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Control of transmembrane ion fluxes to select halorhodopsin-deficient and other energy-transduction mutants of *Halobacterium halobium*

(bacteriorhodopsin/chemiosmotic circuits/sodium pump/membrane potential)

ELENA N. SPUDICH AND JOHN L. SPUDICH

Department of Anatomy, Albert Einstein College of Medicine, Bronx, New York 10461

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ABSTRACT **We describe a selection method for mutants altered in the generation and regulation of transmembrane ion flux in *Halobacterium halobium*. The method is based on experimental control of ion fluxes by a combination of light, ionophore, and external pH to generate an imbalance in the cells' proton circulation through their membranes. The steady-state proton circulation is increased by the introduction of a small inward proton leak with a protonophore. The cells are then illuminated to excite halorhodopsin, which hyperpolarizes the membrane and drives protons into the cells. As a result, wild-type cells suffer cytoplasmic acidification, which causes a dramatic loss of motility and suppresses their growth. These properties can be used to select for mutants that escape cytoplasmic acidification because either they lack halorhodopsin function or they have a greater capacity to eject protons during the illumination. In a population selected by this method, 97% of the individual cells were demonstrably altered in ion flux properties. Cells were selected with alterations in the halobacterial rhodopsins, specifically with deficiencies in membrane potential generation by halorhodopsin and with increased cellular proton ejection by bacteriorhodopsin. We describe properties of one of the halorhodopsin-deficient strains, Flx37.**

Transmembrane currents of protons and other ions are central to energy transduction in halobacteria (cf. reviews in refs. 1 and 2). The cell ejects protons through the light-driven proton pump bacteriorhodopsin and through respiration (3). The resulting inwardly directed protonmotive force (4) drives ATP synthesis (5) and flagellar motion (6) and generates high-capacity Na^+ and K^+ gradients (7, 8). Proton influx is coupled to Na^+ ejection through a Na^+/H^+ antiporter (8). The resulting inwardly directed Na^+-motive force sustains Na^+ inflows, which drive several amino acid-uptake systems (9). An additional component of this "chemiosmotic" circuitry has been discovered and named halorhodopsin (10). Like bacteriorhodopsin, halorhodopsin contains a retinal chromophore (11, 12) and generates an outside-positive membrane potential, apparently by light-driven Na^+ ejection (13, 14). The visible absorption and action spectrum of halorhodopsin is shifted to longer wavelengths by ≈20 nm compared to that of bacteriorhodopsin (11, 12, 15).

A promising approach to understanding energy transduction in halobacteria would be the isolation and study of strains genetically altered in their chemiosmotic circuitry. We have developed a method for the isolation of ion flux mutants in *Halobacterium halobium*, altered in their proton- and other ion-current properties. Isolation of one class of ion flux mutants, deficient in halorhodopsin function, is described in this communication.

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RATIONALE

The basic idea of the method is to perturb the steady-state flux of protons into the cells by introducing a small proton leak with a protonophore. With the cells poised in this state, the inwardly directed protonmotive force is abruptly increased by hyperpolarizing the membrane by photoexcitation of halorhodopsin. This drives protons into the cells, overcoming the cells' proton-ejection capacity and acidifying their cytoplasm. The method selects for mutants that escape cytoplasmic acidification because either they lack halorhodopsin function or they have a greater capacity to eject protons during the photoexcitation.

In reference to the flow chart of Table 1 and the diagram of Fig. 1, the rationale of each step of the method is as follows.

The incubation in step I allows the cells to adjust their ion currents to the low pH of the procedure. The cells are evidently able to adapt well to pH 5.5 because they will grow to high densities if left at step I.

Step II induces a H^+ influx, which can be partially compensated for by cellular H^+ efflux. Motility, which is expected to require continuous proton influx through the flagellar motor, is not eliminated by the treatment, demonstrating that the cells are able to establish a new steady-state H^+ current in the presence of the additional ionophore-induced influx. The loss of flagellar reversals, observed in other bacteria to occur at intermediate protonmotive force (16), insures that the ionophore-induced proton leak is a nonnegligible component in the cells' chemiosmotic circuitry. Poising the cells in this intermediate state of partially compensated, increased H^+ permeability is critical to the method.

The orange light used at step III excites halorhodopsin, which generates an outside-positive electrical potential across the cell membrane, presumably by Na^+ ejection (13, 14). In response to the membrane potential, protons are driven into the cell. Because the wild-type cell's capacity to eject protons has been nearly titrated by the inward proton leak, the halorhodopsin-mediated proton influx overcomes the cell's proton-ejection capacity. As a result, wild-type cells suffer cytoplasmic acidification, which causes loss of motility and suppresses their growth. These properties can be used to select for mutants that escape cytoplasmic acidification. Step IV enriches for these resistant cells, which are isolated and screened in steps V and VI.

MATERIALS AND METHODS

Strains. Strain S9, which contains carotenoids and all known retinal-dependent functions of halobacteria, was obtained from W. Stoeckenius (University of California, San Francisco). We isolated strains S9-O (orange) and S9-P (purple) by visual

Abbreviation: CCCP, carbonyl cyanide-*m*-chlorophenylhydrazone.

Table 1. Isolation of ion flux mutants

Step	Procedure
I	Incubate cells at pH 5.5 in complete medium for 1 hr
II	Add a concentration of CCCP sufficient to inhibit flagellar reversals but not motility
III	Illuminate
IV	Remove CCCP and grow at pH 5.5
V	Plate for single-colony isolation
VI	Examine individual colonies for ion flux properties

Scheme for the isolation of ion flux mutants. Details of steps I–VI are in *Materials and Methods*.

screening for the indicated color of individual colonies grown from strain S9 on an agar surface. Strain L-33, a carotenoid-deficient, bacteriorhodopsin-deficient derivative of S9, was isolated in a similar manner by J. Lanyi (17). Two retinal-deficient strains were used: WT1, which we isolated from S9, and W5002-7, which was from H. J. Weber (University of California, San Francisco). Strains were grown in peptone medium as described (18).

For mutagenesis, 2×10^8 cells in 1 ml of basal salt (19) were incubated for 40 min with 0.25 mg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Sigma). Killing by the mutagen was ≈25% for both S9-O and L-33 strains.

All ion flux mutants isolated with the selection method described in this paper have been given the designation Flx (e.g., Flx3 is a halorhodopsin-deficient mutant selected by the method from S9-O).

Light-Shock Selection Procedure. Aliquots (1 ml) of cell suspension at 2×10^9 cells per ml were removed from a culture grown as described (18). The cells were pelleted by 2 min of microfuge centrifugation at room temperature and resuspended in 4 ml of peptone medium made pH 5.5. After 1 hr of incubation at 37°C without agitation (step I in Table 1), carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP) from Sigma was added to 0.5-ml aliquots of the suspension to determine a concentration of CCCP that permits the cells to swim but eliminates the induction of swimming reversals by a strong repellent light stimulus (20). For strains S9-O and L-33 in our conditions, 10 μM CCCP eliminated reversals without immobilizing the cells.

For step II (Table 1), CCCP from a 10 mM ethanolic stock solution was added to a 10-mm rectangular plastic cuvette containing 0.5 ml of the cell suspension. The cuvette was placed in a cuvette holder thermostatted to 37°C, and the unstirred cell suspension was illuminated for 45 min (step III) with orange light (total of 1.2×10^7 ergs·cm^{-2}·sec^{-1}) delivered from three sides by xenon XBO 150-W and 300-W tungsten/halogen lamp beams extensively heat-filtered with dichroic heat-reflecting mirrors, heat-absorbing glass, and water and color-filtered with Schott OG550 and Corning 3-69 glass. To remove the CCCP (step IV), the cells were pelleted by microfuge centrifugation for 2 min and resuspended in fresh pH 5.5 medium at 5×10^7 cells per ml. After growth to 2×10^9 cells per ml, the cells were microfuged, resuspended at 2×10^8 cells per ml in pH 7.4 peptone medium, and grown to stationary phase. Single colony isolates were obtained by diluting and spreading the culture on peptone medium (pH 7.4) containing 1.5% agar.

Measurement of Photoinduced Proton Uptake. Cells were pelleted by Microfuge centrifugation, washed with basal salt containing 0.1 mM 2(*N*-morpholino)ethanesulfonic acid (pH 6.9 at 37°C), repelleted, and resuspended at 3.0×10^9 cells per ml. This suspension (2 ml) was placed in a 10-mm plastic cuvette containing a magnetic stirrer. Vigorous stirring at 37°C was maintained throughout the following sequence. After incubation in the dark for 1–2 hr, CCCP was added (final concentration, 20 μM). After 5 min of incubation in the dark, the suspension was illuminated for 5 min with orange light (2.0×10^6 ergs·cm^{-2}·sec^{-1}) with the optical and heat filters described in ref. 21. After a second 5-min period of dark incubation, the suspension was again illuminated. Data collection was begun 1 min prior to the second illumination. The pH was monitored with an Altex 4500 digital pH meter with a Beckman 39505 combination electrode inserted into the cuvette suspension immediately after CCCP addition.

RESULTS

Effects of Light-Shock. A population of S9-O cells was taken through steps I, II, and III of the procedure (Table 1). After steps I and II, microscopic examination showed >90% of the cells to be motile. Cells maintained at step II remained motile (with reduced swimming speed) for at least 6 hr, but dramatic

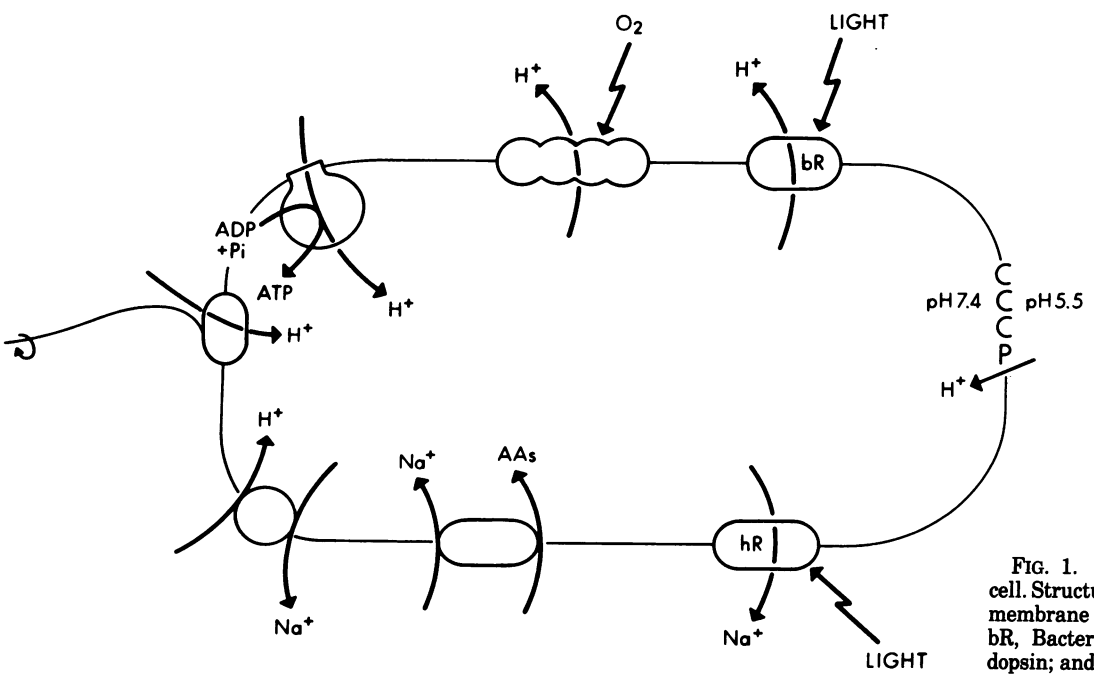

FIG. 1. Diagram of a halobacterial cell. Structures diagrammed in the cell membrane are discussed in the text. bR, Bacteriorhodopsin; hR, halorhodopsin; and AAs, amino acids.

loss of motility occurred after 45 min of illumination in step III. Microscopic examination showed this "light-shocked" population to be <1% motile. This immobilization effect was very useful as a simple and rapid assay for the effectiveness of light-shock under various conditions.

A second effect of the light-shock on the S9-O population was a pronounced inhibition of growth when the cells were washed and resuspended in fresh pH 5.5 peptone medium (Fig. 2, S9-O illuminated). Cells maintained in the dark as a control during the illumination were able to grow in the pH 5.5 peptone medium (Fig. 2, S9-O dark control).

The orange light of step III excites halorhodopsin, which hyperpolarizes the membrane and drives protons into the cell. This proton influx is facilitated by proton ionophores such as CCCP (13, 14). Several observations indicate that intracellular acidification from this proton influx causes the immobilization and growth suppression.

(*i*) Illumination in the light-shock procedure did not cause growth suppression or immobilization unless a proton ionophore (e.g., CCCP) was present, indicating proton flux is required for these effects.

(*ii*) The retinal mutant, W5002-7, which lacks light-induced proton fluxes, is resistant to light-shock as indicated by the growth curve of Fig. 2 and by its resistance to light-shock immobilization.

(*iii*) The immobilization and growth suppression persisted in medium at pH 5.5 but could be reversed by suspending the cells in medium at pH 7.4. This result is consistent with our interpretation because adjustment of the external pH to 7.4 will generate an outwardly directed pH gradient, facilitating a return of the acidified cell cytoplasm to its normal near neutral pH.

(*iv*) Bacteriorhodopsin protects cells from light-shock. Bacteriorhodopsin uses the orange light of the illumination at step III (Table 1) to pump protons out of the cell. Therefore, bacteriorhodopsin should protect the cells against internal acidification at the illumination step. If our interpretation is correct, bacteriorhodopsin should prevent the immobilization and growth suppression by light-shock. We tested this by comparing the effect of light-shock on the low bacteriorhodopsin strain S9-O and the purple strain S9-P, which has a high content of bacteriorhodopsin. Both strains were isolated as spontaneous color variants of the same parent (S9). We observed S9-P to be totally resistant to light-shock immobilization, and the data in Fig. 2 show that S9-P is resistant to growth suppression by light-shock, whereas S9-O is sensitive.

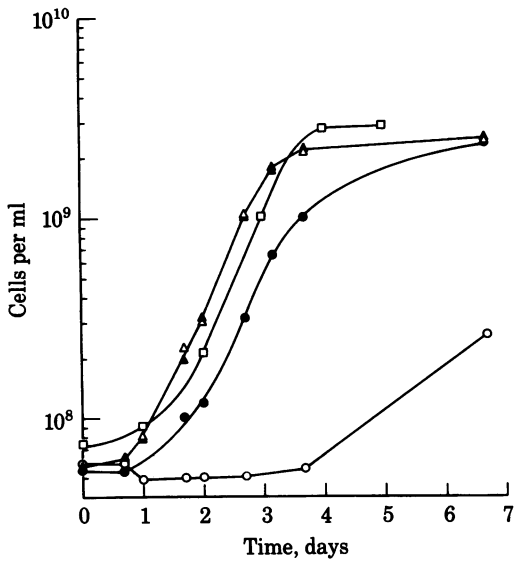

FIG. 2. Light-shock effects on growth. Cell density was determined by Petroff–Hausser counts after the indicated strains were taken through steps I–III (Table 1) of the light-shock procedure (illuminated) or incubated in the dark at step III (dark control). Time 0 is the time of removal of CCCP at step IV of the procedure (Table 1). ○, S9-O illuminated; ●, S9-O dark control; △, S9-P illuminated; ▲, S9-P dark control; and □, the retinal mutant W5002-7 illuminated.

Selection of Ion Flux Mutants. As shown in Fig. 2, growth suppression of shock-sensitive cells persisted for at least 3.5 days. Because shock-resistant cells can grow under these conditions, one would expect enrichment for resistant cells to occur during incubation. To determine the extent of enrichment, a population of mutagen-treated S9-O was divided into two aliquots. One, S9-O-L, was taken through the light-shock selection procedure outlined in Table 1. The control culture, S9-O-D, was treated identically except that it was maintained in the dark during step III (Table 1). To determine whether the light-shock method was effective in selecting a population of cells with altered ion flux properties, we measured the rate of photoinduced uncoupler-insensitive proton uptake. Such proton uptake, measured by following the alkalization of a cell suspension, has been shown to be a consequence of halorhodopsin-generated membrane potential (13, 14). The light-shocked population (S9-O-L) showed a proton uptake rate about one-fourth the rate of the control (S9-O-D) (Fig. 3), which was similar to that of the original strain (S9-O).

To analyze further the differences between the light-shocked and control populations, we plated each on an agar surface for examination of individual colonies. The nonshocked control showed 3% variation from the usual orange pigmentation, appearing either purple or with reduced pigmentation ("white") (Table 2). From our interpretation of light-shock effects, one would expect the purple (high bacteriorhodopsin content) variants of S9-O to be shock-resistant and, therefore, to be enriched by the light-shock method. Also those of the white variants, which have lost halorhodopsin (e.g., retinal mutants), should be resistant and enriched as well. Table 2 shows that application of the method did enrich considerably the purple and white variants.

The orange color in the colonies is due to carotenoid rather than retinal pigments. Therefore, to find mutants specifically deficient in halorhodopsin (and not in general pigmentation), one should examine the orange colonies from S9-O-L (Table 2). Accordingly, we examined 22 individual orange colonies from the light-shocked population (S9-O-L) and 12 individual orange colonies from the control (S9-O-D) for comparison. All of the control colonies showed large proton-uptake rates, predominantly producing large alkalizations between 0.30 and 0.45 pH units (Fig. 4), but only 1 of the 22 light-shocked colonies produced a pH increase in this range. Eight of the 22 S9-O-L colonies showed no detectable photoinduced proton uptake (these strains are included in the 0–0.05 range of the histogram). By the same criteria applied to strain Flx37 in the following section, none of the eight strains are mutants in the retinal synthesis pathway.

Considering as mutants all of the purple and white colonies and the orange colonies with proton uptake <0.20 pH units in Fig. 4, one can calculate that 97% of the individual cells in the light-shocked population are ion flux mutants by this definition. Because growth occurred during the selection, mutants of the same phenotype do not necessarily derive from independent mutations. Separate isolations are required to insure that mutants of a given phenotype derive from independent mutational events with this method.

To examine the mutant strains further, one would like to test for the lack of halorhodopsin absorbance and for its ion trans-

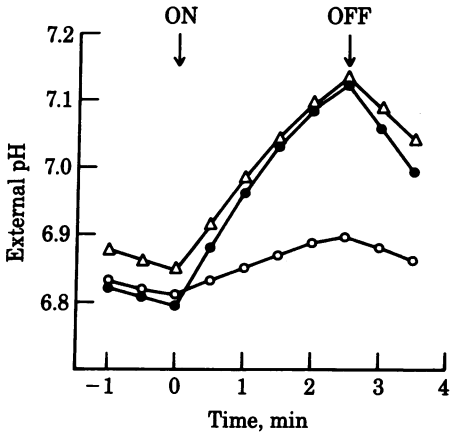

FIG. 3. Selection for a population of reduced photoinduced proton uptake by the light-shock procedure. A population of mutagen-treated S9-O cells were divided into two aliquots. One (S9-O-L) was processed through steps I–VI in Fig. 1 (○). The other (S9-O-D) was treated identically except that it was maintained in the dark during step III (●). Photoinduced proton uptake in the presence of uncoupler was measured as described in the text for the two processed populations and for an untreated population (S9-O) (△).

location function. The presence in these strains of carotenoid pigments is a disadvantage for spectroscopic measurements. Accordingly, we applied the light-shock procedure to L-33, a carotenoid-deficient ("white") strain (17), and isolated ion flux mutants showing reduced proton uptake as we had done for S9-O. One of these ion flux mutant strains, named Flx37, showed photoinduced proton uptake of <10% that of L-33 when assayed as in Fig. 3.

Properties of Flx37. The deficiency in photoinduced proton influx in Flx37 suggested that this mutant was deficient in halorhodopsin activity. Therefore, we examined a Flx37 membrane preparation to determine if this strain lacks the halorhodopsin absorption. In comparison with control strain membranes, Flx37 membranes are deficient in a pigment with maximum absorbance in the range of 580–590 nm (Fig. 5), in agreement with the absorption spectrum of halorhodopsin (11, 12, 15).

Two lines of evidence indicate that the halorhodopsin deficiency in Flx37 is not due to a defect in retinal synthesis. First, Flx37 retains full phototactic sensitivity, which depends on retinal (23). When examined microscopically, Flx37 shows swimming reversals in response to increases in blue light and decreases in red light. The sensitivities of Flx37 are quantitatively similar to those of L-33 when assayed by individual cell tracking (20). Because Flx37 is deficient in both halorhodopsin and bacteriorhodopsin, this result suggests the presence of at least one more retinal pigment, functioning as a photosensory receptor in *H. halobium*. It is important to note that, except for retinal-deficient isolates, all of the halorhodopsin-deficient mutants described in this paper retain full phototactic sensitivity.

The second evidence that Flx37 is not a retinal mutant is that addition of retinal to the growth medium does not restore photoinduced proton uptake assayed as in Fig. 3 (data not shown). Retinal addition to a retinal mutant (WT1) restored its photoinduced proton uptake under the same conditions.

Table 2. Color distribution in cell populations derived from S9

Population	Bacteriorhodopsin content, fg/cell*	% colonies†		
		Orange	Purple	White
S9-P	8.0	0.03	99.9	0.06
S9-O	<0.5	97.0	1.5	1.5
S9-O-D	<0.5	97.3	1.1	1.6
S9-O-L	1.4	60.5	32.3	7.2

* Determined by the light/dark-adaptation method as described (22).
† Based on >500 colonies per population.

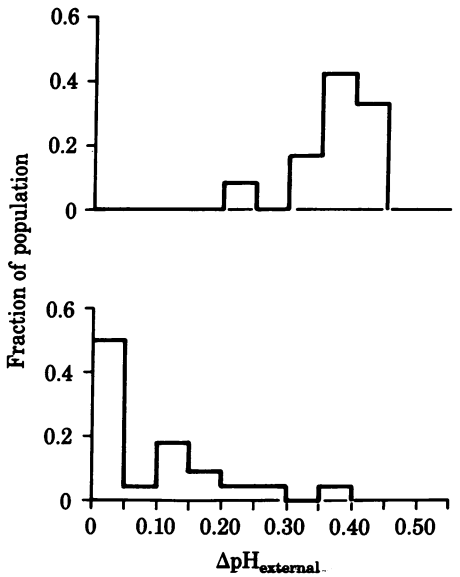

FIG. 4. S9-O population histograms of photoinduced proton uptake. Individual colonies were isolated from the S9-O-D (dark control) (*Upper*) and S9-O-L (light-shocked) (*Lower*) populations described in the text and in Fig. 3. For cultures prepared from each colony, photoinduced proton uptake was determined and expressed as the increase of external pH after 2.5 min of illumination.

DISCUSSION

The results presented above characterize a method for the selection of mutant *H. halobium* altered in membrane potential and H^+-current generation and regulation. The procedures described provide an enrichment and selection method for en-

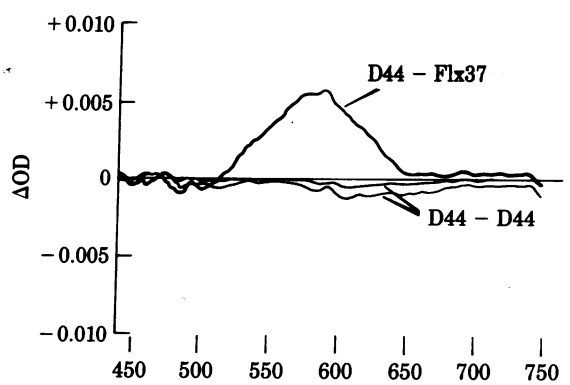

FIG. 5. Flx37 and D44 membrane absorption-difference spectrum. The control strain D44 was isolated from a non-light-shocked mutagen-treated L-33 population. Membranes containing 1.6 mg of protein per ml in 4 M NaCl were prepared as described (19). Difference spectra were determined in a Hitachi 110 spectrophotometer with the spectral band width set at 2.0 nm and the scanning rate set at 200 nm/min. A baseline from 438 nm to 750 nm was established by placing identical D44 membrane preparations in the sample and reference 10-mm-path-length cuvettes. To generate the D44 − Flx37 difference spectrum, the sample cuvette was washed with 4 M NaCl and filled with the Flx37 membrane preparation adjusted to have the same absorbance as D44 membranes at 700 nm. The two D44 − D44 difference spectra indicate error introduced by washing and refilling the sample cuvette.

ergy-transduction mutants of this organism.

Until now, the only variant *H. halobium* strains available have been those with pigmentation and refractility (gas vacuole variants) differences dramatic enough to be identified without an enrichment method by screening large numbers of colonies. "Colorless" forms, in particular, occur with a high frequency. Two classes of colorless strains have been particularly useful in energy-transduction studies: (*i*) those that, due to retinal synthesis defects, lack all light-driven ion-pumping activity unless cultured in the presence of exogenous retinal (11, 17, 24) and (*ii*) those that have negligible bacteriorhodopsin but contain other retinal pigments such as halorhodopsin (11, 15, 17, 25). Until now, no mutant deficient in halorhodopsin that was not a retinal mutant had been isolated. Initial application of the light-shock method reported here has led to the isolation of several halorhodopsin-deficient mutants that evidently are not retinal mutants. These mutants probably will be valuable in efforts to identify and isolate the halorhodopsin protein.

Mutants with several types of alterations in their ion flux properties would be predicted from the interpretation of the selection method in *Rationale*. Mutants defective in halorhodopsin function are expected because such cells would not hyperpolarize their membranes at step III (Table 1) and, therefore, would not be traumatized by a subsequent H^+ influx. We have shown that one such dysfunctional mutant, Flx37, is deficient in absorbance attributable to halorhodopsin and is not lacking other retinal pigments, such as those mediating phototaxis responses. One of the potentially interesting applications of the method is to produce a large number of different strains with variant dysfunctional halorhodopsins.

A second class of expected mutants are those with increased bacteriorhodopsin because such cells should be better able to maintain their internal pH during the light-shock. Confirming our interpretation of the method, our results show a 30-fold enrichment of strains with high bacteriorhodopsin content from a single application of light-shock selection. One can envision modifications of the method to use this protective effect of bacteriorhodopsin to select mutants with altered bacteriorhodopsin function.

In addition to these light-driven ion pump mutants, several other types of mutants with altered H^+-current regulation may escape light-shock. We might expect variants with increased permeability to Na^+ or other ions that can dissipate the hyperpolarized membrane state at step III before traumatic H^+ influx occurs. Mutants with increased H^+-ejection rates through respiration or the H^+-translocating ATPase might also be light-shock resistant. Similarly, activity of a Na^+/H^+ antiporter (8) could alleviate the effects of light-shock if it could couple proton ejection to Na^+ influx in the light-shock conditions. Finally, mutants in previously unidentified components involved in the regulation of the H^+ current may be selected by this method.

We have isolated a number of mutant strains partially or completely resistant to light-shock. Study of such mutants may clarify the relationship between the various interconnected energy transducers of these cells and aid the identification and isolation of the components of *H. halobium* chemiosmotic circuitry.

Note Added in Proof. The deficiency of halorhodopsin in Flx mutant membranes has now been confirmed in collaboration with R. A. Bogomolni by sensitive flash spectroscopic measurements made in the laboratory of W. Stoeckenius. Flx37 contains 8% and Flx3 contains <1% of wild-type levels of halorhodopsin photocycle intermediates. Flash spectroscopy has also revealed the presence of another photochemically reactive retinal-containing pigment in Flx mutant membranes lacking both bacteriorhodopsin and halorhodopsin.

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