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Genome Analysis

Microbial rhodopsins: functional versatility and genetic mobility

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The type 1 (microbial) rhodopsins are a diverse group of photochemically reactive proteins that span the three domains of life. Their broad phylogenetic distribution has motivated conjecture that rhodopsin-like functionality was present in the last common ancestor of all life. Here, we discuss the evolution of the type 1 microbial rhodopsins and document five cases of lateral gene transfer (LGT) between domains. We suggest that, thanks to the functional versatility of these retinylidene proteins and the relative ease with which they can complement the existing energy-generating or photosensory repertoires of many organisms, LGT is in fact the principal force that determines their broad but patchy distribution.

The microbial rhodopsins

The microbial rhodopsins are typically seven-pass transmembrane proteins that use a retinal chromophore attached at a conserved lysine residue to absorb light energy for ion transport or photosensory functions (Box 1). They are classified as type 1 rhodopsins according to conserved residues in the retinal binding pocket that distinguish them from the (possibly non-homologous) type 2 rhodopsins found in the eyes of animals [1,2]. These retinylidene pigments were first discovered in haloarchaea during the early 1970s in the form of the light-driven proton pump bacteriorhodopsin. This finding led the way to the discovery of additional rhodopsin paralogs in this archaeal lineage (halorhodopsin and the sensory rhodopsins; Box 1) [3–8].

Phylogenetic distribution

Genomic and metagenomic sequencing reveal that the microbial rhodopsins are not restricted to the haloarchaea. Homologs involved in ion transport and sensory functions are present in many disparate eukaryotes and bacteria (Figure 1). The eukaryotic type 1 rhodopsins include those found in the green algae *Chlamydomonas reinhardtii* [9–11] and *Acetabularia acetabulum* [12], the cryptomonads *Guillardia theta* and *Cryptomonas* [13], the alveolate *Pyrocystis lunula* [14] and many fungal species from Basidiomycota and Ascomycota [15]. Recently, the wide taxonomic distribution of type 1 rhodopsins has attracted general attention through the discovery of rhodopsin-based phototrophy in marine bacteria. In this case, sequence-based analyses of a metagenomic fragment linked a 16S rRNA gene from an uncultivated γ-proteobacterium (the Sar86 lineage) to a rhodopsin homolog that was subsequently characterized as the light-driven proton pump, proteorhodopsin [16]. Further analyses of marine picoplankton established that proteorhodopsin functionality is widespread both phylogenetically and biogeographically in the oceans [17,18], a finding also supported by the large number of rhodopsin homologs (~800) found during the Sargasso Sea metagenome study [19]. Although such broad and yet patchy distribution could indicate that the last common ancestor of all life had a type 1 rhodopsin gene that has been independently lost in many lineages, there are now several well-supported phylogenetic trees in which lateral gene transfer (LGT) across great evolutionary distances is unquestionably the better explanation.

Documented cases of interdomain LGT

Two instances of LGT of rhodopsins have been reported within the last year. First, the sequenced genome of the hyperhalophilic bacterium *Salinibacter ruber* encodes four rhodopsin homologs, three of which display clear specific affinity to haloarchaeal genes, although the direction in which the transfer occurred could not be resolved (*S. ruber* to haloarchaea or vice versa) [20]. The fourth rhodopsin of *S. ruber* (xanthorhodopsin) was characterized as a proton pump [21] and did not display close relation to any haloarchaeal protein. Second, metagenomic analyses of the prokaryotic community present in the photic zone of the North Pacific subtropical gyre found multiple instances of a proteorhodopsin-like gene that was linked to a 16S rRNA gene belonging to an uncultured lineage of Archaea (group II marine euryarchaeotes) [22]. Phylogenetic analyses clearly demonstrated the bacterial origin of this gene in these Thermoplasmatales-related organisms, which represents yet another case of interdomain transfer among this diverse group of multifunctional proteins.

Here, we present an evolutionary analysis of the type 1 rhodopsins. The versatility of these proteins is discussed in light of the likely instances of independent evolution of sensory function from an ion transport background across the three domains of life. Phylogenetic analyses of a large set of microbial rhodopsins that spans all three domains indicate that the versatile light-based functionality contained within this single group of proteins provides a basis for frequent LGT.

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Box 1. The two functional categories of type 1 microbial rhodopsins

There are two main functional categories of the type 1 (microbial) rhodopsins: transporters and receptors. Both use a retinal chromophore to absorb light energy.

(i) Transporters

Proton transport (H^+): Light energy is used to create proton electrochemical potential for ATP production, flagellar rotation and other energy-requiring processes. This functionality is present in haloarchaea (bacteriorhodopsin) [3], group II marine Archaea (euryarchaeotic rhodopsins) [22] and Bacteria (proteorhodopsin [16] and xanthorhodopsin [21]). The cellular function of the proton pump present in the fungus *Leptosphaeria maculans* is unclear: it might be used for energetic purposes or light-induced acidification of cellular compartments [27].

Chloride transport (Cl^-): The chloride pump halorhodopsin accentuates the electrochemical gradient through the electrogenic transport of chloride ions into the cytoplasm, hyperpolarizing the cell membrane [33]. It was once believed to be unique to haloarchaea [4] but was recently discovered in the halophilic bacterium *Salinibacter ruber* [20].

(ii) Receptors

Phototaxis: Rhodopsin-mediated phototaxis in response to specific wavelengths of light has been demonstrated in archaeal [6,8] and eukaryotic organisms [9,13]. It also occurs in Bacteria, as deduced from the *S. ruber* two-gene pair that is homologous to the SRI-taxis transducer gene pair. The presence of sensory rhodopsins in all three domains of life in conjunction with a variety of signal transduction mechanisms is a hallmark of the versatility of these proteins (for specific examples, see the review by Spudich [55], this issue).

Photoadaptation: The use of light to regulate gene expression or protein function. For example, characterization of the rhodopsin present in *Anabaena* (*Nostoc*) sp. PCC7120 suggests that this protein might be responsible for modulating chromatic adaptation in photosynthesis (triggering differential biosynthesis of light-absorbing pigments based on the quality of light) [54] (also discussed by Spudich [55], this issue).

Inferences based on phylogeny: independent evolution of sensory functions

Haloarchaea produce multiple rhodopsins that span transport and sensory functions. Previous work by Ihara *et al.* [23] suggested that the rhodopsins in the haloarchaea originated as transporters and that their two sensory rhodopsins (SRI and SRII, used for phototaxis under alternative wavelengths) were derived through an ancient duplication event. This order of derivation is consistent with our phylogenetic analysis in Figure 2 that used non-archaeal (and non-fungal) sequences as an outgroup. Assuming that successive gene duplications produced the several classes of archaeal rhodopsins that occur within this lineage, then SRI and SRII proteins probably derive from an ancestor that had a function in ion (possibly H^+) transport. Additional instances of the evolution of sensory rhodopsins from transporters might be inferred, although each requires further testing through refined phylogenetic and structural analyses and *in vitro* assays of function (Figure 1).

Fungal sensory rhodopsins

In 1999, Bieszke *et al.* [24,25] suggested that the evolution of sensory function occurred independently in the haloarchaea and the fungi. This hypothesis was based on the initial characterization of a sensory-like rhodopsin (Nop-1) in *Neurospora crassa* and its evolutionary placement with regards to the haloarchaea. More recently, Brown and Jung [26] inferred (from phylogenetic and admittedly limited functional analyses) that the fungal rhodopsins, which are monophyletic in both their tree and ours (Figure 1 and Figure 2), comprise three clusters that are probably functionally distinct. Most basal in our rooted tree (Figure 2) are the light-driven proton-pumping types, for which *Leptosphaeria maculans* provides the physically characterized example [27]. The possibility that the ancestral fungal rhodopsins might have been transporters is also most convincingly shown in Figure 2, in which the fungal rhodopsin clade is strongly supported as sister to the haloarchaeal Cl^--pumping rhodopsins, with the exclusion of the sensory types.

From our analyses, the second group of fungal rhodopsins (Nop-like) seems to be more recently derived than the light-driven proton-pumping types (Figure 1 and Figure 2) and includes the *Neurospora Nop-1* gene product, which shares primary sequence features with the proton-pump bacteriorhodopsin but is much more like sensory rhodopsin II in its photochemical behavior [24,25,28,29]. The retinal-dependent phototaxis demonstrated by zoospores of the fungus *Allomyces reticulatus* [30] provides further support for a sensory function for certain fungal rhodopsins. The absence of haloarchaeal-like taxis transducer genes in the genomes of fungi [15] could also be an indicator that these two groups of organisms have converged on this mode of sensory function from a common protein structure.

The third cluster of fungal rhodopsins has not been characterized functionally and is more closely related to the fungal paralogs that seem to have lost the ability to bind to a retinal chromophore. Both of these sequence types are represented in Figure 1 by the longer branching fungal sequences but have been excluded in Figure 2 for various reasons (stated later).

Bacterial sensory rhodopsins

Additional examples of conversion to sensory function can be found within the large group of proteorhodopsins identified by environmental sequencing. In the accompanying article in this issue of *Trends in Microbiology*, **Spudich** [55] argues for the sensory function of a subset of the proteorhodopsins from the (surface water) Sargasso Sea metagenome project based on: (i) the absence of a conserved carboxylate residue always found in proton transporters; and (ii) the presence of signal transduction machinery immediately downstream of these genes on the metagenomic scaffolds (see **Spudich** [55], this issue). In Figure 1, this subset of sequences forms a cluster within the much larger group of bacterial proteorhodopsins, which, on the basis of their sequences (and in some cases functional characterization), are likely to be proton transporters.

Another potential example of sensory function conversion concerns the blue-light-absorbing proteorhodopsins (BPRs), which were discovered by metagenomic analyses of samples from deep-ocean, low-light-intensity regions of the photic zone. BPRs have slow photochemical reaction cycles and receive low light fluence because of their

presence at greater oceanic depths. These factors prompted Wang *et al*. [31] to suggest that this group of proteorhodopsins could also represent an independent case of conversion to sensory function (Figure 1), using electrogenic proton transport itself as a cellular signal. The absence of cultivated organisms containing BPR hampers a direct test of this suggestion and precludes inquiry into the signaling machinery that BPR might regulate.

A study by Bielawski *et al.* [32] used Bayesian modeling methods to identify amino acid sites in BPR that were under positive selection. The majority of these sites were mapped to the exterior of the protein structure and the results suggested that BPR might interact with a membrane-bound protein involved in signal transduction. Functional characterization of the proteorhodopsins from group II marine euryarchaeotes should prove interesting in this regard because they were obtained from similar depths in the water column [22].

Inferences based on phylogeny: interdomain LGT

The utility of light-based functionality combined with structural versatility in a single protein (and further features highlighted later) make type 1 rhodopsins good candidates to proliferate and evolve by the process of LGT. The relationships of the rhodopsins from the domains Bacteria and Eukarya to those in the haloarchaea can be seen in Figure 1. Many features from previous phylogenetic analyses enable new conclusions to be drawn about the evolution of this group of proteins and allow one to be more decisive regarding certain previous conjectures.

Features of the three-domain phylogeny

With some exceptions, the tree in Figure 1 highly supports the separation of bacterial and archaeal (or more precisely, haloarchaeal) type rhodopsins. These exceptions include the green alga *C. reinhardtii* and the cryptomonads (*G. theta* and *Cryptomonas*), which do not clearly align with each other or with either domain. These sequences contribute to long branch attraction (LBA) artifacts within the haloarchaeal type rhodopsins that weaken the phylogenetic signal (and therefore, they are excluded from Figure 2). The presence of rhodopsins in the green algae and the cryptomonads could suggest that these organisms acquired their rhodopsins through plastid endosymbiosis, although it should be considered that many losses of rhodopsins would have to be invoked among other photosynthetic eukaryotic lineages to explain the acquisition of these proteins through this process.

The *Anabaena* sensory rhodopsin does not form a clade with the other cyanobacterial sequence (*Gloeobacter violaceus*) or other bacterial types, which suggests that this rhodopsin might be of foreign origin. Phylogenetic analyses support a relationship between the *Anabaena* rhodopsin and the haloarchaeal type rhodopsins (data not shown; available from the author on request), although the unstable nature of this rapidly evolving sequence also causes LBA artifacts within this grouping and, as a result, more extensive phylogenetic analyses are required to verify this initial finding. (*Anabaena* has been excluded from Figure 2.) The rest of these exceptions to the bacterial–haloarchaeal dichotomy are explained by five instances of strongly supported LGT that we discuss here.

Salinibacter ruber and Haloarchaea

As mentioned earlier, two examples of interdomain LGT events have recently been reported – proteorhodopsins into group II euryarchaeota and the haloarchaeal–*S. ruber* exchange. The relationship between the rhodopsins from group II marine Archaea and bacterial proteorhodopsins is indeed recovered in our tree, and a stronger conclusion can now be made about the direction of the haloarchaeal–*S. ruber* exchange. Figure 2 supports the hypothesis that haloarchaea were the donors of the halorhodopsin (Cl^-) and (independently) the ancestor of the sensory rhodopsin homologs found in *S. ruber*. The *S. ruber* homologs clearly group within the haloarchaea and form well-supported clades with their functional counterparts. The acquisition of these proteins probably had an important role in the adaptation of *S. ruber* to hypersaline environments, where rhodopsins function in the efficient capture of solar energy in the micro-oxic conditions that are characteristic of such settings [33].

The ability of xanthorhodopsin (the H^+ pump of *S. ruber*) to absorb across a greater light wavelength spectrum by using carotenoid antennae in addition to retinal enables this organism to compete efficiently with haloarchaea for light used in the environment [21]. Interestingly, xanthorhodopsin provides evidence for a third independent LGT event. Although its bacterial nature could be taken as evidence that this gene was present in the ancestor of *S. ruber* before it acquired the haloarchaeal rhodopsins, xanthorhodopsin does not exhibit a close evolutionary relationship with other rhodopsins that are known to originate from members of the *Cytophaga–Flexibacter–Bacteroides* (CFB) phylum to which this organism belongs (Figure 2).

Fungi and Haloarchaea

A possible sister-group relationship between halorhodopsin (Cl^-) and fungal rhodopsins was observed in previous phylogenetic analyses [18,22,34,35] but a role for LGT was not proposed, perhaps as a result of inadequate sampling and low statistical support, and because Bieszke *et al.* [24] previously discounted this hypothesis. However, LBA artifacts potentially affect the more rapidly evolving fungal rhodopsin paralogs, the photochemistry and functions of which remain unknown (for a detailed review of the fungal rhodopsins, see Ref. [15]). Indeed, numerous fungal paralogs lack the conserved lysine residue that is necessary to bind retinal, which indicates a change in function that might drive increased rates of evolution (a similar phenomenon has been observed for paralogs in the haloarchaeon *Natronomonas pharaonis*, which was also excluded from our analyses).

As shown in Figure 2, removal of these rapidly evolving sequences results in a substantial increase in the bootstrap values, supporting a haloarchaeal–fungal clade. We believe that this evolutionary relationship between the rhodopsins found in fungi and the haloarchaeal Cl^- pumps represents an ancient LGT event from haloarchaea into the ancestor of the fungal groups representing Basidiomycota and Ascomycota (although Basidiomycota are not

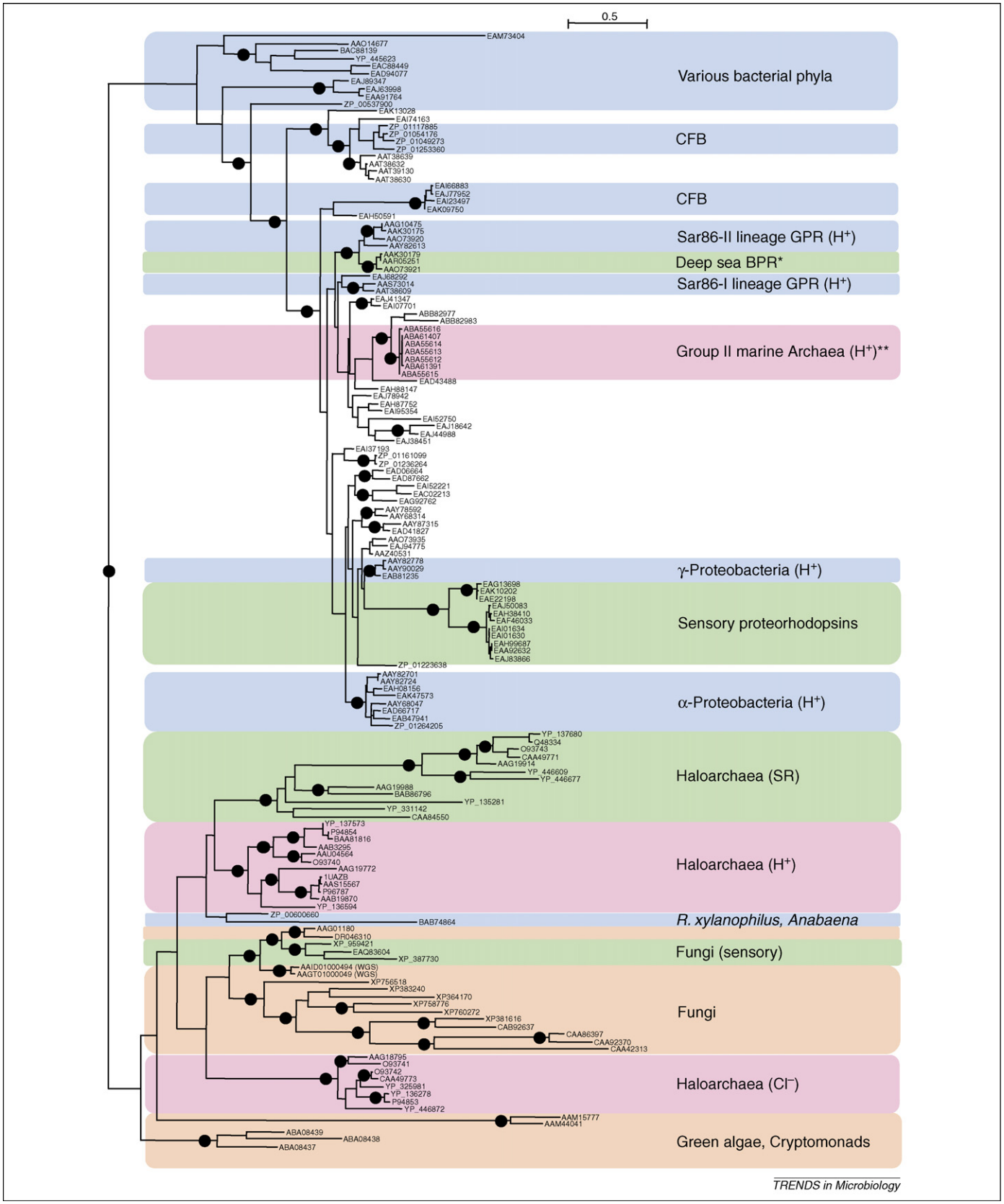

Figure 1. Best maximum likelihood tree of the type 1 (microbial) rhodopsins, created using PHYML [48,49] and JTT [50] and an estimated shape-parameter α with eight rate categories plus invariable sites for 153 amino acid positions. Sequence groupings are color-coded by domain: Bacteria (blue), Archaea (pink) and Eukaryotes (orange), with the exception of suggested cases for the independent evolution of sensory function within these domains (green). Bootstrap values were also obtained with PHYML and only those values that are $\geq$70% are shown (black dots). Cultivated organisms in the group labeled 'various bacterial phyla' are: *Salinibacter ruber* (CFB), *Kineococcus radiotolerans* (Actinobacteria), *Gloeobacter violaceus* (Cyanobacteria), *Exiguobacterium sibiricum* (Firmicutes). This group also includes the eukaryote *Pyrocystis lunula* (a dinoflagellate) as a result of LGT (see text). Sequences were retrieved from NCBI using BLASTP on the NR database in GenBank and the Sargasso Sea metagenome database in Genome BLAST. Additional fungal sequences were obtained from the fungal genome database at Stanford University (http://seq.yeastgenome.org/cgi-bin/blast-fungal.pl). Multiple sequences from each domain of life were used as query sequences for their respective domains to obtain a thorough representation of the

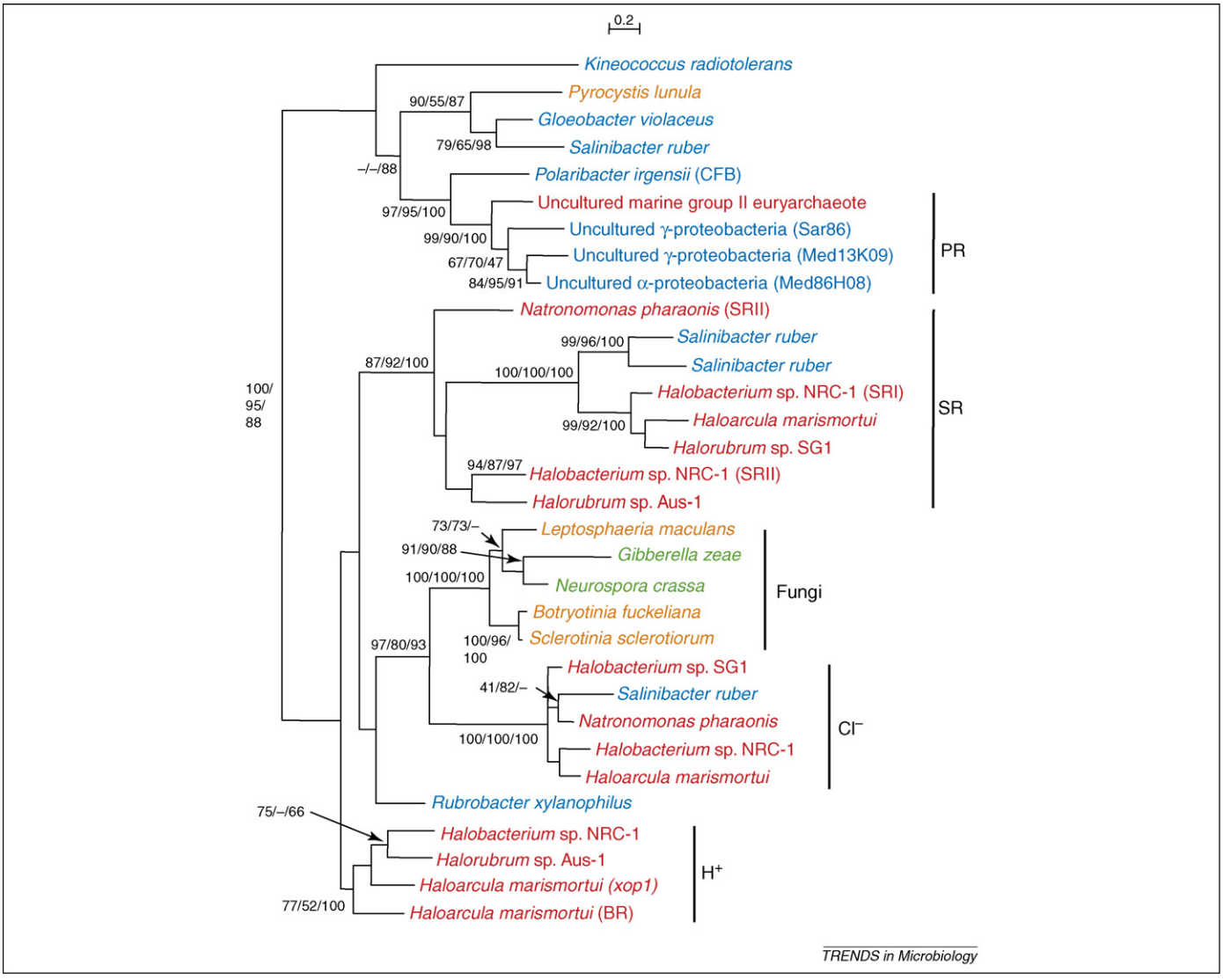

Figure 2. Best maximum likelihood tree of selected type 1 (microbial) rhodopsins (created as described for Figure 1 but with 160 amino acid positions). Sequence groupings are color-coded by domain: Bacteria (blue), Archaea (red) and Eukaryotes (orange), with the exception of a suggested case for the independent evolution of sensory function within the Fungi (green). A separate automated and manual alignment was performed as described in Figure 1. Abbreviations: BR, bacteriorhodopsin; CFB, *Cytophaga–Flexibacter–Bacteroides*; Cl^-, chloride pumps; H^+, proton pumps; PR, proteorhodopsins; SR, sensory rhodopsins. Only proton pumps are represented from the proteorhodopsins. Bootstrap values were calculated using PHYML, Important Quartet Puzzling and NNI [52] and neighbor joining [53], and are shown in this order in the tree.

present in Figure 2, the monophyly of the fungi is clearly represented in Figure 1 and further unpublished analyses). This interdomain transfer is also supported by both structural and functional considerations. Brown [15] had previously commented upon the high similarity of the retinal-binding pocket between the rhodopsins of fungi and haloarchaea. Furthermore, biochemical studies of Nop-1 indicate that it shares strong photochemical characteristics with both sensory rhodopsin II and bacteriorhodopsin [25,36].

Rubrobacter xylanophilus and Haloarchaea

A fourth strongly supported and not previously described interdomain transfer involves actinobacteria. The genomes of the radiation-resistant bacteria *Kineococcus radiotolerans* and *Rubrobacter xylanophilus* are currently being sequenced by the Joint Genome Institute (http://www.jgi.doe.gov/), and each of these organisms harbors a rhodopsin gene. Phylogenetic analyses of the protein sequence indicate differing origins of their respective rhodopsins: the sequence from *K. radiotolerans* is clearly bacterial and the *R. xylanophilus* rhodopsin looks to be haloarchaeal, which implicates this archaeal lineage as donors in yet another interdomain transfer (Figure 2). Bootstrap values for this grouping are high, although there is little support for the formation of a *R. xylanophilus* clade with any specific haloarchaeal paralog. Both actinobacterial homologs contain the

diversity contained within the databases. Automated alignment was performed using ClustalX [51], after which, the alignment was manually adjusted using previous alignments [23,35] to remove hypervariable loops and other unaligned regions. A subset of the sequences obtained from these searches was chosen for phylogenetic analyses based on known phylogenetic affiliation and removal of metagenomic sequences that shared >97% amino acid identity. The single asterisk (*) denotes rhodopsins of potential sensory function, based on photochemical characterization (see main text). The double asterisk (**) denotes that some of these proteorhodopsins were obtained from approximately the same depth in the water column as the deep-sea BPRs. Abbreviations: BPR, blue-absorbing proteorhodopsins; CFB, the bacterial phyla *Cytophaga–Flexibacter–Bacteroides*; Cl^-, chloride pumps; GPR, green-absorbing proteorhodopsins; H^+, proton pumps, SR, sensory rhodopsins.

conserved carboxylate residues that are necessary for proton transport [26]. *R. xylanophilus* belongs to the most deeply branching lineage within the actinobacteria [37] and the absence of this homolog in *K. radiotolerans* suggests that the transfer event took place after the divergence of *R. xylanophilus* from the rest of the actinobacteria.

Pyrocystis lunula and Bacteria

Yet still a fifth case of interdomain LGT involves *P. lunula.* A previous study implicated the rhodopsin of this marine dinoflagellate in a transfer event with proteobacteria but data available at that time did not permit a definite conclusion to be drawn concerning direction of the transfer [34]. The authors logically reasoned that because rhodopsins were present in green algae, cryptomonads and dinoflagellates, which share a common ancestor with red algae, the more probable direction of transfer was into the proteobacteria. Here, Figure 2 demonstrates with certainty that this protein was transferred from Bacteria to *P. lunula*.

Rhodopsin genes as exemplars of LGT

The rhodopsin literature was already rich before the genomics-engendered resurgence of interest in LGT as a major evolutionary force. Indeed, the similarities of type 1 and type 2 rhodopsins have long driven speculation as to whether both derive from a retinylidene pigment in the last common ancestor of all life, or instead are stunning examples of convergence at the molecular level [38]. Within the first-discovered type 1 rhodopsins – those of haloarchaea – the patchy distribution of bacteriorhodopsin (which is present in some species and not others) and the relative frequency of spontaneous and fully viable BR^{-} mutants make it an especially likely candidate for LGT [39,40]. Our own preliminary comparative metagenomic work (A.K. Sharma, unpublished) documents at least one case of *BR* gene LGT between haloarchaeal genera.

As genomic and metagenomic data expand the known phylogenetic distribution of the microbial rhodopsins, some authors have used this phylogenetic breadth itself to argue that rhodopsins were present in the last universal common ancestor (LUCA) of all three domains, and have been variously lost multiple times within each domain [34,41,42]. But as shown here, and as argued recently by Frigaard *et al.* [22], there are instances in which LGT can be conclusively shown to be the cause of such patchy distribution. As these authors point out, LGT of rhodopsin-based energy-generating photosystems might be relatively frequent and often advantageous because the only components needed to produce a functional light-absorbing protein are the rhodopsin gene itself and a single gene that produces retinal from a carotenoid [43,44]. In haloarchaea, fungi and proteobacteria, these two genes are often closely linked [45–47] (A.K. Sharma, unpublished).

Rhodopsins that serve sensory functions must, in addition, effectively couple to transducers but the fact that these can differ greatly between the various lineages that harbor sensory rhodopsins highlights the 'plug-and-play' versatility of this coupling. Indeed, although the two *S. ruber* rhodopsin genes that cluster within the haloarchaeal SR1 clade are, like their haloarchaeal progenitor, closely linked to signal transducer loci, these transducer genes are of bacterial rather than haloarchaeal origin [20]. (See **Spudich**, this issue, for further examples of such versatility.) The proton pump xanthorhodopsin represents yet another variation on this theme of versatility: its ability to form functional complexes with membrane carotenoids to harvest light further expands the repertoire of this diverse set of proteins. Moreover, as is apparent both from phylogenetic analyses and from many structural and mutational studies, functional interconversions are not difficult [33]. So rhodopsins that are acquired by LGT to serve one purpose can be readily co-opted for another.

Although LUCA might well have harbored a type 1 rhodopsin gene, the current broad but patchy distribution of rhodopsins of both the archaeal and bacterial subtypes is not evidence for such antiquity. Indeed, if this distribution is to be explained without LGT, we must assume the existence of a LUCA with many rhodopsins of both subtypes encoded in its genome. Although an ancient origin of this gene family is certainly possible, its popularity among microbes that use light for energy and direction is most easily accounted for by LGT. To expand on the conclusion of Frigaard *et al.* [22] concerning proteorhodopsin, the microbial rhodopsins in general could 'represent a category of 'cosmopolitan genes' whose broad phylogenetic distribution is driven in part by LGT, which further influences the recipient lineage's evolution and speciation'. Indeed, they might be among the best examples of this evolutionary mode.

Concluding remarks and future perspectives

Since their discovery in the early 1970s, the microbial rhodopsins, with their simple structure and complex function, looked to be excellent model systems for structural biologists. As the multiple light-dependent functions of the several haloarchaeal rhodopsin types were uncovered, they increasingly attracted the attention of microbial physiologists. Now, as the diversity of lifestyles and of phylogenetic affinities of the Bacteria, Archaea and Eukarya that employ microbial rhodopsins for one or another light-responsive functions is revealed through genomics and metagenomics, new disciplines such as ecology, biogeography and phylogenetics are also engaged. For those comparative genomicists who are fascinated by the role of LGT in microbial adaptation and divergence, these proteins and the relatively simple (and thus, easily transferred) cluster of genes involved in their synthesis and regulation will be a particularly rich resource. As microbiologists from all fields use molecular and metagenomic methods to look at microbial diversity in the biosphere in more breadth and depth, more and more profound surprises and insights can be expected.

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