Improved Preparation Procedure for the Endogenous 115 kb Plasmid of *Rhodobacter capsulatus* AD2 and Analysis of Restriction Pattern

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None of six published preparation procedures for circular bacterial plasmids was satisfactory to purify the endogenous plasmid of a nitrate-reducing strain (AD2) of the phototrophic bacterium *Rhodobacter capsulatus.* By modifying the method of N. T. Hu and B. L. Marrs (1979), Arch. Microbiol. **121,** 61–69, the 115 kb plasmid of the latter strain was prepared to a highly pure state. Digestion of the plasmid with restriction endonuclease *Eco* RI yielded 21 fragments with sizes ranging from 0.3 to 18.4 kb. Except for the three largest ones (18.4 kb, 16.9 kb and 11.2 kb), all fragments were cloned into the vector plasmid pUC8, amplified in *E. coli* JM83 and characterized by *Hind* III restriction analysis.

Introduction

The majority of strains of purple non-sulfur bacteria investigated so far *(Rb. capsulatus, Rb. sphaeroides, R. rubrum, Rf. fermentans, Rps. palustris)* were found to contain one or more endogenous plasmids with sizes ranging from 10 to 130 kb (Willison *et al.,* 1987 *[Rb. caps.];* Fornari *et al.,* 1984 *[Rb. sphaer.];* Kawamukai *et al.,* 1990 *[R. rub.];* A. Hougardy and J.-H. Klemme, unpublished *[Rf. fer., Rps. pal.]*). Although, for the 55 kb plasmid of *R. rubrum,* an involvement in polysaccharide synthesis was postulated (Ideguchi *et al.,* 1993), the cellular functions of most of these cryptic plasmids are unknown yet. In a previous paper we have shown that nitrate and nitrite reductase genes are located and, probably, clustered on the endogenous 115 kb plasmid of *Rb. capsulatus* AD2 (Koch and Klemme, 1994). To facilitate analyses of the large cryptic plasmids of AD2 and other NO_3^--reducing *Rb. capsulatus* strains (Richardson *et al.,* 1994), a reliable purification procedure was required. This paper describes a modification of the procedure of Hu and Marrs (1979) allowing the preparation of microgram quantities of the plasmid and gives data on the number and sizes of the DNA fragments obtained by restriction nuclease (*Eco* RI and *Hind* III) treatment.

Abbreviations: R., Rhodospirillum; Rb., Rhodobacter; Rf., Rhodoferax; Rps., Rhodopseudomonas; SDS, sodium dodecyl sulfate.

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Materials and Methods

Rb. capsulatus strains AD2 and B10, *Rb. sphaeroides* 130, *R. rubrum* S1 and *E. coli* strain JM83 (as host for the vector plasmid pUC8) are kept in the culture collection of the institute. Phototrophic bacteria were grown and maintained under photosynthetic conditions (30 °C; illumination with tungsten lamps at about 2500 lux) in the RCV malate–ammonium sulfate medium described by Weaver *et al.* (1975). *E. coli* JM83 was grown at 37 °C in LB medium (Maniatis *et al.,* 1982). Digestion of plasmid with *Eco* RI and *Hind* III restriction endonucleases, cloning of *Eco* RI-plasmid fragments in vector pUC8, transformation of *E. coli* JM83 with recombinant plasmids and agarose gel electrophoresis were performed according to standard techniques (Maniatis *et al.,* 1982). Agarase and lysozyme were obtained from Boehringer, Mannheim; agarose from Serva, Heidelberg; Magic Miniprep from Promega, Madison, Wis. (U.S.A.); restriction enzymes from Gibco, Gaithersburg, Pa. (U.S.A.); and all other chemicals from E. Merck, Darmstadt.

Results and Discussion

As shown in previous publications, the nitrate-reducing *Rb. capsulatus* strain AD2 contains a single 115 kb plasmid which is lacking in certain nitrate reductase-negative mutants (Willison, 1990; Witt and Klemme, 1991). To isolate the 115 kb plasmid from photosynthetically grown cells in microgram quantities, six preparation procedures published by other laboratories (including the methods of Bazaral and Helinski, 1968; Birnboim and Doly, 1979; and Hu and Marrs, 1979) were tested for their suitability. Although

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the method of Bazaral and Helinski (1968) yielded satisfactory results with plasmids from photosynthetically grown cells of *Rb. sphaeroides* strain 130 (130 kb plasmid) and *R. rubrum* S1 (55 kb plasmid) it failed with *Rb. capsulatus* AD2.

By modifying the method of Hu and Marrs (1979), the AD2 plasmid was prepared in sufficient quantities and purity. 1. Cells from 100 ml of a photosynthetic culture were harvested by centrifugation and resuspended in 1.5 ml icecold sucrose solution (25% [w/v] sucrose in 50 mM Tris-HCl, pH 8). 2. Cell lysis was initiated by addition of 25 mg lysozyme and gentle agitation for 10 min at low temperature (< 10 °C). 3. After addition of 1 ml of 0.25 M EDTA (pH 8), the tubes were inverted 4 times to mix the contents and stored on ice for 10 min. 4. Cellular lysis was completed by addition of 3.5 ml of a 10% (w/v) SDS solution (mixing and incubation as above). 5. Chromosomal DNA was precipitated from the lysate by addition of 8 ml of 5 M NaCl and incubation on ice for 2 h. The precipitate was removed by centrifugation (20,000×*g*; 4 °C; 30 min) and discarded. 6. After addition of 3 volumes of 96% ethanol and glycogen (5 μg/ml) the supernatant was incubated for 2 h at −20 °C. This step effectively removed the membraneous and proteinaceous contaminations of the plasmid preparation. 7. The precipitated plasmid DNA, collected by centrifugation (see above) was dissolved in TE buffer (10 mM Tris-HCl, pH 8, plus 1 mM EDTA) and then subjected to electrophoresis in a gel of low-melting agarose.

Residual DNA contaminations were removed by a another electrophoretic step. For this purpose, the plasmid-containing bands were cut out of the gel and subjected to a second agarose gel electrophoresis. Plasmid DNA was isolated from these gels by enzymatic digestion with agarase: 1. The agarose-slice was incubated for 2×20 min in three volumes of incubation buffer (10 mM EDTA; 100 mM NaCl; 50 mM Tris-HCl, pH 8) and subsequently heated at 68 °C for 10–15 min. 2. After temperature adaptation (37 °C, 15 min), agarase was added (20 U/ml) and the mixture incubated for 6–8 h at 37 °C with gentle agitation. 3. Plasmid DNA was precipitated by addition of three volumes of 96% ethanol (incubation at −20 °C for 2 h) and finally collected by centrifugation (see above).

Table I. Restriction fragments of 115 kb plasmid of *Rhodobacter capsulatus* AD2. Plasmid DNA was treated with *Eco* RI restriction nuclease and then subjected to agarose gel electrophoresis. The *Eco* RI fragments were cloned in pUC8, transformed to *E. coli* JM83 and amplified. The recombinant plasmids were purified from *E. coli* JM83 with the Magic Miniprep system (Promega, Madison) by following the instructions of the manufacturer and subjected to *Eco* RI/*Hind* III restriction analysis. Numbers represent sizes of primary *Eco* RI fragments and sizes of *Hind* III fragments of the latter (numbers in parentheses).

Fragment	Size [kb]	Fragment	Size [kb]
pHK1	10 (8.4; 1.5)	pHK10	3.1 (2.2; 1.0)
pHK2	9.5 (9.5)	pHK11	2.5 (1.7; 0.45; 0.4)
pHK3	6.8 (2.9; 1.7; 1.0)	pHK12	2.2 (1.7; 0.5)
pHK4	6.8 (4.4; 1.5; 1.0)	pHK13	2.0 (1.9; 0.1)
pHK5	6.3 (6.3)	pHK14	2.0 (2.0)
pHK6	4.8 (4.8)	pHK15	1.9 (1.0; 0.8)
pHK7	3.8 (3.8)	pHK16	1.8 (1.8)
pHK8	3.5 (1.8; 1.7)	pHK17	0.9 (0.6; 0.3)
pHK9	3.5 (3.5)	pHK18	0.3 (0.3)

Using the procedure outlined above, highly purified plasmid preparations with yields of 0.1–1 μg/100 ml culture were obtained. Treatment of the plasmid with *Eco* RI restriction endonuclease gave 21 fragments with sizes ranging from 0.3 to 18 kb. From these fragments, 18 were cloned in pUC8, amplified in *E. coli* JM83 and subjected to *Hind* III restriction analysis (Table I). Three of the *Eco* RI fragments (18.4; 16.9 and 11.2 kb, respectively) could not be cloned yet. Adding up the sizes of all single *Eco* RI fragments gave a total size of 118 kb. This value is in good agreement with that (115 kb) obtained for the native plasmid (Willison, 1990; Witt and Klemme, 1991).

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