A Self-Transmissible, Narrow-Host-Range Endogenous Plasmid of 
*Rhodobacter sphaeroides* 2.4.1: Physical Structure, Incompatibility 
Determinants, Origin of Replication, and Transfer Functions

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Received 5 August 1991/Accepted 12 December 1991

*Rhodobacter sphaeroides* 2.4.1 naturally harbors five cryptic endogenous plasmids (C. S. Fornari, M. Watkins, and S. Kaplan, Plasmid 11:39–47, 1984). The smallest plasmid (pRS241e), with a molecular size of 42 kb, was observed to be a self-transmissible plasmid which can transfer only to certain strains of *R. sphaeroides*. Transfer frequencies can be as high as $10^{-7}$ to $10^{-2}$ per donor under optimal mating conditions in liquid media in the absence of oxygen. pRS241e, designated the S factor, was also shown to possess a narrow host range, failing either to replicate or to be maintained in *Escherichia coli*, *Agrobacterium tumefaciens*, and *Rhizobium meliloti*. It was further revealed that one of the remaining four endogenous plasmids, pRS241d, was also transmissible at a frequency similar to that of the S factor. As a coconjugate with pSU283, S was maintained in *E. coli*, providing sufficient DNA from which a physical map of S could be constructed. Progressive subcloning of S-factor DNA, in conjunction with assays of plasmid transfer, led to the localization and identification of orf1 (IncA, IncB, and the putative oriT locus. The DNA sequence of the 427 bp containing orf1 revealed topological similarity to other described oriT sequences, consisting of an A-T-rich DNA region, several direct and inverted repeats, and putative integration host factor (IHF)-binding sites, and was shown to be functional in promoting plasmid transfer.

*Rhodobacter sphaeroides* is an orange nonsulfur phototrophic bacterium able to grow aerobically as a chemo- 
heterotroph, by anaerobic respiration, fermentation, and 
anoxicogenic photosynthesis, the latter either phototetero-

trophically or photoautotrophically. In addition, this bacte-

rion is also able to grow diazotrophically. The remarkable 
metabolic diversity of this organism and its ability to syn-

thesize photosynthetic membrane invaginations (intracyto-

plasmic membranes) when grown anaerobically have made it 
and related bacteria, such as *R. capsulatus*, excellent model 
systems for the study of complex biological and biophysical 
phenomena. For example, photosynthetic membrane bio-
genesis (18), carbon dioxide fixation (22), nitrogen fixation 
(20), biophysical and structural studies of the light 
responses of photosynthesis (5, 19, 35), pigment biosynthesis (1, 31), 
and hydrogen metabolism (49), to mention only a few, are 

fertile areas of research employing these facultative photo-
synthetic bacteria.

All natural strains of *R. sphaeroides* which have been 
examined harbor at least one endogenous plasmid, and some 
strains carry as many as six different plasmid species, with 
sizes ranging from 42 to 140 kb (10). Despite their stable 
maintenance and relatively large sizes, these plasmids (as 
well as the endogenous plasmids of several other species of 
anoxicogenic photosynthetic bacteria) are cryptic. However, 
given the above-described physiological diversity, it is logi-
cal to attempt to relate phenotypes to some of these plas-

mids.

*R. sphaeroides* 2.4.1 harbors five endogenous plasmids 
(originally designated pRS241a, -b, -c, -d, and -e) with a total 
DNA content of approximately 450 kb (10), which comprises 

somewhat more than 10% of the total genomic DNA (38, 39). 
pRS241e is the smallest endogenous plasmid with a size of 42 
kb. This plasmid shows cross-hybridization to one of the 
larger endogenous plasmids of strain 2.4.1, as well as to the 
smaller (pRS241e-like) plasmids present in other *R. sphaeroides* strains (10). In addition a preliminary physical 
map of pRS241e has been constructed (10). However, con-
struction of the complete map has been hampered by diffi-
culty in purifying pRS241e from the larger endogenous 
plasmids (9).

Since genetic manipulation of many bacteria depends to a 

large extent on plasmids and their derivatives, the chance 
observation that pRS241e is self-transmissible provided the 
possibility that a detailed analysis of this plasmid could lead 
to its utilization for genetic analysis of the *R. sphaeroides* 
genome.

In this study, we demonstrated that at least two of the five 
endogenous plasmids of *R. sphaeroides* 2.4.1 (pRS241e and 
d) are transmissible, pRS241e, which we designated the S 
factor, was completely mapped. It is a narrow-host-range 
plasmid which is transferred and replicates only in certain 
strains of *R. sphaeroides* and failed to replicate in *Esche-

richia coli*, as well as the closely related bacteria *Rhizobium 
meliloti* and *Agrobacterium tumefaciens*.

(Part of this work was presented at the 90th Annual 
Meeting of the American Society for Microbiology, Ana-
heim, Calif., 1990.)

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and 
plasmids used in this study and their relevant characteristics 
are described in Table 1.

E. coli and *R. sphaeroides* strains were routinely grown in 
Luria-Bertani (LB) medium at 37°C and Sistrom's minimal

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<td>WS8</td>
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<td>RS2</td>
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<td>MS2III-48</td>
<td>Green (Ct’), pRS241c::Tn5</td>
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<td>MS2III-17</td>
<td>Green (Ct’), pRS241c::Tn5</td>
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<td>MS2-14</td>
<td>Green (Ct’), pRS241b::Tn5</td>
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<td>MS2-F</td>
<td>Orange, ‘he’ , pRS241c::Tn5</td>
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<td>PitB</td>
<td>pRS241c::SpSm’</td>
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<tr>
<td>ΔS</td>
<td>2.4.1-ΔTnd of pRS241e</td>
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<tr>
<td>ΔSB</td>
<td>PitB™ cured of pRS241e</td>
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<td>ΔS2, ΔS3, ΔS19</td>
<td>ΔS, Tn-Δ125 in pRS241e, each in different position</td>
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<tr>
<td>ΔS6, ΔS9, ΔS12, ΔS17</td>
<td>ΔS, Tn-Δ125 in pRS241e, each in different position</td>
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<td>F’ lacZAM15 recA1 hsdR17</td>
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<td>HB101</td>
<td>Res™ Mod’ recA3 Sm’</td>
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<td>SI7-1</td>
<td>Pro™ Res’ Mod’ recA integrated plasmid RP4-Tc::Mo-Km::Tn7</td>
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<td>pRK415</td>
<td>IncP Tc’</td>
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<td>ColE1 r placon, Tra’ of RK2, Km’</td>
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<td>pSUP203</td>
<td>pBR325 derivative; mob” Tc’ Cm’ Ap’</td>
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<tr>
<td>pUC4K</td>
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<td>pHP4501</td>
<td>Source of SpSm’ cartridge</td>
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<td>pHP4501 Km</td>
<td>Same as pHP4501; Km’ cassette instead of SpSm’</td>
<td>33</td>
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<td>pBS</td>
<td>pUC19 derivative with T3 and T7 promoters</td>
<td>Stratagene</td>
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<td>pUB800</td>
<td>pSUP202 B:6::TnpK Howell (source of Tn5 for pAS303 construction)</td>
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<td>pSOC240</td>
<td>–3 kb LNA fragment (presumably oriV’ in pBR322 derivative; Km’</td>
<td>H.-C. Yen</td>
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<td>pAS103</td>
<td>3.2 kb ori’ from pSOC240 cloned into pSUP203; Tc’ Ap’</td>
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<td>pAS301</td>
<td>2.9 kb βgfl fragment (Tn5) of pU100 cloned into pUC4K·BamHI</td>
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<td>pAS302</td>
<td>2.9 kb EcoRI fragment of pAS301 cloned into pSUP203·EcoRI</td>
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<td>pAS307</td>
<td>12.4 kb βgflI fragment of pAS303 (containing oriP) cloned into pSUP203·EcoRI; βgflI ends were converted into EcoRI ends by intermediate cloning into pUC4K·BamHI</td>
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<td>pAS321</td>
<td>–14 kb βgflI XbaI fragment (including the internal βgflI site) of pAS303 cloned into pRK415 with Km’ (BamHI fragment) insert:d in the outside BgflI site</td>
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<td>pAS323</td>
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<td>pAS328</td>
<td>–2.0 kb PshI fragment of SpSm’ cartridge from pU1001 inserted into pAS303·PshI</td>
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<td>pAS332</td>
<td>–3.4 kb Sall fragment of pAS328 cloned into pUC4K·Sall</td>
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<td>pAS341A</td>
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<td>pAS336</td>
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<td>pAS346</td>
<td>3.2 kb Smal-EcoRI fragment of pAS341A (including SpSm’) cloned into pUC19·Smal-EcoRI</td>
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<td>pAS354</td>
<td>1.3 kb TdiI fragment of pAS346 cloned into pUC4K·Sall</td>
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<td>pAS370</td>
<td>0.7 kb PstI-Stai fragment of pAS354 cloned into pBS/PstI·Stai</td>
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<td>pAS371</td>
<td>0.3 kb BamHI·HindIII deletion of pAS370 followed by filling in and religation</td>
<td>This work</td>
</tr>
<tr>
<td>pAS372</td>
<td>0.4 kb EagI·EcoRI deletion of pAS370 followed by filling in and religation</td>
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medium lacking glutamate and asparagine; pH 7.2 (Sis), at 32°C (24), respectively, unless otherwise stated. LB medium was made essentially as previously described (34), except that this medium contained only 5 g of NaCl per liter and the pH was adjusted to 7.2 by adding KOH.

Molecular techniques. Standard methods were used for plasmid isolation, restriction endonuclease analysis, ligati- tion, and other accessory techniques. For a molecular cloning (34). Large DNA fragments (more than 30 kb) were purified from low-melting-point agarose by using a β-agarase method (4, 34). Medium-size DNA fragments (10 to 30 kb) were purified from agarose gels using Spin-Gel columns containing 0.45-μm-pore-size cellulose acetate filter (Costar) or electroelution (34), and the smallest fragment (less than 10 kb) were purified by using a Gene Clean Kit (Bio 101 Inc., La Jolla, Calif.). Southern hybridization analysis was performed as described prevously (8), by using stringent washing conditions at 55°C twice for 15 min each time and detected by using a chemiluminescent method (Photogene detection system; BRL).

R. sphaeroides endogenous-plasmid analysis. Small-scale preparation for analysis of R. sphaeroides endogenous plasmids was done essentially as for the alkaline lysis method (2) with slight modifications as follows. R. sphaeroides was grown in Sis containing 10% (v/vol.) LB medium supplemented with the appropriate antibiotic(s) with shaking at 32°C for 24 h or to approximately 180 to 220 Klett units (1 Klett unit = 107 cells per ml) as measured with a Klett-Summerson colorimeter with a no. 66 filter (24). The cell pellet was suspended in glucose-EDTA-EDTA containing 8 mg of lysozyme per ml, lysed was accomplished by adding 210 μl of a sodium dodecyl sulfate-NaOAc solution at room temperature for 10 min, and the extract was placed on ice for 10 min before addition of a potassium acetate-acetic acid solution. The DNA was picnic extracted and ethanol precipitated as indicated in the standard protocol (34), except that we used concentrated phenol equilibrated in 1% NaCl without chloroform and isoamyl alcohol. The total endogenous plasmid DNA was dissolved in sterile distilled water without RNase and digested with either Ascl, SpeI, or Ascl-SpeI restriction endonucleases(s). Usually, plasmid DNA obtained from a 1.5-l culture volume was sufficient for one digestion or two.

Digestions were performed in 1x KGB (20) for 2 to 4 h. For loading onto a transverse alternating-field electrophoresis (TAFE) gel (38), the digested plasmid DNA was mixed with an equal volume of 1% low-melting-point agarose in 1× TE buffer, pH 8.0 (34) and then carefully pipetted into the well of a TAFE gel apparatus, sealed with 1% low-melting-point agarose as described previously, and then subjected to electrophoresis.

Running gel conditions were usually performed as follows: stage 1, 1-s pulse, 10 min, 175 mA; stage 2, 6-s pulse, 8 h; stage 3, 4-s pulse, 6 h; stage 4, 2-s pulse, 3 h. All other stages, except stage 1, were 155 mA in 1× TAFE buffer at 12 ± 1°C, and the gel was 1% Seakem GTO (FMC Bioproducts).

Bacterial mating. Conjugation from E. coli to R. sphaeroides was done either di- or trip parentally. For diparental mating, we used S17-1 as the E. coli donor (36). In most cases, we used triparental mating with HE101(pRK2013) as a helper (7). The exconjugants were selected on Sis containing the appropriate antibiotic(s), which was usually tetracycline supplemented with 10 μg of K2TeO4. Potassium tellurite was used to kill E. coli while allowing normal growth of R. sphaeroides (29) exconjugants harboring pRK415 and its derivatives (S-factor DNA fragments cloned into the multiple cloning sites of pRK415).

R. sphaeroides donors and recipients were grown to the log phase (80 to 100 Klett units) in Sis supplemented with 10% (v/vol.) LB medium (pH 7.2) containing the appropriate antibiotic(s). Each 1.5 ml of the donor and recipient was pelleted in a microcentrifuge at maximum speed for 1 min, and then the cells were washed with 0.8 ml of Sis medium, recentrifuged, and again suspended in 30 to 40 μl of Sis before being spotted onto a filter (1-cm diameter; pore size, 0.45 μm; Millipore) on an LB plate (pH 7.2). For liquid matings, the cells were suspended in glucose-dimethyl sulfoxide (DMSO) broth (8) or in Sis plus 50% (v/vol.) LB medium supplemented with DMSO (56 μl of DMSO per 100 ml of medium) and kept in the dark, or mated photosynthetically in LB (pH 7.2) under medium light (10 W/m2) (8). Photosynthetic matings were performed in 8-ml screw-cap glass vials (Wheaton), while DMSO mating in the dark was conducted in either a glass vial or an Eppendorf tube. The bacteria were incubated at 32°C for 12 to 18 h, unless otherwise stated.

At the end of the mating period, the filter containing the bacterial mixture was transferred to a 1.5-ml Eppendorf tube containing 0.7 ml of Sis. The mixture was suspended by using a Vortex mixer until all of the filter surface was clear. That suspension was used to calculate the transfer frequency by spreading it onto the appropriate selective media. In the case of liquid matings, the bacteria were spread and counted directly from the mating vials.

Transfer frequency was expressed as the number of exconjugants divided by the number of donors which were indirectly determined by using the appropriate antibiotic markers. The spontaneous resistance of R. sphaeroides to kanamycin (Km), spectinomycin (Sp)-streptomycin (Sm), and tetracycline (Tc) was undetectable (less than 10−3) under these experimental conditions.

Antibiotics were used at the following concentrations: Km, 25 μg/ml; Sp, 50 μg/ml; Sm, 50 μg/ml; rifampin, 50 μg/ml; Tc, 1 μg/ml (for R. sphaeroides) and 15 μg/ml (for E. coli); ampicillin, 150 μg/ml (only for E. coli).

Curing of the S Factor. Both IncA and IncB regions of the S factor were used to eliminate the native S factor. IncB, which is present on pAS336 (Table 1), was routinely used to cure S, since this region exhibited much stronger incompatibility with S than did that of IncA (oriS). The procedure for curing was as follows. pAS336 was introduced into R. sphaeroides 2.4.1 or its derivatives by triparental mating as described in Bacterial Mating. The exconjugants were selected for Tc and analyzed for loss of S by using plasmid fingerprinting (see Results for details). Usually, the Tc exconjugants had lost the S factor. The cured strain was then grown in Tc-free medium to screen for cells which had lost pAS336, since pRK415 is not stably maintained in R. sphaeroides without marker selection.

DNA sequencing. A 700-bp oriS-containing DNA fragment from pAS34 was cloned into plasmid pBS (Stratagene, La Jolla, Calif.) and yielded pAS370 (Table 1). Deletion of pAS370 yielded two plasmid derivatives, i.e., pAS371 and pAS372 (Table 1), which were employed as double-stranded templates for DNA sequencing as follows.

DNA fragments in pAS371 and pAS372 were initially sequenced by using T3 and T7 as dye primers for automated DNA sequencing (Applied Biosystem 370A/373A). Manual double-stranded (plasmid) DNA sequencing was employed to determine the ambiguous bases and also to verify the DNA sequence obtained for pAS371 by automated DNA
sequencing. For manual DNA sequencing, T3 and T7 universal primers (Stratagene) were used, as well as two oligonucleotide primers, 5'-AGGCGCATCGAGCGCT and 5'-AGGCGATCGAGCGCT, located in the middle of the DNA fragment contained in pAS371, which were deduced after alignment of the DNA sequences originally obtained by automated sequencing. These oligonucleotides were synthesized by Genosys, Woodlands, Tex. Two sequencing reactions, i.e., dTTP and 7-deaza-dGTP reaction versions from the Sequenase version 1 sequencing kit (USB, Cleveland, Ohio) and the 7-deaza-dGTP reaction version from the Bst-DNA sequencing kit (Bio-Rad Laboratories, Richmond, Calif.), were employed for each primer.

Nucleotide sequence accession number. The nucleotide sequence of the S. pneumoniae 705-bp PstI-Stul restriction endonuclease fragment described in this report has been deposited in GenBank under accession no. M77658.

RESULTS

R. sphaeroides 2.4.1 endogenous plasmid fingerprints. Since R. sphaeroides 2.4.1 harbors five endogenous plasmids with a total size of approximately 450 kb, plasmid identification and analysis of plasmid rearrangements by conventional approaches were complicated and tedious [10]. Therefore, it was necessary to develop a simple routine method which could be consistently performed and which was reliably able to detect any changes in plasmid profiles.

Thus, we used pulsed-field gel (PFGE) electrophoresis to separate the plasmids in supercoiled form, as shown in Fig. 1. Plasmid cointegrations and some insertions (such as Tn5) can be readily detected by this method. However, for smaller insertions and for separation of similar-size plasmids, this method alone was not sufficient. As shown in Fig. 1, the four largest plasmids of strain 2.4.1 were represented by only two ethidium bromide-staining bands, because the sizes of two of these plasmids were very similar. To improve the sensitivity of detection while attempting to simplify the analysis, we modified a plasmid miniprep procedure, described in Materials and Methods, digested the plasmids with rarely cutting restriction enzymes, and separated the resulting fragments by PFGE electrophoresis.

This analysis enabled us to develop "fingerprints" of the R. sphaeroides 2.4.1 endogenous plasmids, which are schematically depicted in Fig. 2. When we used AaeI, two of the endogenous plasmids (pRS241b and -c) were not digested, while each plasmid has at least one SpeI site. This fingerprinting technique has provided a suitable reference for probing of plasmid rearrangements and has also been employed to detect plasmid loss, addition, and insertion. Moreover, plasmid fingerprinting and analyses have proven to be a rapid and reliable method for R. sphaeroides strain identification and verification (41).

Transmissibility of pRS241e (S factor). R. sphaeroides MS2111-48 is a green mutant derived from wild-type strain 2.4.1 because of a point mutation in the cytochrome-biosynthetic gene cluster (Crt⁺) (41) which also possesses a Tn5 insertion in pRS241e (38). Strain PrkB has an SpSm' cartridge inserted in the prkB gene and is Crt⁺ (Table 1). When MS2111-48 was mated with PrkB on solid medium and exconjugants were selected for on SpSm- and Km'-containing medium, we observed exconjugants at a frequency of 10⁻⁶ to 10⁻⁷ per donor and all were Crt⁺. This was the first evidence for transfer of pRS241e. In subsequent experiments, it was found that the transfer frequency of pRS241e (designated the S factor) was as high as 10⁻³ to 10⁻² per donor. Furthermore, genome analysis indicated that S:Tn5 was found in the recipients (strains 2.4.1 and L) and concomitantly appeared to exclude the native S factor in strain 2.4.1 or the S-like element in strain L (Fig. 1).

Several other strains of R. sphaeroides, namely, RS2, L, WS8, 2.4.7, and 630; R. capsulatus 310; and Paracoccus denitrificans were made R' and then mated to MS2111-48 by solid-filter mating. The spontaneous resistance of R. sphaeroides to 50 μg of rifampin per ml was 10⁻⁶ to 10⁻⁷. However, since MS2111-48 is Crt⁺, spontaneous R' derivatives of MS2111-48 were readily distinguished. The results showed that S could transfer at the same rate to certain strains of R. sphaeroides, namely, L and RS2, but transfer to strains 630, and 2.4.7, R. capsulatus, and P. denitrificans was undetectable (less than 10⁻¹) in contrast to strain 2.4.1. However, S did transfer at a low frequency (10⁻⁸ to 10⁻⁹) to R. sphaeroides WS8 under liquid photosynthetic mating conditions.

Anaerobic liquid mating is more favorable for S-factor transfer. To optimize the conditions for plasmid transfer, S-factor transfer from MS2111-48 to ΔSB' was examined under different mating conditions. The results of these studies are summarized in Table 2. These and numerous other studies (41) clearly demonstrated that anaerobic photosynthetic conditions of growth for both the donor and the recipient, as well as mating, yielded the highest frequency of S-factor transfer.
Asel | Spel | Asel-Spel
---|---|---
110 | 110 | 110 (2x)
97 | 105 | 73
63* | 73 | 63*
63** | 45* | 42**
52* | 31* (2x) | 17

S-factor

FIG. 2. Schematic drawing of *R. sphaericus* 2.4.1 endogenous plasmid fingerprints. For an example of the actual data obtained from this analysis, see Fig. 6. Two circles indicate the Asel digestion pattern represent the two plasmids not digested by Asel. These plasmids migrated as faint bands in PFG with a apparent molecular size larger than their linear form, which is also true for any other pulse conditions normally employed in this analysis. Thickened bars represent a doublet. The numbers at the right side of each DNA fragment indicates the molecular size of the corresponding DNA fragment. Single and double asterisks identity portions of pRS241 and S, respectively. The S DNA fragment was readily detected as a bright band, since this plasmid appears to present in three or four copies per chromosome. The plasmid preparation and electrophoresis conditions used are described in Materials and Methods.

Physical mapping of the S factor. The dual observations that the frequency of S-factor transfer was high and the apparent displacement of the endogenous S factor by the exogenous factor suggested that this system might provide the basis for the construction of a genetic system in *R. sphaericus*. Thus, we required more information as to the physical and genetic structure of the S factor. Because we were unable to introduce the S factor directly into *E. coli*, we employed an entrapment strategy as follows. The internal *BglII* fragment of Tn5 was excised and cloned into pSUP203, a strain deficient derivative which can be mobilized into but by itself cannot replicate in *R. sphaericus* and this recombinant plasmid (pAS302) was introduced into *E. coli* strains as a conjugal mating as explained in Materials and Methods.

TABLE 2. Frequency of S transfer under different mating conditions

<table>
<thead>
<tr>
<th>Mating condition</th>
<th>Frequency (exconjugants/ donor)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solid-filter mating, aerobic</td>
<td>$10^{-5}$-10^{-7}</td>
</tr>
<tr>
<td>Solid-filter mating, anaerobic</td>
<td>$10^{-6}$-10^{-8}</td>
</tr>
<tr>
<td>Solid-filter mating, anaerobic</td>
<td>$10^{-6}$-10^{-8}</td>
</tr>
</tbody>
</table>

*The values shown are averages of three repeat experiments.*

Conjugants were simultaneously selected for Km' (from Tn5 in the S factor) and Tc' (from pSUP203). This selection enriched for strains which could be used for any other pulse conditions normally employed in this analysis. The plasmid preparation and electrophoresis conditions used are described in Materials and Methods.

**FIG. 3.** Physical map and some genetic loci of S. The numbers in parenthesis indicate relative distances (0.1 kb units). Not all Sall and PstI sites in the circular map are shown. The enlarged region shows the locations of orf T and IncB DNA-containing fragments.

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**Host range of S.** As described above, we were unable to demonstrate the transfer or maintenance of S in *E. coli*. However, because of the physiological similarity of *R. sphaericus* to *Agrobacterium* and *Rhizobium* spp., we attempted to transfer S to these bacteria. pAS303 was mobilized into A. tumefaciens CS8 and R. meliloti 1021 via diparental mating using *E. coli* S17 (pAS303), and the resulting Km’ Tc’ exconjugants were analyzed for plasmid content. All of the representative exconjugants failed to show the presence of pAS303, although the results indicated that transfer of the chimeric plasmid did occur. Hybridization analysis revealed that pSUP203 was integrated into
either the chromosomese or megaplasmids of these bacteria. The presence of pSU203 in the Rhizobium and Agrobacterium genomes was most likely generated by transposition of all of pSU203 (see map of pSU303, Fig. 3) to the chromosomal or megaplasmid DNA or, possibly, by recombination between pSU303 and the genomic DNA of A. tumefaciens or R. meliloti.

Mobilization of pSU303 from S17-1 into R. sphaeroides 2.4.7 and WSS, which are not competent of respond poorly as recipients in conjugal R. sphaeroides-p. sphaeroides strain (presumably strain 2.4.1) which appeared to contain an origin of replication. We determined further that this particular DNA fragment (designated oriV) was derived from strain 2.4.1, specifically from S1 DNA. Introduction of pSU303 containing the oriV fragment from S1 DNA cloned into pSU203 (Table 1) into strain 2.4.1 generated conjugants which gained pSU303 (because of selection for Tc) but excluded the endogenous S factor. Moreover, plasmid fingerprinting analysis confirmed that pSU303 was present as an independent replicon. These results suggested that oriV was able to function in strain 2.4.1.

We also observed that pSU303 was not stably maintained in R. sphaeroides in the absence of antibiotic selection (Fig. 4). However, if maintenance of pSU303 was forced by growing the cells in Tc-containing medium for 18 to 20 generations, approximately 1 to 5% of the population lost the native S factor, indicating that the DNA fragment containing oriV also contains at least one incompatibility determinant unique to S. This incompatibility phenotype was designated IncA. oriV (IncA) was localized on the S physical map as shown in Fig. 3. Since all of the 2.4.1 endogenous plasmids, including S, are very stably maintained, we exploited this observation to cure S on the basis of incompatibility with the resulting petite plasmid (pSU303) by introduction of pSU303 and subsequent elimination of pSU303 by growing the cells in the absence of Tc. In subsequent work, we found a much more effective way to cure S, by using a second incompatibility determinant (IncB) (Fig. 3).

R. sphaeroides 2.4.1, which lacks the S factor (strain AS), grow normally under photosynthetic, anaerobic-with-DMSO, or aerobic growth conditions. The strain is genetically prototrophic and was also resistant to tellurite to the same level as is the wild-type strain. We have observed no significant phenotype associated with S other than conjugal transfer activities.

Transfer capability of the other four R. sphaeroides 2.4.1 endogenous plasmids. We have one strain each containing an insertion of Tn5 in plasmids pRS241c, pRS241b (MS214), and pRS241c (MS2-F) (S4), four strains with a Tn5-B125 (14) insertion in pRS241c (AS6, AS9, AS12, and AS17) (Table 1), and three strains with Tn5-B125 present in pRS241d (AS2, AS5, and AS9) (Table 1).

When each of these strains was mated with strain ASB and selected conjugants which were SpSm KmR, we observed exconjugants only by using AS5, AS5, and AS9 as donors, with a frequency of 10−3 to 10−4 per donor (solid mating). These results suggested that pRS241d (molecular size, ca. 95 kb) was a second transmissible plasmid present in R. sphaeroides 2.4.1, and since it was in a stable background this observation also indicated that pRS241d (or simply plasmid D) was capable of transfer independently of S. More recent work has shown that plasmid D can be transferred at a frequency of 10−6 to 10−8 per donor. Since we isolated five independent insertions of either Tn5 or Tn5-B125 into pRS241c and observed no detectable exconjugants, it is probable that pRS241d is not a transmissible plasmid. The other two plasmids (pRS241a and -b) contain only one representative Tn5 insertion. Therefore, we cannot make a firm conclusion regarding the transfer ability of these two plasmids, since it is possible that the Tn5 insertion somehow interfered with their transfer ability were such an ability present.

When either AS2(D::KmR) or AS3(D::KmR) (Table 1) was used as a donor in a mating with an R′ derivative of pRS241b (MS214), and pRS241c (MS2-F) (S4), four strains with a Tn5-B125 (14) insertion in pRS241c (AS6, AS9, AS12, and AS17) (Table 1), and three strains with Tn5-B125 present in pRS241d (AS2, AS5, and AS9) (Table 1).

Cloning and localization of the S-factor oriT and IncB regions. S-factor DNA from pSU303 was progressively subcloned into pRK415 as described in the legend to Fig. 5. Although pRK415 can be stably maintained in R. sphaeroides in the presence of antibiotic selection, by itself it cannot transfer between strains of R. sphaeroides. Each of these recombinant plasmids was introduced into strain AS and in some cases into strain pSU303, as described in Materials and Methods. Each AS and/or pSU303 strain containing

**FIG. 4.** Plasmid segregation analysis. pSU303 (Tc') was introduced into strain MS211-48. Selection with Tc and Km yielded cells harboring both pSU303 and S: Tn5. Plasmid segregation was evaluated after growing Km' Tc' cells in antibiotic-free medium for several generations. Percentages of segregants were calculated from the total number of colonies appearing on the medium without antibiotics. The number of generations was calculated by using the generation time. The line connecting the squares escribes plasmid segregation analysis when selection was made on plate containing both Km and Tc.
pRK415 carrying portions of S DNA was diparentally mated with strain AB155. The number of donors, recipients, and exconjugants was scored for each of the different matings, and the transfer frequency was calculated from at least two repeated experiments for each recombinant plasmid. These observations are tabulated in Fig. 5.

Both pAS317 and pAS318 were transferable, but the transfer frequency of pAS318 was 100-fold lower than that of pAS317. Assuming that S has only a single oriT region, as is the case for other conjugative or mobilizable plasmids (47), we had to determine which of these plasmids carried the oriT region. Plasmid fingerprinting of the representative exconjugants revealed that transfer of pAS318 was the result of conjoint formation between this plasmid and plasmid D. In contrast, pAS317 was capable of transfer independently of plasmid D. pAS325 contains two BamHI sites, which can be divided, following digestion, into three DNA fragments. Cloning of these fragments into pRK415 yielded recombinant plasmids pAS336, pAS325-10, and pAS325-2. When each of these strains, ΔS(pAS336), ΔS(pAS325-10), and ΔS(pAS325-2), was mated with ASB', none of these matings yielded exconjugants. The simplest interpretation of these results is that one or more of the BamHI sites might be part of a cis-acting region important for transfer or they might be important for expression of a trans-acting gene product required for transfer.

To determine whether one of these three plasmids required a complete trans-acting gene product(s) for transfer proficiency, we introduced each of these plasmids into MS2III-48. Since MS2III-48 carried S::Tn5, then MS2III-48(pAS330), MS2III-48(pAS325-10), and MS2III-48(pAS325-2) would be expected to be Km' Tc'. However, when we grew each of these strains in Tc-Km-containing medium, one strain, i.e., MS2III-48(pAS330), failed to grow. Plasmid fingerprinting showed that MS2III-48(pAS336) contained pAS335 but had lost S::Tn5. This was the first evidence of a second incompatibility determinant associated with S. Subsequent experiments indicated that introduction of pAS336 and concomitant selection for Tc' excluded the native S. This strong incompatibility determinant was designated IncB (to distinguish it from the IncA-oriV region) and routinely employed to cure the S factor as described in Materials and Methods. The evidence of plasmid introduction and S-factor curing was verified by plasmid fingerprinting as shown in Fig. 6. Both MS2III-48(pAS325-2) and MS2III-48(pAS325-10) contain the endogenous S::Tn5 but failed to yield any exconjugants, pAS342 and its derivatives were further assayed for plas-

FIG. 5. Plasmids used in the molecular and genetic analysis of the oriT region. A linear map of pAS303 was constructed from Fig. 3. All of these S-derived DNA fragments were obtained from pAS303 and cloned into pRK415. An open triangle under the bar indicates a Km' (from pUC4K) insertion in that particular DNA fragment. An arrow under the triangle indicates the relative orientation of Km'. III Km indicates insertion of Km' from pHPS11Km, and the arrowheads show the direction of the tac2-Tc promoters of pRK415 relative to the S DNA in those recombinant plasmids. A transfer frequency of 10^2 to 10^3 per donor (solid-filter mating) or 10^4 to 10^6 per donor (liquid aneural mating) was evaluated as positive, and a transfer frequency of <10^4 per donor (in either solid or liquid mating) or transfer mediated by conjugation to D (asterisk) was evaluated as negative. Representative S DNA fragment sizes from each group of expanded regions are indicated by the numbers of kilobases under the solid bars of those DNA fragments. The numbers in parentheses following some restriction enzyme sites indicate relative distances (in 0.1-kb units for pAS303 and in base pairs for the pAS342 enlargement).
strated, the evidence for self-transmissibility is less clear because of the presence of plasmid D and the ability of DNA sequences cloned from S to recombine into plasmid D. The presence of the other four plasmids serves as a further complication to the demonstration of S transfer in a ΔS background. Any of these four plasmids might be the facilitator or promoter of S mobilization. On the other hand, we were not able to cure any of these four endogenous plasmids and, like S, they are very stable (41).

To analyze the self-transmissibility of S, we introduced S (pAS303) and some of its derivatives into R. sphaeroides WS8 through diparental mating from E. coli S17-1. The resulting WS8 exconjugants, i.e., WS8(pAS303), WS8 (pAS308), WS8(pAS331), and WS8(pAS17), were used as donors to be mated with ΔSΔB-.
	pAS303 was transferred from WS8(pAS303) to ΔSΔB-, as shown by the presence of Sp'/Sm'/Te' exconjugants, and the presence of pAS303 was further verified in both the donor and the exconjugants by plasmid fingerprinting (41). pAS308, pAS331, and pAS17 are pAS303 derivatives cloned into pJRK415 and were mobilized from ΔS to ΔSΔB-. However, in the WS8 background these plasmids were not mobilizable to ΔSΔB-. This result suggested that these pAS303 derivatives lacked an essential function(s), which is present on pAS303, needed for transfer proficiency. These data also demonstrated that strain WS8 was not able to provide transfer functions to S, which indirectly proves the self-transmissibility of S.

Since pAS303 also failed to be transferred from WS8(pAS303) to ΔSΔB-, the possibility that psUP203 contributes to the transfer of pAS303 was ruled out. Moreover, psUP203 itself is not able to replicate in R. sphaeroides.

DNA sequence of orfT. A 705-bp PstI-Stul R. sphaeroides DNA fragment containing orfT was sequenced as described in Materials and Methods. The DNA sequence and a preliminary analysis of this sequence are described in Fig. 7. The location of the PstI-Stul fragment on the S-factor physical map is described in Fig. 3.

Our previous work determined that a 400-bp EcoGI-Stul fragment contains a functional orfT sequence, and from chromosome transfer experiments (40) we inferred that the orientation of orfT transfer is such that the PstI or EcoGI sites will be among the last DNA sequences to enter the recipient cells. Several interesting features have been identified in the region between EcoGI and Stul. For example, the region between bp 551 and 644 is 56% A-T, which is significantly A-T rich for the R. sphaeroides genome, with an average of 30 to 32% A-T (25). Three inverted repeat sequences and three putative integration host factor (IHF)-binding sites have been identified, in addition to several direct repeats. Each of these three putative IHF-binding sites has a two-nucleotide mismatch compared with the consensus IHF-binding site sequence (22). Overall, this 400-bp region contains the topological feature, associated with other orfT sequences (11, 15).

DISCUSSION

Replication and incompleteness of narrow-host-range plasmids have been thoroughly examined in plasmids of E. coli or in plasmids which can replicate in E. coli (22). However, replication and incompleteness determination of narrow-host-range plasmids in nonenteric bacteria is largely unstudied, although the replication regions of some narrow-host-range plasmids, such as pBE-2 of Methanothermobacter thermautotrophicus (21),
pMMC7105 of Pseudomonas syringae (32), and pTAR of A. tumefaciens (12), have been localized or cloned. The S factor has been shown to be a narrow-host-range plasmid which fails to replicate in E. coli, R. meliloti, and A. tumefaciens on the basis of the behavior of S:Trc. However, we have not been able to rule out the possibility of another region(s) necessary for replication in other bacterial hosts which might have been inactivated by insertion of Ti plasmid.

Two incompatibility determinants have been cloned and localized on the physical and genetic map of S, one closely linked to the origin of replication, orI-S, and the other linked to the origin of transfer, orT. Most studies of incompatibility by have been conducted with plasmids which can replicate in E. coli. By analogy to E. coli, the five endogenous plasmids of R. sphaeroides 2.4.1 are clearly compatible with each other, implying that they are in different incompatibility groups. Since plasmids belonging to IncP, IncQ, and IncW can be stably maintained in R. sphaeroides, we imagine that these endogenous plasmids represent different incompatibility groupings. By employing the DNA fragment containing the IncB determinant, we can readily cure the native S factor or its derivatives from strains of R. sphaeroides. Loss of S reveals no apparent phenotypic effect.

Two conjugal plasmids were identified in this study. All of the evidence suggests that S is a self-transmissible plasmid, even in the presence of D, while D was transmitted only regardless of the presence or absence of S. Subcloning analysis indicated that a 427-bp DNA fragment of S present in pRK415 was able to promote plasmid transfer between R. sphaeroides 2.4.1 and strains lacking the S factor. It seems unlikely that this 427-bp orf1 DNA fragment contains all of the necessary information for DNA transfer, including conjugal transfer, since for most conjugative plasmids a relatively large segment of DNA is required for conjugal transfer, including plus formation (3, 46, 48). However, if the 427-bp DNA fragment contains only the cis-acting component(s) required for transfer, then it is essential to have a region elsewhere in the genome to provide the Tra functions in trans for conjugal activity. The most likely candidate providing these Tra functions appears to be plasmid D.

Although we did not define the Tra region of the factor, it is possible that those DNA sequences of S having substantial homology to plasmid D could represent all or a portion of the Tra region. We raise this possibility because it is likely that plasmid D can provide these functions in trans to orf1 and we know that conjugate formation between S and plasmid D is readily demonstrable (10, 41).

As far as we know, there is no information about the presence of an R. sphaeroides-specific sex pilus and to our knowledge this is the first report of conjugal transfer of any endogenous plasmid of a member of the family Rhodospirillaceae. Transfer of the S factor is more efficient in liquid than in mating on a solid surface. This might suggest either a short, flexible pilus or, perhaps, no pilus involvement. Comparison of liquid mating under aerobic versus anaerobic conditions indicated that the S factor transfers much more efficiently under anaerobic conditions than under aerobic conditions. Therefore, anaerobiosis appears to be essential for optimal conjugal transfer of S. Whether anaerobiosis might activate the Tra genes directly or stimulate transient derepression of naturally repressed pilin functions (16) which indirectly give rise to high transfer frequencies remains to be determined.

Sequence analysis revealed that orf1 has an organization similar to that of F (11) or R100 (15). This region should contain only the cis-acting elements required for transfer. These DNA sequences include the three putative IHF-binding sites within an A-Rich tract with several inverted repeat sequences located to one side of the A-Rich region. Assuming that at least one of the three putative IHF-binding sites is bound by IHF, then the nick site(s) of orf1 would be expected to be somewhere between bp 640 and 713 (Strai site) with an orientation such that the IHF-binding site(s) is the last to enter recipient cells (15).

The small orf1 region of S (orf1s), which can be efficiently mobilized by a second endogenous plasmid of R. sphaeroides 2.4.1, has given us the advantage of being able to examine the transfer of orf1-containing plasmids or chromosomes in the absence of the entire S factor. By inserting orf1 into the chromosomes of R. sphaeroides, we have been able to detect chromosomal transfer and by using this approach we have been able to generate Hz-like strains of R. sphaeroides which will be extremely useful for genetic analysis, as well as manipulation of the genome, of this photosynthetic bacterium (40).

ACKNOWLEDGMENTS

We thank H.-C. Yen for communicating unpublished data and sending plasmids pSC204 and pJOC241 and his R. sphaeroides strain; Greg Shipley for performing preliminary DNA sequencing of orf1, DNA using an automated DNA-sequencing machine; and J. K. Lee, E. Nidele, and M. Wood for suggestions and technical assistance in manual DNA sequencing. We are grateful to M. F. Hynes, S. K. Farrand, and T. Jacobs for providing particular bacterial strains and plasmids used in this study.

This work was supported by grant GM 31667 to S.K. and by the Indonesian Second University Development Project (World Bank XVII) (A.S.).
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