Plasmidless, Photosynthetically Incompetent Mutants of 
Rhodospirillum rubrum

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Ethyl methanesulfonate rendered a high percentage of Rhodospirillum rubrum cells plasmidless and photosynthetically incompetent (Kuhl et al., J. Bacteriol. 156:737–742, 1983). By probing restriction endonuclease-digested chromosomal DNA from these plasmidless strains with 32P-labeled R. rubrum plasmid DNA, we showed that no homology exists between the plasmid and the chromosomal DNA of the mutant. Loss of the plasmid in all the nonphotosynthetic isolates was accompanied by the synthesis of spirilloxanthin under aerobic growth conditions, resistance to cycloserine and HgCl₂, and loss of ability to grow fermentatively on fructose. Changes in both the protein and lipid composition of the membranes and the impaired uptake of 203HgCl₂ in the plasmidless strains (compared with the wild type) suggest either that membrane modification occurs as a result of plasmid loss, accounting for several of the acquired phenotype characteristics of the cured strains, or that both membrane modification and plasmid loss are part of the same pleiotropic mutation.

It has been suggested that plasmids, owing to their frequent occurrence in photosynthetic organisms, may have a role in coding for genes that are needed for the formation of the photosynthetic apparatus (7). Saunders et al. (21) treated Rhodopseudomonas sphaeroides with sodium dodecyl sulfate and obtained a photosynthetically incompetent mutant whose plasmid profile was altered in that one of its three plasmids had increased in molecular weight from 28 \times 10^6 to 34 \times 10^6. These workers suggested that the photosynthetic process was inactivated by a genetic rearrangement, perhaps as a result of an insertion sequence. Although Marrs (15) showed the presence of linkage in Rhodopseudomonas capsulata between DNA coding for the photopigments and known chromosomal genes, such as those for tryptophan biosynthesis and rifampin resistance, he also found that the gene transfer agent carrying genes for the photopigments hybridized with plasmid DNA (8) which did not rule out the possibility of a plasmid role in the expression of photosynthesis. Hu and Marrs (8) suggested that a unifying theory to explain these observations would require the existence of an episequence that either be extrachromosomal or integrated into the chromosome.

In the purple nonsulfur photosynthetic bacteria, two or more plasmids have been isolated from Rhodopseudomonas sphaeroides (7, 21) and Rhodopseudomonas capsulata (8); however, these plasmids are cryptic in that no function has yet been associated with them. The only published report dealing with the isolation of plasmid DNA from Rhodospirillum rubrum was that of Kuhl et al. (12), who showed that each strain of R. rubrum examined had a single 55-kilobase plasmid, and that all were nearly identical in their restriction endonuclease profiles. Plasmidless (cured) strains are unable to grow under photosynthetic conditions (Psg-) (12). In the present report, these Psg- plasmidless strains were characterized, and their chromosomal DNA was analyzed for integrated R. rubrum plasmid sequences.

MATERIALS AND METHODS

Bacterial strains and growth conditions. R. rubrum S1-G and S1-A were obtained from H. Gest (Indiana University, Bloomington, Ind.) and D. I. Arnon (University of California at Berkeley), respectively; the strains were given different designations because they have different drug resistance phenotypes. Plasmidless strains of R. rubrum were obtained by methods described previously (12). Strain KY11 was a plasmidless strain obtained by treatment of the wild type (S1-G) with ethidium bromide (50 \mu g ml⁻¹). It was selected as a pink colony on an aerobic plate of K medium containing 25 mM CaCl₂, where the wild-type colonies were initially colorless and then turned red in the center as the colony became anaerobic. Strains KY115 and KY116 are kanamycin-resistant (50 \mu g ml⁻¹) and rifampin-resistant (50 \mu g ml⁻¹) mutants of this strain, respectively. Strains KY51 and KY60 were obtained by treatment of strains S1-G and S1-A, respectively, with ethyl methanesulfonate (EMS), as described previously (12).

R. rubrum and the plasmidless mutants were grown on minimal maltate medium (16) or K medium (12), either aerobically in the dark or photosynthetically. (The plasmidless strains grow only aerobically.)

Chemicals. All antibiotics, DNase (grade I), salmon sperm DNA, lysozyme (grade I), Triton X-100, EMS, ethidium bromide, and polyvinylpyrrolidone were obtained from Sigma Chemical Co. St. Louis, Mo. Bio-Gel P-60 was obtained from Bio-Rad Laboratories, Richmond, Calif. Radioactively labeled 32P-deoxyribonucleotides were obtained from New England Nuclear Corp., Boston, Mass.

EMS mutagenesis was done by the same procedures used to produce cured strains (12), except that after growth on high-calcium medium, both photosynthetically grown cells and those grown aerobically in the dark were treated with EMS. To screen for mutants, colonies on K medium were replicated onto K medium supplemented with either streptomycin (50 \mu g ml⁻¹) or cycloserine (50 \mu g ml⁻¹) and incubated aerobically.

Isolation of DNA. Plasmid DNA (pKY1) was isolated from R. rubrum, and agarose gel electrophoresis was done as described previously (12). Chromosomal DNA was isolated...
from 25 ml of cells (with a turbidity of 250 to 300 Klett units) grown aerobically in the dark. After harvesting, the cells were washed with 0.85% NaCl. They were frozen to −20°C for 1 h, after which the thawed pellet was suspended in 1.2 ml of 50 mM Tris-EDTA buffer (pH 8.0) containing 15% sucrose and incubated at 37°C for 30 min in the presence of 0.5 mg of lysozyme. The culture was then passed repeatedly through a 1.0-ml hypodermic syringe equipped with a 22-gauge needle to further disrupt the cells. Sodium dodecyl sulfate (0.1%) was added, and the cell extract was kept at 70°C for 15 min. Then 0.5 M potassium acetate was added, and the temperature was lowered to 4°C for 1 h. The sediment which formed was pelleted by centrifugation at 9,000 × g for 15 min, and 1 ml of ethanol (−20°C) was then added to the supernatant fluid and sedimented as before. This mixture was kept at room temperature for 30 min while the DNA precipitated. After the DNA was suspended in 200 μl of 300 mM sodium acetate in 50 mM Tris (pH 8.0), ethanol (400 μl at −20°C) was again added, and this mixture was incubated at room temperature for 30 min. The DNA was purified by repeating this DNA precipitation procedure; contaminating substances were further removed by phenol-chloroform extraction. The resulting DNA was ethanol precipitated overnight at −10°C, centrifuged, and finally suspended in 50 μl of sterile water.

Hybridization procedures. Southern transfer (23) of EcoRI restriction endonuclease-digested R. rubrum chromosomal DNA from agarose gels to nitrocellulose filters, nick translation (20) of the plasmid pKY1, and hybridization of the 32P-labeled pKY1 plasmid to the restriction endonuclease-digested chromosomal DNA on the nitrocellulose filters were done essentially as described by Davis et al. (3).

Figure 1. Analysis of R. rubrum lysates for plasmid DNA. Lanes: 1. R. rubrum S1-G; 2. R. rubrum KY11. Crude plasmid DNA was isolated and analyzed by agarose gel electrophoresis as previously described (12). chm, Chromosomal DNA fragments.

RESULTS

Hybridization of R. rubrum plasmid to chromosomal DNA. Although plasmid DNA could not be isolated from the cytoplasm of a Psg mutant (Fig. 1), the possibility remained that the plasmid was not lost from the cell but was integrated into the chromosome. Therefore, genomic material from several plasmidless strains was examined by Southern blot hybridization to determine whether homology existed between them and plasmid pKY1 from the wild type. When EcoRI-digested total cellular DNA was subjected to electrophoresis, transferred to nitrocellulose, and hybridized to 32P-labeled pKY1 DNA, the autoradiogram (Fig. 2) showed that there was no hybridization between the wild-type plasmid (pKY1) and the chromosome of the cured strains (lanes 2 through 5). The nick-translation probe (pKY1 DNA) hybridized to total DNA from the wild-type strain (lane 1) as well as to itself (lane 6). These results show that there is no sequence homology between total DNA isolated from the plasmidless strains and plasmid pKY1.

Growth characteristics of plasmidless strains. While screening for plasmid markers, we found that plasmidless strains would not grow photosynthetically, but that they did grow satisfactorily in the dark under aerobic conditions (Fig. 3A). The difference in anaerobic growth responses between the plasmidless (KY11) and the wild-type (S1-G) strains under light (photosynthetic) (Fig. 3B) and dark (fermentative)
growth conditions (Fig. 3C) is also apparent. The cured strain (KY11) was unable to grow fermentatively but the wild-type strain (S1-G) grew as expected (22). Psg− mutants have been reported to arise after long-term anaerobic growth in the dark and, in two cases, have had decreased levels of bacteriochlorophyll and carotenoids (14, 24). \textit{R. rubrum} C and G1, two of these Psg− mutants found by Uffen et al. (24), however, have the normal 55-kilobase plasmid (12). In K medium, the wild-type strain always grew to a greater turbidity than did the cured strains (Fig. 3A and see Fig. 5), but recently we have found that they grow to the same level in minimal malate medium (16) supplemented with 0.3% yeast extract.

The plasmidless strains were further characterized by an examination of their carbon metabolism. Since \textit{R. rubrum} is the only member of the \textit{Rhodospirillaceae} family that grows on malate and fructose but not on glucose (17), the carbon utilization profiles of the wild-type and a cured strain (KY116) were compared. The growth pattern of the KY116 strain was identical to that of the S1-G strain.

**Pigments produced by the plasmidless strains.** The absorption spectra of purified carotenoid (spirilloxanthin) isolated from photosynthetically grown wild-type (S1-G) cells and the pigment isolated from the aerobically grown cured strain (KY11) were exactly superimposable with maxima at 540, 505, 475, and 393 nm (in benzene) and 525, 491, and 465 nm (in acetone-methanol [7:2]), suggesting that these two pigments are identical. Additional evidence that strain KY11 produces spirilloxanthin, the primary carotenoid of \textit{R. rubrum}, is presented in Table 1. Benzene extraction of both the wild-type strain (S1-G) and the cured strain (KY11) yielded carotenoid that migrated on the Silica Gel TLC system with an $R_f$ value of 0.97. When the pigments were eluted from the TLC plate and dissolved in benzene, the absorption spectra of the two carotenoid bands were again identical (data not shown). Only about one-half of the carotenoid of the KY11 strain was found in this band, however; the other half was distributed in four bands that show a higher degree of polarity (lower $R_f$ values) than spirilloxanthin (Table 1). Because of their polarity, these four carotenoid components could be extracted from the KY11 strain with methanol (Table 1); the remaining “authentic” spirilloxanthin could then be extracted with benzene. Although the chemical modification that produce this heterogeneity in spirilloxanthin is not understood, it is not reflected in the spectrum of the form having an $R_f$ value of 0.43, since it was indistinguishable in acetone-methanol (7:2) from that of the wild-type cells (data not shown).

Very little spirilloxanthin was produced by the wild-type cells during aerobic growth in the dark in liquid medium (Fig. 4A), but an equivalent mass of cells of cured strain KY115 produced enough of this pigment (four times more) to give the culture a distinct pinkish appearance. The peak at 418 nm observed for the wild-type strain has not been identified, but could represent the Soret band of some extracted protoheme (5). The aerobically grown strain S1-G produced a small amount of bacteriochlorophyll, but the cured strain produced virtually none (Fig. 4B). The bacteriochlorophyll peak at 772 nm in the photosynthetically grown cells provides a qualitative standard from which to make this determination. On aerobic agar plates, colonies of the cured
strains had a pinkish-orange appearance, whereas those of wild-type cells were red as a result of the full derepression of carotenoid (and bacteriochlorophyll) when the center of the colony became anaerobic. This difference in colony color was used to select for putative plasmidless mutants.

**Drug and heavy-metal phenotypes of the plasmidless strains.** In searching for plasmid-borne markers, we noted that cured strains derived from strain S1-G became sensitive to streptomycin and resistant to cycloserine and HgCl₂ (Fig. 5). Strain KY11 grew well in the presence of 100 μM HgCl₂, although growth of the wild-type strain was inhibited at concentrations beyond 0.1 μM (Fig. 5C).

Since the cured strains had gained a number of new characteristics not possessed by the wild type, it was of interest to determine the genetic relationship between them. Treatment of *R. rubrum* S1-G with EMS under aerobic growth conditions in the dark produced only three mutants (0.2% of total cells) that were photosynthetically incompetent (Psg⁻ Sm⁺ Cy⁻) (Table 2). Conversely, when photosynthetically grown cells were treated with EMS, 90% of the surviving cells were changed in all three phenotypes to Psg⁻ Sm⁺ Cy⁺; without exception, they were also resistant to mercury (Hg²⁺). Analysis of 10 of these mutants (selected randomly) showed that all were plasmidless, strongly suggesting that a relationship exists between the genetic markers Psg⁻, Sm⁺, Cy⁺, and Hg²⁺ and the plasmid in *R. rubrum*.

Because one or more of these characteristics might be explained by membrane changes leading to decreased uptake of various substances such as cycloserine or HgCl₂, the uptake of ²⁰³Hg²⁺ was examined. ²⁰³Hg²⁺ uptake by two different cured strains (KY116 and KY51) was very low compared with uptake by the parent strain (Fig. 6). Substantial changes in protein composition occurred in both the outer and the cytoplasmic membranes of the plasmidless strains (C. Deal and S. Kaplan, personal communication), which is consistent with the idea that some of these phenotype changes might be due to changes in their membranes.

**Lipid analysis.** The amount and proportion of the lipids in the cured strain (KY11) and the wild-type strain (S1-G) were examined (Table 3). The lipid composition of wild-type *R. rubrum* (8.6%, dry weight) agrees well with that reported by Imhoff et al. (9%) (9). Strain KY11 has ca. 21% less total lipid and a considerably higher ratio of neutral lipid to phospholipid than the wild-type strain has.

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**TABLE 1. TLC of *R. rubrum* carotenoids**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Extraction solvent</th>
<th>Rf</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1-G</td>
<td>Chloroform</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>Benzene</td>
<td>0.97</td>
</tr>
<tr>
<td>KY11</td>
<td>Benzene</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>0.97</td>
</tr>
</tbody>
</table>

* a Grown photosynthetically (all other cells were extractions of cells grown aerobically in the dark).
* b Spirilloxanthin was extracted and purified by the method of Polgár et al. (18) and used as a standard.

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**FIG. 4.** Comparison of carotenoid and bacteriochlorophyll contents of the wild-type strain and a plasmidless mutant grown aerobically in the dark. (A) Carotenoid levels: ——, plasmidless mutant (KY115); ——, wild type (S1-G). (B) Bacteriochlorophyll levels: △, strain KY115; ○, strain S1-G; and ■, strain S1-G control grown photosynthetically.

**FIG. 5.** Effect of streptomycin, cycloserine, and Hg²⁺ on aerobic growth in the dark of wild-type *R. rubrum* and the plasmidless mutant. Cells were grown in K medium supplemented with the inhibitor concentrations indicated.
The most striking difference in the lipid compositions of the two strains was in the distribution of phospholipids (Fig. 7). PC was the major phospholipid in strain KY11, in which it represented half the total phospholipid; however, it was undetectable in the wild-type strain (Fig. 7). Production of PC by R. rubrum cells is uncertain since it has been detected in this organism by some researchers but not by others (see review by Kenyon [11]). High-pressure liquid chromatographic analysis of the phospholipids showed that the wild-type strain had four times more PE and nearly seven times more PG (on a dry weight basis) than the cured derivative had (Table 3). When acid hydrolyzed and then analyzed for amino acids, phospholipid fractions from both strains showed the presence of ornithine and substantial amounts of what appears to be alanine (data not shown). An alanine phospholipid has previously been found in Clostridium welchii (13) but not in photosynthetic bacteria.

DISCUSSION

Saunders et al. (21) have suggested the involvement of a plasmid in photosynthetic growth. Since we found no plasmidless cells that were capable of growing in the light (12), it also appeared that the plasmid in R. rubrum may be required for photosynthetic growth. The relationship may not be as simple as this, however, since we have recently isolated a single plasmidless Psg strain revertant from a cured Psg strain. This observation was confirmed by probing the total DNA complement of this revertant with pKY1, the wild-type plasmid of another strain.

**TABLE 2. Effect of growth conditions on EMS-induced mutagenesis of R. rubrum S1-G**

<table>
<thead>
<tr>
<th>No. of cells undergoing mutations under the following growth conditions:</th>
<th>Phenotype*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic, dark</td>
<td>Photosynthetic</td>
</tr>
<tr>
<td>0'</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>0</td>
<td>424</td>
</tr>
<tr>
<td>1,239</td>
<td>36</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Cells were grown in high-calcium K broth (12) for several generations, and transferred to either K broth for continued aerobic growth or to minimal malate medium (16) for several passages of photosynthetic growth before treatment with EMS.

**TABLE 3. Lipid composition of aerobically grown strains of R. rubrum**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Amt of lipid (mg g⁻¹ [dry wt]) in strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S1-G</td>
</tr>
<tr>
<td>Total lipid</td>
<td>86</td>
</tr>
<tr>
<td>Neutral lipid</td>
<td>12.9</td>
</tr>
<tr>
<td>Phospholipid*</td>
<td>73.9</td>
</tr>
<tr>
<td>PC</td>
<td>0</td>
</tr>
<tr>
<td>PE</td>
<td>21.9</td>
</tr>
<tr>
<td>PG</td>
<td>38.0</td>
</tr>
</tbody>
</table>

* Minor lipid components, including several that were tentatively identified as ornithine and alanine phospholipid and cardiolipin, were not detectable on our high-pressure liquid chromatography system; this resulted in less than 100% recovery of phospholipids.

![Fig. 6. Uptake of ²³⁴Hg²⁺ by wild-type R. rubrum and plasmidless mutants.](http://jb.asm.org/) Cells (10 ml, after 24 h of aerobic growth) were harvested, washed, and suspended in 5 ml of 50 mM Tris buffer (pH 7.5) containing 5 mM HgCl₂. After a 15-min equilibration at 20°C, 10 μM ²³⁴Hg²⁺ (specific activity of 1 mCi/mg of Hg) was added, and 0.5-ml samples were removed at various intervals and filtered through 0.45-μm-pore filters (Millipore Corp., Bedford, Mass.). After the filters were washed twice with 4 ml of 50 mM Tris buffer (pH 7.5), the radioactivity on each filter was determined by liquid scintillation counting. Symbols: •, wild-type strain; ○, strain KY116; and ▲, strain KY51.

![Fig. 7. TLC of phospholipids from the wild-type and plasmidless strains of R. rubrum.](http://jb.asm.org/) Lanes 1, 4, and 5 contained 20 μg each of the authentic standards PE, PC, and PG, respectively. Lanes 2 and 3 contained 100 μg of the phospholipid fraction from the wild-type strain (S1-G) and the plasmidless cured strain (KY11), respectively. The trace of phospholipid running near the front (lanes 2 and 3) was cardiolipin. Phospholipids were separated on Silica Gel 60H TLC plates (Brinkmann Instruments, Inc., Westbury, N.Y.), developed with a solvent (9) of chloroform-methanol-acetic acid-water (85:15:10:3.5). The phospholipids were located by spraying the plate with 5% phosphomolybdic acid in 95% ethanol.
plasmid, and finding no homology. Although this type of reversion from Psg- to Psg+ is very rare (one isolate), the existence of even one plasmidless Psg+ strain suggests that plasmid loss may not be responsible for the Psg+ phenotype, but that loss of the plasmid and the Psg+ phenotype (with its membrane alterations) could both be the result of a pleiotropic mutation.

Alternatively, we cannot rule out at this time a rare second mutation that could have reversed the effect of plasmid loss on this isolate. The possibility therefore remains that the acquired resistance to drugs and HgCl2 and the changes in lipid composition are determined by the plasmid. This phenomenon has been observed previously where the presence of colicin (4) or resistance (10) plasmids not only affected the drug sensitivity of the host, but also mediated substantial changes in the composition of the cell envelope. For example, moving the RPl plasmid into Pseudomonas aeruginosa has resulted in cells with outer membrane PC levels decreased more than fivefold compared with those of the plasmidless wild type (10). The parallel to the PC-plasmid relationship in R. rubrum is striking. Judging from the drastically altered lipid composition in the cured R. rubrum cells (Table 3), membrane modifications could be responsible for the acquired resistance to HgCl2 and cycloloserine (Fig. 5), just as the altered membranes are believed to have affected the drug sensitivity patterns of P. aeruginosa (10).

We previously reported the isolation of plasmid-carrying Psg+ revertants arising from plasmidless (Psg-) cultures (S. A. Kuhl and D. C. Yokoh, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, H126 p. 134), but they were also extremely rare (only two were obtained), and the method used to isolate them did not produce any more. We could not be certain, therefore, that these so-called revertants were not, in fact, wild-type contaminants, especially since the DNA of the cured strains from which they appeared to arise did not contain any plasmid sequence homology (Fig. 2). The available evidence suggests that the plasmid is involved in the sequence of events resulting in photosynthetic incompetence in the plasmidless R. rubrum mutants, but its exact role in this process is unclear and is currently under investigation.

ACKNOWLEDGMENTS

We thank C. Formari and S. Kaplan for providing us with a method for isolating chromosomal DNA from R. rubrum, J. Schultz and P. Weaver for communicating to us the fact that the plasmidless strains of R. rubrum would not grow fermentatively on fructose, and T. Donohue for stimulating discussions about the lipids of the Rhodopseudomonaceae family.

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