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Characterization of a *Rhodospirillum rubrum* Plasmid: Loss of Photosynthetic Growth in Plasmidless Strains

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A single plasmid of 55 kilobases was found in crude cell lysates of each of nine strains of *Rhodospirillum rubrum*. Restriction endonuclease analysis showed identical fragment patterns with a given nuclease for all plasmids except one, for which an additional *Eco*RI site was observed. Elimination of the plasmid required that the cells be passaged several times in 25 mM calcium-containing medium, followed by at least two passages under photosynthetic growth conditions in low-calcium medium before treatment with ethyl methanesulfonate. The resulting plasmidless mutants only grew aerobically and were all incapable of pigment formation and photosynthetic growth, suggesting that plasmid DNA is required for photosynthetic competence in *R. rubrum*.

The occurrence of plasmids in various photosynthetic procaryotes has raised questions about their role in the cell, in particular their possible involvement in coding for proteins required for photosynthesis. Since the initial report by Suyama and Gibson (16) of satellite DNA in *Chromatium* sp. and *Rhodopseudomonas sphaeroides*, Gibson and Niederman (5) have determined that *R. spaeroides* NC1B 8327 has two covalently closed circular plasmids having masses in the range of 70 to 75 megadaltons. Saunders et al. (15) found that a second strain of *R. sphaeroides* (2.4.1) had three plasmids and estimated their masses to be 28, 66, and 75 megadaltons, whereas *Rhodopseudomonas capsulata* BH9 was shown by Hu and Marrs (8) to have two plasmids of 74 and 94 megadaltons. Although these members of the *Rhodospirillaceae* were shown to have two or more plasmids, the traditional functions associated with plasmids, such as bacteriocin production, drug and heavy metal resistance, and gene transfer functions, have not yet been linked to any of them.

The only published study dealing with an attempt to isolate plasmid DNA from *Rhodospirillum rubrum* yielded negative results, in that Suyama and Gibson (16) were unable to detect satellite DNA in CsCl density gradients. We report here the discovery of a single, highly conserved plasmid in *R. rubrum*, outline some of its major characteristics, and show that its presence is essential for photosynthetic growth.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *R. rubrum* S1 was obtained from both H. Gest and D. I. Arnon; because the two strains proved not to be identical, they have been designated S1-G and S1-A, respectively. *R. rubrum* G-1980, a wild-type isolate, and G-9, a carotenoidless mutant originally isolated from strain S1 by J. Newton, were also obtained from H. Gest. Other wild-type *R. rubrum* strains, ATCC-11170, FR1, and S-4, were obtained from R. H. Burris, P. Weaver, and E. M. Carney, respectively. Mutant C and strain G1 were obtained from R. Uffen. *Escherichia coli* J53 *pro met* λ^+ (RP4 Tc^r AP^r Km^r) was obtained from B. E. Ely.

R. rubrum was grown in the dark aerobically (in shake flasks or roll tubes) at 32°C in K medium (0.3% peptone, 0.3% yeast extract, 20 mM sodium malate, 25 mM $CaCl_2 \cdot 2H_2O$). The calcium chloride was autoclaved separately to prevent precipitation. Cells were grown photosynthetically at 32°C in stoppered bottles completely filled with the minimal malate medium of Ormerod et al. (13) that were placed between two banks of fluorescent lights (140 $\mu E\ m^{-2}\ s^{-1}$). A 20% inoculum was routinely used for all growth experiments.

Chemicals. Lysozyme (grade I), sodium dodecyl sulfate, Triton X-100, polyethylene glycol 6000, RNase (grade I), phage lambda DNA, ethyl methanesulfonate, and ethidium bromide were obtained from Sigma Chemical Co., St. Louis, Mo. Cesium chloride (technical grade) was obtained from Kawecki Berylco, Revere, Pa. Restriction endonucleases *Hin*dIII and *Eco*RI were obtained from New England Biolabs, Beverly, Mass., and *Sma*I was purified and kindly provided by R. P. Lawther.

Purification of plasmid DNA. Plasmid DNA was purified from 1 liter of photosynthetically grown cells (turbidity, 250 to 300 Klett units) by the basic procedures of Guerry et al. (6) and Humphreys et al. (9). Washed, frozen (−70°C) cells (2 to 3 g [wet weight]) suspended in 50 ml of 50 mM Tris-hydrochloride (pH 8) containing 25% sucrose and 5 mg of lysozyme were incubated for 30 min at 37°C, after which 70 mM EDTA was added, and the incubation was continued for another 30 min. RNase (10 μg) and 25 ml of the

Triton-sucrose solution of Holmes and Quigley (7) were then added, and the incubation temperature was raised to 55°C; the cell suspension was held at this temperature for 10 to 15 min with intermittent swirling.

Centrifugation at 48,000 × *g* for 30 min resulted in a cleared lysate (ca. 150 ml) to which was added 50 ml of a 30% polyethylene glycol 6000 PEG solution containing 1.5 M NaCl. After standing overnight at 4°C, the extract was centrifuged at 1000 × *g* for 3 min, the supernatant was discarded, and the pellet (the plasmid DNA) was dissolved in 8.7 ml of TEN buffer (50 mM Tris, 5 mM EDTA, 50 mM NaCl [pH 8.0]). Solid CsCl (8.3 g) was added, and the mixture was incubated at 37°C for 20 min, during which time a reddish precipitate floated to the top of the mixture. After the floating precipitate was removed (using a separatory funnel), ethidium bromide (4 mg) and CsCl (refractive index adjusted to 1.390) were added to the cleared solution, which was then centrifuged at 42,000 rpm for 60 h at 20°C in a Beckman type 65 rotor. After the DNA bands were removed from the tube by syringe, ethidium bromide was removed (2), and 3 volumes of TEN buffer was added. The plasmid DNA was precipitated by the addition of 6 volumes of isopropanol and allowing the solution to stand at −20°C overnight (2). The DNA was removed by centrifugation (12,100 × *g* for 20 min at −10°C), air dried, dissolved in 0.1 ml of 10 mM Tris–4 mM EDTA (pH 8.0), and stored at −20°C. The concentration of purified *R. rubrum* plasmid DNA was estimated (2) to be about 10 μg ml^{-1}.

Restriction endonuclease digestion and agarose gel electrophoresis. Purified plasmid DNA (0.1 μg) was digested with 1 U of the appropriate restriction endonuclease for 3 h at 37°C according to the directions supplied by the manufacturer. Lambda DNA treated with *Hin*dIII (17) was used as a standard in determining fragment size. Slab gel electrophoresis was performed on 1% agarose as described by Meyers et al. (12). Electrophoresis was performed at 30 mA and 125 V for 2.5 h or until the dye band reached the bottom of the gel (12 by 13 by 3 mm).

Plasmid curing. Elimination of the *R. rubrum* plasmid with ethyl methylsulfonate (EMS) required that the cells be preconditioned by growth in a high-calcium (25 mM) medium such as K medium, followed by photosynthetic growth in a low-calcium medium. After four or more transfers in K medium, the culture was transferred to low-Ca^{2+} minimal medium (13) in screw-capped tubes and incubated in low light until growth and pigment development began (4 to 10 days). The light was increased as the culture developed; when a turbidity of 150 to 250 Klett units was reached, it was transferred for a second passage in the light. After this culture developed, 0.1 ml of culture was diluted to 1 ml in 100 mM phosphate buffer containing 10 μl of EMS and incubated on a rotary shaker for 30 min at 32°C. The EMS-treated cells were then diluted into 50 ml of K medium and placed in a shaker flask for recovery. When the culture reached 150 to 250 Klett units, serial dilutions were made onto plates of K medium. Colonies that grew (many of which were orange in color) were transferred to a grid on the Ormerod et al. (13) medium supplemented with 0.3% yeast extract and 10 mM $(NH_4)_2SO_4$. These isolates were then replica plated and incubated in an illuminated GasPak anaerobe chamber (BBL Microbiology Systems, Cockeysville, Md.) to test for photosynthetic growth (Psg). Isolates that did not grow (Psg^-) were selected from the aerobic control plate, purified, and examined for plasmids by the rapid-screen method of Holmes and Quigley (7).

RESULTS

***R. rubrum* plasmids.** Although plasmids had not originally been observed in *R. rubrum* (16) with other techniques, crude DNA was isolated from strain S1-G and reexamined for plasmid DNA by agarose gel electrophoresis. A single plasmid (pKY1) was found which comigrated with the 55-kilobase (kb) *Pseudomonas aeruginosa* plasmid RP4 (Fig. 1). Gel electrophoresis of plasmid DNA from a number of other *R. rubrum* strains revealed that each had a single plasmid which comigrated with plasmid pKY1 of strain S1-G (Fig. 2). Not shown are *R. rubrum* "mutant C" and G1 (18), which had plasmids of identical size. In several of the strains examined, the plasmid appeared on gels as a doublet (Fig. 2, lanes 1, 2, and 5). Treatment of purified plasmid DNA with restriction endonucleases (discussed below) and UV radiation (data not shown) suggests that the uppermost band of the doublet represents the open circular form of the plasmid, and the lower part of the doublet represents the covalently closed circular form. Finally, the size of plasmid pKY1 calculated from mobility measurements of the intact plasmid in comparison with plasmids of known size was 55 kb; this agreed fairly well (54 kb) with available restriction endonuclease data (shown below).

Restriction endonuclease analysis of plasmids. The *R. rubrum* strains examined had a single plasmid that comigrated with RP4. Restriction patterns of the *R. rubrum* plasmids, when com-

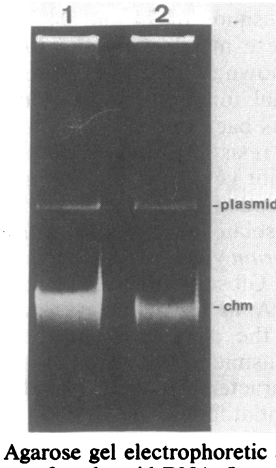

FIG. 1. Agarose gel electrophoretic analysis of *R. rubrum* lysates for plasmid DNA. Lane 1, *R. rubrum* S1-G(pKY1); lane 2, *E. coli* J53(RP4). Crude plasmid DNA was isolated by the method of Holmes and Quigley (7). chm, Chromosomal fragments.

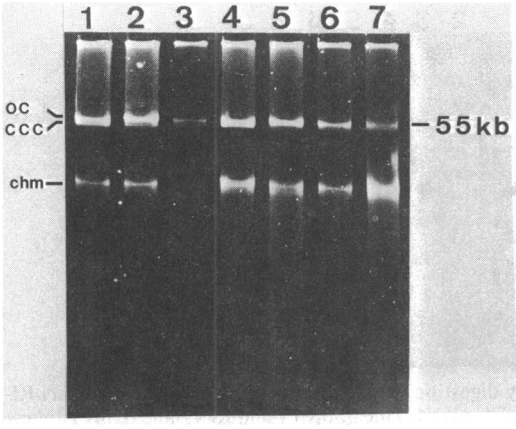

FIG. 2. Plasmid composition of several *R. rubrum* strains by agarose gel electrophoresis. Plasmid DNA was purified by banding on a cesium chloride-ethidium bromide equilibrium density gradient as described in the text. Lane 1, S1-G(pKY1); lane 2, S1-A(pKY2); lane 3, G-9(pKY3); lane 4, ATCC 11170(pKY4); lane 5, FR1(pKY5); lane 6, S-4(pKY6); lane 7, G-1980(pKY7). Uppermost bands are open circular (OC) and covalent closed circular (CCC) forms of plasmid DNA, and lower bands are chromosomal fragments (chm).

pared to those of RP4 (3), showed no similarity.

Because two isolates of the commonly used *R. rubrum* S1 gave different drug phenotypes and cultural characteristics (unpublished data), a restriction analysis was carried out on purified plasmid DNA from these two strains, designated S1-G(pKY1) and S1-A(pKY2). Treatment of plasmids pKY1 and pKY2 with restriction endonucleases *Hin*dIII and *Sma*I (Fig. 3) gave identical patterns of fragments, unique to the nuclease employed. Treatment of these plasmids with *Eco*RI, however, yielded a slightly different pattern (Fig. 3C). Plasmid pKY2 was missing the 11.7-kb fragment but had gained two smaller fragments, the sum of whose sizes was very close to that of the 11.7-kb fragment. This indicates that pKY2, with an extra *Eco*RI site, has at least one minor difference in its nucleotide sequence from plasmid pKY1.

Plasmids from other strains were also digested with these enzymes. The patterns which resulted from digesting these plasmids with *Eco*RI and *Hin*dIII (Fig. 4) were the same as those obtained on digestion of pKY1 with these enzymes. A partial digestion of several of these plasmids resulted, however, with *Hin*dIII. The *Eco*RI digestion of an S1-G strain containing both the native plasmid pKY1 and RP4 showed an extra high molecular weight fragment (top band), which is RP4 with its single *Eco*RI cut (Fig. 4A, lane 5). Endonucleases *Kpn*I, *Sac*I, *Xba*I, and *Xho*I cut plasmid pKY1 at five or six sites each (data not shown). A summary of the endonuclease restriction of *R. rubrum* plasmid pKY1 is given in Table 1.

Plasmidless strains. To cure *R. rubrum* of its plasmid, the previous growth history of the culture proved to be important. This discovery was made by a series of fortuitous observations, which are discussed below. Only after several passages of the cells in a high-Ca^{2+} preconditioning medium, followed by two passages in low-Ca^{2+} medium under photosynthetic growth conditions, was EMS effective in eliminating the plasmid from these organisms (Table 2). Following this protocol, we observed that a high percentage of the survivors that were able to grow in the dark aerobically were, however, incapable

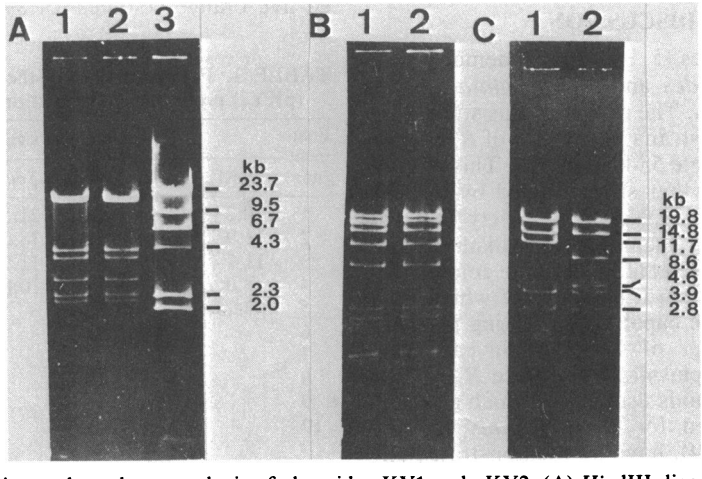

FIG. 3. Restriction endonuclease analysis of plasmids pKY1 and pKY2. (A) *Hin*dIII-digested plasmids: lane 1, pKY1; lane 2, pKY2; lane 3, lambda phage DNA. (B) *Sma*I-digested plasmids: lane 1, pKY1; lane 2, pKY2. (C) *Eco*RI-digested plasmids: lane 1, pKY1; lane 2, pKY2.

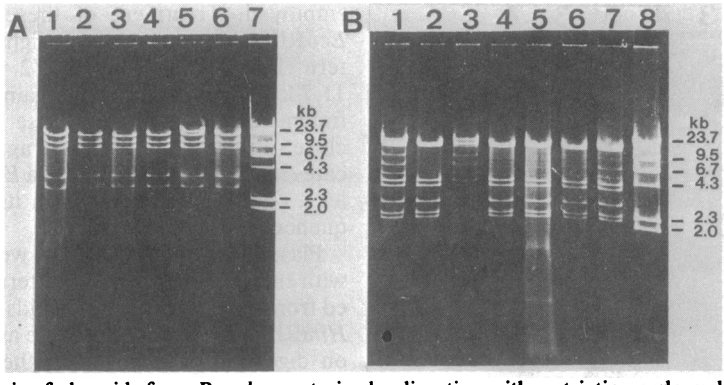

FIG. 4. Analysis of plasmids from *R. rubrum* strains by digestion with restriction endonucleases. (A) *Eco*RI-digested plasmids: lane 1, pKY5; lane 2, pKY6; lanes 3 and 4, pKY1; lane 5, pKY1 and RP4; lane 6, pKY4; lane 7, *Hin*dIII-digested phage lambda. (B) *Hin*dIII-digested plasmids: lanes 1 and 7, pKY1; lane 2, pKY2; lane 3, pKY3; lane 4, pKY4; lane 5, pKY5; lane 6, pKY6; lane 7, phage lambda DNA.

of photosynthetic growth (Psg^-). A rapid screen analysis of these Psg^- mutants for plasmid DNA (7) showed that all were plasmidless and that the curing rate by this technique was between 83 and 90%. A random survey of the Psg^+ cells showed that all contained the 55-kb plasmid pKY1. Hybridization analysis showed no plasmid sequence homology with the chromosomal DNA of the plasmidless (cured) strains (S. A. Kuhl and D. C. Yoch, manuscript in preparation), indicating that the plasmid had not simply been lost from the cytoplasm by incorporating into the chromosome.

When *R. rubrum* was grown aerobically in the dark instead of photosynthetically after preconditioning in high-Ca^{2+} medium, the rate of curing was much lower (2%). This low rate is significant, however, since we have never seen any cures in the controls or occurring spontaneously.

DISCUSSION

Previous studies (5, 8, 15) have demonstrated that *R. sphaeroides* and *R. capsulata* possess multiple plasmids. The results of this study have shown, in contrast, that all strains of *R. rubrum* tested have a single 55-kb plasmid. This conclusion has been further substantiated by the fact that preparation of DNA by a variety of crude lysis procedures, including the alkaline lysis procedure of Casse et al. (1) and the colony lysis procedure of Eckhardt (4), both of which have been shown to be capable of isolating plasmids with a wide range of sizes, has in each case yielded a single plasmid from these *R. rubrum* strains. The methods used here, which are similar to those used for *Pseudomonas* sp. and *Rhizobium* sp. (14), have not demonstrated any other plasmids in *R. rubrum*.

The fact that all the *R. rubrum* strains examined have one 55-kb plasmid of identical size suggests the possibility that a single plasmid has been disseminated throughout the strains of *R. rubrum*, as has been suggested for *Streptococcus mutans* (10). Evidence for a high degree of relatedness is provided by restriction enzyme analysis. Plasmids pKY1 and pKY2 are not identical, even though both are derived from the same strain (S1) of *R. rubrum*. However, since the *Eco*RI restriction patterns differ by only one restriction site, it appears that only a small degree of sequence divergence has occurred.

Finding the conditions necessary for efficient curing of *R. rubrum* and the accompanying loss of photosynthetic competence required a series of fortuitous observations. The high $CaCl_2$ levels and aerobic growth mode used in the initial curing experiments were used in previous (unsuccessful) experiments to develop a gene transfer system in *R. rubrum*. In one of the curing experiments, ethidium bromide treatment yielded five orange colonies (of 899 isolates), all of

TABLE 1. Fragment sizes of the *R. rubrum* plasmid (pKY1) produced by restriction endonucleases[a]

Fragment no.	Fragment size (kb)						
	*Eco*RI	*Hin*dIII	*Sma*I	*Soc*I	*Xba*I	*Xho*I	*Kpn*I
1	20.2	19.4	9.3	21.5	16.5	21.5	18.0
2	14.9	16.6	7.9	18.0	10.0	15.5	16.5
3	11.7	5.3	6.4	11.5	9.6	10.0	13.5
4	4.0	4.4	4.8	10.0	6.6	3.4	9.6
5	3.1	2.8	3.1		4.4	1.0	
6		2.1	1.6				
7		2.0	1.4				
8			1.2				
9			1.1				
10			Others <1.0				

[a] Fragment size was determined by agarose gel (1.0%) electrophoresis with *Hin*dIII-restricted lambda phage fragments as standards.

TABLE 2. Effect of growth conditions on EMS-induced loss of plasmid DNA from *R. rubrum*

Ca^{2+} in preconditioning aerobic medium (mM)	Mode of growth after transfer[a]	No. of colonies examined	Plasmid loss (%)
0.5	Photosynthetic	575	0
	Dark aerobic	526	0
25	Not transferred[b]	76	0
	Photosynthetic	108	83
	Dark aerobic	96	2

[a] To low (0.5 mM)-$CaCl_2$ medium.
[b] Cells for EMS treatment were taken directly from the preconditioning medium.

which were incapable of photosynthetic growth and subsequently proved to be plasmidless. An extensive analysis (Kuhl and Yoch, manuscript in preparation) showed these isolates to be *R. rubrum*. Because wild-type colonies become red due to the full complement of carotenoids that are derepressed as the colony becomes anaerobic, the orange pigment of the mutants, which appeared to be a low concentration of spirilloxanthin, the major carotenoid of *R. rubrum*, was useful in selecting for cures. Extensive efforts to reproduce the curing phenomenon with ethidium bromide were unsuccessful until, in an unrelated series of experiments designed to isolate auxotrophs, we again noted the appearance of orange Psg^- colonies in a culture that had been grown photosynthetically for a short time before the mutagen, in this case EMS, was added. These isolates all proved to be plasmidless and had a phenotype identical to the ethidium bromide-induced cures. About 10 to 15% of the total cells still had the pKY1 plasmid and the wild-type phenotype. In a detailed series of experiments (Table 2), we found that both the initial high-calcium aerobic growth mode and subsequent photosynthetic growth in low-calcium Ormerod medium (13) supplemented with 0.3% yeast extract were necessary before *R. rubrum* could be cured by EMS treatment.

At this time curing of *R. rubrum* of its plasmid remains an empirical observation. Since we observe only about a 10% survival rate when cells are transferred from the high-calcium aerobic growth mode to photosynthetic conditions, some type of preselection (perhaps a membrane modification) may have occurred which makes these cells more susceptible to EMS mutagenesis.

No biological function has yet been ascribed to any known plasmid of the photosynthetic bacteria, but there is suggestive evidence, both from our work (Table 2; Kuhl and Yoch, manuscript in preparation) and that of Saunders et al. (15) that plasmid DNA is required for photosynthetic competence. This suggests (although does not prove) that genes essential to photosynthetic growth reside on the plasmid. In contrast, in *R. capsulata*, Marrs (11) has demonstrated linkage between DNA coding for all photosynthesis genes and known chromosomal genes such as *trpA20* (tryptophan biosynthetic) and rifampin resistance, which suggests that in *R. capsulata* all photosynthesis genes are coded by chromosomal DNA.

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