta	Eukaryota	9
Tgo4	*Toxoplasma gondii* VEG	665	221505087	Apicomplexa	Eukaryota	11
Bsa1	*Bodo saltans*	526	206598109	Bodonidae	Eukaryota	10
Lma1	*Leishmania* major strain Friedlin	511	157873729	Trypanosomatidae	Eukaryota	10
Mbr1	*Monosiga brevicollis* MX1	512	167521960	Codonosigidae	Eukaryota	9

Abbreviation	Organism	Protein Size	GenBank No.	Phylum	Domain	No. of TMSs
Pte6	*Paramecium tetraurelia* strain d4-2	491	145483119	Oligohymenophorea	Eukaryota	9
Pte8	*Paramecium tetraurelia* strain d4-2	473	145514235	Oligohymenophorea	Eukaryota	9
Pte4	*Paramecium tetraurelia* strain d4-2	424	145501808	Oligohymenophorea	Eukaryota	10
Pte1	*Paramecium tetraurelia* strain d4-2	441	145493138	Oligohymenophorea	Eukaryota	10
Tth2	*Tetrahymena thermophila*	570	118348626	Oligohymenophorea	Eukaryota	9
Pte7	*Paramecium tetraurelia* strain d4-2	430	145531341	Oligohymenophorea	Eukaryota	10
Tth1	*Tetrahymena thermophila*	505	146183328	Oligohymenophorea	Eukaryota	11
Tth3	*Tetrahymena thermophila*	503	118395416	Oligohymenophorea	Eukaryota	10
Tth4	*Tetrahymena thermophila*	1325	118401229	Oligohymenophorea	Eukaryota	8
Pte5	*Paramecium tetraurelia* strain d4-2	400	145528512	Oligohymenophorea	Eukaryota	10
Pte2	*Paramecium tetraurelia* strain d4-2	406	145538953	Oligohymenophorea	Eukaryota	9
Osa2	*Oryza sativa* Japonica Group	465	222625716	Viridiplantae	Eukaryota	5
Cre1	*Chlamydomonas reinhardtii*	929	159479540	Viridiplantae	Eukaryota	6
Gla2	*Giardia lamblia* ATCC 50803	748	159117352	Hexamitidae	Eukaryota	10
Tgo2	*Toxoplasma gondii* VEG	299	221501858	Apicomplexa	Eukaryota	2
Cre2	*Chlamydomonas reinhardtii*	1854	159469083	Viridiplantae	Eukaryota	9
Pte3	*Paramecium tetraurelia* strain d4-2	454	145493226	Oligohymenophorea	Eukaryota	7
Tgo1	*Toxoplasma gondii* GT1	1659	221487433	Apicomplexa	Eukaryota	8
		Average Size= 572 +/− 312 (all)				
		Average Size= 476 +/− 71 (w/out Tth4, Cre1, Gla2, Cre2, Tgo1)				
Cluster 2						
Ssp3	*Sphingomonas sp.* SKA58	259	94497264	Alphaproteobacteria	Bacteria	7
Rsp4	*Ruegeria* sp. TM1040	252	99082858	Alphaproteobacteria	Bacteria	5
Pas1	*Photorhabdus asymbiotica* subsp. *asymbiotica* ATCC 43949	260	211638062	Gammaproteobacteria	Bacteria	7
Vpa2	*Variovorax paradoxus* S110	270	239813891	Betaproteobacteria	Bacteria	7
Dno1	*Dichelobacter nodosus* VCS1703A	258	146329063	Gammaproteobacteria	Bacteria	6
Sna1	*Stackebrandtia nassauensis* DSM 44728	256	229865833	Actinobacteria	Bacteria	6
Par1	*Psychrobacter arcticus* 273-4	251	71066392	Gammaproteobacteria	Bacteria	5
Hca1	*Helicobacter canadensis* MIT 98-5491	250	224418685	Epsilonproteobacteria	Bacteria	7
Eta1	*Edwardsiella tarda*	280	158512112	Gammaproteobacteria	Bacteria	6
Msp4	*Marinomonas* sp. MED121	258	87120732	Gammaproteobacteria	Bacteria	7

Abbreviation	Organism	Protein Size	GenBank No.	Phylum	Domain	No. of TMSs
Orf2	*gamma proteobacterium*	250	90416226	Gammaproteobacteria	Bacteria	6
Cje1	*Campylobacter jejuni* RM1221	254	57238492	Epsilonproteobacteria	Bacteria	7
Orf5	*bacterium Ellin514*	268	223939838	Verrucomicrobia	Bacteria	6
Msp1	*Marinomonas* sp. MED121	253	87118707	Gammaproteobacteria	Bacteria	8
Ftu1	*Francisella tularensis* subsp. *holarctica* FSC200	366	167010238	Gammaproteobacteria	Bacteria	8
Avi1	*Azotobacter vinelandii* DJ	289	226943937	Gammaproteobacteria	Bacteria	7
Psp4	*Psychromonas* sp. CNPT3	274	90407709	Gammaproteobacteria	Bacteria	8
Nar1	*Novosphingobium aromaticivorans* DSM 12444	256	87200262	Alphaproteobacteria	Bacteria	6
Ade1	*Anaeromyxobacter dehalogenans* 2CP-C	254	86157393	Deltaproteobacteria	Bacteria	7
Dpi1	*Desulfovibrio piger* ATCC 29098	259	212704568	Deltaproteobacteria	Bacteria	7
Fva1	*Fusobacterium varium* ATCC 27725	276	253583632	Fusobacteria	Bacteria	7
Tps5	*Thermoanaerobacter pseudethanolicus* ATCC 33223	253	167038325	Firmicutes	Bacteria	7
Bmu1	*Brachyspira murdochii* DSM 12563	255	227999578	Spirochaetes	Bacteria	6
Vpa1	*Veillonella parvula* DSM 2008	264	227372642	Firmicutes	Bacteria	8
Ban1	*Bacillus anthracis* str. A2012	263	65318350	Firmicutes	Bacteria	8
Taf1	*Thermosipho africanus* TCF52B	254	217077973	Thermotogae	Bacteria	7
Tde1	*Treponema denticola* ATCC 35405	262	42525707	Spirochaetes	Bacteria	7
Cbo6	*Clostridium bolteae*	251	160940895	Firmicutes	Bacteria	7
Cac1	*Cloacamonas acidaminovorans*	257	218961280	none	Bacteria	7
Psy1	*Pseudomonas syringae pv. oryzae*	258	237801487	Gammaproteobacteria	Bacteria	6
Ttu1	*Teredinibacter turnerae* T7901	256	237685094	Gammaproteobacteria	Bacteria	7
Orf4	*uncultured marine bacterium* 439	252	40062756	none	Bacteria	7
Gbe1	*Granulibacter bethesdensis* CGDNIH1	253	114328287	Alphaproteobacteria	Bacteria	7
Lho1	*Laribacter hongkongensis* HLHK9	310	226942144	Betaproteobacteria	Bacteria	6
Rco1	*Ricinus communis*	301	223512929	Viridiplantae	Eukaryota	8
Nmu1	*Neisseria mucosa* ATCC 25996	256	225367635	Betaproteobacteria	Bacteria	7
Sli1	*Spirosoma linguale* DSM 74	254	229867512	Bacteroidetes	Bacteria	6
Cps1	*Corynebacterium pseudogenitalium* ATCC 33035	260	227490282	Actinobacteria	Bacteria	8
Jde1	*Jonesia denitrificans* DSM 20603	269	227383462	Actinobacteria	Bacteria	7
Pac1	*Propionibacterium acnes* KPA171202	255	50842975	Actinobacteria	Bacteria	6

Abbreviation	Organism	Protein Size	GenBank No.	Phylum	Domain	No. of TMSs
		Average Size= 264 +/− 21 (all)				
Cluster 3						
Mka1	*Methanopyrus kandleri* AV19	252	20093583	Euryarchaeota	Archaea	7
Dde1	*Desulfovibrio desulfuricans*	379	220904085	Deltaproteobacteria	Bacteria	9
Dth2	*Desulfonatronospira thiodismutans* ASO3-1	254	225199785	Deltaproteobacteria	Bacteria	7
Rca1	*Roseiflexus castenholzii*	251	156743559	Chloroflexi	Bacteria	8
Orf6	*uncultured bacterium*	654	239787713	none	Bacteria	9
		Average Size= 358 +/− 174 (all)				
		Average Size= 252 +/− 2 (w/out Orf6, Dde1)				
Cluster 4						
Iho1	*Ignicoccus hospitalis* KIN4/I	240	156936864	Crenarchaeota	Archaea	7
Bsp1	*Beggiatoa* sp. PS	787	153869281	Gammaproteobacteria	Bacteria	10
Cbu3	*Coxiella burnetii Dugway* 5J108-111	274	209364180	Gammaproteobacteria	Bacteria	8
Cgl1	*Chryseobacterium gleum* ATCC 35910	505	227369714	Bacteroidetes	Bacteria	8
Gla1	*Giardia lamblia* ATCC 50803	520	159115095	Hexamitidae	Eukaryota	10
Min1	*Methylacidiphilum infernorum* V4	267	189218632	Verrucomicrobia	Bacteria	8
		Average Size= 432 +/− 214 (all)				
		Average Size= 260 +/− 18 (w/out Bsp1, Cgl1, Gla1)				
Cluster 5						
She1	*Slackia heliotrinireducens* DSM 20476	277	229879562	Actinobacteria	Bacteria	8
Ele1	*Eggerthella lenta* DSM 2243	307	227411437	Actinobacteria	Bacteria	8
Rxy1	*Rubrobacter xylanophilus* DSM 9941	267	108803101	Actinobacteria	Bacteria	8
Sfu1	*Syntrophobacter fumaroxidans* MPOB	269	116750841	Deltaproteobacteria	Bacteria	7
Ptr3	*Phaeodactylum tricornutum*	2798	219127009	Bacillariophyta	Eukaryota	4
Mch1	*Microcoleus chthonoplastes*	267	224407624	Cyanobacteria	Bacteria	8
Ter2	*Trichodesmium erythraeum*	305	113475233	Cyanobacteria	Bacteria	8
Ssp1	*Synechococcus* sp. JA-3-3Ab	317	86606127	Cyanobacteria	Bacteria	8
Pca1	*Pyrobaculum calidifontis* JCM 11548	244	126458964	Crenarchaeota	Archaea	8
		Average Size= 561 +/− 839 (all)				
		Average Size= 282 +/− 25 (w/out Ptr3)				
Cluster 6						

Abbreviation	Organism	Protein Size	GenBank No.	Phylum	Domain	No. of TMSs
Sus1	*Candidatus Solibacter usitatus* Ellin6076	281	116624708	Acidobacteria	Bacteria	8
Sth2	*Symbiobacterium thermophilum* IAM 14863	279	51892120	Firmicutes	Bacteria	8
Bsu1	*Brucella suis* 1330	289	23500891	Alphaproteobacteria	Bacteria	8
Ooe1	*Oenococcus oeni* PSU-1	283	116491798	Firmicutes	Bacteria	8
Pto1	*Picrophilus torridus* DSM 9790	333	48478318	Euryarchaeota	Archaea	8
Sth1	*Sphaerobacter thermophilus*	282	229877687	Chloroflexi	Bacteria	8
Mth2	*Moorella thermoacetica*	271	83589239	Firmicutes	Bacteria	8
Kcr1	*Candidatus Korarchaeum cryptofilum* OPF8	285	170290371	Korarchaeota	Archaea	8
Mxa1	*Myxococcus xanthus* DK 1622	260	108758495	Deltaproteobacteria	Bacteria	7
Dra1	*Deinococcus radiodurans* R1	255	15805571	Deinococcus-Thermus	Bacteria	8
Sma1	*Staphylothermus marinus* F1	250	126465319	Crenarchaeota	Archaea	8
Dac1	*Denitrovibrio acetiphilus*	274	227423788	Deferribacteres	Bacteria	7
Emi1	*Elusimicrobium minutum* Pei191	275	187251557	candidate division TG1	Bacteria	7
Hbu2	*Hyperthermus butylicus*	255	124028506	Crenarchaeota	Archaea	8
		Average Size= 277 +/− 20 (all)				
Cluster 7						
Bad1	*Bifidobacterium adolescentis* ATCC 15703	292	119026567	Actinobacteria	Bacteria	8
Gva1	*Gardnerella vaginalis* ATCC 14019	267	227507357	Actinobacteria	Bacteria	8
		Average Size= 280 +/− 18 (all)				
Cluster 8						
Asa1	*Aliivibrio salmonicida*	279	16605593	Gammaproteobacteria	Bacteria	8
Rru1	*Rhodospirillum rubrum*	276	83592684	Alphaproteobacteria	Bacteria	8
Rsp3	*Roseovarius* sp. HTCC2601	274	114764120	Alphaproteobacteria	Bacteria	8
Rsp1	*Ruegeria* sp. TM1040	278	99080207	Alphaproteobacteria	Bacteria	7
Msp3	*Magnetococcus* sp. MC-1	265	117925601	Proteobacteria	Bacteria	6
Fpe1	*Fulvimarina pelagi* HTCC2506	275	114707272	Alphaproteobacteria	Bacteria	7
Bja2	*Bradyrhizobium japonicum* USDA 110	287	27375621	Alphaproteobacteria	Bacteria	6
Hne1	*Hyphomonas neptunium* ATCC 15444	314	114797241	Alphaproteobacteria	Bacteria	9
Cbu2	*Coxiella burnetii* RSA 331	275	161831015	Gammaproteobacteria	Bacteria	7
Lsp1	*Limnobacter* sp. MED105	278	149925520	Betaproteobacteria	Bacteria	7
Nmo1	*Nitrococcus mobilis* Nb-231	266	88811005	Gammaproteobacteria	Bacteria	8

Abbreviation	Organism	Protein Size	GenBank No.	Phylum	Domain	No. of TMSs
Mca1	*Methylococcus capsulatus* str. Bath	294	53802665	Gammaproteobacteria	Bacteria	6
Pir1	*Polaribacter irgensii* 23-P	281	88803086	Bacteroidetes	Bacteria	7
Kko1	*Kangiella koreensis* DSM 16069	268	227997603	Gammaproteobacteria	Bacteria	8
Har1	*Herminiimonas arsenicoxydans*	287	134096092	Betaproteobacteria	Bacteria	7
Swo1	*Shewanella woodyi* ATCC 51908	268	170728324	Gammaproteobacteria	Bacteria	8
Ptu1	*Pseudoalteromonas tunicata* D2	269	88860323	Gammaproteobacteria	Bacteria	8
Ama2	*Alteromonas macleodii 'Deep ecotype'*	268	196158505	Gammaproteobacteria	Bacteria	7
Sbe1	*Shewanella benthica* KT99	267	163752420	Gammaproteobacteria	Bacteria	8
Msu1	*Mannheimia succiniciproducens* MBEL55E	266	52424462	Gammaproteobacteria	Bacteria	8
Psp3	*Photobacterium* sp. SKA34	267	89072545	Gammaproteobacteria	Bacteria	8
Afe1	*Acidithiobacillus ferrooxidans* ATCC 23270	264	218665563	Gammaproteobacteria	Bacteria	8
Pne1	*Polynucleobacter necessarius* subsp. *asymbioticus* QLW-P1DMWA-1	272	145589361	Betaproteobacteria	Bacteria	8
Lsp2	*Limnobacter* sp. MED105	289	149926219	Betaproteobacteria	Bacteria	8
Eco1	*Eikenella corrodens* ATCC 23834	270	225024689	Betaproteobacteria	Bacteria	7
Ama1	*Acaryochloris marina*	278	158336922	Cyanobacteria	Bacteria	9
Tsp1	*Thioalkalivibrio* sp. K90mix	268	224818668	Gammaproteobacteria	Bacteria	8
Rso1	*Ralstonia solanacearum*	273	17549483	Betaproteobacteria	Bacteria	8
Ppe1	*Proteus penneri* ATCC 35198	271	226330327	Gammaproteobacteria	Bacteria	8
Iba1	*Idiomarina baltica* OS145	264	85713215	Gammaproteobacteria	Bacteria	8
		Average Size= 275 +/− 11 (all)				
Cluster 9						
Epe1	*Endoriftia persephone 'Hot96_1+Hot96_2'*	287	167948520	Gammaproteobacteria	Bacteria	5
Cluster 10						
Pal1	*Providencia alcalifaciens* DSM 30120	271	212712467	Gammaproteobacteria	Bacteria	8
Cluster 11						
Orf3	*uncultured archaeon* GZfos34A6	276	52549977	none	Archaea	8
Mma1	*Methanosarcina mazei* Go1	270	21228951	Euryarchaeota	Archaea	8
Mma2	*Methanococcus maripaludis* S2	270	45358505	Euryarchaeota	Archaea	8
		Average Size= 272 +/− 3 (all)				
Cluster12						
Tko1	*Thermococcus kodakarensis* KOD1	254	57640914	Euryarchaeota	Archaea	7

Abbreviation	Organism	Protein Size	GenBank No.	Phylum	Domain	No. of TMSs
Tba1	*Thermococcus barophilus* MP	251	223475524	Euryarchaeota	Archaea	8
Mbo1	*Methanoregula boonei 6A8*	269	154149849	Euryarchaeota	Archaea	8
Sma2	*Staphylothermus marinus* F1	265	126466107	Crenarchaeota	Archaea	8
		Average Size= 260 +/− 9 (all)				
Cluster 13						
Tte1	*Thermobaculum terrenum* ATCC BAA-798	255	227375491	none	Bacteria	8
Cbe1	*Clostridium beijerinckii* NCIMB 8052	272	150017843	Firmicutes	Bacteria	8
Vdi1	*Veillonella dispar* ATCC 17748	264	238018311	Firmicutes	Bacteria	8
Nma1	*Nitrosopumilus maritimus* SCM1	257	161528556	Crenarchaeota	Archaea	7
Bbr2	*Brevibacillus brevis*	274	226314422	Firmicutes	Bacteria	7
Gka1	*Geobacillus kaustophilus* HTA426	300	56421519	Firmicutes	Bacteria	8
Bcl1	*Bacillus clausii* KSM-K16	272	56964722	Firmicutes	Bacteria	8
Psp2	*Paenibacillus* sp. JDR-2	272	251794851	Firmicutes	Bacteria	8
Oih1	*Oceanobacillus iheyensis* HTE831	285	23099829	Firmicutes	Bacteria	8
Sau1	*Staphylococcus aureus* subsp. *aureus Mu50*	275	15923912	Firmicutes	Bacteria	8
		Average Size= 273 +/− 13 (all)				
Cluster 14						
Bja1	*Bradyrhizobium japonicum* USDA 110	380	27376265	Alphaproteobacteria	Bacteria	8
Ssp5	*Sphingomonas* sp. SKA58	304	94498747	Alphaproteobacteria	Bacteria	7
Pth1	*Pelotomaculum thermopropionicum* SI	299	147678596	Firmicutes	Bacteria	7
Dau1	*Desulforudis audaxviator*	394	169832116	Firmicutes	Bacteria	8
Dre4	*Desulfotomaculum reducens* MI-1	426	134299284	Firmicutes	Bacteria	9
Abo1	*Aciduliprofundum boonei* T469	254	223473124	Euryarchaeota	Archaea	8
Lbi1	*Leptospira biflexa serovar Patoc strain 'Patoc 1 (Paris)'*	325	183219704	Spirochaetes	Bacteria	8
		Average Size= 340 +/− 61 (all)				
		Average Size= 296 +/− 30 (w/out Bja1, Dau1, Dre4)				
Cluster 15						
Orf1	*synthetic construct*	284	62258462	none	Unclassified	8
Vsp2	*Verrucomicrobium spinosum* DSM 4136	264	171915322	Verrucomicrobia	Bacteria	8
Rme1	*Ralstonia metallidurans* CH34	268	94311333	Betaproteobacteria	Bacteria	8

Abbreviation	Organism	Protein Size	GenBank No.	Phylum	Domain	No. of TMSs
Pre1	*Providencia rettgeri* DSM 1131	264	223992411	Gammaproteobacteria	Bacteria	8
Sso1	*Sulfolobus solfataricus* P2	293	15899038	Crenarchaeota	Archaea	8
Mmu1	*Mobiluncus mulieris* 35243	361	227876711	Actinobacteria	Bacteria	9
Aau1	*Arthrobacter aurescens* TC1	300	119952309	Actinobacteria	Bacteria	8
Lmo1	*Listeria monocytogenes* EGD-e	246	16802663	Firmicutes	Bacteria	8
Ste2	*Sebaldella termitidis* ATCC 33386	246	229881273	Fusobacteria	Bacteria	7
Bsp2	*Bacillus* sp. B14905	282	126650500	Firmicutes	Bacteria	8
Cph2	*Chlorobium phaeobacteroides*	408	189499528	Chlorobi	Bacteria	9
Afu1	*Archaeoglobus fulgidus*	325	11499708	Euryarchaeota	Archaea	7
Dha1	*Desulfitobacterium hafniense* DCB-2	312	219669180	Firmicutes	Bacteria	7
Dre3	*Desulfohalobium retbaense*	569	227420936	Deltaproteobacteria	Bacteria	7

Average Size= 316 +/− 86 (all)

Average Size= 280 +/− 26 (w/out Mmu1, Cph2, Dre3)

Table 2

Summary of functional predictions made for representative members of each phylogenetic cluster (Figure 1).

Cluster #	Proposed Functions
1	Transport of sulfur-based compounds; FeS cluster assembly.
2	Transport of peptides/amino acids/glycerate. Nitrogen-based compound transport;
3	Arsenate/arsenite resistance; Oxidative, heat and metabolic stress response.
4	Transport of sulfur-based compounds.
5	Transport of sulfur-based compounds/ions/peptides/amino acids; FeS cluster assembly; Stress response.
6	Transport of carbon or sulfur-based compounds.
7	Stress response and virulence.
8	Transport of sulfur-based compounds.
9–11	None identified
12	Transport of amino acids.
13	Transport of sulfur-based compounds/Iron/peptides/amino acids.
14	Transport of sulfur-based compounds.
15	Transport of sulfur-based compounds; Extrusion of sulfite.

Table 3

Currently recognized families of transporters included within the Rhodopsin Superfamily. The family names, abbreviations, TC numbers and dominant topologies are presented. This superfamily also includes the non-transporter eukaryotic 7 TMS proteins that include vertebrate and invertebrate rhodopsins, G-protein coupled receptors (GPCRs), a variety of hormone receptors, and invertebrate odorant receptors among others (MA Shlykov, DC Yee, VS Reddy, S Aurora, JS Chen, EI Sun and MH Saier Jr., manuscript in preparation).

Family Name	Family Abbreviation	TC #	# TMSs
Ion-Translocating Microbial Rhodopsin	MR	3.E.1	7
Sweet	Sweet	9.A.58	3 or 7
Branched Chain Amino Acid Exporter	LIV-E	2.A.78	7 or 8
Nicotinamide Ribonucleotide Uptake Permease	PnuC	4.B.1	7 or 8
4-Toluene Sulfonate Uptake Permease	TSUP	2.A.102	7–9
Ni^{2+}-Co^{2+} Transporter	NiCoT	2.A.52	6–8
Organic Solute Transporter	OST	2.A.82	7
Phosphate:Na^+ Symporter PNaS	PNaS	2.A.58	8 or 9
Lysosomal Cystine Transporter	LCT	2.A.43	7
Disulfide Bond Oxidoreductase D	DsbD	5.A.1	6–9