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Isolation of a *Rhodobacter capsulatus* Mutant That Lacks *c*-Type Cytochromes and Excretes Porphyrins

SUSAN W. BIEL AND ALAN J. BIEL*

Department of Microbiology, Louisiana State University, Baton Rouge, Louisiana 70803

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A *Rhodobacter capsulatus* mutant lacking cytochrome oxidase activity was isolated by Tn*5* mutagenesis. Difference spectroscopy of crude extracts and extracted *c*-type cytochromes demonstrated that this mutant completely lacked all *c*-type cytochromes. The strain did, however, synthesize normal amounts of *b*-type cytochromes and nonheme iron. This mutant also excreted large amounts of coproporphyrin and protoporphyrin and synthesized reduced amounts of bacteriochlorophyll, suggesting a link between the synthesis of *c*-type cytochromes and the expression of the tetrapyrrole biosynthetic pathway.

The photosynthetic bacterium *Rhodobacter capsulatus* uses a single branched pathway to synthesize two major tetrapyrrole end products, heme and bacteriochlorophyll, and two minor tetrapyrrole end products, siroheme and vitamin B_{12}. Since bacteriochlorophyll levels vary greatly with growth conditions, whereas heme levels remain relatively constant, some mechanism must exist to monitor and independently control the levels of these two tetrapyrroles. Studies in *Rhodobacter sphaeroides* have suggested that carbon flow over the common tetrapyrrole pathway leading to protoporphyrin is controlled by heme (25), and that heme may feedback inhibit aminolevulinate synthase (36). Recently, there have been some intriguing observations suggesting a link between cytochrome synthesis and pigment formation. Mutants lacking the *R. capsulatus* cytochrome bc_1 complex showed increased pigmentation (12), whereas mutants with reduced capacity to synthesize *c*-type cytochromes excreted an uncharacterized pigment (17). These observations suggest that the regulation of these two branches may be more complicated than previously thought.

We attempted to probe the possible link between cytochrome *c* biosynthesis and expression of the tetrapyrrole pathway by isolating a mutant that completely lacks *c*-type cytochromes. Several years ago an *R. capsulatus* strain, MT113, was isolated (38) and subsequently shown to have reduced levels of all *c*-type cytochromes (13). This strain was isolated during a screen for normally pigmented mutants that lack cytochrome oxidase activity (38). In this report we describe the use of the cytochrome oxidase assay to isolate a Tn*5* insertion mutant that completely lacks *c*-type cytochromes. In addition to lacking *c*-type cytochromes, this strain excretes coproporphyrin and protoporphyrin and synthesizes reduced amounts of bacteriochlorophyll. The isolation of this strain and the cloning of the gene responsible for the pleotropic phenotype will allow us to investigate the connection between cytochrome *c* synthesis and the expression of the tetrapyrrole biosynthetic pathway.

MATERIALS AND METHODS

Media. *R. capsulatus* was routinely grown in either a malate-minimal salts medium (RCV) (33) or in 0.3% Bacto-Peptone–0.3% yeast extract (PYE; Difco Laboratories, Detroit, Mich.). RCV$^+$ medium was RCV medium supplemented with 0.6% glucose–0.5% pyruvate–0.05 M dimethyl sulfoxide. *Escherichia coli* was grown in L broth (4), which was modified by omitting glucose and decreasing the sodium chloride concentration to 0.5%. Solid media contained 1.5% agar (Difco). Antibiotics, when necessary, were added at the following final concentrations: kanamycin, 10 μg/ml; tetracycline, 1 μg/ml; spectinomycin, 5 μg/ml.

Pigment determinations. Porphyrins were extracted from culture supernatants as described previously (28). Coproporphyrin was quantitated by using a millimolar extinction coefficient of 489 at 401 nm, and protoporphyrin was quantitated using a millimolar extinction coefficient of 262 at 408 nm (28). The bacteriochlorophyll concentration was determined by extracting the cell pellet with acetone-methanol (7:2) (10). A millimolar extinction coefficient of 76 at 770 nm was used (9).

Difference spectroscopy. Cultures (10 ml) were grown in RCV medium overnight with gentle shaking. The cells were harvested and suspended in 20 mM 3-morpholinopropanesulfonic acid (pH 7.0)–100 mM potassium chloride. The suspension was sonicated three times for 20 s each and clarified by centrifugation for 20 min at 27,000 × *g*. Protein concentrations of the extracts were measured by the dye-binding method of Bradford (7). Difference spectra of sodium dithionite reduced minus potassium ferricyanide oxidized extracts were recorded in a Perkin-Elmer Lambda 3B dual-beam spectrophotometer. The concentrations of the cytochromes were determined by measuring the areas under the peaks.

Cytochromes *c* were extracted from the crude extracts by the method of Rieske (26). The proteins were precipitated three times with 5 ml of cold acetone–0.05 ml of 2.4 N hydrochloric acid. The pellet was then suspended in 1 ml of pyridine; 1 ml 0.2 N potassium hydroxide was then added.

Cytochromes *b* were extracted by precipitating the proteins from the crude extract with 10 ml of cold acetone and homogenizing successively with 10 ml of cold chloroform-methanol (2:1), 10 ml of cold acetone, and then three times with 5 ml cold acetone–0.05 ml of 2.4 N hydrochloric acid. The acetone was evaporated to near dryness; the residue was suspended in 1.5 ml of pyridine, 1.5 ml of 0.2 N potassium hydroxide was added (26).

Cytochromes *b* and *c* in whole cells were determined by difference spectra of dithionite reduced minus oxidized extracts (22). Total iron was determined by atomic absorption. Protein was determined by adding 0.1 ml of culture to 1 ml of 0.2 N sodium hydroxide. The samples were boiled for

* Corresponding author.

3 min, and the protein concentration was determined by the method of Lowry et al. (19) with crystalline bovine serum albumin (Pentex) as the standard. All samples and standards contained 0.02 N sodium hydroxide.

Polyacrylamide gel electrophoresis. Cultures were grown semiaerobically in RCV$^+$, harvested, and suspended in 0.1 M potassium phosphate (pH 7.6)–10 mM β-mercaptoethanol. The cell suspension was passed through a French pressure cell at 18,000 lb/in^2 and spun at 27,000 × *g* for 15 min. The supernatant was spun for 90 min at 150,000 × *g*. The pelleted membrane fraction was suspended and subjected to lithium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (32), except that electrophoresis was carried out at 25°C instead of 4°C to reduce the staining of *b*-type cytochromes. The gel was stained for heme-dependent peroxidase activity with 3,3′,5,5′-tetramethylbenzidine (31).

Southern hybridizations. Isolation of chromosomal DNA, preparation of Southern blots, and hybridization conditions were as previously described (34).

RESULTS

Isolation of Tn*5* insertion mutant. Strain PAS100 (*hsd-1 str-2*) (30) was mutagenized by using the Tn*5* insertion mutagenesis system of Simon et al. (27). The mutagenized culture was plated on RCV agar containing kanamycin, and the resulting colonies were screened for cytochrome oxidase activity by using the NADI reaction (20). Among the colonies lacking cytochrome oxidase activity was one that excreted an orange pigment. Examination of the colony under longwave UV light (366 nm) revealed that the pigment was strongly fluorescent. Although this strain, designated AJB530, grew at a normal rate aerobically in the dark, it would not grow photosynthetically.

Characterization of porphyrins excreted by AJB530. A 10-ml culture of AJB530 was grown in RCV$^+$ without shaking for 2 days. The cells were harvested and extracted with acetone-methanol. The extract was spotted on a silica gel thin-layer chromatography plate along with uroporphyrin, coproporphyrin, and protoporphyrin standards (Porphyrin Products, Logan, Utah). The plate was developed with 2,6-lutidine–0.05 N ammonium hydroxide (10:7). Two fluorescent bands were observed in the AJB530 lane. One comigrated with protoporphyrin (R_f, 0.82), whereas the other comigrated with coproporphyrin (R_f, 0.73).

The porphyrins were quantitated by differential extraction of the porphyrins from 1 ml of culture supernatant as described by Tait (28). Strain AJB530 accumulated 1.8 nmol of coproporphyrin and 0.9 nmol of protoporphyrin per ml of culture. In contrast, the parental strain PAS100 accumulated only 0.1 nmol of each porphyrin per ml of culture.

Extraction of a second portion of the RCV$^+$-grown cultures with acetone-methanol revealed that, whereas PAS100 produced 8.6 nmol of bacteriochlorophyll per ml of culture, AJB530 produced only 1.6 nmol of bacteriochlorophyll per ml of culture.

Characterization of cytochromes produced by AJB530. Difference spectroscopy of cytochromes extracted from AJB530 indicated that the strain lacked the 552-nm peak due to *c*-type cytochromes but had *b*-type cytochromes, as evidenced by a peak at 560 nm (Fig. 1). Since the *b*-type cytochromes of PAS100 appeared as a shoulder on the cytochrome *c* peak, it was difficult to compare the amounts of *b*-type cytochromes in PAS100 and AJB530. We therefore extracted the *b*-type cytochromes from both strains; we found that AJB530 had a normal level of *b*-type cytochromes (data not shown).

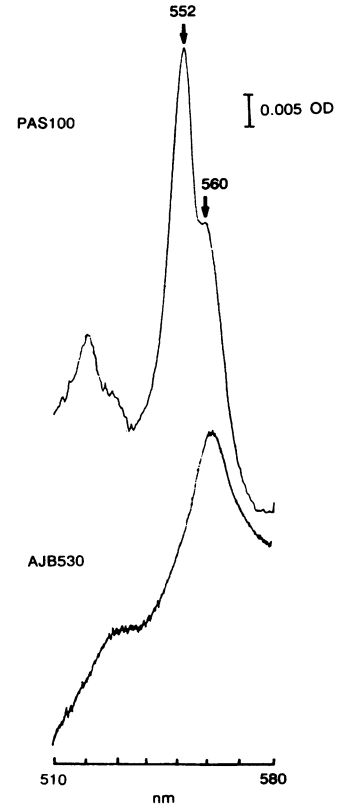

FIG. 1. Difference spectra of total cytochromes from PAS100 and AJB530. Each sample contained 1.25 mg of protein. OD, Optical density.

The difference spectrum of AJB530 is reminicent of the difference spectrum of another *R. capsulatus* strain, MT113 (38). It has recently been demonstrated that MT113, which is NADI negative, has greatly reduced levels of all *c*-type cytochromes (13). Membrane fractions were prepared from AJB530 and PAS100 and solubilized in lithium dodecyl sulfate, and the proteins were separated on a lithium dodecyl sulfate-polyacrylamide gel. After tetramethyl benzidine staining, nine bands were visible in the PAS100 lane, whereas no bands were visible in the AJB530 lane, even after the lane was overloaded 10-fold (data not shown). Studies in *R. capsulatus* and *R. sphaeroides* have demonstrated the presence of up to 10 *c*-type cytochromes (13, 21); the roles of many of these cytochromes have not been determined.

Since staining of heme-containing bands may not be sensitive enough to detect low levels of *c*-type cytochromes, we used the method of Rieske (26) to extract total *c*-type cytochromes and then quantitated the pyridine ferrohemochromes by difference spectroscopy (Fig. 2). Using this procedure, we were unable to detect any *c*-type cytochromes in AJB530. However, MT113 had 29% of the level of *c*-type cytochromes found in PAS100.

The presence of normal amounts of *b*-type cytochromes in AJB530 suggests that it is proficient in heme synthesis but lacks the ability to form mature *c*-type cytochromes. It is also possible, however, that AJB530 has some impairment in its ability to obtain iron and that, under conditions of iron limitation, *R. capsulatus* preferentially synthesizes *b*-type cytochromes. We tested the ability of AJB530 to obtain iron

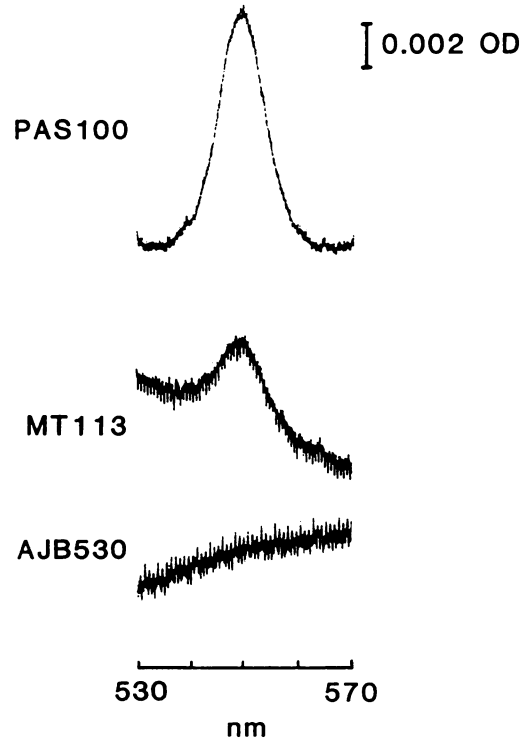

FIG. 2. Difference spectra of extracted *c*-type cytochromes. Each sample contained 0.5 mg of protein. OD, Optical density.

from the medium by measuring total intracellular iron in AJB530 and PAS100. The two strains were grown in RCV medium, the cells were harvested, and the pellets were washed twice with 10 mM potassium phosphate buffer (pH 7.0). Total iron was determined by atomic absorption. The concentrations of *b*- and *c*-type cytochromes were estimated by difference spectroscopy of whole cells (22). The concentration of nonheme iron was derived by subtracting the concentration of *b*- and *c*-type cytochromes from the total intracellular iron concentration (22). Although AJB530 lacked *c*-type cytochromes, it had normal levels of *b*-type cytochromes and nonheme iron (Table 1), indicating that the defect in AJB530 is not in iron uptake or heme synthesis.

Cloning of the gene responsible for the AJB530 phenotype. Before we could clone the gene responsible for the pleotropic phenotype of AJB530, we had to be certain that the lack of *c*-type cytochromes and the overproduction of porphyrins were due to a single mutation. DNA was isolated from strains PAS100 and AJB530, cut with *Eco*RI, separated on an agarose gel, and blotted to nitrocellulose. The blots were hybridized to nick-translated pSUP2021 DNA. This plasmid is a pBR325 derivative that contains Tn*5* (27). pSUP2021 did not hybridize to PAS100 but hybridized to a single band of

TABLE 1. Heme and non-heme iron in AJB530 and PAS100

Strain	Fe (nmol/mg of protein)		
	Cytochrome *b*	Cytochrome *c*	Nonheme Fe[a]
AJB530	0.36	<0.07[b]	14.8
PAS100	0.29	0.64	11.1

[a] Total iron concentration minus total heme iron concentration.
[b] Minimum concentration of cytochrome *c* that could have been detected in this experiment.

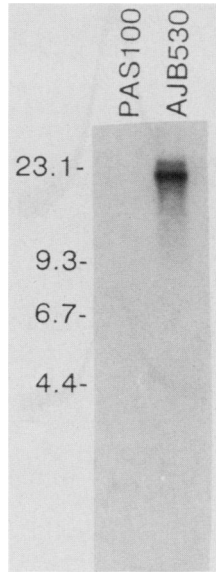

FIG. 3. Hybridization of PAS100 and AJB530 to Tn*5*. Southern blots of *Eco*RI-digested chromosomal DNA from PAS100 and AJB530 were hybridized to pSUP2021.

approximately 20 kilobases (kb) in AJB530 (Fig. 3). This suggests that the Tn*5* inserted into a 14-kb *Eco*RI fragment and produced a new fragment of 20 kb in AJB530.

We used the cosmid library constructed by Avtges et al. (2) to isolate a cosmid that complemented the mutation in AJB530. Photosynthetically competent strains were selected by using a triparental mating procedure described previously (2). The resulting colonies were purified on RCV agar containing kanamycin and tetracycline to select for the presence of the Tn*5* and the cosmid, respectively.

Cosmid DNA was isolated from one of the exconjugants and used to transform *E. coli* MC1061 to tetracycline resistance. One of the resulting transformants was mated with AJB530, and tetracycline-resistant colonies were selected for on RCV agar. We screened for complementation of the mutation in AJB530 by viewing the exconjugants under longwave UV light. If the plasmid corrected the mutant phenotype of AJB530, the colonies would not accumulate coproporphyrin and would therefore not fluoresce. None of the resulting colonies was fluorescent, indicating that the cosmid corrected the defect in AJB530. Restriction digests of cosmid pCAP21 with *Eco*RI indicated the presence of five insert bands with sizes of 13.5, 8.8, 2.9, 2.0, and 0.4 kb. The size of the Tn*5*-containing the chromosomal *Eco*RI fragment in AJB530 (approximately 20 kb) suggested that the gene of interest was on the 13.5-kb *Eco*RI fragment of pCAP21. Due to the low efficiency of cloning large fragments into small plasmids, the fragment was subcloned from the cosmid into lambda EMBL3. The 13.5-kb *Eco*RI fragment could then be subcloned from the phage into pCAP59. Plasmid pCAP59 is derived from pSUP202 and contains the omega cartridge (24), which confers spectinomycin resistance. Plasmids derived from pBR322 or pBR325, such as pSUP202, require the presence of a selectable marker, like the omega cartridge, to be stably maintained in *R. capsulatus* (30). The presence of such a marker allows these vectors to be used for complementation analysis (14, 35). The resulting plasmid, pCAP61, was mated into strain AJB530, and spectinomycin-resistant exconjugants were selected. Several hundred spectinomycin-resistant colonies were obtained, none of which was

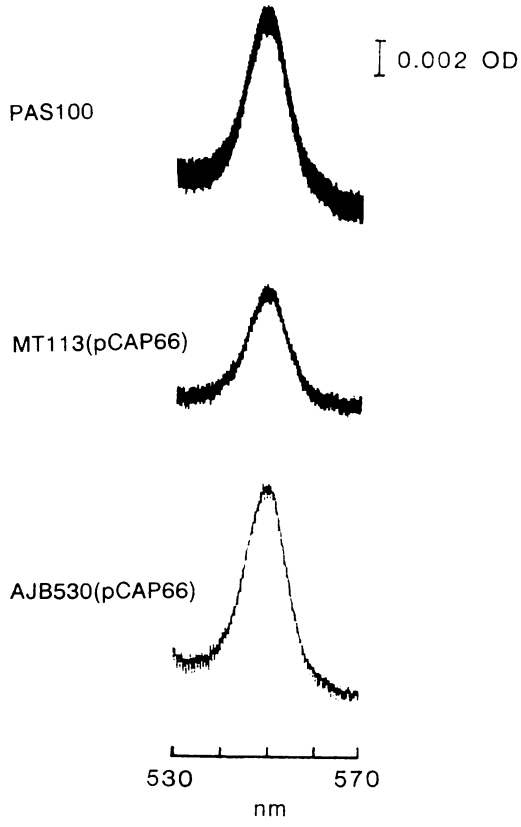

FIG. 4. Difference spectra of *c*-type cytochromes extracted from strains containing pCAP66. Each sample contained 0.5 mg of protein. OD, Optical density.

fluorescent, indicating that the gene of interest was on the 13.5-kb *Eco*RI fragment.

Restriction digests of pCAP61 indicated that the *R. capsulatus* insert contained three *Pst*I sites, yielding fragments of 4.4, 3.4, and 0.6 kb in addition to the two fusion fragments. The two larger fragments were subcloned and tested for complementation of AJB530. The 4.4-kb *Pst*I fragment in pCAP59, designated pCAP66, complemented AJB530, whereas a plasmid containing the 3.4-kb *Pst*I fragment did not complement the mutant. As expected, when pCAP66 complemented the mutation, AJB530 carrying pCAP66 was nonfluorescent, $NADI^+$, and photosynthetically competent. Total cytochrome *c* was extracted from strains AJB530(pCAP66) and MT113(pCAP66). AJB530(pCAP66) contained 95% and MT113(pCAP66) contained 55% of the level of *c*-type cytochromes found in PAS100 (Fig. 4). The increased cytochrome *c* content of MT113(pCAP66) suggests that the mutations in AJB530 and MT113 are in the same gene. It is unclear, however, why pCAP66 only increased the level of *c*-type cytochromes in MT113 twofold. Perhaps it is due to strain differences between PAS100 and the parental strain of MT113, SB1003.

To confirm that the gene we had cloned was the same one that had been mutated in AJB530, we cloned the Tn*5* insertion from AJB530. Chromosomal DNA from AJB530 was digested with *Bam*HI and ligated into pSUP202. The DNA was used to transform *E. coli* MC1061, and kanamycin-resistant colonies were selected. The resulting plasmid, pCAP51, had a 3.2-kb *Bam*HI fragment containing *R. capsulatus* DNA and the left half of Tn*5*. The plasmid was nick

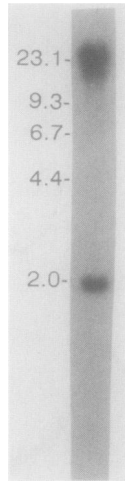

FIG. 5. Hybridization of pCAP51 to pCAP21. A Southern blot of *Bam*HI-digested pCAP21 was hybridized to nick-translated pCAP51.

translated and hybridized to *Bam*HI-digested pCAP21. A band of approximately 1.95 kb was found (Fig. 5), which corresponded to the smallest *Bam*HI fragment in pCAP21. The large band in Fig. 5 is due to hybridization between pSUP202 and pLAFR1. The 1.95-kb fragment was subcloned from pCAP21 into pCAP59, and the resulting plasmid (pCAP71) was mated into strain AJB530. The exconjugants were still fluorescent, suggesting that the *Bam*HI fragment does not contain the entire gene. This 1.95-kb *Bam*HI fragment did, however, hybridize only to the 4.4-kb *Pst*I fragment in pCAP66, confirming our evidence that the entire gene is present on pCAP66. A restriction map of these plasmids, along with the location of the Tn*5* insertion, is presented in Fig. 6.

DISCUSSION

Over the past several years, a considerable effort has been made to study the regulation of bacteriochlorophyll biosynthesis. Most of the attention has been devoted to regulation by oxygen tension (6, 8, 15, 39), light intensity (1, 5, 23), DNA supercoiling (40), and the effects of the recently discovered *pufQ* gene (3, 16). Relatively little attention has been paid to the interrelationships between heme and bacteriochlorophyll syntheses. It has been known for some time that heme feedback inhibits aminolevulinate synthase, thereby preventing excess heme synthesis (36), but recent observations suggested that the link between heme and bacteriochlorophyll syntheses is much more complicated than previously suspected. When Daldal et al. (12) isolated an *R. capsulatus* strain lacking the cytochrome bc_1 complex, they found that the colonies were more pigmented than normal. When the lesion was complemented by a plasmid containing the *pet* genes, the pigmentation returned to normal. Additionally, mutants with lesions in the *helA*, *helB*, and *helC* genes have less than 20% of the normal level of *c*-type cytochromes and excrete an orange pigment (17).

Our idea was to isolate a mutant that completely lacked *c*-type cytochromes and to determine the effect of this mutation on pigment synthesis. Since it has been shown that the reduction in *c*-type cytochromes in MT113 leads to a $NADI^-$ phenotype, we decided that the NADI reaction could be a useful indicator of the presence of *c*-type cy-

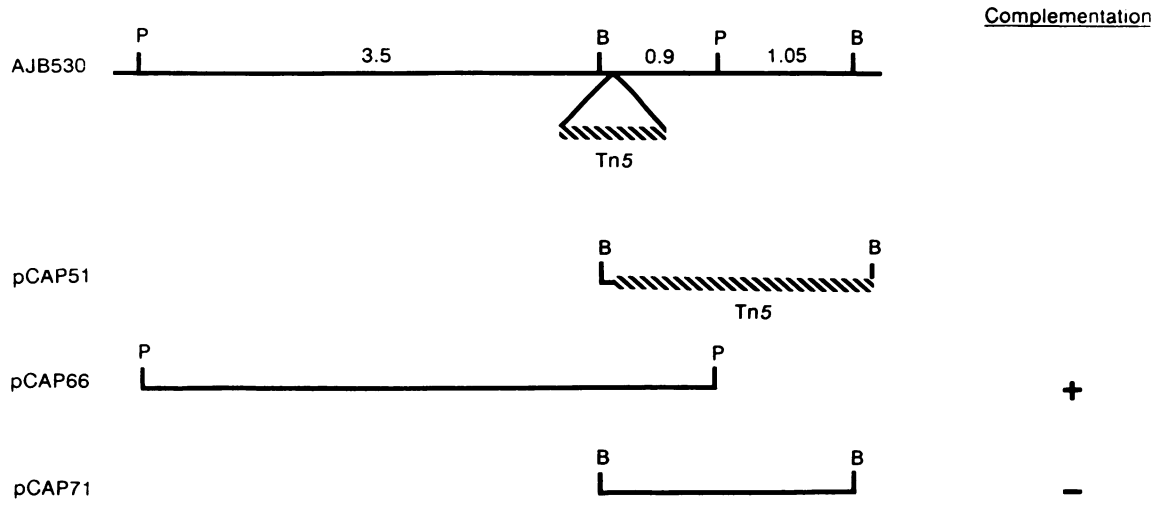

FIG. 6. Restriction map of region surrounding Tn5 insertion in AJB530. Tn5 regions are not shown to scale. B, *Bam*HI; P, *Pst*I. Complementation refers to the ability of the indicated plasmid to allow AJB530 to produce *c*-type cytochromes.

tochromes. Among the NADI$^-$ strains isolated was one, AJB530, that excreted an orange pigment. Other strains with the same phenotype have subsequently been isolated.

Based on the known electron transport chains in *R. capsulatus*, a strain lacking all *c*-type cytochromes should be able to use the alternate electron transport branch for respiratory growth, since that branch does not contain any *c*-type cytochromes (37). Such a strain should, however, lack the cytochrome bc_1 complex and therefore should be incapable of photosynthetic growth (12, 37). AJB530 grew heterotrophically, but not photosynthetically, as would be predicted if it lacked *c*-type cytochromes.

Further analysis of AJB530 indicated that it did, indeed, lack all detectable *c*-type cytochromes. The 552-nm peak was missing in difference spectra of dithionite reduced minus ferricyanide oxidized extracts (Fig. 1), and tetramethyl benzidine staining failed to reveal any cytochrome *c*-containing bands. Because tetramethyl benzidine staining may not be sensitive enough to detect low levels of *c*-type cytochromes, and because the presence of *b*-type cytochromes may mask the presence of low levels of *c*-type cytochromes in difference spectra, we extracted the *c*-type cytochromes from AJB530. Even using this technique, we were unable to detect any *c*-type cytochromes in AJB530, although the reduced level of *c*-type cytochromes in MT113 was readily detectable (Fig. 2). Extraction of *b*-type cytochromes from AJB530 and PAS100 indicated that the mutant had normal levels of *b*-type cytochromes.

Although these results suggest that AJB530 can synthesize heme but is unable to form mature *c*-type cytochromes, they did not completely rule out the possibility that AJB530 has some defect that reduces iron transport or metabolism. Measurements of total intracellular iron showed that AJB530 has the same nonheme iron concentration and cytochrome *b* concentration as PAS100, indicating that AJB530 does not have a defect in iron metabolism. Additionally, it has been shown that in *R. sphaeroides* iron starvation results in drastically reduced levels of nonheme iron while only moderately reducing the levels of *b*- and *c*-type cytochromes (22). It therefore appears that AJB530 has a mutation in a gene for cytochrome *c* maturation.

The most striking aspect of this strain is the excretion of coproporphyrin and protoporphyrin and the reduced synthesis of bacteriochlorophyll. The tetrapyrrole excretion by this strain is much more than that observed in *bchD* and *bchH* mutants, which are unable to convert protoporphyrin to magnesium-protoporphyrin monomethyl ester, and is very similar to what is seen when *R. capsulatus* or *R. sphaeroides* is starved for iron (18; unpublished observations). It is tempting to speculate that somehow *R. capsulatus* monitors the level of *c*-type cytochromes and increases expression of the common tetrapyrrole pathway in an attempt to synthesize *c*-type cytochromes. It appears that, although the total amount of *c*-type cytochromes in MT113 is greatly reduced, the mutation is not stringent enough to cause excretion of porphyrins (Fig. 2) (13, 38).

We have cloned a 4.4-kb *Pst*I fragment (pCAP66) that complements the mutation in AJB530. Although the introduction of pCAP66 into MT113 doubled the total amount of *c*-type cytochromes produced by the strain, it did not completely restore them to normal levels. Because of this, we cannot be sure that the mutations in MT113 and AJB530 are in the same gene, although this seems plausible.

The results reported here indicate that AJB530 is capable of accumulating iron and synthesizing heme but is unable to make mature *c*-type cytochromes. Mutants deficient in the biosynthesis of *c*-type cytochromes, designated *hel* mutants, have been described, and the *hel* gene region has been cloned from strain SB1003 (17). The *Bam*HI, *Eco*RI, and *Pst*I restriction maps of the *hel* gene region are not at all similar to those of the clone we have isolated. The restriction maps of this clone are also not the same as those of the *pet* genes (cytochrome bc_1 complex) or the *cycA* gene (cytochrome c_2) (11, 12). Because of the extensive restriction site differences between the region we cloned and the *hel*, *pet*, or *cycA* genes, it seems unlikely that the Tn5 insertion in AJB530 is located in any of those cloned regions, and it therefore appears that AJB530 has an insertion in a previously unknown gene. We cannot rule out the possibility that this insertion is in a *hel* gene not previously discovered. It is possible that AJB530 lacks cytochrome *c* synthetase (29), the enzyme that links heme to apocytochrome *c* in yeast. However, with at least four different genes being involved in the maturation of *c*-type cytochromes, the identification of the enzyme lacking in this strain will have to await detailed biochemical analysis.

ACKNOWLEDGMENTS

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