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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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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^{-2} to 10^{-3} 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 cointegrate with pSUP203, 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 *oriV* (IncA), IncB, and the putative *oriT* locus. The DNA sequence of the 427 bp containing $oriT_S$ 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 α-purple nonsulfur photosynthetic eubacterium able to grow aerobically as a chemoheterotroph, by anaerobic respiration, fermentation, and anoxygenic photosynthesis, the latter either photoheterotrophically or photoautotrophically. In addition, this bacterium is also able to grow diazotrophically. The remarkable metabolic diversity of this organism and its ability to synthesize photosynthetic membrane invaginations (intracytoplasmic 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 biogenesis (18), carbon dioxide fixation (42), nitrogen fixation (20), biophysical and structural studies of the light reactions 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 photosynthetic 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 anoxygenic photosynthetic bacteria) are cryptic. However, given the above-described physiological diversity, it is logical to attempt to relate phenotypes to some of these plasmids.

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, construction of the complete map has been hampered by difficulty 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 *Escherichia 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, Anaheim, 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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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Source or reference
Rhodobacter sphaeroides strains		
2.4.1	Wild type	43
2.4.7	Wild type	43
WS8	Wild type	37
630	Wild type	Laboratory collection
L	Wild type	Laboratory collection
RS2	Wild type	27
2.4.1-Yen	Wild type	H.-C. Yen
Strain 2.4.1 derivatives		
MS2III-48	Green (Crt$^-$), pRS241e::Tn*5*	38
MS2III-17	Green (Crt$^-$), pRS241a::Tn*5*	38
MS2I-14	Green (Crt$^-$), pRS241b::Tn*5*	38
MS2-F	Orange, Phe$^-$, pRS241c::Tn*5*	38
PrkB$^-$	*prkB*::Sp^r/Sm^r	13
ΔS	2.4.1 cured of pRS241e	This work
ΔSB$^-$	PrkB$^-$ cured of pRS241e	This work
ΔS2, ΔS5, ΔS19	ΔS, Tn*5*-B12S in pRS241d, each in different position	This work
ΔS6, ΔS9, ΔS12, ΔS17	ΔS, Tn*5*-B12S in pRS241c, each in different position	This work
Rhodobacter capsulatus B10	Wild type	45
Paracoccus denitrificans	Wild type	Laboratory collection
Rhizobium meliloti 1021	Wild type, Sm^r	T. Jacobs
Agrobacterium tumefaciens C58	Wild type	S. K. Farrand
Escherichia coli strains		
DH5α	F$^-$ *lacZ*ΔM15 *recA1 hsdR17*	34
HB101	Res$^-$ Mod$^-$ *recA13* Sm^r	34
S17-1	Pro$^-$ Res$^-$ Mod$^+$ *recA* integrated plasmid RP4-Tc::Mu-Km::Tn*7*	36
Plasmids		
pMH1701	Suicide plasmid carrying Tn*5*-B125	14
pRK415	IncP Tc^r	17
pRK2013	ColE1 replicon, Tra$^+$ of RK2, Km^r	7
pSUP203	pBR325 derivative; *mob*$^+$ Tc^r Cm^r Ap^r	36
pUC4K	Source of Km^r cartridge	44
pHP45Ω	Source of Sp/Sm^r cartridge	33
pHP45ΩKm	Same as pHP45Ω; Km^r cassette instead of Sp/Sm^r	33
pBS	pUC19 derivative with T3 and T7 promoters	Stratagene
pUI800	pSUP203 *bla*::Tn*phoA* (source of Tn*5* for pAS303 construction)	28
pSOC240	~3-kb DNA fragment (presumably *oriV*) in pBR322 derivative; Km^r	H.-C. Yen
pAS103	3.2-kb *oriV* from pSOC240 cloned into pSUP203; Tc^r Ap^r	This work
pAS301	2.9-kb *Bgl*II fragment (Tn*5*) of pUI800 cloned into pUC4K-*Bam*HI	This work
pAS302	2.9-kb *Eco*RI fragment of pAS301 cloned into pSUP203-*Eco*RI	This work
pAS303	Cointegrate of pAS302 into S::Tn*5*; Tc^r Km^r Ap^r	This work
pAS307	12-kb *Bgl*II fragment of pAS303 (containing *oriV*) cloned into pSUP203-*Eco*RI; *Bgl*II ends were converted into *Eco*RI ends by intermediate cloning into pUC4K-*Bam*HI	This work
pAS321	~14-kb *Bgl*II-*Xba*I fragment (including the internal *Bgl*II site) of pAS303 cloned into pRK415 with Km^r (*Bam*HI fragment) inserted in the outside *Bgl*II site	This work
pAS323	~5.5-kb *Sal*I fragment of pAS321 (1 *Sal*I site was derived from Km^r cartridge in pAS321) cloned into pUC4K-*Sal*I	This work
pAS328	~2.0-kb *Pst*I fragment of Sp/Sm^r cartridge from pUI1001 inserted into pAS323-*Pst*I	This work
pAS332	~3.9-kb *Sal*I fragment of pAS328 cloned into pUC4K-*Sal*I	This work
pAS341A	2.1-kb *Pst*I fragment of Sp/Sm^r cartridge from pUI1001 inserted into pAS332-*Pst*I (A orientation)	This work
pAS336	1-kb *Bam*HI fragment containing IncB from S cloned into pRK415	This work
pAS346	3.2-kb *Sma*I-*Eco*RI fragment of pAS341A (including Sp/Sm^r) cloned into pUC19-*Sma*I-*Eco*RI	This work
pAS354	1.3-kb *Sal*I fragment of pAS346 cloned into pUC4K-*Sal*I	This work
pAS370	0.7-kb *Pst*I-*Stu*I fragment of pAS354 cloned into pBS/*Pst*I-*Sma*I	This work
pAS371	0.3-kb *Bam*HI-*Hin*dIII deletion of pAS370 followed by filling in and religation	This work
pAS372	0.4-kb *Eag*I-*Eco*RI deletion of pAS370 followed by filling in and religation	This work

medium lacking glutamate and aspartate, 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, ligation, and other accessory techniques used in 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-X centrifuge columns containing 0.45-μm-pore-size cellulose acetate filter (Costar) or electroelution (34), and the smallest fragments (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 previously (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% (vol/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 = 10^8 cells per ml) as measured with a Klett-Summerson colorimeter with a no. 66 filter (24). The cell pellet was suspended in glucose-EDTA solution containing 8 mg of lysozyme per ml, lysis was accomplished by adding 210 μl of a sodium dodecyl sulfate-NaOH 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 phenol extracted and ethanol precipitated as in the standard protocol (34), except that we used neutralized phenol equilibrated in 3% NaCl without chloroform and isoamyl alcohol. The total endogenous plasmid DNA was dissolved in sterile distilled water without RNase and digested with either *Ase*I, *Spe*I, or *Ase*I-*Spe*I restriction endonuclease(s). Usually, plasmid DNA obtained from a 1.5-ml culture volume was sufficient for one digestion or two.

Digestions were performed in 1× KGB (26) 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 wells 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, 8-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 GTG (FMC Bioproducts).

Bacterial mating. Conjugation from *E. coli* to *R. sphaeroides* was done either di- or triparentally. 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 K_2TeO_3. 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% (vol/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 Sis plus 50% (vol/vol) LB medium supplemented with DMSO (560 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/m^2) (8). Photosynthetic matings were performed in 5-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 in contact 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^{-9}) 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 (*oriV*). 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^r and analyzed for loss of S by using plasmid fingerprinting (see Results for details). Usually, the Tc^r 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 $oriT_S$-containing DNA fragment from pAS354 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'-AGGCCGATCCGAGCGCT and 5'-AGCGCTCGGATCGGCCT, 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., dITP 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 *R. sphaeroides* 705-bp *Pst*I-*Stu*I restriction endonuclease fragment described in this report has been deposited at 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 (PFG) electrophoresis to separate the plasmids in supercoiled form, as shown in Fig. 1. Plasmid cointegrations and some insertions (such as Tn*5*) 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 PFG 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 *Ase*I, two of the endogenous plasmids (pRS241b and -e) were not digested, while each plasmid has at least one *Spe*I site. This fingerprint 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* MS2III-48 is a green mutant derived from wild-type strain 2.4.1 because of a point mutation in the carotenoid-biosynthetic gene cluster (Crt$^-$) (41) which also possesses a Tn*5* insertion in pRS241e (38). Strain PrkB$^-$ has an Sp/Sm^r cartridge inserted in the *prkB* gene and is Crt$^+$ (Table 1). When MS2III-48 was mated with PrkB$^-$ on solid medium and exconjugants were selected for on Sp-Sm- 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^{-2} to

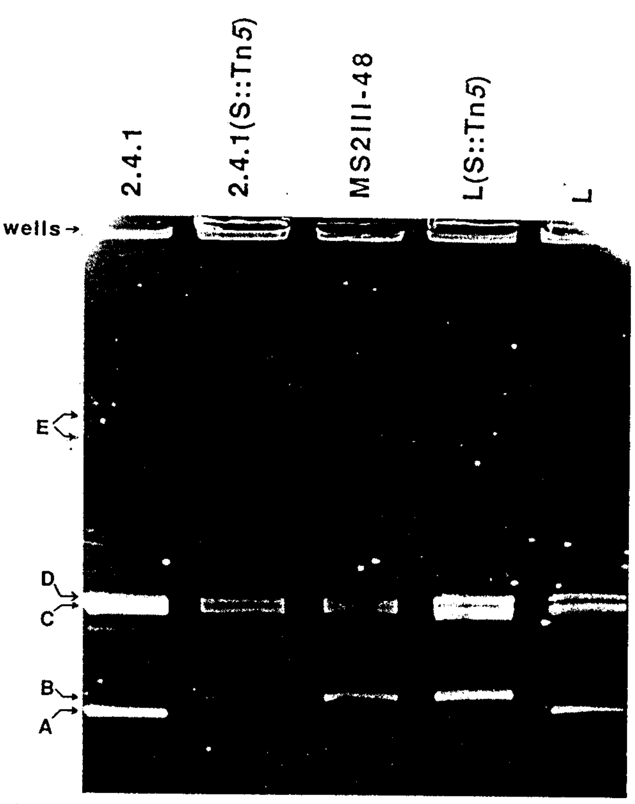

FIG. 1. Pulsed-field gel electrophoresis of undigested genomic DNA used to show the transfer of S::Tn*5* from *R. sphaeroides* MS2III-48 (green) into *R. sphaeroides* 2.4.1 and strain Lascelles (L). (A) The native S factor (in strain 2.4.1) or a S-factor-like element (in strain L). (B) S::Tn*5*. (C) pRS241c and -d. (D) pRS241a and -b. (E) Chromosomes. TAFE was performed with stages 1 (1-s pulse, 10-min run, 175 mA) and 2 (55-s pulse, 18-h run, 155 mA). See the text for details.

10^{-3} per donor. Furthermore, genome analysis indicated that S::Tn*5* 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* B10; and *Paracoccus denitrificans* were made Rf^r and then mated to MS2III-48 by solid-filter mating. The spontaneous resistance of *R. sphaeroides* to 50 μg of rifampin per ml was 10^{-5} to 10^{-6}. However, since MS2III-48 is Crt$^-$, spontaneous Rf^r derivatives of MS2III-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^{-9}) in contrast to strain 2.4.1. However, S did transfer at a low frequency (10^{-5} to 10^{-6}) 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 MS2III-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 matings, yielded the highest frequency of S-factor transfer.

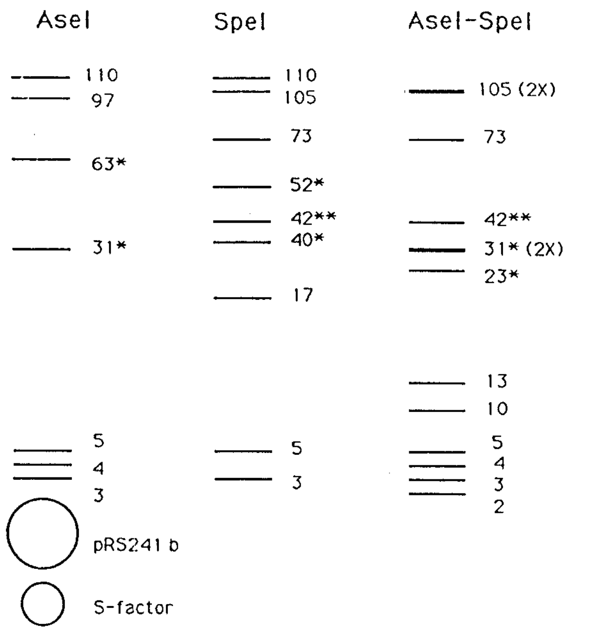

FIG. 2. Schematic drawing of *R. sphaeroides* 2.4.1 endogenous plasmid fingerprints. For an example of the actual data obtained from this analysis, see Fig. 6. Two circles in the *AseI* digestion pattern represent the two plasmids not digested by *AseI*. These plasmids migrated as faint bands in PFG with an 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 identify portions of pRS241d and S, respectively. The S DNA fragment was readily detected as a bright band, since this plasmid apparently is present in three or four copies per chromosome. The plasmid preparation and electrophoresis conditions used are described in Materials and Methods.

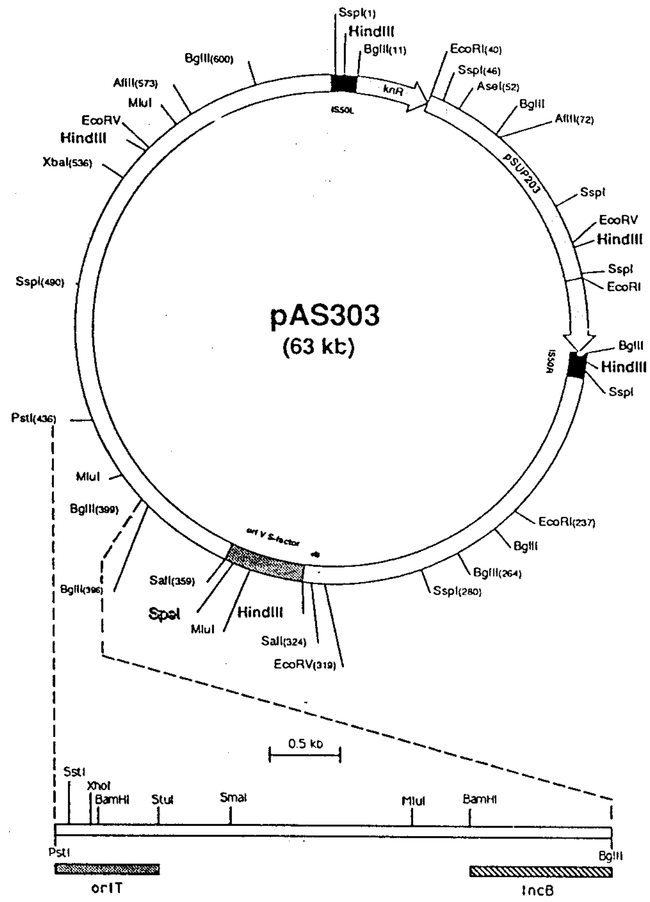

FIG. 3. Physical map and some genetic loci of S. The numbers in parenthesis following some of the restriction enzyme sites indicate relative distances (in 0.1-kb units). Not all *SalI* and *PstI* sites in the circular map are shown. The enlarged region shows the locations of *oriT* and IncB DNA-containing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

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. sphaeroides*. 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 Tn*5* was excised and cloned into pSUP203, a pBR325 derivative which can be mobilized into but by itself cannot replicate in *R. sphaeroides*, and this recombinant plasmid (pAS302) was introduced into MS2III-48 via conjugal mating as explained in Materials and Methods. The exconjugants were simultaneously selected for Km^r (from Tn*5* in the S factor) and Tc^r (from pSUP203). This selection enriches for strains in which pAS302 recombined with S::Tn*5* by using the internal portion of Tn*5* as a homologous crossover region, yielding a cointegrate plasmid (pAS303). Plasmid fingerprinting analysis was done to verify the structure of pAS303. Once the structure of pAS303 was verified, the isolated plasmid was used to transform *E. coli* DH5α. pAS303 should replicate in *E. coli* using pSUP203 (pBR325) *oriV*. A physical map of pAS303 with selected genetic markers is described in Fig. 3. This plasmid served as the main source of all of the DNA involved in the characterization of S.

TABLE 2. Frequency of S transfer under different mating conditions

Mating condition	Frequency (exconjugants/donor)[a]
Solid-filter mating, aerobic	10^{-5}–10^{-7}
Solid-filter mating, anaerobic[b]	10^{-4}–10^{-6}
Liquid DMSO, dark	10^{-3}–10^{-4}
Liquid, photosynthetic (10 W/m^2)	10^{-2}–10^{-3}

[a] The values shown are averages of three repeated experiments.
[b] Same in aerobic solid-filter mating but performed in an anaerobic jar (BBL GasPak).

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. sphaeroides* to *Agrobacterium* and *Rhizobium* spp., we attempted to transfer S to these bacteria. pAS303 was mobilized into *A. tumefaciens* C58 and *R. meliloti* 1021 via diparental mating using *E. coli* S17-1(pAS303), and the resulting Km^r Tc^r 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

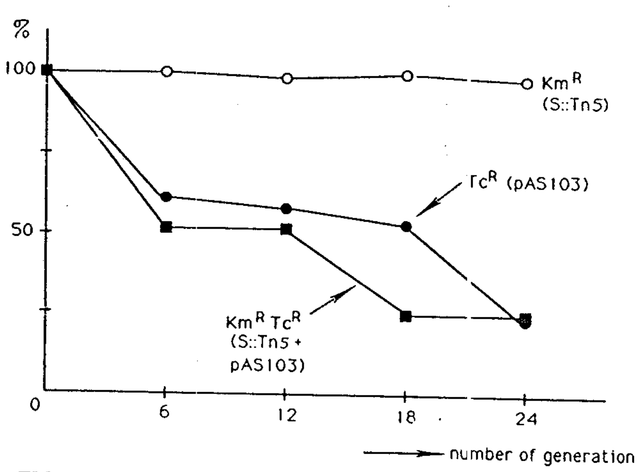

FIG. 4. Plasmid segregation analysis. pAS103 (Tc^r) was introduced into strain MS2III-48. Selection with Tc and Km yielded cells harboring both pAS103 and S::Tn*5*. Plasmid segregation was evaluated after growing Km^r Tc^r cells in antibiotic-free medium for several generations. Percentages of segregants were calculated from the total numbers of colonies appearing on the medium without antibiotics. The number of generations was calculated by using a 4-h generation time. The line connecting the squares describes plasmid segregation analysis when selection was made on a plate containing both Km and Tc.

either the chromosomes or megaplasmids of these bacteria. The presence of pSUP203 in the *Rhizobium* and *Agrobacterium* genomes was most likely generated by transposition of all of pSUP203 (see map of pAS303, Fig. 3) to the chromosomal or megaplasmid DNA or, possibly, by recombination between pAS303 and the genomic DNA of *A. tumefaciens* or *R. meliloti*.

Mobilization of pAS303 from S17-1 into *R. sphaeroides* 2.4.7 and WS8, which are not competent or respond poorly as recipients in conjugal *R. sphaeroides*-*R. sphaeroides* transfer assays, generated exconjugants with a replicative pAS303 plasmid. Thus, it seems that S-factor replication-stability determinants function in a wider-host range than its more restricted host range of transfer. From all of the information available, we infer that S is a narrow-host-range plasmid which is capable of transfer only between selected strains of *R. sphaeroides*.

Localization of *oriV* (IncA), stability analysis, and properties of *R. sphaeroides* 2.4.1 lacking the S factor. Yen (50) isolated a 3.1-kb *Sal*I-*Hin*dIII DNA fragment from an *R. 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 S DNA. Introduction of pAS103 containing the *oriV* fragment from S DNA cloned into pSUP203 (Table 1) into strain 2.4.1 generated exconjugants which gained pAS103 (because of selection for Tc^r) but excluded the endogenous S factor. Moreover, plasmid fingerprinting analysis confirmed that pAS103 was present as an independent replicon. These results suggested that *oriV* was able to function in strain 2.4.1.

We also observed that pAS103 was not stably maintained in *R. sphaeroides* in the absence of antibiotic selection (Fig. 4). However, if maintenance of pAS103 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 (IncA) by introduction of pAS103 and subsequent elimination of pAS103 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 ΔS), grew normally under photosynthetic, anaerobic-with-DMSO, or aerobic growth conditions. The strain was nutritionally 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 Tn*5* in pRS241a (MS2III-17), pRS241b (MS2I-14), and pRS241c (MS2-F) (38), four strains with a Tn*5*-B12S (14) insertion in pRS241c (ΔS6, ΔS9, ΔS12, and ΔS17) (Table 1), and three strains with Tn*5*-B12S present in pRS241d (ΔS2, ΔS5, and ΔS19) (Table 1).

When each of these strains was mated with strain ΔSB$^-$ and selected exconjugants which were Sp/Sm^r Km^r, we observed exconjugants only by using ΔS2, ΔS5, and ΔS19 as donors, with a frequency of 10^{-5} to 10^{-6} 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 ΔS 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^{-2} to 10^{-3} per donor. Since we isolated five independent insertions of either Tn*5* or Tn*5*-B12S into pRS241c and observed no detectable exconjugants, it is probable that pRS241c is not a transmissible plasmid. The other two plasmids (pRS241a and -b) contain only one representative Tn*5* insertion. Therefore, we cannot make a firm conclusion regarding the transfer ability of these two plasmids, since it is possible that the Tn*5* insertion somehow interfered with their transfer ability were such an ability present.

When either ΔS2(D::Km^r) or ΔS5(D::Km^r) (Table 1) was used as a donor in a mating with an Rf^r derivative of several other strains of *R. sphaeroides* (e.g., L, RS2, 630, and WS8), D was transferred to L, RS2, and WS8 but not to 630. Therefore, the host range for plasmid D appears to be similar to that of S.

Thus far, we have observed that S and D are transmissible and, interestingly, these two plasmids share a large extent of sequence homology (10, 41). However, they must have different incompatibility determinants since they can coexist stably in strain 2.4.1. This observation provides a partial explanation for the earlier results observed by Nano and Kaplan (30).

Cloning and localization of the S-factor *oriT* and IncB regions. S-factor DNA from pAS303 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 ΔS and in some cases into strain MS2III-48, as described in Materials and Methods. Each ΔS and/or MS2III-48 strain containing

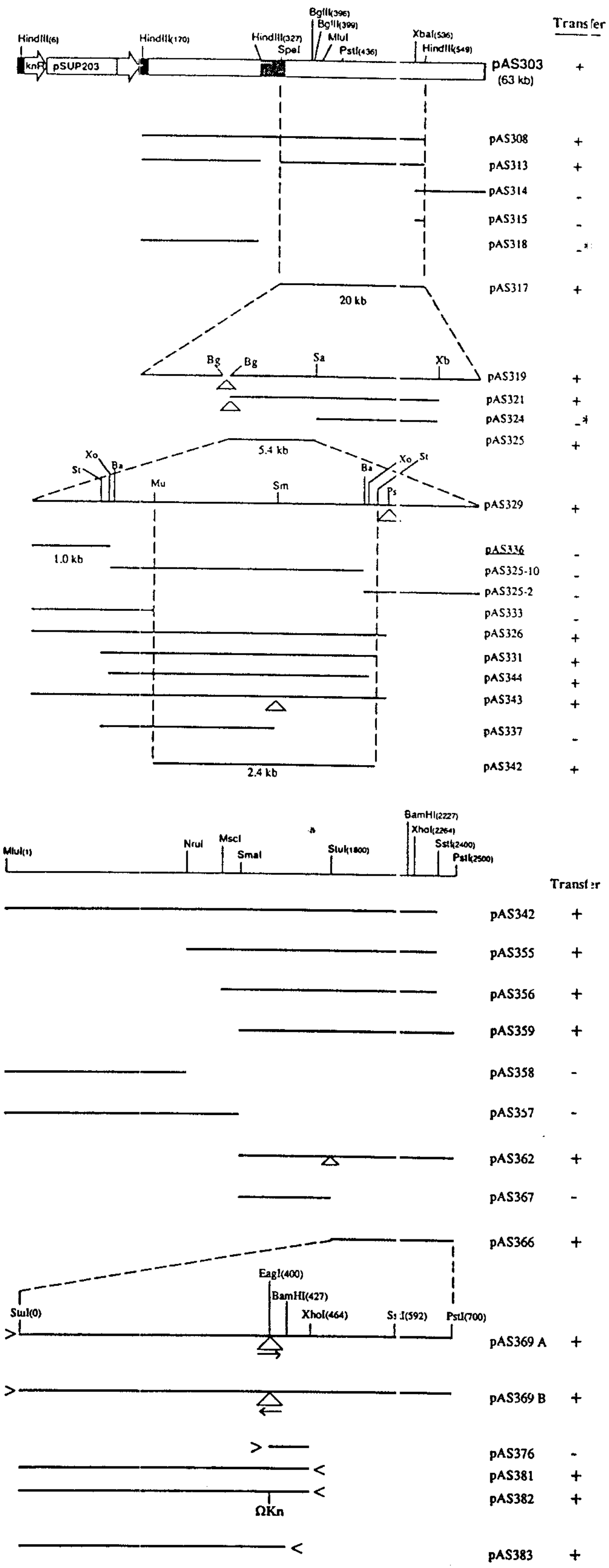

FIG. 5. Plasmids used in the molecular and genetic analysis of the $oriT_S$ region. A linear map of pAS303 was constructed from Fig. 3. All of these S-derived DNA fragments were obtained from pAS303 and cloned into pRK415. An open triangle under the bar indicates a Km^r (from pUC4K) insertion in that particular DNA fragment. An arrow under the triangle indicates the relative orientation of Km^r. ΩKn indicates insertion of Km^r from pHP45ΩKm, and the arrowheads show the direction of the *lacZ*-Tc promoters of pRK415 relative to the S DNA in those recombinant plasmids. A transfer frequency of 10^{-5} to 10^{-6} per donor (solid-filter mating) or 10^{-2} to 10^{-3} per donor (liquid anaerobic mating) was evaluated as positive, and a transfer frequency of $<10^{-9}$ per donor (in either solid or liquid mating) or transfer mediated by cointegration 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).

pRK415 carrying portions of S DNA was diparentally mated with strain ΔSB$^-$. 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 cointegrate formation between this plasmid and plasmid D. In contrast, pAS317 was capable of transfer independently of plasmid D.

pAS325 contains two *Bam*HI 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 ΔSB$^-$, none of these matings yielded exconjugants. The simplest interpretation of these results is that one or more of the *Bam*HI 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::Tn*5*, then MS2III-48(pAS336), MS2III-48(pAS325-10), and MS2III-48(pAS 325-2) would be expected to be Km^r Tc^r. However, when we grew each of these strains in Tc-Km-containing medium, one strain, i.e., MS2III-48(pAS336), failed to grow. Plasmid fingerprinting showed that MS2III-48(pAS336) contained pAS336 but had lost S::Tn*5*. 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^r 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::Tn*5* but failed to yield any exconjugants.

pAS342 and its derivatives were further assayed for plas-

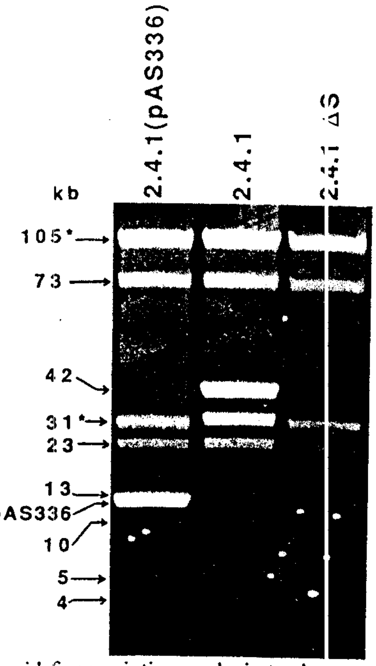

FIG. 6. Plasmid fingerprinting analysis to demonstrate S-factor curing by IncB in pAS336. Total endogenous plasmid DNA was digested with an *Ase*I-*Spe*I double digestion and separated by using PFG as described in Materials and Methods. pAS336 is linearized because of the *Ase*I sites (two *Ase*I sites are separated by 59 bp and located near the multiple cloning site) in pRK415, while *Spe*I digests all of the 2.4.1 endogenous plasmids, so that all of the DNA fragments in this fingerprinting analysis are linear DNA molecules. pAS336 is approximately 11.5 kb long; however, this plasmid DNA banded at an apparent size of 13 kb but is very broad because of the copy number of pRK415, which is about four to six per chromosome (6). Introduction of pAS336 into strain 2.4.1 with selection on Tc generates strain 2.4.1(pAS336), which maintains pAS336 but loses S. pAS336 was cured from strain 2.4.1(pAS336) by allowing the bacteria to grow in Tc-free medium to yield strain 2.4.1 ΔS. An asterisk after the molecular size indicates that that particular DNA fragment was a doublet (see also Fig. 2).

mid transfer. These plasmid derivatives showed either a high frequency of transfer (10^{-2} to 10^{-3} per donor) in liquid photosynthetic mating or no transfer ($<10^{-9}$ per donor) with no complications resulting from cointegration with plasmid D. pAS369A and pAS369B are pAS366 derivatives, each with a Km^r insertion in an *Eag*I site oriented, as shown in Fig. 5. These plasmids were transmissible, indicating that each has a functional *oriT* region.

Transfer assays employing pAS383, which carries a 427-bp *Stu*I-*Bam*HI DNA fragment, demonstrated that a functional *oriT* locus is confined to this region. This result and the data obtained from plasmid transfer assay of pAS369A, pAS369B, and pAS376 strongly suggest that the 400-bp *Stu*I-*Eag*I DNA fragment (Fig. 5) contains a functional *oriT* locus. However, we were not able to resolve why pAS325-10, which includes all of the cloned DNA present in pAS383, repeatedly failed to transfer, although several explanation come to mind. Interestingly, pAS344, which includes all of the cloned DNA present in pAS325-10, showed high transfer proficiency (Fig. 5). The locations of *oriT* and IncB in the S physical map are depicted in Fig. 3.

S-factor transfer is self-transmissible. Although S transfer from *R. sphaeroides* 2.4.1 has been unambiguously demonstrated, the evidence of its 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(pAS313), and WS8(pAS317), were used as donors to be mated with ΔSB$^-$.

pAS303 was transferred from WS8(pAS303) to ΔSB$^-$, as shown by the presence of Sp^r/Sm^r Tc^r exconjugants, and the presence of pAS303 was further verified in both the donor and the exconjugants by plasmid fingerprinting (41). pAS308, pAS313, and pAS317 are pAS303 derivatives cloned into pRK415 and were mobilizable from ΔS to ΔSB$^-$ (Fig. 5). However, in the WS8 background these plasmids were not mobilizable to ΔSB$^-$. 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 pAS103 also failed to be transferred from WS8(pAS103) to ΔSB$^-$, 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 *oriT*$_S$. A 705-bp *Pst*I-*Stu*I *R. sphaeroides* DNA fragment containing *oriT*$_S$ 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 *Pst*I-*Stu*I fragment on the S-factor physical map is described in Fig. 3.

Our previous work determined that a 400-bp *Eag*I-*Stu*I fragment contains a functional *oriT*$_S$ sequence, and from chromosome transfer experiments (40) we inferred that the orientation of *oriT*$_S$ transfer is such that the *Pst*I or *Eag*I sites will be among the last DNA sequences to enter the recipient cells.

Several interesting features have been identified in the region between *Eag*I and *Stu*I. 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 (23). Overall, this 400-bp region contains the topological features associated with other *oriT* sequences (11, 15).

DISCUSSION

Replication and incompatibility 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 incompatibility 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 *Methylomonas clara* (21),

```
                PstI
  1 TTGCATGCCT GCAGGACGAG TTCGATCGAC TGGATGGTCG GATGGTCAAC
                                      a          a
 51 CTCGTCTCTG CGCACAACCA TGGTCTCCGG GGGCTGATGA TGATGACCGG
                  SstI
101 ACAGATGGTT CGCGAGCTCA ACGTCATCCG G[illegible]GATCCTG ATCGTCTCGG
151 CGGCATCGAA CAATGAGCAG GCCGAACGGC TCTACGACAA GGCGGTGCCG
201 GCGATCATGG AGATGCGCGC CACGCTCGCT GTCTGGCGGG AGCGCGAAGG
      XhoI                                   BamHI
251 TCTCGAGGTG CCCGAGTTCT CCTTCGAGCA GGAGGAGCGG ATCCTCCAGC
                   EagI                          b
301 AGCTCGTCCA GAACCCGGCC GAGCACCGCC ATCCCGATCA TGGCGAGGAG
               b
351 CCGGAGACGC GGCGAGGCGC TTGCGGAGCA GGTGCAGCGG ATCGGCGAAG
                          c
401 CCTTCGATCC GAGCCTCACG TTGATGAGCG CCACGAACGT GAAATGGAGA
         c         d                      d
451 TGGGTGACGA TGAGCGGTAA GGCCGATCCG AGCGCTCAGA AGGCCGAAAA
        e          f          e           f
501 CTACCGTGGC ACGTCGTGCC ACGCGCGTGG C[illegible]CACTGTGC CACGGCCCCG
                                       1   1
551 AAAAACAACG TAAATACAGC GCGTTGGGAG C[illegible]GGGGGCAA ATTACGCCCC
                                        2          2
601 CTTTTAAGAC GAAAAGCCTT TATTTATAAA G[illegible]CTTGAAGG CAATGGGCAG
       3          3               ATATTT C[illegible]GAACT...5'
651 CTTGCTGCGT CAGGTGTGTC CTCTTCAGAC C[illegible]GAGAGGCA GCCGTAAGGC
                StuI/SmaI            EcoRI
701 TGCCGATCCG AGGGGGTACC GAGCTCGAAT TC
```

FIG. 7. Nucleotide sequence of S-factor *oriT* region. S-factor DNA is from *Pst*I to *Stu*I sites. Putative IHF-binding sites are indicated by nucleotides inside the boxes. The lower box with the nucleotide sequence represents the putative IHF-binding site of the bottom strand. Nucleotides that do not match the consensus sequence C/TAANNNNTTGATA/T are marked with dots above or below those nucleotides. Inverted repeats are represented by arrows and numbered to facilitate pairing. Direct repeats are represented by lines and lettered to facilitate pairing. The A-T-rich region (>55% A-T) is underlined (nucleotides 551 to 639)

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::Tn*5*. 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 Tn*5*.

Two incompatibility determinants have been cloned and localized on the physical and genetic map of S, one closely linked to the origin of replication, *oriV*, and the other linked to the origin of transfer, *oriT*. Most studies of incompatibility 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 the endogenous plasmids represent still 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 conjugative 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 transmissible 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 *oriT* 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 pilus 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 S 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 $oriT_S$ and we know that cointegrate 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 matings than in mating on a solid surface. This might suggest either a short, flexible pilus or, perhaps, no pilus involvement. Comparison of liquid matings 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 plasmid functions (16) which indirectly give rise to high transfer frequencies remains to be determined.

Sequence analysis revealed that $oriT_S$ 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-T-rich tract with several inverted repeat sequences located to one side of the A-T-rich region. Assuming that at least one of the three putative IHF-binding sites is bound by IHF, then the nick site(s) of $oriT_S$ would be expected to be somewhere between bp 640 and 713 (*Stu*I site) with an orientation such that the IHF-binding site(s) is the last to enter recipient cells (15).

The small *oriT* region of S ($oriT_S$), 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 $oriT_S$-containing plasmids or chromosomes in the absence of the entire S factor. By inserting $oriT_S$ 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 Hfr-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).

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