Extrachromosomal Deoxyribonucleic Acid in Wild-Type and Photosynthetically Incompetent Strains of *Rhodopseudomonas spheroides*

VENETIA A. SAUNDERS,1,* J. R. SAUNDERS,2 AND P. M. BENNETT

Departments of Biochemistry and Bacteriology, Medical School, University Walk, Bristol BS8 1TD, England

Received for publication 8 December 1975

Three covalently closed circular species of extrachromosomal deoxyribonucleic acid have been identified by electron microscopic analysis in strains of *Rhodopseudomonas spheroides*. The weights of these plasmids, as determined from contour length, are about 75 × 10^6, 66 × 10^6, and 28 × 10^6 daltons for both aerobically grown and photosynthetically grown *R. spheroides* strain 2.4.1 (NRS) and for the photosynthetically incompetent strain V-2 (obtained by N-methyl-N-nitro-N'-nitrosoguanidine mutagenesis) and 74 × 10^6, 66 × 10^6, and 34 × 10^6 daltons for a second photosynthetically incompetent strain, SLS I (obtained by incubating strain 2.4.1 [NRS] in medium containing sodium lauryl sulfate). Buoyant densities were found to be 1.717 g/cm^3 (58% guanly plus cytosine) for the plasmids of 66 × 10^6, 28 × 10^6, and 34 × 10^6 daltons in weight and 1.724 g/cm^3 (65% guanly plus cytosine) for those weighing about 75 × 10^6 daltons. Possible functions of these plasmids are discussed.

*Rhodopseudomonas spheroides* is a facultatively anaerobic, photosynthetic bacterium capable of growing anaerobically in the light by photosynthesis and aerobically in darkness by oxidative metabolism. It is possible to obtain either photosynthetically or aerobically incompetent mutants of *R. spheroides* that are still capable of growing in the alternative mode (13, 19, 22). By virtue of this versatility, *R. spheroides* provides a useful test system for investigating the genetic control of differentiation and adaptation of electron transport systems. Considerable interest has therefore been generated in recent years in the genetics of this and related photosynthetic bacteria (1, 14, 15).

There are reports of circular satellite species of deoxyribonucleic acid (DNA) in *R. spheroides* (6, 7, 25), and it has been postulated (7) that this extrachromosomal (plasmid) DNA may be concerned in some way with formation of the photosynthetic apparatus. A variety of agents, for example, acridine orange, ethidium bromide, and sodium lauryl sulfate, are known to eliminate plasmids from bacteria (2, 23, 26, 28). Gibson (6) was, however, unable to induce any stable phenotypic change in strains of *R. spheroides* using acridine derivatives as curing agents.

1 Present address: Department of Biochemistry, University of Liverpool, Liverpool L69 3BX, England.
2 Present address: Department of Microbiology, University of Liverpool, Liverpool L69 3BX, England.

We have obtained cells of *R. spheroides* that are incapable of growing photosynthetically (Pho−) after incubating the wild type, strain 2.4.1 (NRS), in medium containing sodium lauryl sulfate. This paper describes properties of the extrachromosomal DNA found in wild-type and Pho− strains of *R. spheroides* (obtained both by sodium lauryl sulfate treatment and N-methyl-N-nitro-N'-nitrosoguanidine mutagenesis).

MATERIALS AND METHODS

Materials. Sodium lauryl sulfate, ammonium acetate, and isopropyl alcohol were purchased from BDH Chemicals Ltd., Poole, Dorset, England. All other materials were as described previously (8).

Bacterial strains and growth conditions. The bacterial strains used and their origins are shown in Table 1. A multiple drug-resistant strain of *R. spheroides* wild type, strain 2.4.1 (resistant to nalidixic acid, rifampin, and streptomycin, all at 100 μg/ml), designated strain 2.4.1 (NRS), was prepared by plating sequentially about 10^6 cells of *R. spheroides* strain 2.4.1 on agar plates containing the requisite antibiotic and incubating aerobically in the dark at 30°C for about 5 days. Colonies growing on such plates were selected, and the stability of the acquired antibiotic resistance was checked.

A Pho− strain of *R. spheroides* was obtained by inoculating about 10^6 cells of strain 2.4.1 (NRS) into medium (21) containing 0.1% (wt/vol) sodium lauryl sulfate and incubating aerobically without shaking in the dark at 30°C. After about 5 days, cells were spread on agar plates and incubated aerobically in...
the dark at 30 C. Photosynthetically incompetent strains were selected by replica-plating. Identification of such isolates as *R. spheroides* strains was confirmed by the presence of the three resistance markers.

Cells were grown aerobically in the dark at 30 C in the liquid medium of Sistrom (21) as described previously (18). For photosynthetic growth, cells were grown in vessels filled with capacity to medium (21) and incubated in the light at 30 C. Strains were also grown on solid medium (yeast extract, 0.3% [wt/vol]; peptone, 0.2% [wt/vol]; agar, 1.5% [wt/vol]) either aerobically in the dark at 30 C or anaerobically in the light in a glass McIntosh and Fildes' anaerobic jar (containing a room-temperature platinum catalyst) evacuated and filled with hydrogen.

**Labeling of cultures.** Cultures to be labeled with [32P]phosphate were grown in "low-phosphate" medium (i.e., medium of Sistrom modified by reducing the concentration of KH2PO4 to 1.0 mM) until the absorbance at 680 nm was about 0.3. The cultures were diluted between 1:10 and 1:20 with prewarmed low-phosphate medium, [32P]phosphate (5 to 10 μCi/ml) was added, and incubation was continued until the absorbance at 680 nm of cultures was between 0.5 and 1.0. Cultures were then lysed as described previously (8).

Detection and isolation of extrachromosomal DNA. Labeled lysates were analyzed by ethidium bromide-CsCl gradient centrifugation and covalently closed circular (CCC)-DNA was prepared as described in reference 8, except that CsCl-saturated isopropyl alcohol was used to remove ethidium bromide. Samples for electron microscopy were dialyzed against 0.15 M ammonium acetate and, for CsCl gradient centrifugation, against 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0 (SSC buffer). Solutions prepared in this way were stored at −20 C until required.

CsCl density gradient centrifugation. Preparations of CCC-DNA were mixed with 3H-labeled *Escherichia coli* chromosomal DNA (buoyant density, 1.710 g/cm3) as a density marker and subjected to CsCl density gradient centrifugation as described before (17), using a starting density of 1.720 g/cm3. Gradients were fractionated, and the refractive index of each fraction was determined using an Abbé high-accuracy refractometer (Bellingham and Stanley Ltd., London). Buoyant densities were calculated from refractive index values of the fractions, and the method gave an accuracy of ±0.002 g/cm3.

Electron microscopy. DNA-protein films were prepared by the droplet method of Lang and Mitani (12), transferred to carbon-coated grids, rotary shadowed with platinum, and examined under a Hitachi HS7S electron microscope.

**RESULTS**

Isolation of Pho− strains using sodium lauryl sulfate. Pho− strains of *R. spheroides* could be isolated after incubating the parent strain, 2.4.1 (NRS), in medium containing sodium lauryl sulfate. The frequency of recovery of Pho− strains was highly variable, ranging from about 10−9/cell to none at all in replicate experiments. One such Pho− strain, designated SLS I, was selected for further study. No reversion of SLS I to the wild-type phenotype could be detected when cultures of SLS I were incubated anaerobically in the light. In contrast, the Pho− strain V-2, obtained by mutagenesis using N-methyl-N-nitro-N′-nitrostroguanidine, reverted to the wild-type phenotype at a frequency of between 10−2 and 10−3 per cell division.

**Biochemical properties of *R. spheroides* strains.** Both strains SLS I and V-2 are incapable of growing anaerobically in the light on agar plates. Neither bacteriochlorophyll nor the carotenoids characteristic of aerobically grown cells of *R. spheroides* strains 2.4.1 (NRS) were detected in acetone-methanol extracts of either strain SLS I or strain V-2. Preliminary biochemical studies have revealed no apparent differences between strain SLS I and strains V-2 and 2.4.1 (NRS) with respect to potentiometric and spectroscopic analyses of the cytochromes and studies on respiratory activities of membrane fractions (see references 19 and 29).

Ethidium bromide-CsCl gradient centrifugation. Attempts were made to label cultures of *R. spheroides* with [3H]thymidine or with [3H]thymine in the presence and absence of adenosine, but there was insufficient uptake of label into DNA. Therefore, cultures were labeled with [32P]phosphate.

CCC-DNA can be separated from other forms of DNA on ethidium bromide-CsCl gradients (27). Crude lysates of cultures of aerobically and photosynthetically grown strain 2.4.1 (NRS) and of aerobically grown strains SLS and V-2 labeled with [32P]phosphate were analyzed by ethidium bromide-CsCl gradient centrifugation. Two bands of radioactive DNA were found; a minor band of radioactive DNA was present on the dense side of the major, presum-

---

**Table 1. Bacterial strains used**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4.1</td>
<td>Wild type</td>
<td>W. R. Sistrom, Department of Biology, University of Oregon</td>
</tr>
<tr>
<td>2.4.1 (NRS)</td>
<td><em>Nal</em>&lt;sup&gt;b&lt;/sup&gt;, <em>Rif</em>&lt;sup&gt;b&lt;/sup&gt;, <em>Str</em>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Triple drug-resistant mutant of strain 2.4.1</td>
</tr>
<tr>
<td>SLS I</td>
<td><em>Nal</em>&lt;sup&gt;b&lt;/sup&gt;, <em>Rif</em>&lt;sup&gt;b&lt;/sup&gt;, <em>Str</em>&lt;sup&gt;b&lt;/sup&gt;, Pho&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Strain 2.4.1 (NRS) after incubation with sodium lauryl sulfate</td>
</tr>
<tr>
<td>V-2</td>
<td><em>Nal</em>&lt;sup&gt;b&lt;/sup&gt;, <em>Rif</em>&lt;sup&gt;b&lt;/sup&gt;, <em>Str</em>&lt;sup&gt;b&lt;/sup&gt;, Pho&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Strain 2.4.1 (NRS) by NTG mutation (19)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Abbreviations: *Nal*, *Rif*, *Str*, Resistance to nalidixic acid, rifampin, and streptomycin, respectively. Pho−, Inability to grow photosynthetically. NTG, N-methyl-N-nitro-N′-nitrosoguanidine.
ably chromosomal, DNA band in all lysates, indicating the presence of satellite DNA in a CCC form in each culture. The satellite DNA amounts to between 5 and 15% of the chromosomal DNA in all strains tested (Fig. 1a and b and data not shown).

Electron microscopy. Electron microscopic analysis revealed that the extrachromosomal DNA in *R. spheroides* was indeed circular. Three separate species of circular DNA were identified in approximately equal amounts, in preparations from each culture (Fig. 2). The molecular weights for these species, estimated from contour length, are given in Table 2. Species of CCC-DNA with weights of about 75 × 10^6 and 66 × 10^6 daltons are present in strain 2.4.1 (NRS) whether grown aerobically or photosynthetically and in strains SLS I and V-2. A third species of CCC-DNA is present in strains 2.4.1 and V-2, weighing 28 × 10^6 daltons. For strain SLS I the third species is 34 × 10^6 daltons, which we consider to be significantly different from the 28 × 10^6-dalton species found in the other strains.

CsCl gradient centrifugation. Preparations of CCC-DNA from both strains 2.4.1 (NRS) and SLS I were subjected to CsCl density gradient centrifugation. In both cases the DNA formed two bands of buoyant densities, about 1.724 and 1.717 g/cm^3 (Fig. 3). Samples from these bands were examined (where indicated) by electron microscopy to determine the molecular weight of the DNA species in each case. In strains 2.4.1 (NRS) and SLS I the bands of buoyant density of about 1.724 g/cm^3 were composed almost exclusively of circular DNA of about 75 × 10^6 daltons. The bands of buoyant density 1.717 g/cm^3 contained a mixture of circular DNA molecules of about 66 × 10^6 and 28 × 10^6 daltons for strain 2.4.1 (NRS) and 66 × 10^6 and 34 × 10^6 daltons for strain SLS I.

**DISCUSSION**

Gibson and Niederman (7) have reported the presence in *R. spheroides* 2.4.1 of two satellite species of DNA with buoyant densities of 1.724 and 1.718 g/cm^3, both weighing between 70 × 10^6 and 75 × 10^6 daltons. By electron microscopic analysis we have shown the existence of three separate CCC-DNA species of 75 × 10^6, 66 × 10^6, and 28 × 10^6 daltons in this organism. The weights of the two larger species are in good agreement with those obtained by Gibson and Niederman (7) using sedimentation analysis in sucrose gradients. The CCC-DNA species of 28 × 10^6 daltons has not previously been detected in *R. spheroides*. The presence of the smallest plasmid may have been obscured in earlier work using sedimentation analysis, since the CCC-DNA form of a plasmid of about 28 × 10^6 daltons would have a similar sedimentation coefficient (about 50S) to open circular forms of circular DNA molecules of about 70 × 10^6 daltons (5).

We have determined the buoyant density of each of the plasmids and found that the species
of about 75 × 10^6 daltons has a buoyant density of 1.724 g/cm³, corresponding to a guanine plus cytosine (G + C) ratio of 65% (20), and the species of 66 × 10^6 daltons has a buoyant density of 1.717 g/cm³ (58% G + C). This is in close agreement with the results of Gibson and Niedermer (7), who did not, however, assign a specific buoyant density to a plasmid of given molecular weight. The species of 28 × 10^6 and 34 × 10^6 daltons are also of a buoyant density of about 1.717 g/cm³ (58% G + C). This value contrasts with the base composition of the chromosome of *R. spheroides*, which is about 71% G + C (7).

All strains of *R. spheroides* tested contained CCC-DNA amounting to between 5 and 15% of the chromosomal DNA (fig. 1a and b), and all three plasmid species were present in approximately equal amounts. Assuming that this represents the lower limit for the amount of plasmid DNA in these strains and that the weight of the chromosome of *R. spheroides* is 1.6 × 10^9 daltons (7), then each plasmid is present at about one to two copies per chromosome equivalent. This is in good agreement with the known behavior of plasmids of similar molecular weight in the *Enterobacteriaceae* (5).

The Pho⁻ strain SLS I of *R. spheroides* also contains three species of circular DNA. The two larger species of DNA are indistinguishable from the species of 75 × 10^6 and 66 × 10^6 daltons found in the wild-type strain. However, the smallest species of CCC-DNA found in SLS I is significantly larger (34 × 10^6 daltons) than the species of 28 × 10^6 daltons found in the wild-type or V-2 strain of *R. spheroides*, despite having the same buoyant density and base composition. It seems unlikely that such a situation has arisen in SLS I by replacement of the pre-existing plasmid of 28 × 10^6 daltons with a "foreign" plasmid of 34 × 10^6 daltons. It is much more likely that in SLS I the species of 28 × 10^6 daltons found in the parent has been modified, probably by gene duplication and/or rearrangement, to give a molecule of increased size. This could arise by the integration of insertion sequences, which are known to induce mutations and cause loss of gene function in other genomes (for a review see reference 24). If this is the case, then SLS I shows no net loss in its complement of extrachromosomal DNA relative to the parent strain. In contrast, other bacterial strains that have been cured by sodium lauryl sulfate show concomitant loss of plasmid-borne characters and extrachromosomal DNA (17, 26). The induction of photosynthetic incompetence in SLS I by incubation in sodium lauryl sulfate could therefore not have been due to the classical curing action of this chemical.

The production of photosynthetic incompetence in SLS I is accompanied by a change in the smallest plasmid species. That this is not a general property of Pho⁻ mutants of *R. spheroides* is shown by the observation that Pho⁻ strain V-2 has a complement of plasmid DNA species indistinguishable from the wild type and no species of 34 × 10^6 daltons is observed. In addition, the lesion affecting photosynthetic competence in strain V-2 is probably a point mutation, since revertants, capable of growing anaerobically in the light, can be isolated readily from cultures of this strain. In contrast, revertants to the wild-type phenotype from cultures of strain SLS I have never been isolated.

The stability of photosynthetic incompetence in SLS I could therefore be due either to multiple mutation and/or gross genetic rearrangement in this strain.

It has been suggested from experiments with cuprizone-treated rats that deletions or additions to mitochondrial DNA might arise if the structure of the mitochondrial membrane were modified, leading to erroneous DNA replication (9). By analogy, treatment of *R. spheroides* strain 2.4.1 with sodium lauryl sulfate, an agent known to disrupt membranes, might select for strains with altered membrane structure in which (i) photosynthetic pigment synthesis and/or membrane differentiation may be affected and/or (ii) DNA replication may be affected. One might expect such strains to be more resistant to sodium lauryl sulfate than the parent, thus conferring a selective advantage. The variability in recovery of Pho⁻ strains using sodium lauryl sulfate suggests that any selective advantage is not great and indeed that the isolation of such strains by this method may be fortuitous. Furthermore, in view of the absence of gene transfer techniques in this organism, it is not possible at present to determine whether the photosynthetic incompetence of SLS I is due directly to alteration in the properties of the smallest plasmid DNA species. Further experiments using curing agents for the successful elimination of the plasmids from *R. spheroides* may resolve their possible role in specifying parts of the photosynthetic machinery.

No genetic functions have been ascribed to the extrachromosomal DNA elements found in *R. spheroides*. Classically, resistance to heavy metal ions and antibiotics is plasmid borne (16); however, *R. spheroides* 2.4.1 appears to be sensitive to a wide range of antibiotics and to mercuric and arsenate ions (V. A. Saunders,
unpublished data). Genes controlling certain catabolic pathways may also be extrachromosomal (3, 4). It is possible, therefore, in *R. spheroides* that such genes may be carried on the observed plasmids.

It has been suggested in view of the similarity in size of the two larger (66 × 10^6 and 75 × 10^6 daltons) plasmids of *R. spheroides* with the F factor of *E. coli* that these plasmids may be involved in conjugation (7). However, using genetically marked derivatives of strains 2.4.1 and SLS I, transfer of genetic markers by cell-to-cell contact between strains of *R. spheroides* could not be detected (V.A. Saunders, unpublished data). Marrs (14) has reported gene transfer in a related organism, *Rhodopseudomonas capsulata*. The mechanism of transfer appeared unusual, and it was postulated that the gene transfer agent may be a type of detachable sex pilus.

The presence of bacteriophages specific for *R. spheroides* is well established (1, 15). Part of the complement of plasmid DNA in *R. spheroides* 2.4.1 could therefore be composed of tem-
perate bacteriophage(s). We have, however, been unable to detect infective phage particles or bacteriocins active against *R. spheroides* in cultures of *R. spheroides* strains 2.4.1, whether uninduced or induced with mitomycin C or sub-lethal doses of ultraviolet irradiation. Guest (10) has similarly reported few, if any, intraspecific inhibitory interactions between strains of *R. spheroides*. However, *R. spheroides* 2.4.1 does apparently produce a bacteriocin effective against *R. capsulata* (10). The genes specifying bacteriocinogy are often extrachromosomal (16), which may therefore account for at least one of the plasmid species found in strain 2.4.1.

The inability to detect inhibitory interactions between different strains of *R. spheroides* could be explained if all such strains carried the same or similar complement of plasmids and therefore specified immunity to homologous bacteriocins or bacteriophages. Similarly, the inability to detect conjugation in this organism may be due merely to the fact that all the strains of *R. spheroides* used contain homologous sex factors. In such a situation the ability of recipient strains to receive donor genetic material would be greatly impaired (30). It may well be, therefore, that a more fruitful approach to achieving a gene transfer system in this organism would
Table 2. Contour lengths and molecular weights of circular DNA molecules isolated from strains of R. spheroides

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth conditions</th>
<th>Contour length* (μM)</th>
<th>Avg mol wt (× 10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4.1</td>
<td>Aerobic</td>
<td>13.5 ± 0.39</td>
<td>(i) 28.0</td>
</tr>
<tr>
<td></td>
<td>(NRS)</td>
<td>32.0 ± 0.58</td>
<td>(ii) 66.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>36.6 ± 1.3</td>
<td>(iii) 75.7</td>
</tr>
<tr>
<td></td>
<td>Photosynthetic</td>
<td>13.8 ± 0.53</td>
<td>(i) 28.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>32.1 ± 0.7</td>
<td>(ii) 66.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>36.3 ± 1.0</td>
<td>(iii) 75.1</td>
</tr>
<tr>
<td>SLS I</td>
<td>Aerobic</td>
<td>16.4 ± 0.68</td>
<td>(i) 34.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>31.9 ± 0.68</td>
<td>(ii) 66.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>35.9 ± 1.1</td>
<td>(iii) 74.3</td>
</tr>
<tr>
<td>V-2</td>
<td>Aerobic</td>
<td>13.8 ± 0.3</td>
<td>(i) 28.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>31.8 ± 0.4</td>
<td>(ii) 65.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>36.2 ± 0.9</td>
<td>(iii) 75.0</td>
</tr>
</tbody>
</table>

* Contour lengths were obtained by measuring 10 to 30 molecules of each species. Results are given as mean ± standard error.

be to isolate as recipient strains derivatives of R. spheroides that lack one or more of the resident plasmid species found in strain 2.4.1.

Acknowledgments

V. A. Saunders wishes to acknowledge support from a Science Research Council Postdoctoral Fellowship. We would like to thank M. H. Richmond and O. T. G. Jones for additional support.

Literature Cited