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Construction, Expression, and Localization of a CycA::PhoA Fusion Protein in *Rhodobacter sphaeroides* and *Escherichia coli*

AMY R. VARGA† AND SAMUEL KAPLAN†*

Department of Microbiology, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

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We demonstrated the utility of *Escherichia coli* alkaline phosphatase, encoded by *phoA*, as a reporter molecule for genetic fusions in *Rhodobacter sphaeroides*. A portion of the *R. sphaeroides cycA* gene was fused to *phoA*, yielding a fusion protein comprising the putative signal sequence and first 10 amino acids of the cytochrome c_2 apoprotein joined to the sixth amino acid of alkaline phosphatase. The fusion protein was efficiently transported to the periplasm of *R. sphaeroides* as determined by enzyme activity, Western immunoblot analysis, and immunogold electron microscopy. We also documented the ability of an *R. sphaeroides* mutant, RS104, with gross defects in photosynthetic membrane morphology to efficiently recognize and translocate the fusion protein to the periplasmic compartment. The inclusion of 500 base pairs of *R. sphaeroides* DNA in *cis* to the *cycA* structural gene resulted in a 2.5-fold increase in alkaline phosphatase activity in photosynthetically grown cells compared with the activity in aerobically grown cells, demonstrating that the fusion protein is regulated in a manner similar to that of cytochrome c_2 regulation. We also constructed two pUC19-based plasmids suitable for the construction of translational fusions to *phoA*. In these plasmids, translational fusions of *phoA* to the gene under consideration can be made in all three reading frames, thus facilitating construction and expression of fusion protein systems utilizing *phoA*.

Rhodobacter sphaeroides is an ideal procaryote for the study of protein targeting. Depending upon the growth conditions, it can have either two or three distinct membrane systems: the outer membrane, cytoplasmic membrane, and, under photosynthetic growth conditions, intracytoplasmic membrane (ICM) vesicles (8, 20, 33). Each of these membranes possesses a specific subset of proteins which must be properly targeted and inserted for normal cell growth (see references 12 and 22 for reviews). As is the case in other gram-negative bacteria, *R. sphaeroides* possesses two soluble compartments: cytoplasm and periplasm. The internal volume of the ICM vesicles (ICM lumen) has previously been demonstrated to be structurally contiguous with the periplasm (8, 33).

In recent years a number of genetic techniques have been developed to follow and dissect the process of protein localization (see reference 36 for a review). One such technique involves the genetic fusion of genes encoding reporter molecules such as *lacZ* (1, 2), *galK* (32), or *cat* (16) to the gene(s) of interest to monitor the regulation of expression and localization of a particular protein. Specific fusions to a known region of the gene encoding the protein of interest may then allow genetic dissection of a protein into its structural and functional domains to reveal those regions of a protein which are required for specific targeting to one of the cellular membrane systems or compartments (2, 15, 27).

Recently the utility of *Escherichia coli* alkaline phosphatase, encoded by *phoA*, as a reporter for gene fusions was demonstrated (3, 18, 27, 28). Alkaline phosphatase is translocated across the cytoplasmic membrane and localized in the periplasm of *E. coli* (6, 19), and the DNA sequence of *phoA* has been determined (7). By utilizing *phoA* gene fusions, the expression of a number of proteins have been investigated in *E. coli*, both translocated proteins (3, 18, 27) and membrane proteins with regions exposed to the periplasmic side of the cytoplasmic membrane (28).

In order to test the application of this particular experimental approach to *phoA* gene fusions in *R. sphaeroides*, cytochrome c_2 (cyt c_2), a known, periplasmically localized protein, was chosen for study. cyt c_2 is expressed in both aerobically and photosynthetically grown *R. sphaeroides* cells and is localized to the periplasm (or ICM lumen) under both growth conditions (35). The DNA sequence of the *R. sphaeroides cycA* gene encoding cyt c_2 is known (13), and the gene has been cloned and studied in detail (4, 13, 14). From the DNA sequence data, a 21-amino-acid N-terminal signal sequence has been proposed (13). Although processing of the signal sequence has not been directly demonstrated in vivo, in vitro transcription-translation studies have revealed a 15.5-kilodalton [kDa] polypeptide species, whereas a 13.5-kDa species is found in vivo (13). Recently, export of the mature *R. sphaeroides* cyt c_2 to the periplasm of *E. coli* was demonstrated, but only under anaerobic growth conditions (31). The expression of the *cycA* gene in *R. sphaeroides* has been shown to be inversely regulated by oxygen tension at both the transcriptional and translational level (4, 13, 14). Therefore fusions were made between *cycA* and *phoA* in vitro and reintroduced into *R. sphaeroides* in *trans*, employing the previously established vector systems pRK404 (9, 11) and pRK415 (9, 21) to demonstrate the utility of the *phoA* system for studies of gene regulation and protein targeting in *R. sphaeroides*.

MATERIALS AND METHODS

Bacterial strains and growth. The *E. coli* and *R. sphaeroides* strains used are listed in Table 1. *E. coli* cells were grown aerobically on a gyratory shaker at 37°C in Luria broth medium supplemented with the appropriate antibiotics for plasmid selection. *R. sphaeroides* 2.4.1. was grown in Sistrom minimal medium A (25) at 32°C. Chemoheterotrophic cells were grown either by vigorous shaking on a gyratory shaker

* Corresponding author.
† Present address: University of Texas Medical Center, Department of Microbiology, P.O. Box 20708, F.B. 1.765, Houston, TX 77225.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference or source
E. coli		
CC118	*phoA* deletion strain	27
C600	*thr-1 leu-6 thi-1 supE44 lacY1 tonA*, host for omega plasmids	34
S17-1	Pro$^-$ Res$^-$ Mod$^+$ *recA*, integrated plasmid RP4-Tc::Mu-Km::Tn*7*	37
R. sphaeroides		
2.4.1.	Wild type	41
RS104	B800/850$^-$, *crt*	23
Plasmids		
pUC19	Ap^r	42
pRK404	Tc^r, *oriTlacZ*	11
pRK415	Derivative of pRK404, Tc^r	21
pC2P2.71	*cycA* gene within 2.7-kb *R. sphaeroides* DNA insert in pUC19	13
pCH39	*phoA* fusion to *bla*, Tc^r	18
pHP45Ω	Spc/Sm omega cartridge	34
pUI300	*phoA*[a] in 2.3-kb DNA fragment in pUC19, Ap^r, (+)[b]	This study
pUI302	2.95-kb insert containing *cycA*::*phoA* fusion[c] in pUC19, Ap^r, (+)	This study
pUI303	2.95-kb insert containing *cycA*::*phoA* fusion[c] in pUC18, Ap^r, (−)[d]	This study
pUI304	2.5-kb insert containing *cycA*::*phoA* fusion[e] in pRK404, Tc^r, (+)	This study
pUI314	2.95-kb insert containing *cycA*::*phoA* fusion[c] in pRK415, Tc^r, (+)	This study
pUI304Ω	Omega cartridge inserted 5′ of *cycA*::*phoA* fusion of pUI304	This study
pUI314Ω	Omega cartridge inserted 5′ of *cycA*::*phoA* fusion of pUI314	This study
pUI310	1.47-kb insert containing *phoA*[f] in pUC19, Ap^r, (+)	This study
pUI320	Identical to pUI310, but *Bam*HI site deleted from vector polylinker	This study
pUI322	1.47-kb insert derived from pUI322 in pRK415, Tc^r, (+)	This study

[a] *phoA* gene fragment lacking coding region for signal sequence of alkaline phosphatase.
[b] (+), Orientation of the insert was the same as that of the *lac* promoter of the vector.
[c] Insert contains 500 bp of *R. sphaeroides* DNA 5′ to the *cycA* start.
[d] (−), Orientation of the insert was the opposite of that of the *lac* promoter.
[e] Insert contains 100 bp of *R. sphaeroides* DNA 5′ to the *cycA* start.
[f] *phoA* fragment lacking the coding region for signal sequence of alkaline phosphatase and 900 bp of DNA 3′ to the *phoA* terminator.

or by sparging with a mixture of 25% O_2, 74% N_2, and 1% CO_2. Photoheterotrophic cells were grown at 10 W/m^2 illumination either in completely filled screw-cap tubes or by sparging with 95% N_2–5% CO_2. *R. sphaeroides* RS104 was grown as previously described (23). Tetracycline (1 μg/ml) was added to *R. sphaeroides* cultures containing the plasmids.

Plasmid construction and molecular biological methods. A *phoA* gene fragment lacking DNA encoding a Shine-Dalgarno and amino-terminal signal sequence was derived as a *Pst*I-*Xho*I restriction endonuclease fragment from pCH39, a *bla*::*phoA* fusion plasmid (18), which was a gift from Colin Manoil. A *Pst*I-*Stu*I *cycA* DNA fragment was derived from pC2P2.71 (13). The streptomycin/spectinomycin DNA fragment was derived by digestion with *Hin*dIII from pHP45Ω (34). DNA fragments were cloned into pUC19 (42), pRK404 (11), or pRK415 (21). Plasmid DNA was purified either by large-scale CsCl gradient centrifugation (29) or small-scale preparations by alkaline sodium dodecyl sulfate lysis (26). DNA fragments were isolated from agarose or acrylamide gels as described elsewhere (26). Restriction endonucleases and additional DNA modification enzymes were used according to the manufacturers' specifications.

Genetic selection. Plasmid DNA was introduced into *R. sphaeroides* via diparental mating from *E. coli* S17-1 as previously described (9). Selection and screening for cells expressing CycA::PhoA fusion proteins was performed by plating *R. sphaeroides* exconjugants onto plates containing 1 μg of tetracycline per ml and 40 μg of 5-bromo-4-chloro-3-indolyl phosphate (XP; Sigma Chemical Co., St. Louis, Mo.) per ml and picking blue (XP-positive) colonies.

Cell fractionation. *E. coli* CC118 cells were fractionated into periplasmic and combined membranous and cytoplasmic (spheroplast lysate) fractions by the osmotic shock method of Koshland and Botstein (24). *R. sphaeroides* 2.4.1. and RS104 cells were fractionated into periplasmic, membranous, and cytoplasmic components by the lysozyme-induced spheroplast method of Tai and Kaplan (39).

Assays. Alkaline phosphatase activity, in arbitrary units, was determined by the endpoint assay method of Brickman and Beckwith (5). For periplasmic and total alkaline phosphatase levels, periplasmic and total units were determined on the basis of total cell number. Specific activity was determined on the basis of periplasmic or total protein levels. Protein was assayed, with bovine serum albumin as a standard, by the method of Markwell et al. (30). In the case of cell fractions containing sucrose or high salt, the Pierce BCA method (Pierce Chemical Co., Rockford, Ill.) was employed; added lysozyme in periplasmic fractions was subtracted to determine protein amounts for specific activity determinations.

Immunochemical methods. Antibodies to electrophoretically purified *E. coli* alkaline phosphatase were raised in New Zealand White rabbits as described by Deal and Kaplan (10) for use in Western blotting (immunoblotting) and immunoelectron microscopy. Purification of antibodies to *R. sphaeroides* cyt c_2 was as described previously (13). Cell fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 12.5% acrylamide gels and transferred to nitrocellulose (0.2-μm pore size) for immunoblotting (8 to 10 V, 12 h, 4°C). Goat anti-rabbit immunoglobulin conjugated to mammalian alkaline phosphatase was used to visualize bound primary antibody by following the protocol for ProtoBlot (Promega Biotec, Inc., Madison, Wis.).

Electron microscopy. *R. sphaeroides* cells were fixed in 1% glutaraldehyde for 1 h at room temperature (followed in some cases by 1% OsO_4 for 1 h at room temperature), dehydrated in ethanol, and then embedded in LR White resin. Thin sections were labeled with primary antibody and reacted with goat anti-rabbit immunoglobulin G conjugated to 15-nm gold particles as previously described (38).

Materials. Restriction endonucleases, T4 DNA polymerase, and mung bean nuclease were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). Nitrocellulose was from Schleicher & Schuell Co. (Keene, N.H.). Purified *E. coli* alkaline phosphatase was from Sigma. Goat anti-rabbit immunoglobulin G conjugated to 15-nm gold particles was from Janssen Life Sciences (Piscataway, N.J.).

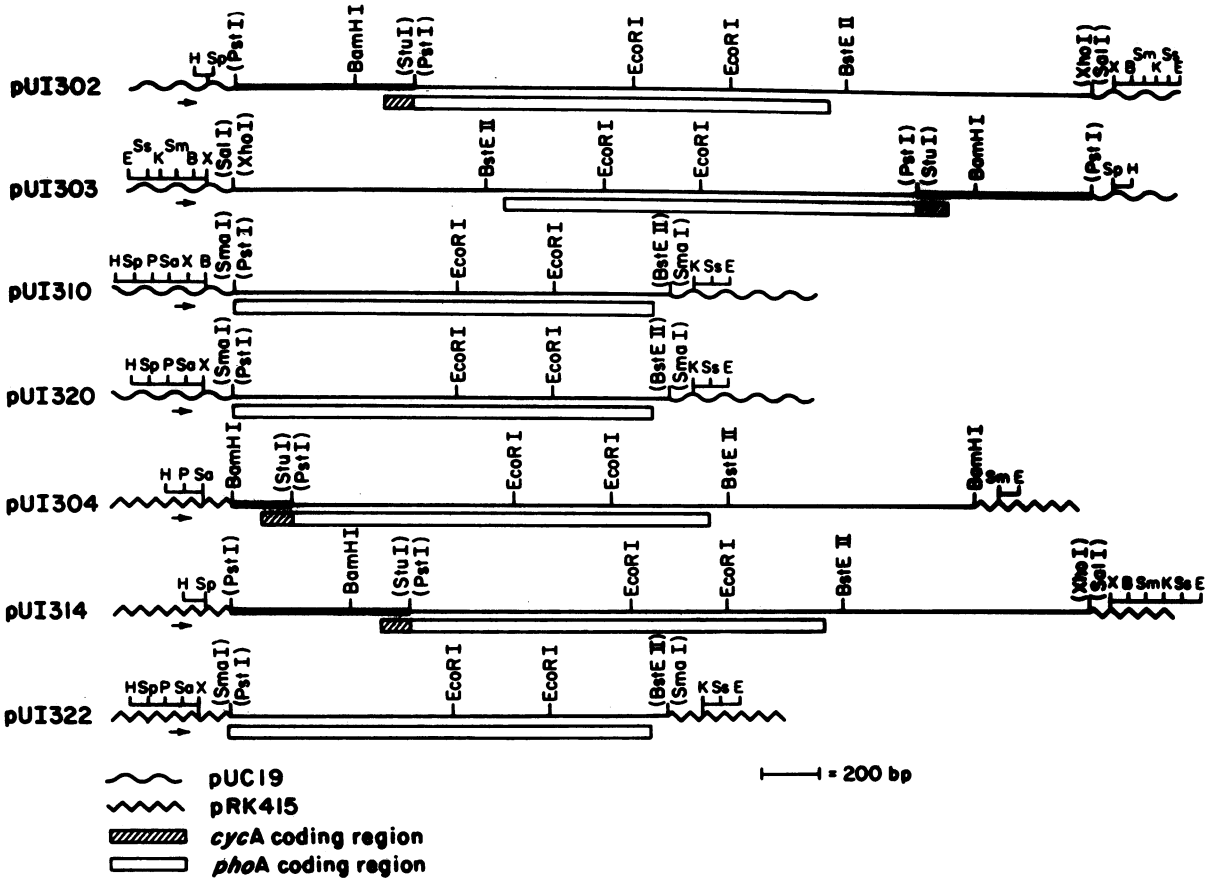

FIG. 1. Plasmid restriction maps. Thin lines represent vector DNA: either pUC19 (pUI302, pUI310, pUI320), pRK404 (pUI304), or pRK415 (pUI314). Shaded boxes (▨) represent *cycA* DNA derived from pC2P2.71; open boxes (□) represent *phoA* DNA derived from pCH39. Restriction sites in vector polylinkers are not drawn to scale. Restriction sites within parentheses were lost in plasmid construction. In all cases, the *lac* promoter of the vector is to the left (upstream) of the insert DNA and the direction of transcription of *lacP* is left to right.

Electron microscopy-grade glutaraldehyde, OsO_4, and LR White resin were from EMCorp (Chestnut Hill, Mass.). Goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase was from Promega Biotec or Sigma. All other chemicals were of reagent grade.

RESULTS

Construction of *cycA*::*phoA* fusions in pUC-based plasmids. The structural gene of *E. coli* alkaline phosphatase (*phoA*) lacking the coding sequence for the alkaline phosphatase signal sequence was derived from pCH39 (18) as a *Pst*I-*Xho*I restriction fragment. This 2.35-kilobase (kb) restriction fragment was ligated into the polylinker of pUC19, which had been digested with *Pst*I and *Sal*I, yielding plasmid pUI300. To generate the *cycA*::*phoA* fusion, a 600-base-pair (bp) DNA fragment containing the coding region for the presumptive cyt c_2 signal sequence and the first 10 amino acids of the mature protein in addition to 500 bp of upstream DNA was isolated from pC2P2.71 (13) via a *Pst*I-*Stu*I double digestion. The *Pst*I end of the DNA fragment was blunt ended by utilizing the 3′-5′ exonuclease activity of T4 DNA polymerase (26), as was a *Pst*I digest of the above-described *phoA*-containing plasmid, pUI300. The *cycA*-derived fragment was ligated into pUI300 to yield a plasmid with an in-frame fusion of the *cycA* sequence with the *phoA* gene fragment and was designated pUI302 (Fig. 1). pUI302 was transformed into *E. coli* CC118 and grown on screening plates (50 μg of ampicillin per ml plus 40 μg of XP per ml), where the cells were observed as intensely blue colonies. Since the sequences of *cycA* (13) and *phoA* (7) have been determined and restriction endonuclease sites have been mapped, the orientation of the *cycA*::*phoA* insert in pUI302 was confirmed by restriction endonuclease digestion (data not shown) and determined to be in the same orientation as the *lac* promoter of the vector.

Previous studies have demonstrated that *R. sphaeroides* genes are only expressed in *E. coli* if they are aligned with an external, plasmid-borne promoter such as *lac* or *tet* (22). Therefore, as a control, a plasmid with the *cycA*::*phoA* fusion oriented opposite to the *lac* promoter was also constructed by insertion of the *cycA*::*phoA* insert from pU1302 into pUC18, yielding pU1303 (Fig. 1). Cells that contained pUI303 were pale on XP plates. Expression of the CycA::PhoA fusion protein in pUI302 and pUI303 is discussed below.

Plasmids for the rapid construction of *phoA* fusions in *E. coli*. To compare expression of *phoA* with and without the fused *cycA* sequence coding for the putative N-terminal signal sequence, two additional vectors were constructed, pUI310 and pUI320 (Fig. 1), both derivatives of pUC19. These two plasmids also make suitable cloning vectors for

construction of *phoA* fusions and rapid manipulation in *E. coli*.

pUI310 contained a 1.47-kb *Pst*I-*Bst*EII restriction fragment isolated from pCH39. This fragment contained the *phoA* gene fragment, less the coding region for the signal sequence, with its proposed transcriptional terminator (7) as in the above-described constructions, but nearly 900 bp of extraneous downstream DNA was deleted. The fragment ends were made blunt and then ligated into the *Sma*I site of the pUC19 polylinker to yield pUI310 (Fig. 1). This *phoA* translational fusion vector had four unique restriction endonuclease sites in the polylinker upstream of the *phoA* gene (*Bam*HI, *Xba*I, *Pst*I, *Sal*I, and *Hin*dIII) and two unique sites downstream (*Kpn*I and *Sst*I). pUI320 (Fig. 1) was constructed by digestion of pUI310 with *Bam*HI, treatment with mung bean nuclease to remove the 5′ extensions (17), and religation of the blunt-ended *Bam*HI site. These procedures eliminated one possible cloning site but generated alternate translational reading frames for fusion to *phoA*, utilizing the remaining upstream restriction endonuclease sites in the polylinker (*Xba*I, *Pst*I, *Sal*I, and *Hin*dIII). By employing pUI310 and pUI320, all three translational reading frames could be used to construct *phoA* fusions, and blue-white screening of colonies on XP plates for alkaline phosphatase-positive colonies was possible.

Construction of plasmids for the expression of *phoA* fusions in *R. sphaeroides*. Since the ColE1 origin of replication of pUC19 does not function in *R. sphaeroides* (40), DNA fragments containing the *cycA*::*phoA* fusions were ligated into the broad-host-range vector pRK404 or pRK415 to examine expression of the fusion proteins in *R. sphaeroides* (9). The two vectors are very similar: pRK404 contains the pUC9 polylinker (11), whereas pRK415 contains the pUC19 polylinker, and a single *Eco*RI site in pRK415 has been deleted (21). pUI304 (Fig. 1) was constructed by digestion of pUI302 with *Bam*HI, isolation of the 2.5-kb restriction fragment containing the *cycA*::*phoA* fusion, and ligation into the *Bam*HI site of pRK404. As a result, pUI304 contained only 100 bp of DNA upstream of the initiation codon for the putative cyt c_2 signal sequence. pUI314 (Fig. 1) was constructed by removal of the *cycA*::*phoA* fusion from pUI302 after a double digestion with *Hin*dIII and *Sst*I. This 2.9-kb DNA fragment was ligated into pRK415, also digested with *Hin*dIII and *Sst*I. pUI304 and pUI314 differed only in the amount of DNA upstream of the start of the *cycA* structural gene, 100 and 500 bp, respectively. The effects of the amount of upstream DNA on the expression of the fusion protein is discussed below. The inserts in both pUI304 and pUI314 were in the same orientation (+) as the *lac* and *tet* promoters of the vector. pUI304 and pUI314 were further modified by the insertion of an omega cartridge, consisting of an antibiotic resistance gene (spectinomycin-streptomycin) and transcription and translation stop signals (34). The insertion of the omega cartridge into the *Hin*dIII site between the *lac* promoter and the *cycA*::*phoA* insert blocked expression from any vector promoters and allowed analysis of possible *R. sphaeroides* promoters carried on the *cycA* DNA insert.

As a control for the importance of *cycA* sequences in the expression of *phoA* in *trans*, a plasmid (pUI322) was constructed that contained *phoA* without the coding region for a signal sequence or a start codon. The 1.47-kb DNA fragment derived from a *Hin*dIII-*Sst*I double digest of pUI320 was ligated into pRK415 to yield pUI322 (Fig. 1).

Alkaline phosphatase activity assays. Both *E. coli* CC118 and *R. sphaeroides* cell fractions were assayed for alkaline phosphatase activity. The results of assays of *E. coli* CC118 are given in Table 2. Very high levels of activity were detected in the periplasmic fractions from cells containing plasmids with *cycA*::*phoA* fusions. *E. coli*(pUI302) showed total alkaline phosphatase levels over 600-fold higher than those in cells carrying pUC19. *E. coli* carrying pUI304 and pUI314 had enzyme levels 14- and 13-fold greater, respectively, than those in control cells carrying pRK415. The high levels of activity in cells carrying pUI302 compared with those in pUI304 or pUI314 most likely arose from the difference in copy number of the plasmids. pUI302 is a pUC19-derived plasmid with a high copy number, whereas pUI304 and pUI314 are low-copy-number plasmids derived from pRK404 and pRK415, respectively.

TABLE 2. Alkaline phosphatase activity in *E. coli* CC118

Plasmid	Periplasm		Total		XP colony color[c]	Total APase relative to control[d]
	APase/cell[a]	Sp act[b]	APase/cell	Sp act		
pUC19	0.12	92	0.265	40	Pale	1.0
pUI310	4.31	2,300	6.9	1,000	Pale	26.0
pUI320	0.23	140	0.875	130	Pale	3.3
pUI302	121.4	109,250	166.0	24,900	Dark blue	627.0
pUI303	2.43	1,760	3.15	472	Pale	12.0
pRK415	ND[e]	ND	1.4	208	Pale	1.0
pUI304	12.5	11,435	19.5	2,930	Blue	14.1
pUI314	13.6	7,033	18.4	2,760	Blue	13.3
pUI304Ω	0.26	256	0.35	52	Pale	0.25
pUI314Ω	0.25	250	0.49	74	Pale	0.35

[a] Value × 10^{-7} = units per cell.
[b] Units per minute per milligram of protein.
[c] Colony color on XP indicator plates after plating of 200 CFU per plate and incubation for 12 to 14 h at 37°C.
[d] pUI310, pUI320, pUI302, and pUI303 relative to pUC19; pUI304, pUI314, pUI304Ω, and pUI314Ω relative to pRK415.
[e] ND, Not detectable.

Cells with plasmids which contained *phoA* without a coding region for an N-terminal signal sequence showed variable levels of activity. The total activity of cells carrying pUI310 was 20-fold greater (although still low) than that in pUC19-containing cells, whereas pUI320-containing cells showed a minimal increase over the background level. This difference was probably due to the reading frame of the *phoA* insert relative to the *lacZ* sequence in the vector polylinker. *phoA* present in pUI310 remained in frame, whereas pUI320 was out of frame with *lacZ* because of the deletion of the *Bam*HI site in the polylinker. Importantly, cells carrying the plasmid with the *cycA*::*phoA* fusion oriented opposite to the *lac* promoter of the vector (pUI303) showed activity that was only minimally above background activity, indicating very poor expression of the fusion protein and supporting previous observations that *R. sphaeroides* regulatory regions appear to function poorly or not at all in *E. coli*. The constructions containing the omega cartridge (pUI304Ω and pUI314Ω) further demonstrated the inability of *E. coli* to utilize *R. sphaeroides* regulatory sequences. In plasmids in which expression from vector promoters was blocked, the levels of CycA::PhoA fusion protein activity dropped to the level of the pRK415 control.

It was important to note the colony color of *E. coli* cells harboring the various plasmids. Upon overnight incubation of plates containing XP, only cells with a total cell activity of about 8 × 10^{-7} or more would exhibit the blue colony phenotype, i.e., those carrying pUI302, pUI304, or pUI314. Cells with minimal activity, such as those carrying pUI310 or pUI303, would develop color when left for 7 to 14 days at 4°C. Cells carrying pUC19, pRK415, pUI320, pUI322,

TABLE 3. Alkaline phosphatase activity in *R. sphaeroides* 2.4.1. and RS104

Strain and plasmid	Aerobic growth				Photosynthetic			
	Periplasm		Total		Periplasm		Total	
	APase/cell[a]	Sp act[b]	APase/cell	Sp act	APase/cell	Sp act	APase/cell	Sp act
2.4.1.(pRK415)	0.53	35.3	2.5	39.9	0.82	90.1	2.7	156
2.4.1.(pUI304)	59.8	7,970.0	70.7	1,080.0	86.5	9,123.0	89.7	5,595
2.4.1.(pUI314)	84.0	11,150.0	90.3	4,830.0	207.0	34,220.0	214.0	17,900
2.4.1.(pUI322)	ND[c]	ND	1.3	104.0	ND	ND	1.8	160
2.4.1.(pUI304Ω)	19.0	5,686.0	21.2	1,596.0	11.0	5,420.0	13.5	1,410
2.4.1.(pUI314Ω)	55.0	15,770.0	57.0	5,800.0	11.8	55,700.0	12.2	13,200
RS104(pRK415)	1.4	480.0	2.0	190.0	1.9	590.0	5.0	240
RS104(pUI314)	102.0	20,880.0	106.0	10,100.0	104.0	29,700.0	174.0	8,700

[a] Value × 10^{-8} = units per cell.
[b] Units per minute per milligram of protein.
[c] ND, Not detectable.

pUI304Ω, or pUI314Ω remained pale even upon extended storage.

In *E. coli* cells expressing high levels of alkaline phosphatase activity, export of the fusion protein to the periplasm appeared to be efficient: *E. coli*(pUI302) showed 73%, *E. coli*(pUI304) showed 64%, and *E. coli*(pUI314) showed 74% of the activity in the periplasm.

The results of alkaline phosphatase assays on *R. sphaeroides* 2.4.1. and the mutant RS104 are shown in Table 3. Very little endogenous alkaline phosphatase activity was found in control *R. sphaeroides* containing pRK415 or the *phoA*-containing plasmid pUI322 grown either aerobically or photosynthetically, and no alkaline phosphatase antigenic activity could be detected in these cells (see below). High levels of activity were seen in the periplasmic fractions of cells carrying pUI304 or pUI314. *R. sphaeroides* 2.4.1.(pUI304) and 2.4.1.(pUI314) grown aerobically showed total cell alkaline phosphatase activity that was 55- and 70-fold greater, respectively, than that of the 2.4.1.(pUI322) control cells. When grown photosynthetically, 2.4.1.(pUI304) and 2.4.1.(pUI314) showed a level of activity that was 49- and 118-fold greater, respectively, than that of 2.4.1.(pUI322) control cells. When aerobically grown 2.4.1. cells carrying pUI304 and pUI314 were compared, 2.4.1.(pUI314) showed a 1.4-fold greater activity than 2.4.1.(pUI304) in periplasmic activity levels. Photosynthetically grown 2.4.1.(pUI314) cells had approximately 2.4 times the periplasmic alkaline phosphatase levels in 2.4.1.(pUI304) cells. 2.4.1. cells carrying pUI304 (100 bp of DNA 5′ to the *cycA* start) showed about the same level of activity under both aerobic and photosynthetic growth conditions. In contrast, 2.4.1. carrying pUI314 (500 bp of DNA 5′ to the *cycA* start) grown under photosynthetic conditions showed 2.5 times the periplasmic alkaline phosphatase activity as cells grown aerobically. The levels of enzyme activity measured in *R. sphaeroides* cells carrying the omega-cartridge-containing plasmids pUI304Ω and pUI314Ω were similar to those of their parent plasmids. When compared with pUI304, pUI304Ω showed a 70% decrease in total alkaline phosphatase activity in aerobically grown cells and a 85% decrease in photosynthetically grown cells (Table 3). In contrast, pUI314Ω showed only a 40% decrease in expression compared with that in pUI314 (Table 3), but a similar level of induction in activity in photosynthetically grown versus aerobically grown cells (2.5-fold for pUI314, 2.1-fold for pUI314Ω).

R. sphaeroides RS104 cells containing pUI314 showed a similar increase in alkaline phosphatase activity, with a 1.6-fold increase in total activity in photosynthetically grown cells as compared with that in aerobically grown cells. The increase in alkaline phosphatase activity in both 2.4.1.(pUI314) and RS104(pUI314) correlates to the known induction of cyt c_2 when *R. sphaeroides* cells are shifted from aerobic to photosynthetic growth conditions (4, 13, 14) but is only seen in cells carrying the plasmid with the greater amount of upstream DNA.

The efficiency of translocation of the CycA::PhoA fusion protein to the periplasmic compartment of *R. sphaeroides* was very high. In aerobically grown 2.4.1. cells carrying pUI304 and pUI314, 85 and 93%, respectively, of the activity was periplasmic; aerobically grown RS104 cells carrying pUI314 showed 96% of the activity in the periplasm. In photosynthetically grown 2.4.1. cells containing either pUI304 or pUI314, 96 to 97% of the activity was in the periplasmic fraction. In photosynthetically grown RS104 cells carrying pUI314, 60% of the activity was periplasmic. When RS104 photosynthetic membrane fractions were treated with lauryl dimethyl amine oxide (LDAO), 30 to 38% additional activity was made available (data not shown), indicating that fractionation of these cells is less efficient than that of 2.4.1., probably because of their highly aberrant photosynthetic membrane structure (23) (see below), leading to an artificially lowered efficiency of translocation.

Immunoblot analysis of *R. sphaeroides*. Western immunoblot analysis, utilizing alkaline phosphatase antibody, of periplasmic and membrane fractions derived from 2.4.1. cells grown under aerobic and photosynthetic conditions is shown in Fig. 2A. In all immunoblot analyses, equal protein loads were used for each lane. Although this approach does not take into account the differences in distribution of the antigen(s) under study, it minimizes distortions due to protein overloading and nonspecific antibody binding. Further, because the periplasmic volume of photosynthetic cells reflects the continuity of the periplasm with the lumen of the ICM equal protein is the most practical method to compare the periplasmic fractions from aerobic and photosynthetic cultures. A major 48-kDa polypeptide species was detected in the periplasmic fractions from cells carrying pUI314 grown either aerobically (Fig. 2A, lane 2) or photosynthetically (lane 4) or, similarly, in cells carrying pUI304 (lanes 6 and 8). The predicted size of the CycA::PhoA fusion protein, based upon DNA sequence data and assuming the processing of the putative cyt c_2 signal sequence, was 47.9 kDa, in close agreement with the size of the major immunoreactive polypeptide detected. A minor band of 47 kDa was detected in these fractions as well, most evident in photosynthetically grown 2.4.1. cells containing pUI314 (Fig. 2A, lane 4). No

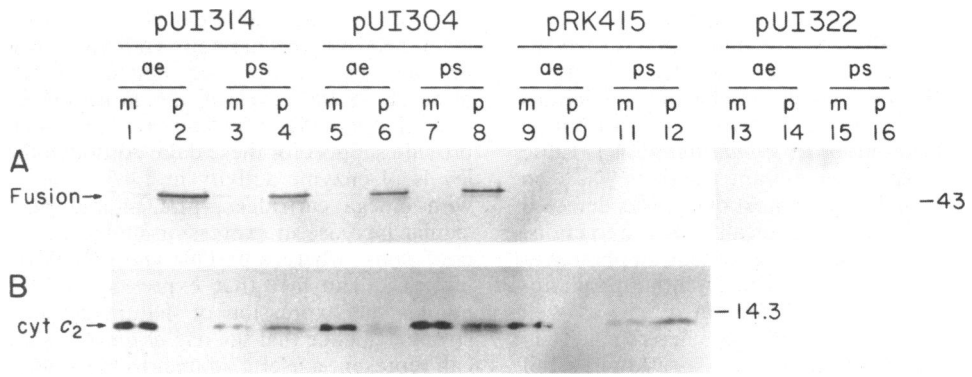

FIG. 2. Western immunoblots of periplasmic (p) and membrane (m) fractions from *R. sphaeroides* 2.4.1. containing the indicated plasmids grown aerobically (ae) and photosynthetically (ps) and probed with anti-alkaline phosphatase antibody (A) or anti-cyt c_2 antibody (B). The position of fusion protein (48 kDa) in panel A is indicated by the arrow; a minor fusion protein species (47 kDa) can also be seen. Samples of 10 μg of protein were run in each lane. The membrane association of cyt c_2 can be seen clearly in panel B.

immunoreactive polypeptides were detected in fractions from cells carrying the vector pRK415 (Fig. 2A, lanes 9 through 12) or from cells carrying the *phoA*-containing plasmid pUI322 (lanes 13 through 16).

All *R. sphaeroides* cells contained a chromosomal copy of the wild-type *cycA* gene; therefore cell fractions from 2.4.1.(pRK415) were compared with the *cycA::phoA* fusion carried in *trans* on pUI304 or pUI314 by probing with cyt c_2 antibody (Fig. 2B). In aerobically grown cells, the majority of immunodetectable cyt c_2 was found in the membrane fractions (Fig. 2B, lanes 1, 5, and 9). In photosynthetically grown cells, cyt c_2 was found in both membrane fractions (Fig. 2B, lanes 3, 7, 11) and periplasmic fractions (lanes 4, 8, and 12).

Figure 3 shows a further analysis of fractions from aerobically grown *R. sphaeroides* 2.4.1. probed with cyt c_2 antibody. No cyt c_2 was detected in the cytoplasmic fractions (Fig. 3, lanes 1 and 6). To determine the membrane association of cyt c_2, two membrane fractions were compared: the first was derived from lysed spheroplasts (Fig. 3, lanes 2 and 7), and the second was derived from spheroplasts washed with 1 M NaCl (lanes 3 and 8). The untreated membrane fraction contained immunodetectable cyt c_2 (Fig. 3, lanes 2 and 7), whereas the washed membrane fraction was depleted of cyt c_2 (lanes 3 and 8); however, cyt c_2 was found in the 1 M NaCl supernatant (lanes 4 and 9). Clearly, the membrane association of cyt c_2 was peripheral. The membrane association of cyt c_2 is not likely to be an artifact associated with lysozyme treatment, since both cyt c_2 and lysozyme are strongly positively charged proteins; thus, lysozyme treatment might be predicted to prevent rather than stimulate the association of cyt c_2 with the cell membrane. A low level of cyt c_2 was detected in the periplasmic fractions (Fig. 3, lane 5); in addition, in cells carrying pUI314, an immunoreactive species of 48 kDa was detected in the periplasmic fraction (lane 5). This cross-reactive polypeptide corresponded precisely to the mature CycA:: PhoA fusion protein detected with alkaline phosphatase antibody, which protein should contain 10 cyt c_2-derived amino acids corresponding to the amino-terminal end of the mature protein.

A similar analysis of the mutant *R. sphaeroides* RS104 containing pRK415 or pUI314 showed both similarities to and differences from wild-type *R. sphaeroides* 2.4.1. containing the same plasmids. Figure 4A shows an immunoblot analysis of RS104-derived fractions probed with alkaline phosphatase antibody. As in the wild-type cells, a 48-kDa species was detected in the periplasmic fraction from both

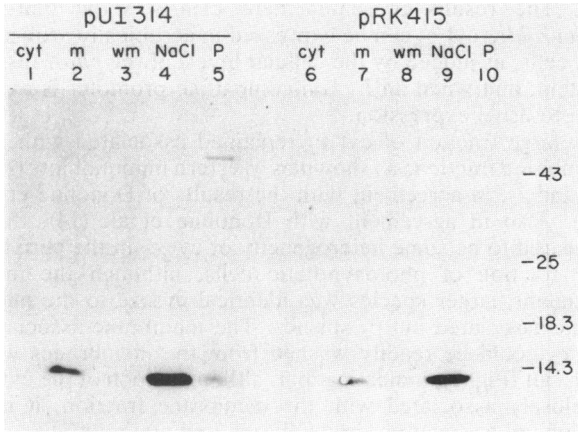

FIG. 3. Western immunoblot of fractionated aerobic *R. sphaeroides* 2.4.1. containing the indicated plasmids probed with cyt c_2 antibody. Cells were separated into the following fractions: cytoplasm (c), membrane (m), 1 M NaCl-washed membranes (wm), 1 M NaCl supernatant (NaCl), and periplasm (P). Samples of 10 μg of protein were run in each lane. Fusion protein in the periplasm cross-reacted with cyt c_2 antibody (lane 5).

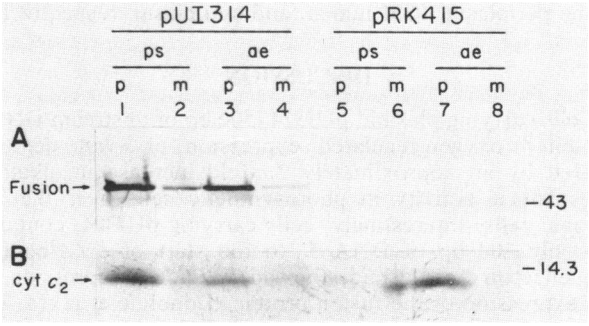

FIG. 4. Western immunoblot of fractionated *R. sphaeroides* RS104 containing the indicated plasmids probed with alkaline phosphatase antibody (A) and cyt c_2 antibody (B). The position of the fusion protein is indicated in panel A. Samples of 10 μg of protein were run in each lane. Abbreviations are as in Fig. 2.

aerobically (Fig. 4A, lane 3) and photosynthetically (lane 1) grown RS104(pUI314) cells. No immunoreactive species was found in cells carrying pRK415 (Fig. 4A, lanes 5 through 8). When probed with cyt c_2 antibody (Fig. 4B), fractions from RS104(pRK415, pUI314) exhibited a cyt c_2 distribution somewhat different from that of *R. sphaeroides* 2.4.1. Little association of cyt c_2 with the membrane fractions was seen (Fig. 4B, lanes 2, 4, 6, and 8), and most cyt c_2 was detected in the periplasm, particularly in aerobically derived fractions (Fig. 4B, lanes 3 and 7) and, to a lesser extent, in photosynthetically derived fractions (lanes 1 and 5), although photosynthetic membrane fractions did contain cyt c_2 as well (lanes 2 and 6). There appeared to be some heterogeneity in cyt c_2 in RS104 membrane fractions, seen as a low level of two forms (lanes 2, 4, and 6); however, in contrast to that in 2.4.1., the minor species in RS104 was slightly larger than the predominant form found in both the periplasm and the membrane. Although we can offer no basis for these observations in the mutant RS104, it is possible that the altered distribution of cyt c_2 is associated with the altered ICM structure in these cells.

Electron microscope observations. To confirm localization of the CycA::PhoA fusion protein in situ, we utilized electron microscopy and on-section immunogold labeling with the alkaline phosphatase antibody to detect the fusion protein in both wild-type and mutant *R. sphaeroides* (Fig. 5). Although on-section immunogold labeling is not a quantitative assay for protein levels due to cell-to-cell variation in antigen exposure, general localization of antigens can be determined. Gold particles found in the periplasmic compartment (periplasm or periplasm plus ICM lumen) were counted and compared with the total label of the cells to estimate the specificity of localization. Aerobically grown 2.4.1. cells containing pUI314 (Fig. 5B) showed 86% periplasmic specificity and an average label density of 9 particles per cell; photosynthetically grown 2.4.1. cells (Fig. 5A) showed 94% periplasmic specificity, with 43% of the label distributed over the periplasm and 51% distributed over the ICM vesicle lumen, and an average label density of 18 particles per cell. Photosynthetically grown RS104 cells carrying pUI314 (Fig. 5C and D) showed 97% periplasmic specificity, with the label distributed over the large irregular vesicles and the membrane tubes (57%) found in these cells as well as the peripheral periplasmic region (40%) with a label density of 17 particles per cell, indicating that translocation was as efficient in RS104(pUI314) cells as in 2.4.1.(pUI314) cells. Control RS104 cells carrying pRK415 (Fig. 5E) showed low label density (1.8 particles per cell) and little localization specificity, with 40, 20, and 40% of the label in the periplasm, ICM lumen, and cytoplasm, respectively.

DISCUSSION

Cells carrying plasmid pUI314 (500 bp of upstream DNA) exhibited oxygen-regulated expression of *cycA* demonstrated by an approximately 2.6-fold increase in alkaline phosphatase activity in photosynthetic cells over that in aerobic cells. Interestingly, cells carrying pUI304, containing only 100 bp of DNA 5′ to the start of *cycA*-coding sequence on the plasmid, showed no such derepression of the expression of the fusion protein. Donohue et al. (4, 13) previously demonstrated the presence of two cyt c_2-specific mRNAs: a large mRNA (920 nucleotides) originating between the *Pst*I and *Bam*HI sites, which is present in pUI314, and a smaller mRNA (740 nucleotides) originating near the *Bam*HI site, present in both pUI314 and pUI304. They also demonstrated that, although there is an overall increase in *cycA* mRNA levels in photosynthetic growth conditions, the increase in cyt c_2 expression appears to result from a marked increase in the level of the large *cycA* mRNA upon a reduction in oxygen tension (4). The results presented here provide support for these data. Although there were lowered levels of enzyme activity in 2.4.1. cells carrying plasmids with omega cartridges, pUI314 and pUI314Ω showed a similar increase in expression under photosynthetic growth conditions, whereas pUI304 and pUI304Ω showed no such increase. The fact that expression of the fusion protein mimics the expression of mature cyt c_2 (4, 13) provides strong evidence that the use of *phoA* fusions described here can represent a useful adjunct to the studies of gene expression in *R. sphaeroides*. However, it is necessary to be cautious when considering cyt c_2 expression in light of the fusion results reported here, since the expression of mature cyt c_2 is dependent upon heme attachment (31), which is not relevant for the *phoA* fusion results reported here.

In contrast, the expression of the CycA::PhoA fusion protein in *E. coli* appeared to be dependent upon vector promoters. pUI304 and pUI314 yielded enzyme levels 14- and 13-fold greater than background levels, respectively, whereas the corresponding plasmids where transcription from the vector promoters was blocked, pUI304Ω and pUI314Ω, showed essentially no increase compared with control cells carrying the vector pRK415 alone. In the high-copy-number pUC19-derived plasmids, pUI302 and pUI303, a similar trend was seen. pUI302-containing cells, in which the fusion protein can be expressed from the vector *lac* promoter, exhibited high activity (600-fold over background), whereas in pUI303-containing cells, in which the *cycA*::*phoA* insert is oriented opposite to the *lac* promoter, the enzyme level was only 12 times the background level of pUC19. The presence of large amounts of the fusion protein in the periplasmic fraction of *E. coli* cells supports recent evidence (31) that the putative cyt c_2 signal sequence is functional in *E. coli*. Further, the high level of fusion protein, which does not contain the cyt c_2 heme-binding site, supports the notion that the expression of mature *R. sphaeroides* cyt c_2 in *E. coli* is limited by the expression of *E. coli* heme lyase, present only under anaerobic growth conditions (31), and is not limited by transcription or protein processing. The results presented here clearly show that *R. sphaeroides* cyt c_2 can be expressed in aerobically grown *E. coli* cells, as judged by the cellular levels of the *phoA* fusion protein, and when an *E. coli*-compatible promoter is available to drive expression.

A large fraction of cyt c_2 remained associated with the membrane fractions as shown by Western immunoblots (Fig. 2B and 3), in agreement with the results of Donohue et al. (14). Also in agreement with Donohue et al. (14), there appeared to be some heterogeneity of cyt c_2 in the periplasmic fraction of photosynthetic cells, although the most abundant, larger species was identical in size to the membrane-associated cyt c_2 species. The membrane-associated cyt c_2 could be readily washed from the membranes with high salt (Fig. 3), indicating that, although much of the cyt c_2 is closely associated with the membrane fraction, it is a peripheral association, probably via ionic interactions.

The translocation of the CycA::PhoA fusion protein to the periplasmic compartment of *R. sphaeroides* was very efficient. The fusion protein (48 kDa) was clearly seen in the periplasm of *R. sphaeroides* 2.4.1. and RS104 carrying pUI304 or pUI314, although there appeared to be a smaller species (47 kDa) of the fusion protein in the periplasm of

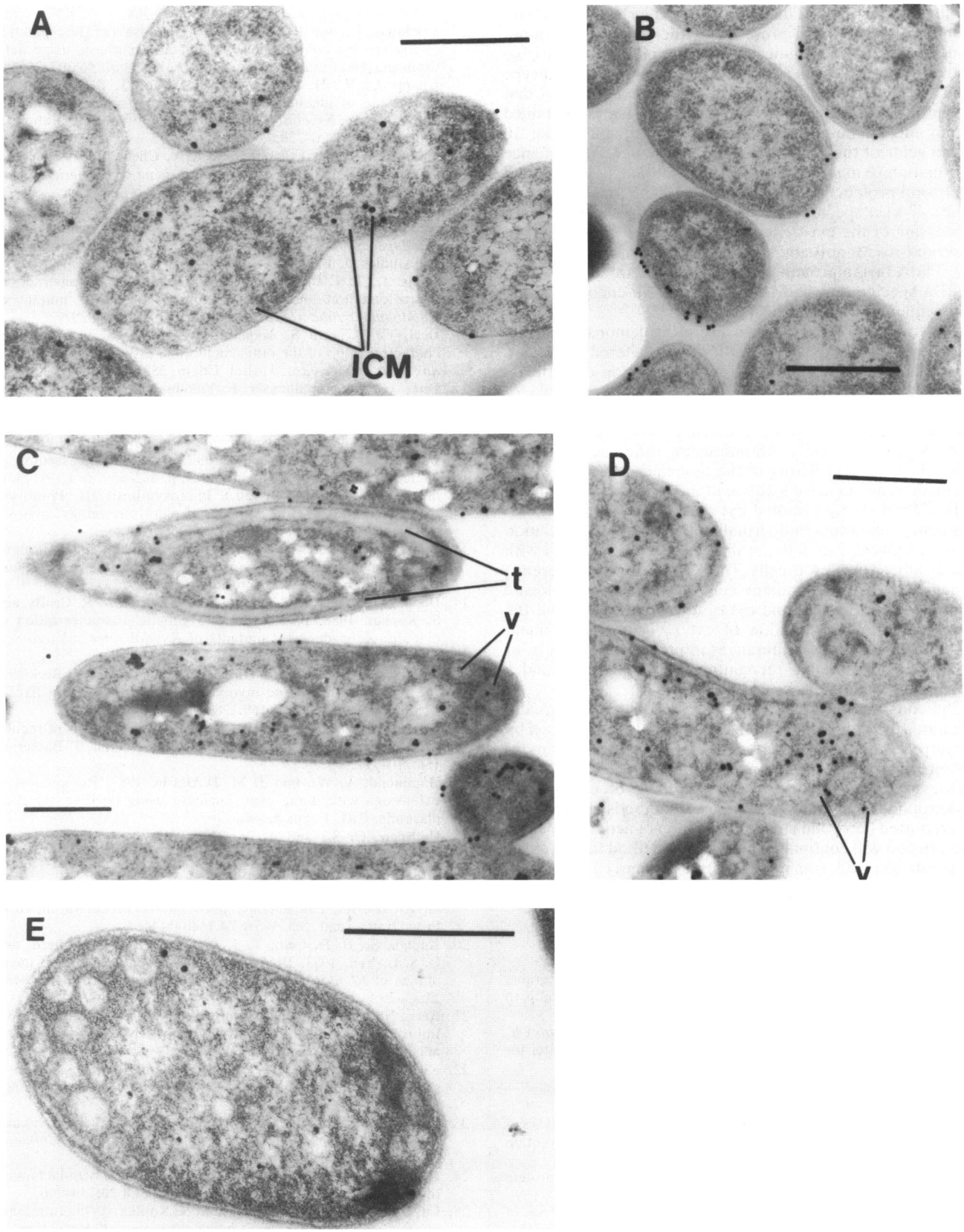

FIG. 5. Immunogold electron microscopy of *R. sphaeroides* 2.4.1.(pUI314) and RS104(pUI314) grown photosynthetically and labeled with alkaline phosphatase antibody, showing localization of the fusion protein in situ. (A) 2.4.1.(pUI314) photosynthetically grown. (B) 2.4.1.(pUI314) aerobically grown. (C and D) RS104(pUI314) labeled with alkaline phosphatase antibody, illustrating the aberrant membrane morphology of the mutant cells with membrane tubes (t) and irregular vesicles (v). (E) RS104(pRK415) control cells exhibit low label density. Bars, 0.5 μm.

photosynthetic cells. The size of the major species, corresponding to the fusion protein less the putative 21-amino-acid signal sequence, indicated that the putative processing of the fusion protein is similar to that of cyt c_2 in wild-type cells, yielding a major and minor soluble species, and that the information necessary for this processing is contained within the amino-terminal signal sequence and initial 10 amino acids of the cyt c_2 apoprotein, although the sequence information to maintain peripheral membrane association of the fusion protein is apparently absent. The presence of the minor 47-kDa species may be either a result of secondary processing of the cyt c_2 sequence or due to a phenomenon observed by Manoil and Beckwith (27), who noted that a Bla::PhoA fusion protein eventually degenerated to a stable 47-kDa species that had lost the fused *bla*-encoded amino acid sequence, leaving PhoA alone.

The fusion protein was also useful in demonstrating the competence of mutant cells with greatly altered photosynthetic membrane morphology for translocation of periplasmic proteins. RS104(pUI314) cells exhibited specificity of immunolabel for the fusion protein in the periplasmic compartment (periplasm plus ICM lumen) that was similar to that of wild-type 2.4.1. cells. An interesting difference was seen between 2.4.1. and RS104 in the association of cyt c_2 with the membranes in aerobically and photosynthetically grown cells; RS104 showed limited cyt c_2 association with photosynthetic membranes and virtually no membrane association in aerobically grown cells, in direct contrast to results with aerobically grown 2.4.1. cells. The reasons for this difference remain unclear. The mutant cells clearly can translocate both the fusion protein and cyt c_2 to the periplasm, but the close membrane association of cyt c_2 is not maintained. Whether this is due to differences in processing of cyt c_2 or to the lack of possible recognition between cyt c_2 and a membrane component is not known.

The construction of plasmids pUI310 and pUI320 greatly facilitates the utilization of *phoA* fusion analyses in *E. coli* by permitting the construction of the fusion junction in any of the three possible translational reading frames.

Due to the simple assay method, lack of endogenous background, available immunological methods, fusion vectors reported here, and strong expression of activity, specific *phoA* fusion will continue to be a useful method for the study of regulation and transport of cytochrome c_2 and other periplasmic or specific membrane proteins in *R. sphaeroides*.

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