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The Photoactive Yellow Protein from *Ectothiorhodospira halophila* as Studied with a Highly Specific Polyclonal Antiserum: (Intra)cellular Localization, Regulation of Expression, and Taxonomic Distribution of Cross-Reacting Proteins

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A rabbit antiserum was raised against the photoactive yellow protein (PYP) from *Ectothiorhodospira halophila* and purified by adsorption experiments to obtain a highly specific polyclonal antiserum. This antiserum was used to obtain the following results. (i) In *E. halophila*, PYP can be isolated from the fraction of soluble proteins. In the intact cell, however, PYP appeared to be associated with (intra)cytoplasmic membranes, as was concluded from analysis of immunogold-labelled thin sections of the organism. (ii) The regulation of expression of PYP was studied by using dot blot assays, Western blotting (immunoblotting), and rocket immunoelectrophoresis. Under all conditions investigated (light color, salt concentration, and growth phase), PYP was expressed constitutively in *E. halophila*. However, when *Rhodospirillum salexigens* was grown aerobically, the expression of PYP was suppressed. (iii) A large number of prokaryotic microorganisms contained a single protein, with an apparent size of approximately 15 kDa, that cross-reacted with the antiserum. Among the positively reacting organisms were both phototrophic and chemotrophic, as well as motile and nonmotile, organisms. After separation of cellular proteins into a membrane fraction and soluble proteins, it was established that organisms adapted to growth at higher salt concentrations tended to have the cross-reacting protein in the soluble fraction. In the cases of *R. salexigens* and *Chromatium salexigens*, we have shown that the cross-reacting protein involved is strongly homologous to PYP from *E. halophila*.

Membrane-bound photoactive proteins are known to function in *Halobacterium halobium*. In this organism and in related bacteria, retinal-containing proteins have a role in light energy transduction and in phototactic responses (for reviews, see references 29 and 31). A few years ago, the presence of a water-soluble protein of 14 kDa with a distinct yellow color was described in the purple sulfur eubacterium *Ectothiorhodospira halophila* (15). This protein is photoactive: it displays a photocycle that is quite similar to the photocycle of the sensory rhodopsins (10, 20, 22, 23). Its three-dimensional structure, which is unrelated to the structure of the bacterial rhodopsins, has been elucidated at 2.4-Å (0.24-nm) resolution (14). It consists of two perpendicular plates of β sheet, forming a β-clam structure, also observed in a number of eukaryotic proteins. Evidence indicating that the protein functions as the photoreceptor in a negative phototactic response has been obtained (28). Despite the photochemical and functional similarities between the photoactive yellow protein (PYP) and the sensory rhodopsins, the chromophoric group in PYP is not retinal (33).

Recently, PYPs were purified from *Rhodospirillum salexigens* (19) and *Chromatium salexigens* (18). Therefore, PYP is now known to be present in halophilic representatives of three purple phototrophic bacterial families. However, little is known about the presence of related proteins in other phototrophic and chemotrophic bacteria. Detection of these proteins may become obscured in those organisms, which contain large amounts of pigmented proteins. This may happen particularly in organisms in which such a protein would remain bound to the membrane(s) of the cell. In that case, the protein would copurify with the pigmented proteins that have a role in photosynthesis and/or electron transfer. Therefore, we have examined the presence of proteins that are immunologically related to PYP from *E. halophila* in a number of microorganisms, using a specific antiserum against this protein.

Below we describe the preparation of a specific antiserum against PYP and its use to characterize the subcellular localization and physiological regulation of expression of this protein. In addition, we demonstrate the presence of proteins that specifically cross-react with this antiserum in several prokaryotic organisms.

MATERIALS AND METHODS

Growth of cells and preparation of subcellular fractions. The strains described in Table 1 were grown in batch culture in complex media (when possible phototrophically) at their optimum growth temperature and harvested at the end of logarithmic growth. Cells were collected by centrifugation. For screening purposes, cells were dissolved in sample buffer (13) and analyzed via Western blotting (immunoblotting) (32).

R. salexigens was grown in the medium described in reference 19. The cells were grown anaerobically at 39°C in a

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TABLE 1. Proteins that cross-react with the specific antiserum against PYP from *E. halophila*

Organism[a]	Apparent molecular size (kDa)[b]	Localization[c]
Achromobacter xyloseoxidans	14	
Acinetobacter calcoaceticus BD413	17	
Anacystis nidulans M6301	[18]	
Azotobacter vinelandii UW38	17	S, M
Bacillus subtilis OG1	–	
Chromatium gracile HOL1	17	
C. minutissimum	15	
C. salexigens	20	
C. vinosum D	18	
Chlorobium thiosulfatophilum Tassajara	15	
C. limicola	18	
Chloroflexus aurantiacus	–	
Desulfovibrio vulgaris	20	
Ectothiorhodospira abdelmalekii	34	
E. halochloris	34	
E. halophila BN9626	21	S
E. halophila SL1	21	
E. mobilis BN9903	35, 38	
E. shaposhnikovii	19	
Escherichia coli NM538	17	M
Halobacterium halobium NRL S9	–	
Klebsiella pneumoniae NCTC418	17	
Methanobacterium thermoautotrophicum (DSM2133 or strain Marburg)	–	
Microcystis aeruginosa	[16]	
Prochlorotrix hollandica	19	
Pseudomonas stutzeri	17	M
Rhodobacter adriaticus GII	18	S, [M]
R. capsulatus 2.3.1	18	
R. capsulatus Kb1	18	M, [S]
R. sphaeroides 2.4.1	17	
R. sulfidophilus 2.11.1	18	
R. sulfidophilus BSW8	18	
R. sulfidophilus W4	18	
Rhodocyclus gelatinosus DSM 1709	14	M
R. purpureus	24	
R. tenuis DSM 109	14	M, [S]
R. tenuis 230	14	M
Rhodopila globiformis	17	
Rhodomicrobium vannielii	16	
Rhodopseudomonas palustris 121	14	M
R. viridis 170	14	M, [S]
R. viridis NTHC 133	14	
R. marina sin 8C (162)	15	S, [M]
Rhodospirillum rubrum DSM 107	18	
R. salinarum 40	35	S, [M]
R. salexigens 260	18	S
Streptococcus cremoris SK110	17	M
Synechocystis strain PC 6701	16	
Thiocapsa roseopersicina	–	

[a] When not indicated otherwise, the type strain was used.
[b] –, no cross-reaction. Brackets indicate the presence of minor quantities.
[c] S, soluble proteins; M, membrane proteins. Brackets refer to the presence of minor quantities.

completely filled screw-cap tube and illuminated with 60-W tungsten light bulbs. When grown aerobically, the cells were grown on a rotary shaker at 37°C. After each growth experiment, the cultures were screened on agar plates for the presence of infections.

To separate membrane-bound and soluble proteins, cells were disrupted with a French press (at 140 MPa) or by sonication (four to six bursts of 30 s each, with intermittent cooling on ice) with a tip sonicator. After removal of debris by low-speed centrifugation (3,000 × *g*, 20 min, 4°C), the two fractions were separated by ultracentrifugation (200,000 × *g*, 2 h, 4°C).

Immunization. Antiserum against purified PYP from *E. halophila* (15) was obtained by injecting New Zealand White rabbits with 100 μg of protein in Freund complete adjuvant (Difco Laboratories, Detroit, Mich.). Subsequent injections of 100 μg of protein in Freund incomplete adjuvant were given at weekly intervals for several weeks. Rabbits were bled 5 and 9 days after the last injection, and the blood was left to clot for 1 h at room temperature. After storage overnight at 4°C, aggregated material was removed by centrifugation, and the supernatant was used as crude antiserum. Serum collected prior to the immunization served as a control. PYPs from *E. halophila*, *R. salexigens*, and *C. salexigens* were purified as described previously (15, 18, 19).

Analytical procedures. Protein was determined by the method of Bradford (2), using bovine serum albumin as a standard. Immunodecoration of Western and spot blots was performed with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G, obtained from Bio-Rad (Richmond, Calif.).

SDS-PAGE. Analytical polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) was carried out as described by Laemmli (13) in a Bio-Rad mini-slab gel apparatus. Preceding electrophoresis, the samples were incubated with SDS at 100°C for 10 min. The gels were stained with Coomassie brilliant blue G250 or by silver staining (37). Phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa) were routinely used as molecular mass markers (Pharmacia, Uppsala, Sweden). In some experiments, horse heart cytochrome *c* (12.5 kDa) also was used.

Western blotting and immunodetection. Proteins from an SDS gel were blotted onto nitrocellulose and subsequently immunodecorated by the method of Towbin et al. (32) in a Bio-Rad trans-blot cell. Serum with the primary antibody was diluted between 250- and 2,000-fold. The secondary antibody was used at a dilution of 1:3,000. When optimal sensitivity was required, the hybridizations with both the primary and secondary antibodies were performed at room temperature for at least 2 h instead of 1 h.

For dot blot assays of PYP, dilution series of protein samples and of a standard with purified PYP were spotted on dry nitrocellulose. After evaporation of the water in the samples on the nitrocellulose, the blots were treated in the same manner as the Western blots. The lowest dilution which still resulted in a positive signal was determined. When total cell extracts were used, first a chloroform pigment extraction was performed, in order to prevent a strong background staining of the spots due to the photosynthetic pigments. This was not necessary when ultracentrifugation supernatant (200,000 × *g*, 2 h, 4°C) was used.

Immunoelectrophoresis. Rocket immunoelectrophoresis was performed as described previously (3, 12) in a Multiphor 2117 apparatus (LKB, Bromma, Sweden). The gels (3.2 ml) were poured on glass plates measuring 4.9 by 4.9 cm. Purified PYP was used as a standard; the concentration of PYP was calculated from the A_{446}, using an extinction coefficient of 45,500 M^{-1} cm^{-1} (21). Crossed immunoelectrophoresis was performed essentially as described by Smyth et al. (26) and Elferink et al. (6). The conditions for electrophoresis in the second dimension were those used for rocket immunoelectrophoresis.

Immunogold labelling and electron microscopy. Immunocy-

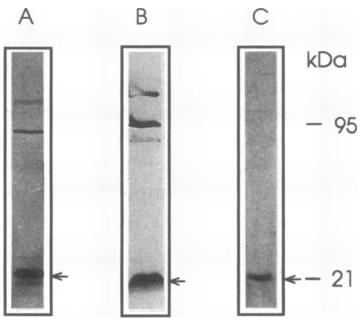

FIG. 1. Preparation of a specific antiserum against PYP from *E. halophila* by removal of nonspecific antibodies from the crude antiserum. Western blots of total soluble protein from *E. halophila* after immunodetection with unadsorbed (A), immunoeluted (B), and twice-adsorbed (C) antiserum against PYP. The primary antisera were diluted approximately 300- to 500-fold in the immunodecoration of the Western blots. The band that comigrated with pure PYP is indicated by an arrow.

tochemistry was performed on ultrathin sections of Lowicryl-embedded cells essentially as described by Douma et al. (5), using a 1:100 dilution of the crude antiserum.

RESULTS

A specific antiserum against PYP. An antiserum against PYP from *E. halophila* was raised by repeated immunization of a rabbit with purified yellow protein from *E. halophila*. When the resulting serum was tested in a Western blot of the soluble protein fraction from *E. halophila*, a number of proteins reacted positively (Fig. 1A). The two predominant bands migrated with apparent sizes of approximately 95 and 20 kDa, respectively. Minor bands were sometimes visible at the positions of 70- and 120-kDa proteins. The cross-reacting protein with the highest molecular mass was only rarely observed (compare Fig. 1 and 4B) and may be an aggregation product of the 95-kDa protein. No additional bands were observed when total cell preparations of *E. halophila* were used for SDS-PAGE.

The 20-kDa band often appeared in Western blots as a doublet or triplet. The dominant band comigrated with Coomassie blue-stained pure PYP, however. The ratio of intensities of the bands was variable. Immunodetection of Western blots with preimmune serum did not show any staining.

To confirm that the 20-kDa protein identified in the Western blot was identical to purified PYP, rocket immunoelectrophoresis and crossed immunoelectrophoresis were performed. One unique precipitation arc was visible after crossed immunoelectrophoresis of the soluble protein fraction of *E. halophila* (Fig. 2A). The protein that gave rise to this arc was identical to the yellow protein, as can be concluded from the fusion of the two arcs in a tandem rocket electrophoresis experiment (Fig. 2B). These results further imply that the 95-kDa protein does not form a precipitation arc in crossed immunoelectrophoresis. The same conclusions could be drawn from immunodiffusion (3) experiments (data not shown).

The molecular weight of PYP, as determined by electrospray mass spectroscopy, is 14,021 (33). The discrepancy between this value and the value of 20 kDa as observed with SDS-PAGE can be explained by an anomalous mobility of PYP in SDS-polyacrylamide gels. This was concluded from a Ferguson analysis (8) of the molecular weight of PYP. The apparent molecular weight of this protein decreased with the percentage of acrylamide in the gel (Fig. 3A), possibly because of an excess of negative charges on the protein. Halophilic organisms generally tend to have an excess of negatively charged amino acid residues in their proteins (35), and this is also true for PYP (15). When the apparent molecular weight of PYP was calculated from the slope of the plot of relative mobility versus the percentage of acrylamide (8), an apparent molecular size of 9 kDa was obtained (Fig. 3B). Apparently, this procedure resulted in an underestimation of the molecular weight.

To obtain a specific antiserum uniquely directed against PYP, experiments were set up to remove antibodies from the antiserum that cross-reacted with other proteins from the soluble protein fraction of *E. halophila*. These other proteins did not react with the antiserum in crossed immunoelectrophoresis (Fig. 2A). In agreement with this finding, it was observed that the specificity of the antiserum could not be increased by straightforward absorption experiments (i.e., by immunoaggregation) with a complex protein mixture from *E. halophila* that lacked PYP (see below). This may be due to the fact that the 95-kDa protein and other weakly cross-reacting proteins contained only a single epitope that is recognized by the antiserum. Therefore, experiments were set up to purify PYP as described previously (15). The soluble protein fraction from *E. halophila* was adsorbed to a DEAE-DE52 cellulose anion-exchange resin in 20 mM Tris-HCl (pH 8.0) and eluted with an NaCl gradient. Western blots of the resulting fractions showed that PYP eluted at about 190 mM NaCl. During this chromatography, PYP clearly separated from the rest of the soluble proteins of *E. halophila* (Fig. 4). These other proteins were pooled, concentrated by ammonium sulfate precipitation, and resuspended at 10 mg/ml. This concentrated protein fraction, which contained the 95-kDa protein, was covalently attached to activated CH-Sepharose 4B (Pharmacia) according to the manufacturer's instructions. Absorption of the antiserum with this modified Sepharose resulted in a specific

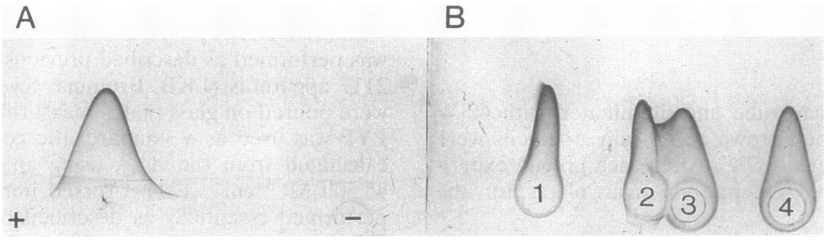

FIG. 2. Identification of the dominant protein from *E. halophila* that cross-reacts with the crude antiserum as the PYP. (A) Crossed immunoelectrophoresis of 50 μg of the soluble protein fraction from *E. halophila*. Thirty microliters of the unadsorbed antiserum was dissolved in the agarose gel prior to solidification. Further details were as described in Materials and Methods. (B) Tandem rocket electrophoresis of purified PYP (100 ng in wells 1 and 2) and the soluble protein fraction from *E. halophila* (0.1 mg in wells 3 and 4); 50 μl of serum was added to the gel.

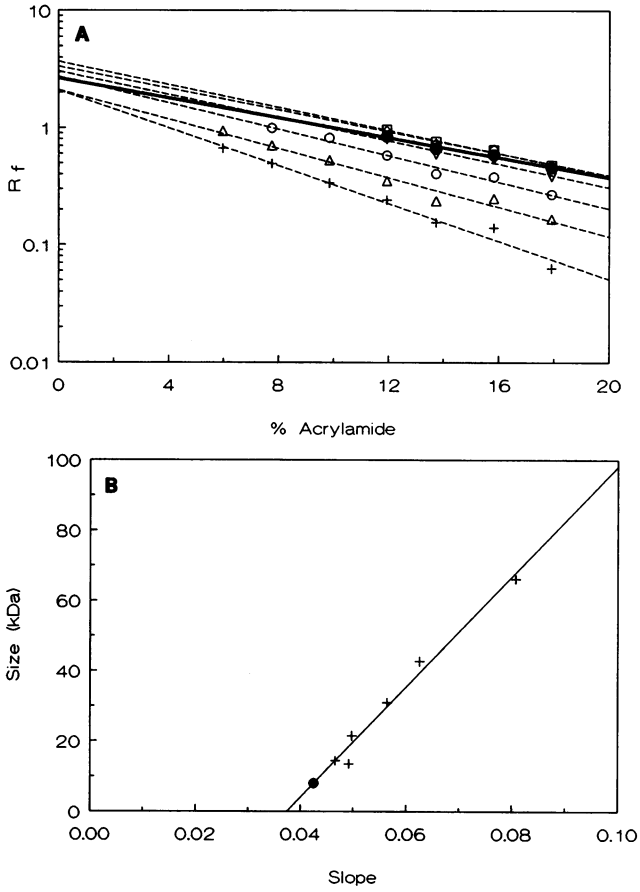

FIG. 3. Ferguson analysis of the molecular weight of PYP from *E. halophila*. (A) The relative mobilities of PYP and marker proteins were analyzed as a function of the percentage of polyacrylamide. Marker proteins (symbols other than filled circles) were bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme, and horse heart cytochrome *c*. Filled circles represent data obtained with PYP. (B) The size of PYP as determined from the retardation of the protein by polyacrylamide during gel electrophoresis. Crosses, marker proteins; filled circle, PYP. Experimental conditions were as described in Materials and Methods.

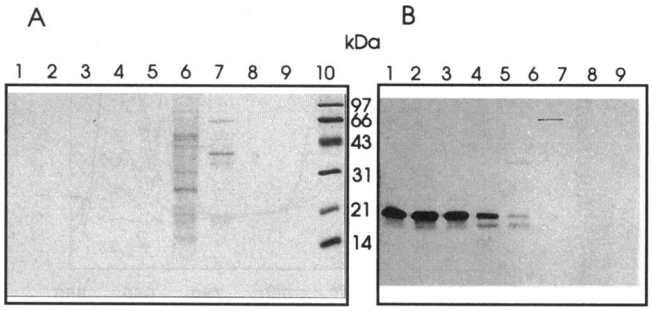

FIG. 4. Separation of PYP from *E. halophila* from proteins cross-reacting with the crude antiserum. (A) Coomassie blue-stained SDS-polyacrylamide gel (12% acrylamide) of fractions from a DEAE-DE52 cellulose column, run as described elsewhere (15). Lanes 1 to 9 contain 5-μl portions of fractions 100, 105, 110, 140, 170, 200, 220, 250, and 280, respectively, from this chromatography. Lane 10 contains molecular mass markers. (B) Western blot of the same gel.

removal from the antiserum of antibodies not uniquely directed against PYP. Repetition of this treatment resulted in an antiserum that, of all soluble proteins in *E. halophila*, reacted exclusively with PYP (Fig. 1C). Staining of PYP in cell extracts from *E. halophila* with this antiserum could easily be accomplished with a 6,000-fold dilution of the antiserum. But even when the absorbed antiserum was used in a very concentrated form (i.e., a 500-fold dilution; Fig. 1C), cross-reaction with only PYP was observed. The antibodies that bound to the derivatized Sepharose were eluted with a carbonate buffer of pH 10.8. This fraction was enriched in antibodies recognizing the 95-kDa protein (Fig. 1B).

Subcellular localization of PYP in *E. halophila*. The majority of PYP in *E. halophila*, upon fractionation of the cells at low ionic strength, was recovered in the soluble protein fraction (Table 1). However, this does not exclude the possibility that the protein in vivo is actually bound to the (intracytoplasmic) membrane. It might have dissociated from the membrane upon lysis of the cells in a medium with a low salt concentration or with a pH that differs significantly from the physiological pH. To elucidate the function of PYP, it is important to determine its in vivo localization. We therefore used the antiserum to analyze the subcellular localization of PYP in *E. halophila* by immunocytochemical methods. However, this experiment was complicated by the fact that the cellular content of PYP was so low that in one thin section of a cell, not more than a few molecules could be expected to become labelled. An intact cell contains no more than a few hundred copies of PYP (15). The labelling patterns obtained indicated that in a thin section of an intact cell, these few molecules indeed were associated with the membrane, most notably with the intracytoplasmic part (Fig. 5). Although the labelling was weak, it was reproducible, and the same pattern could be observed in all cells studied. We cannot exclude the possibility that the 95-kDa protein is responsible for the labelling, but two arguments favor the interpretation that the labelling is caused by PYP. First, the majority of the antibodies in the antiserum is directed against PYP; and second, many proteins from other organisms that cross-reacted with the specific antiserum against PYP were also membrane associated (see below).

Regulation of the expression of PYP. Dot blot and rocket immunoelectrophoresis techniques were used to investigate the regulation of PYP expression in *E. halophila*. The effects of

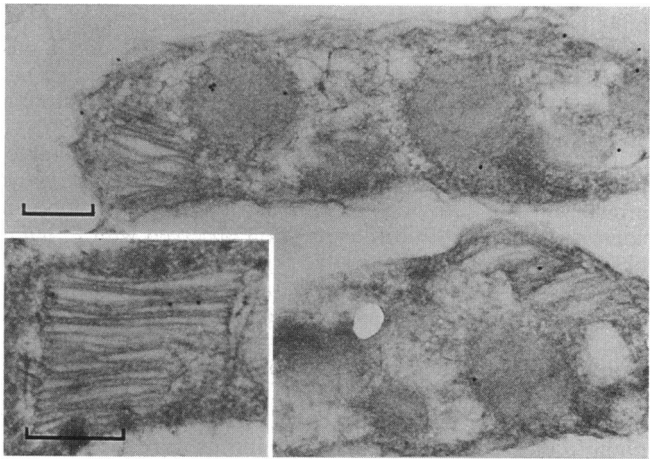

FIG. 5. Subcellular localization of the PYP in *E. halophila* with immunogold labelling of intact cells. Labelling was performed with the crude antiserum as described in Materials and Methods. The bar indicates 1 μm.

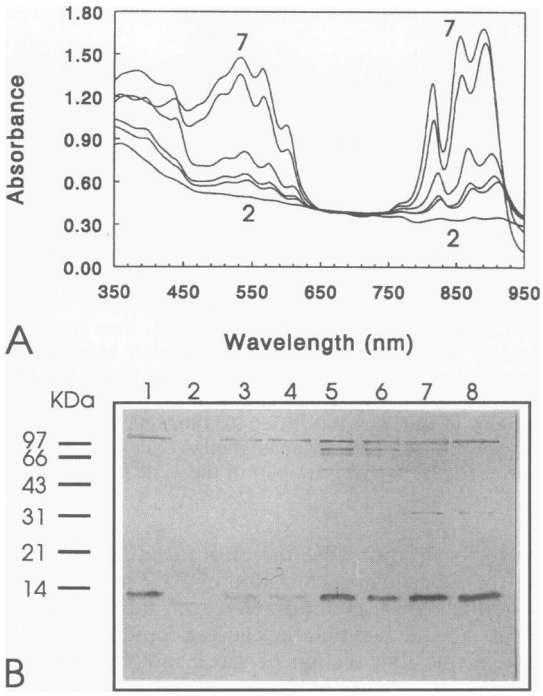

FIG. 6. Expression of PYP in *R. salexigens* during transitions from anaerobic to aerobic growth. Anaerobically grown *R. salexigens* was diluted to different degrees in fresh medium and incubated under aerobic conditions. When the cells had entered the stationary phase, the cultures were examined with in vivo absorbance spectroscopy (A) for the presence of photosynthetic pigments and Western blotting (B) to detect the presence of PYP. The cells applied in lane 2 were grown and regrown (after dilution) aerobically. The cells in lanes 3 to 7 were inoculated with 1, 4, 5, 6, and 10 ml, respectively, of anaerobically grown cells in a final volume of 20 ml of fresh medium and then grown aerobically. In lane 1 and 8, cell material was used from cultures that were grown anaerobically starting with an inoculum of anaerobically grown cells. The numbering of the spectra in panel A corresponds to the lane numbers in panel B.

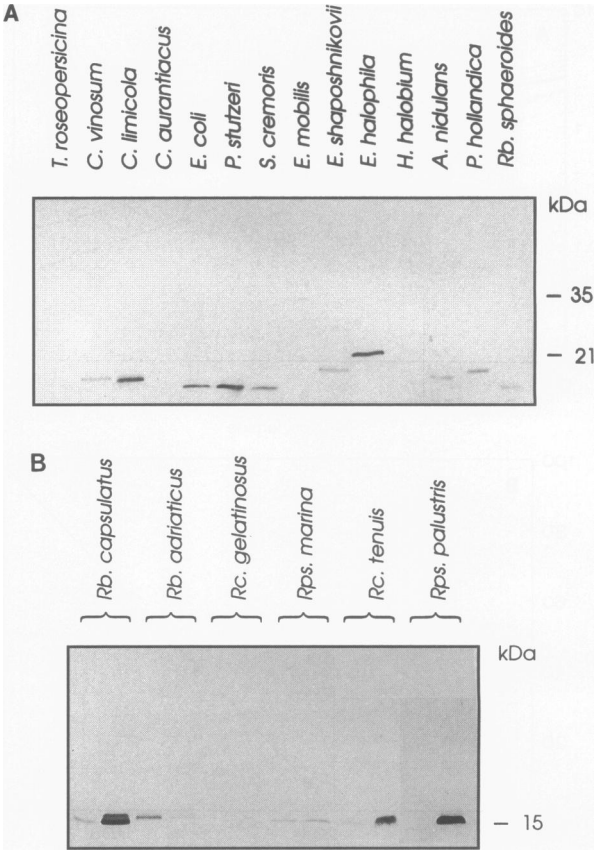

FIG. 7. Western blot of cells, membrane proteins, or soluble proteins from a number of prokaryotic microorganisms, obtained with a specific antiserum against PYP from *E. halophila*. Cells were grown, harvested, and fractionated as described in Materials and Methods. Gels were made of 15% acrylamide. Immunodetection and Western blotting were performed with the specific, adsorbed antiserum as described in Materials and Methods. About 10 μg of protein was layered in each lane. In panel A, total cellular protein was tested of a number of phototrophic and chemotrophic eubacteria. In panel B, a number of members of the family *Rhodospirillaceae* were tested, after separation of their proteins in a soluble fraction (left) and a membrane fraction (right) by high-speed centrifugation.

the following parameters were investigated: growth phase in a batch culture, salt concentration, and light color. To change the composition of the actinic light from a 60-W tungsten light bulb, broad-band blue and red filters and a filter combination transmitting only infrared light were used. All conditions tested resulted in the same level (less than a twofold change) of PYP expression: approximately 0.05% of the total protein (data not shown).

The presence of PYP in *R. salexigens* after both anaerobic, phototrophic growth in the light and aerobic, heterotrophic growth in the dark was examined with Western blots. In contrast to the constitutive expression observed with *E. halophila* (an obligately phototrophic organism [11]), we observed a more than 10-fold suppression of PYP expression during aerobic growth (Fig. 6). The expression of PYP correlated well with the expression pattern of the photosynthetic pigments (compare Fig. 6A and B; results for cells from two separate growth experiments are shown).

Cross-reacting proteins in other organisms. A number of phototrophic and chemotrophic organisms were screened for the presence of proteins immunologically related to PYP from *E. halophila*. The result of this screening showed that in a high percentage of the organisms tested, a single cross-reacting protein was present (Fig. 7). Significantly, such a protein was present in well-characterized organisms like *Escherichia coli* and *Streptococcus cremoris* but not in *H. halobium*, which possesses several retinal-containing photoactive proteins (29, 31).

These results were obtained only when the purified antiserum was used. Immunodetection with the crude antiserum (before adsorption) resulted in the staining of multiple bands in most of the bacteria. Many of the specifically cross-reacting proteins have a size similar to that of PYP of *E. halophila*, i.e., around 15 kDa. The cross-reacting proteins from organisms other than *E. halophila* interacted with the specific antiserum more weakly than PYP did. This could be concluded from the observation that for clear staining of these proteins in Western blots, a dilution of the primary antiserum of no less than 500- to 1,000-fold and long hybridization times were required. This weaker interaction is probably also the explanation for the observed lack of a precipitation reaction (not shown) with any of the cross-reacting proteins (except for PYP from *E. halophila*; Fig. 2) in rocket and crossed immunoelectrophoreses.

The results of the screening are summarized in Table 1.

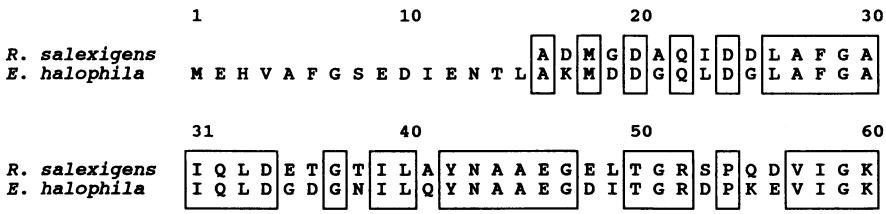

FIG. 8. Alignment of the N-terminal amino acid sequences of the PYPs isolated from *E. halophila* and *R. salexigens*. The alignment of the N-terminal sequence the PYP from *R. salexigens* reported here, as determined by standard automated amino acid sequencing, with that of the amino acid sequence of *E. halophila* (33) shows that the two proteins are strongly homologous and that partial proteolysis of the *R. salexigens* PYP had probably occurred during the isolation of this protein.

Organisms from our screening that explicitly lacked a protein that cross-reacted with the specific antiserum are *Microcystis aeruginosa*, *Chloroflexus aurantiacus*, *Thiocapsa roseopercicina*, and the two archaebacteria *Methanobacterium thermoautotrophicum* and *H. halobium*.

For a number of organisms, we determined whether the cross-reacting protein was a soluble protein or whether it was attached to the cytoplasmic or intracytoplasmic membrane (Table 1 and Fig. 7B). The trend in these results is that in organisms thriving in a salt-rich habitat, the cross-reacting proteins had an increasing tendency to be present in the fraction with soluble proteins, after fractionation of the cells. In the other organisms, the corresponding protein tended to be associated with the membrane.

The key question concerning the cross-reacting proteins described above is whether they are homologous to PYP from *E. halophila*. We have examined this issue with respect to two organisms. *R. salexigens* and *C. salexigens* contain a single cross-reacting protein (Table 1), and PYPs have been isolated from them (18, 19). Flash photolysis experiments have indicated that both proteins have a photocycle very similar to that observed for PYP from *E. halophila* (7, 19). N-terminal sequence analysis of PYP from *R. salexigens* indicated that it is strongly homologous to PYP from *E. halophila* (Fig. 8). The complete amino acid sequences of all three PYPs have been determined and show strong homology over their complete sequences (34). Therefore, both the photophysics and the primary structures of these three proteins are strongly conserved. Western blotting of the three known, homologous PYPs purified from *E. halophila*, *R. salexigens*, and *C. salexigens* showed that the adsorbed antiserum reacted with all three proteins (Fig. 9), which is independent evidence that the antiserum indeed recognizes PYP of *E. halophila* and homologs of this protein. Since Western blot analysis of *R. salexigens* and *C. salexigens* cell material shows a single cross-reacting protein with the same molecular size as those of the PYPs isolated from these organisms (Table 1), it can be concluded that the antiserum specifically recognized PYP homologs in the total cell extracts of these two organisms.

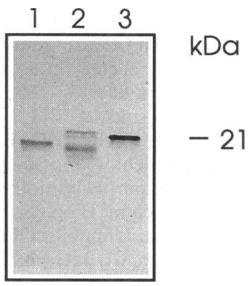

FIG. 9. Selectivity of the specific PYP antiserum, as tested with independently isolated PYPs. Purified PYPs isolated from *C. salexigens* (lane 1), *R. salexigens* (lane 2), and *E. halophila* (lane 3) were blotted and immunodecorated with the adsorbed antiserum. The two bands in lane 2 can be explained by partial proteolysis (see Fig. 8).

To further examine the selectivity of the specific PYP antiserum, it was tested against two fluorescent yellow proteins, which have absorption spectra similar to that of PYP but no photoactivity and a molecular size (approximately 38 kDa) larger than that of PYP, which were partially purified from *Rhodopseudomonas marina* (17) and *Rhodobacter sphaeroides* (16). No cross-reaction was observed with the two fluorescent yellow proteins (not shown). For *Rhodopseudomonas marina* and *Rhodobacter sphaeroides*, the cross-reacting component (Table 1) was smaller than the corresponding fluorescent yellow protein, indicating that these organisms contain both a fluorescent yellow protein and a protein that cross-reacts with the serum directed against PYP. These results independently confirm that a highly specific antiserum directed against a homologous set of PYP has been obtained.

DISCUSSION

In intact *E. halophila* cells, PYP appears to be associated with intracytoplasmic membranes (Fig. 5), although as expected, the labelling was weak. This in vivo localization is supported by the observations regarding cross-reacting proteins in other organisms. Apparently, the proteins involved do not have a transmembrane topology. Preliminary experiments in which *Escherichia coli* membranes, containing the cross-reacting protein after sonification and ultracentrifugation, were incubated with 1 M NaCl resulted in the partial solubilization of this protein (9). In this respect, it is interesting that a hydrophobic patch of aromatic amino acids is present on the surface of PYP and that the acidic and basic residues are localized on opposite sides of the protein (33). These asymmetries may lead to an interaction between PYP and the membrane surface. Alternatively, PYP may specifically interact with a transmembrane protein that forms the next step in a phototactic signal transduction chain leading to the flagellum and resulting in negative phototaxis (28).

Many parallels exist between sensory rhodopsins I and II (SRI and SRII) and PYP (22, 30). The photocycles in these proteins share many characteristics, and all three proteins function as photoreceptors in phototactic responses. The wavelength maxima of SRII and PYP are similar (480 and 446 nm, respectively), while SRI absorbs at 587 nm (15, 29). Functionally, SRII and PYP are closely analogous (both are involved in negative phototaxis). However, the presence of a two-photon photocycle is shared by PYP and SRI (23, 29). Whether this

process is biologically functional in the case of PYP, as it is in SRI, remains to be investigated. With respect to the regulation of expression of PYP, it is interesting that while we have not been able to find conditions in which the expression of PYP was changed in *E. halophila* (an obligatory phototrophic organism), the expression of PYP in *R. salexigens* was suppressed when the organism was grown aerobically. This latter observation is reminiscent of the pattern of gene expression observed for SRI, which is induced under reduced oxygen tensions, while SRII is expressed constitutively (24). Whether the repression of expression of PYP in *R. salexigens* is also triggered by oxygen, in parallel with the photosynthetic machinery (4, 25), should be examined more closely. The adsorbed antiserum did not show any staining of *H. halobium* total cell extracts, consistent with the very large differences in both primary and tertiary structure between PYP and the (sensory) rhodopsins.

The adsorbed antiserum specifically reacts with a single protein of about 15 kDa in a large number of eubacteria (Table 1 and Fig. 7). Although the signals were rather weak, we think that this cross-reaction is significant for a number of reasons. First, rocket and crossed immunoelectrophoreses showed that the antiserum strongly and specifically reacts with a single component from *E. halophila*, which is identical to PYP (Fig. 2). Second, the adsorbed antiserum reacts uniquely with PYP in Western blots of *E. halophila* total cell extracts (Fig. 1C). The fact that the reacting band in Fig. 1C is observed at 20 kDa and not at 14 kDa as expected is the result of an anomalous mobility of PYP in SDS gels (Fig. 3). In addition, the 20-kDa band comigrated with PYP purified from *E. halophila*. Third, the adsorbed antiserum reacts specifically with PYPs isolated from two other organisms (*R. salexigens* and *C. salexigens*) but not with two fluorescent yellow proteins (Fig. 9). The three PYPs have been isolated from purple phototrophic bacteria belonging to three different families (11, 36). Therefore, it is remarkable that the antiserum reacts with all three PYPs, in view of the taxonomic distance between the three organisms involved. These characteristics (both specificity for PYP and cross-reaction with taxonomically distantly related proteins) make the adsorbed antiserum unusually well suited for the taxonomic screening described here. Fourth, the weak cross-reactions in a large number of eubacteria were unique and indicated a protein with a molecular weight similar to that of PYP from *E. halophila*. Also the partially membrane associated nature of the cross-reacting proteins resembles the characteristics of PYP, suggesting that a similar type of protein is involved.

Except for *E. halophila*, none of the organisms tested, including *R. salexigens*, gave rise to a precipitation arc in immunoelectrophoresis experiments. Apparently, only a few epitopes are involved in the staining reactions of the Western blots. A speculative interpretation of this result is that a single very strongly conserved epitope is present in all cross-reacting proteins. The existence and nature of this epitope (possibly involved in signal transduction or cofactor binding) could be examined by further characterization of the cross-reacting proteins. Since specific signals were observed in both phototrophic and chemotrophic, as well as in motile and nonmotile, organisms, a number of speculations about the nature of these proteins are possible: the proteins could be involved in the detection of harmful light, could be involved in light-regulated gene expression (1, 27), or could all have a β-clam structure (chemosensors?). It should be mentioned that PYP is the first eubacterial protein described to have this β-clam structure (14). Purification and characterization of a number of the cross-reacting proteins reported here are necessary to clarify this matter.

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