

SHORT COMMUNICATION

Functional metagenomic screen reveals new and diverse microbial rhodopsins

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Ion-translocating retinylidene rhodopsins are widely distributed among marine and freshwater microbes. The translocation is light-driven, contributing to the production of biochemical energy in diverse microbes. Until today, most microbial rhodopsins had been detected using bioinformatics based on homology to other rhodopsins. In the past decade, there has been increased interest in microbial rhodopsins in the field of optogenetics since microbial rhodopsins were found to be most useful in vertebrate neuronal systems. Here we report on a functional metagenomic assay for detecting microbial rhodopsins. Using an array of narrow pH electrodes and light-emitting diode illumination, we were able to screen a metagenomic fosmid library to detect diverse marine proteorhodopsins and an actinorhodopsin based solely on proton-pumping activity. Our assay therefore provides a rather simple phenotypic means to enrich our understanding of microbial rhodopsins without any prior knowledge of the genomic content of the environmental entities screened.

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Introduction

Microbial rhodopsins function as light-driven (retinal-based) membranal ion pumps, cation channels or light sensors (Heberle and Deupi, 2014). These rhodopsins are found in various microorganisms (Archaea, Bacteria, Eukarya) (Grote and O'Malley, 2011; Béjà *et al.*, 2013; Ernst *et al.*, 2014) and viruses (Yutin and Koonin, 2012; Philosof and Béjà, 2013), and are classified as type I rhodopsins (distinguished from type II animal visual rhodopsins (Spudich *et al.*, 2000)). Microbial rhodopsin ion pumps function as light-driven proton pumps (for example, bacteriorhodopsins, proteorhodopsins and xanthorhodopsins (Oesterhelt and Stoekenius, 1971; Béjà *et al.*, 2000a; Balashov *et al.*, 2005)), chloride pumps (archaeal halorhodopsins and bacterial chloride rhodopsins (Lanyi and Weber, 1980; Lanyi and Oesterhelt, 1982; Yoshizawa *et al.*, 2014)) and bacterial sodium pumps (Inoue *et al.*, 2013).

Current discoveries of novel microbial rhodopsin ion pumps are restricted to homology searches using known rhodopsins as genomic baits. Only one study used phenotypic characterization in order to detect proteorhodopsins in metagenomic libraries (Martínez *et al.*, 2007). Martínez *et al.* screened some 12 000 marine environmental fosmid clones for the characteristic pinkish/orange color of *E. coli* colonies that express microbial rhodopsins. Three colonies were scored positive (based on their color), and they

contained a proteorhodopsin gene as well as a gene cassette coding for a retinal biosynthetic pathway resembling the cassettes reported by Sabeji *et al.* (2005). We envisioned another phenotypic screen for microbial rhodopsins that would monitor ion-pumping activity directly upon illumination of different microbial rhodopsin transporters. Light-driven ion transport by rhodopsins can be measured directly with a pH electrode. Changes in pH indicate outward proton pumping when acidification of the medium is observed, but can also indicate inward chloride pumping or outward sodium pumping when alkalization of the medium is observed (see Inoue *et al.* (2014) for a schematic illustration of different light-driven pH changes behaviors of different proton, chloride or sodium rhodopsin transporters).

Here we report on an assay utilizing a simple device containing an array of narrow pH electrodes that could be illuminated from beneath using light-emitting diode (LED) illumination. Using this device, a subset of an environmental fosmid library from the Red Sea was screened (20 plates, 1920 clones) and 11 clones exhibited light-driven proton-pumping activity. All 11 clones contained a rhodopsin gene (diverse proteorhodopsins and a marine actinorhodopsin), with some fosmids also containing an adjacent gene cassette coding for a retinal biosynthetic pathway.

Results and discussion

A simple device containing eight narrow pH electrodes and LED illumination was designed, which would enable the direct screening of

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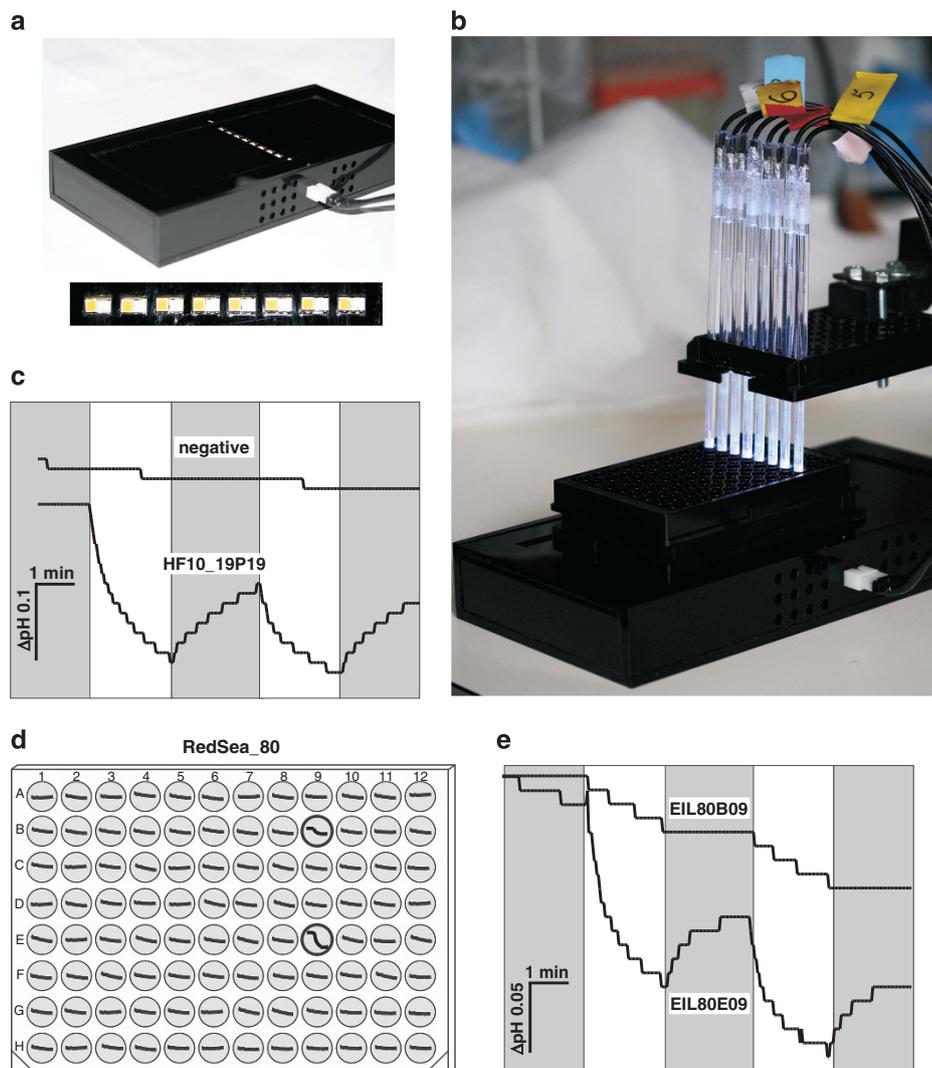


Figure 1 Light-driven transport of protons in the Red Sea fosmid library. (a) LED setup. Upper panel, enclosing of the LED light array; lower panel, close-up on the LED array. (b) Device setup. (c) pH changes in *E. coli* negative control (empty vector) and fosmid HF10_19P19 proteorhodopsin-expressing cells under dark (gray bars) and light (white bars). (d) Light-driven pH changes in the Red Sea fosmid library plate EIL80. Signals from positive wells are highlighted (wells are marked in bold). (e) Light-driven pH changes in positive clones EIL80B09 and EIL80E09. All assays were conducted in the presence of 10 μ M all-*trans* retinal and under induced copy control fosmid conditions.

environmental fosmid libraries in a 96-well format (Figures 1a and b). To adjust our device, we used a positive clone (HF10_19P19) from the study of Martínez *et al.*, 2007 that contained a proteorhodopsin gene and a retinal biosynthesis operon (Figure 1c). The use of narrow electrodes was essential for the simultaneous measurement of eight clones. An environmental fosmid library was constructed from Red Sea surface water (Supplementary methods), and copy control fasmids were used as cloning vectors to construct the library, as light-driven pH changes were observed only after the induction of the plasmid copy number from 1 to ~60 (data not shown).

An example of the screen is shown for plate EIL80 (Figure 1d) under induced copy control conditions and with the addition of external all-*trans* retinal. As seen, light-driven proton-pumping activity was observed in two clones (EIL80B09 and EIL80E09), with clone

EIL80E09 showing stronger proton-pumping activity (Figure 1e). The color of both clones' pellets could not be distinguished clearly from the pale yellowish color of the negative control *E. coli* pellet (see the left panel of Figure 2a). Full sequencing of both fasmids revealed a proteorhodopsin gene on both, and a retinal biosynthetic pathway on clone EIL80B9 (Figure 2a).

Screening overall 20 library plates (20 \times 96 = 1920 clones), 11 clones showed light-dependent pH decrease when all-*trans* retinal was added to the growth medium (Supplementary Table). Light-dependent alkalization, indicative of potential chloride or sodium pumps, was not observed with any of the tested library plates. When retinal was omitted, only clone EIL102C09 retained activity. Clone EIL102C09 was also the only clone to show a colored (orange) cell pellet (left side of Figure 2a), possibly indicative of a strong expression

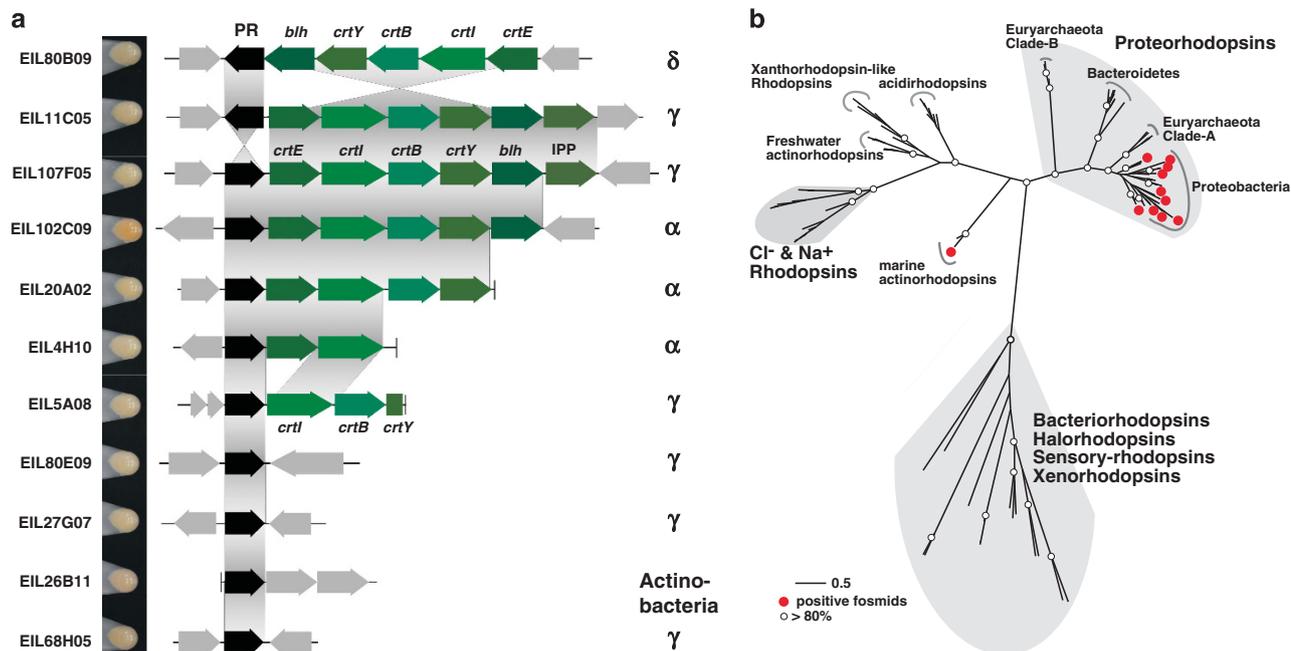


Figure 2 Red Sea microbial rhodopsins. (a) Schematic representation of the rhodopsin gene clusters identified in this work. Rhodopsin genes are marked in black, while genes in the retinal biosynthetic pathway are marked in green. Cell pellets (in the presence of retinal) are shown on the left. Predicted origin (shown on the right) is based on top blastX hits of different open reading frames on each clone (other than the rhodopsin and retinal biosynthetic pathway genes). (b) Unrooted maximum likelihood phylogenetic tree of microbial rhodopsin amino-acid sequences. Rhodopsins identified in this study are indicated with red circles. White circles represent bootstrap values >80%. Trees were constructed using the phylogeny.fr pipeline (Dereeper *et al.*, 2008), which included PhyML v3.0 (Guindon *et al.*, 2010) and the WAG substitution model for amino acids (Whelan and Goldman, 2001). One hundred bootstrap replicates were conducted for each analysis.

of a rhodopsin gene in this clone. All 11 positive fosmids were fully sequenced and contained a microbial rhodopsin gene, with some containing a full or partial retinal biosynthetic pathway (Figure 2a). Four of the positive clones detected did not contain any retinal biosynthesis related genes. Ten of the identified rhodopsins were proteorhodopsins, while one was similar to rhodopsins from the recently reported uncultured low GC and ultra-small marine Actinobacteria (Ghai *et al.*, 2013) (see phylogenetic tree in Figure 2b).

As observed in four of our clones, marine SAR86 bacteria (Dupont *et al.*, 2012) and some freshwater Actinobacteria (Garcia *et al.*, 2013; Keffer *et al.*, 2015) lack a recognizable retinal biosynthesis pathway. These microbes seem to scavenge retinal or structurally related pigments from the environment in order to activate and utilize their rhodopsins (Béjà *et al.*, 2001; Keffer *et al.*, 2015). However, retinal concentrations in sea water and fresh water are unknown.

The host used in our screens is the gamma proteobacteria *E. coli*, and it was therefore not surprising to detect rhodopsins from predicted marine gamma proteobacterial groups (such as SAR86). In addition, our screen detected rhodopsins from various alpha groups (SAR11 and SAR116) and delta proteobacteria (SAR324), and even from a Gram-positive bacterial group (the low GC and ultra-small marine Actinobacteria). Our

assay, therefore, seems elastic enough and is not restricted only to gamma proteobacteria-originating clones.

What does our screen miss?

Our designed screening system is based on the expression of microbial rhodopsins by their native promoters. Therefore, the following options are predicted to be not detected by our system: (i) promoters weakly recognized by *E. coli* will exhibit activity under our detection limits; (ii) promoters that are not recognized by *E. coli* will not be expressed; (iii) expressed but misassembled rhodopsins in *E. coli* membrane (for example, archaeal bacteriorhodopsins) will not exhibit measurable activity; (iv) every environmental DNA that is toxic to *E. coli* will be absent from our fosmid library (Béjà *et al.*, 2000b; Sorek *et al.*, 2007; Feingersch and Béjà, 2009; Danhorn *et al.*, 2012); and, finally, (v) if two rhodopsins with opposite activities are expressed from the same clone (that is, an outward proton transporter and an inward chloride transporter).

With all the above restrictions in mind, we suggest that our designed screening strategy is currently the best option for discovering novel microbial rhodopsins. With more libraries from different environments screened, and with automation of the method, we envision the finding

of diverse rhodopsin groups with new transport activities possibly providing a molecular basis for developing novel optogenetic tools (Boyden *et al.*, 2005; Zhang *et al.*, 2007; Inoue *et al.*, 2014).

Conflict of Interest

The authors declare no conflict of interest.

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