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Chromosome Transfer in *Rhodobacter sphaeroides*: Hfr Formation and Genetic Evidence for Two Unique Circular Chromosomes

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A 600-bp *oriT*-containing DNA fragment from the *Rhodobacter sphaeroides* 2.4.1 S factor ($oriT_S$) (A. Suwanto and S. Kaplan, J. Bacteriol. 174:1124–1134, 1992) was shown to promote polarized chromosomal transfer when provided in *cis*. A Km^r–$oriT_S$–*sacR-sacB* (KTS) DNA cassette was constructed by inserting $oriT_S$–*sacR-sacB* into a pUTmini-Tn*5* Km1 derivative. With this delivery system, KTS appeared to be randomly inserted into the genome of *R. sphaeroides*, generating mutant strains which also gained the ability to act as Hfr donors. An *Ase*I site in the Km^r cartridge (from Tn*903*) and *Dra*I and *Sna*BI sites in *sacR-sacB* (the levansucrase gene from *Bacillus subtilis*) were employed to localize the KTS insertion definitively by pulsed-field gel electrophoresis. The orientation of $oriT_S$ at the site of insertion was determined by Southern hybridization analysis. Interrupted mating experiments performed with some of the Hfr strains exhibited a gradient of marker transfer and further provided genetic evidence for the circularity and presence of two chromosomal linkage groups in this bacterium. The genetic and environmental conditions for optimized mating between *R. sphaeroides* strains were also defined. The results presented here and our physical map of the *R. sphaeroides* 2.4.1 genome are discussed in light of the presence of two chromosomes.

One essential aspect of the biology of *Escherichia coli* which has contributed to the importance and usefulness of this organism was the discovery of gene transfer by Lederberg and Tatum in 1946 (21). In addition, the genetic system itself (17) has been used to deduce the circularity of the *E. coli* chromosome far in advance of any physical mapping techniques (32). For these and other specific reasons dealing with the complexity of genome organization in *Rhodobacter sphaeroides* 2.4.1 (35, 36), we set about the development of a genetic system equivalent to the Hfr system in *E. coli* which could be used in conjunction with the already available physical map of this α-purple, nonsulfur photoheterotrophic bacterium.

Several plasmids with a broad host range, such as IncP plasmids RP4 and RK2, have been investigated, but a low frequency of chromosomal transfer has limited their use (16, 31). High-frequency chromosome transfer in *R. sphaeroides* promoted by a broad-host-range plasmid carrying Tn*501* has been reported and has allowed the first extensive genetic mapping of this bacterium (24). However, a lack of understanding about the initiation and orientation of transfer and the nature of RP1::Tn*501* insertion into the *R. sphaeroides* genome has hampered the completion of this map (24). Blanco et al. (2) reported that a retrotransfer, i.e., chromosomal markers are transferred not only from the donor but also from the recipient, may add another complication to these broad-host-range-mediated mapping experiments. Nevertheless, this method has allowed construction of a circular-linkage map of the *R. capsulatus* B10 chromosome (48).

An improved method to facilitate chromosome transfer in gram-negative bacteria was initiated by cloning the *oriT* or *mob* region from RP4 or RK2 into Tn*5* (13, 30). In a slightly different strategy, Johnson (18) cloned *oriT* and the entire transfer functions of RP4 into Tn*5* and Tn*1*, yielding new transposons ca. 50 kb in size. In this system, the complete conjugal transfer sequences are provided in *cis* relative to *oriT*.

The genome of *R. sphaeroides* 2.4.1 consists of two unique circular chromosomes and five endogenous plasmids (10, 20, 36). The evidence which we have accumulated pointing to the presence of two chromosomes is as follows. (i) the existence of two large (~3,000- and ~900-kb) circular physical DNA structures, (ii) the presence of one *rrn* operon on the large chromosome and two *rrn* operons on the small chromosome, (iii) the presence of tRNA genes on each chromosome, (iv) the 1:1 stoichiometry between chromosomes, and (v) the finding that all strains of *R. sphaeroides* examined by us provided by laboratory collections around the world have two chromosomes comparable in size to the two observed in strain 2.4.1. However, these same strains have variable numbers (from one to six) and sizes of plasmids.

At least two of five endogenous plasmids of *R. sphaeroides* 2.4.1, i.e. the S factor and plasmid D, are transmissible (37). A 427-bp *oriT*-containing DNA fragment has been isolated from the S factor ($oriT_S$) and been shown to confer high-frequency transfer between *R. sphaeroides* strains when $oriT_S$ was cloned into an otherwise nontransmissible plasmid (37).

This study demonstrated the ability of $oriT_S$ to promote polarized chromosomal transfer in either orientation, the construction of Hfr-like strains by random transposition mutagenesis employing a Tn*5* derivative containing $oriT_S$, and the application of these developments for strain constructions, classical genetic mapping, and genetic linkage analysis that have been shown by physical analyses of genome structure in previous work (36).

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tional Symposium of Photosynthetic Procaryotes, Amherst, Mass., 1991 [abstract no. 35A].)

MATERIALS AND METHODS

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

Escherichia coli and *R. sphaeroides* strains were routinely grown in LB (37) at 37°C and Sistrom's minimal medium lacking glutamate and aspartate, pH 7.2 (Sis), at 32°C (23), respectively, unless otherwise stated. An antibiotic(s) and sucrose were supplemented whenever appropriate in the following concentrations: kanamycin (Km), 25 μg/ml; spectinomycin (Sp), 50 μg/ml; streptomycin (Sm), 50 μg/ml; nalidixic acid (Nx), 20 μg/ml; rifampin, 50 μg/ml; tetracycline (Tc), 1 μg/ml (for *R. sphaeroides*) and 15 μg/ml (for *E. coli*); ampicillin, 150 μg/ml; chloramphenicol, 10 μg/ml. The last two antibiotics were used only for *E. coli*. Sucrose was added from a filter-sterilized stock solution into LB or Sis so that the final sucrose concentration was 10%. LB-dimethyl sulfoxide (DMSO) or Sis-DMSO was made by adding 560 μl of DMSO per 100 ml of medium.

Molecular techniques. Standard methods were used for plasmid isolation, *E. coli* transformation, restriction endonuclease analysis, ligation, and other related techniques used in molecular cloning (29). DNA fragments isolated from agarose gels were routinely purified by using a Gene Clean Kit (Bio 101 Inc., La Jolla, Calif.). Southern hybridization analysis was performed as described previously (37), by using stringent washing conditions at 60°C twice for 15 min each time and detection by a chemiluminescence method (Photogene detection system; BRL) or direct exposure to X-ray film (Kodak X-OMAT) when using [α-^{32}P]dCTP-labeled probes.

Bacterial mating. Conjugation to introduce plasmids from *E. coli* to *R. sphaeroides* was done as described previously (37). *R. sphaeroides-R. sphaeroides* matings were routinely performed under photosynthetic condition as follows. Both *R. sphaeroides* donors and recipients were grown in Sis minimal medium containing 10% LB (pH 7.2) (37) supplemented with the appropriate antibiotic(s), incubated aerobically at 32°C for 18 to 24 h (approximately 10^8 to 10^9 cells per ml). Samples (50 μl) of these cultures were inoculated into a 5-ml Whatman clear glass vial containing the same medium used for aerobic culture but lacking antibiotics. These vials were incubated photosynthetically under illumination at 10 W/m^2 (23) for 18 to 20 h. Samples (400 μl each) of the donor and recipient from the preceding photosynthetic culture were placed together in a new 5-ml Whatman glass vial, and LB medium (pH 7.2) was added to fill the entire volume before subsequent photosynthetic incubation for 18 to 20 h unless otherwise stated. At the end of the mating period, the culture was dispensed, agitated briefly but vigorously using a Vortex mixer, plated at the appropriate dilution onto the appropriate antibiotic-containing medium, and incubated aerobically at 32°C for 3 days, and the donor, exconjugants, and in certain cases the recipient were analyzed.

When the *R. sphaeroides* donor or recipient was not able to grow photosynthetically (PS$^-$), then each was pregrown in Sis containing 50% LB supplemented with 560 μl of DMSO per ml and incubated in the dark at 32°C for 3 to 4 days. Matings were conducted by adding 400 μl each of the donor and recipient to a 5-ml Whatman glass vial filled with LB plus DMSO. The mixture was incubated at 32°C in the dark for 20 to 24 h. The exconjugants were calculated as described for photosynthetic matings.

Pulsed-field gel analysis. Intact total genomic DNA of *R. sphaeroides* was isolated by the gel insert techniques; digested with either *Ase*I, *Sna*BI, or *Dra*I; and then separated by transverse alternating field electrophoresis (TAFE) as described previously (35). The TAFE conditions are described for each specific experiment, and unmentioned parameters, such as buffer temperature, stage 1 pulses, and electrical currents, were as described previously (35). A 1× TAFE buffer (Geneline I; Beckman Instruments) was used in all TAFE analyses.

Materials. Nick translation kits were purchased from Bethesda Research Laboratories. [α-^{32}P]dCTP (800 Ci/mmol) was obtained from Amersham Corp., Arlington Heights, Ill. X-ray film was the product of Eastman Kodak Co., Rochester, N.Y. The photogene detection system and Photogene nylon membranes were purchased from BRL. All chemicals were of reagent grade purity and were used without further purification.

RESULTS

Hfr donor strain construction. For initial examination of the ability of *oriT*$_S$ to mobilize the chromosomes of *R. sphaeroides* 2.4.1, we constructed strains with *oriT*$_S$ located in chromosome I in two different orientations by using site-directed mutagenesis as follows.

Strain PUHA1 is an *R. sphaeroides* 2.4.1 derivative which has a Km^r insertion in the *puhA* gene (34). This strain cannot grow photosynthetically but can grow in the dark in DMSO and gives rise to pale red colonies under aerobic plating conditions. We cured the S factor from this strain as described previously (37) to yield strain PUHA1ΔS.

Plasmid pAS332 (Table 1) contains a 4.1-kb *oriT*$_S$-containing *Sal*I fragment cloned into pUC4K. This 4.1-kb *Sal*I fragment was obtained from successive subcloning of the original S-factor DNA (pAS303) as described previously (37; Table 1). A 2.1-kb *Pst*I fragment containing the Sp/Sm^r cartridge was inserted into a unique *Pst*I site in pAS332 to generate pAS341A (Table 1). Therefore, an approximately 6-kb *Eco*RI fragment can be excised as an *oriT*$_S$-Sp/Sm^r cartridge from pAS341A.

pAS348A is a suicide plasmid which carries the *oriT*$_S$-Sp/Sm^r cartridge from pAS341A inserted into the *Xho*I site (in *pufM*) of *pufKBALMX* derived from pUI908 (Fig. 1A and Table 1). *E. coli* HB101(pAS348A) was mated with PUHA1ΔS by using strain HB101(pRK2013) as a helper. The exconjugants were selected on Sis medium supplemented with Sp and Sm. The Tc^r marker in pAS348A was used to detect the occurrence of single or double crossovers indirectly. The resulting double crossover was designated HΔSA. Similarly, introduction of pAS348B into PUHA1ΔS with subsequent screening for the double crossover yielded strain HΔSB. The expected *Ase*I digestion patterns of strains ΔS, PUHA1ΔS, HΔSA, and HΔSB are depicted in Fig. 1B.

Strain ΔS is essentially a wild-type strain of *R. sphaeroides* 2.4.1 lacking the S factor, and since the S factor does not possess an *Ase*I site, it remains intact upon digestion of total genomic DNA with *Ase*I (35, 37). Therefore, *Ase*I digestion of total genomic DNA of strain ΔS exhibits a pulsed-field gel electrophoretic banding pattern identical to that of strain 2.4.1 (35). Throughout this text, the term schizotype refers to the electrophoretic banding pattern of the total genomic DNA, following digestion with rarely cutting restriction endonucleases, of any strain used to

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
R. sphaeroides 2.4.1 strains:		
L188A	*rrnA*::Sp/Sm^r	S. Dryden and S. Kaplan
PUHA1	*puhA*::Km^r	34
ΔS	2.4.1 cured of S	37
MS2III-48	Green S::Tn*5* Nx^r	37
48ΔS	MS2III48 cured of S; Nx^r	This work
PUHA1ΔS	PUHA1 cured of S	This work
LΔS	L188A cured of S	This work
ΔSR	ΔS, *rrnB*::Tc^r	This work
48ΔSR	48ΔS, *rrnB*::Tc^r	This work
HΔSA	PUHA1ΔS, *puf*::*oriT*$_S$-Sp/Sm^r (A orientation)	This work
HΔSB	PUHA1ΔS, *puf*::*oriT*$_S$-Sp/Sm^r (B orientation)	This work
HΔSAX	HΔSA × 48ΔSR exconjugant; green Tc^r Km^r Sp/Sm^s PS$^-$	This work
HΔSBX	HΔSB × 48ΔSR exconjugant; green Tc^r Km^r Sp/Sm^s PS$^-$	This work
Hfr1	ΔS, KTS (I, 861, cw) Aux PS$^+$	This work
Hfr2	48ΔS, KTS (I, 2,650, cw) PS$^+$	This work
Hfr3	ΔS, KTS (I, 2,078, ccw) PS$^+$	This work
Hfr4	48ΔS, KTS (II, 743, cw) PS$^+$	This work
Hfr5	ΔS, KTS (II, 3, ccw) PS$^+$	This work
L9	LΔS, KTS (I, 739, ccw) PS$^+$	This work
L1	LΔS, KTS (I, 2,795, cw) PS$^+$	This work
LΔSf5	LΔS × Hfr5 exconjugant; Sp^r Sm^r KM^r PS$^+$	This work
48ΔSf5	LΔSf5 × 48ΔSR exconjugant; Nx^r Km^r Tc^s PS$^+$	This work
E. coli strains:		
DH5α	F$^-$ *lacZ*ΔM15 *recA1 hsdR17*	29
HB101	Res$^-$ Mod$^-$ *recA13* Sm^r	29
S17-1	Pro$^-$ Res$^-$ Mod$^+$ *recA* integrated plasmid RP4-Tc::Mu-Km::Tn*7*	30
S17-1 (λ*pir*)	Same as S17-1, lysogenized with λ*pir* bacteriophage	14
Plasmids		
pRK2013	ColE1 replicon; Tra$^+$ of RK2, Km^r	6
pSUP203	pBR325 derivative; mob$^+$ Tc^r Cm^r Ap^r	30
pSUP202	Same as pSUP203, but *Mob* is located between Cm^r and Ap^r genes	30
pHP45Ω	Source of Sp/Sm^r cassette	25
pHP45Ω-Tc	Same as in pHP45Ω; Tc^r instead of Sp/Sm^r	25
pUC4K	Source of Km^r cartridge and intermediate vector	45
pBR322	pMB1 replicon; Ap^r Tc^r	29
Ia	Source of *sacR-sacB*; single internal *Eco*RI site in *sac*B eliminated	42
pUC18Not	pUC18 with *Not*I sites flanking its multiple cloning sites	14
pUTmini-Tn*5* Km1	Mini-Tn*5* Km1 in plasmid pUT	14
pUC35	5.7-kb *Eco*RI fragment containing *rrnB* operon cloned into pUC19	S. Dryden and S. Kaplan
pSUPssΩ	*Hin*dIII fragment of Sp/Sm^r cassette inserted into pSUP202-*Hin*dIII	S. Dryden and S. Kaplan
pUI908	4.25-kb *Sph*I fragment containing gene *Q pufKBALMX* cloned into pUC19 such that the direction of *pufBA* transcription opposes that of *lacP*	B. de Hoff and S. Kaplan
pUI389	1.9-kb *Eco*RI-*Alw*NI fragment of pUC19 ligated with 0.82-kb *Eco*RI-*Alw*NI fragment of pUC18	A. Varga, S. Dryden, and S. Kaplan
pUI1001	*Bam*HI fragment of Sp/Sm^r cassette from pHP45Ω cloned into pUI389-*Bam*HI	E. Neidle and S. Kaplan
pUC35-TcA	5.5-kb *Eco*RI fragment containing Tc^r gene from pMH1701 cloned into pUC35-*Eco*RV (A orientation)	This work
pAS404A	11.2-kb *Eco*RI fragment of pUC35-TcA cloned into pSUPssΩ-*Eco*RI	This work
pAS303	Cointegrate of pAS302 into S::Tn*5*; Tc^r Km^r Ap^r	37
pAS321	~14-kb *Bgl*II-*Xba*I fragment (including internal *Bgl*II site) of pAS303 cloned into pRK415 with Km^r (*Bam*HI fragment) inserted in outside *Bgl*II site	This work
pAS323	~5.5-kb *Sal*I fragment of pAS321 (1 *Sal*I site 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
pAS403	3.2-kb *Ase*I fragment of pUI908 cloned into pSUP203-*Ase*I	This work
pAS348A	6-kb *Eco*RI fragment of pAS341A inserted into the *Xho*I site in pAS403 by blunt-end ligation (A orientation)	This work
pAS348B	Same as pAS348A but in B orientation (opposite of A orientation)	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

Continued on following page

TABLE 1—*Continued*

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
pAS375A	2 kb of *sacB* gene (*Bam*HI filled in) from plasmid Ia cloned into pAS354-*Stu*I-*Sma*I (A orientation)	This work
pAS377	2.7-kb *Xba*I-*Sst*I fragment (*sacB-oriT*$_S$) of pAS375A cloned into pUC18NOT-*Xba*I-*Sst*I	This work
pAS378	2.7-kb *Not*I fragment (*sacB-oriT*$_S$) of pAS377 cloned into pUTmini-Tn*5* Km1-*Not*I such that the orientation is KTS	This work
pAS380	2-kb *Sma*I fragment containing ΩTc from pHP45Ω-Tc inserted into pAS378-*Sca*I	This work
pAS384	3.4-kb *Dra*I-*Ssp*I fragment of pBR322 (containing *oriV* and Tc^r gene) inserted into pAS378-*Sca*I	This work

[a] The location and orientation of KTS are indicated in parentheses following KTS. I and II indicate insertion in the large and small chromosomes, respectively, and the number in the middle indicates the relative distance (in kilobases) from *oriT*$_S$ to *puf* (in the large chromosome) or *rrnC* (in the small chromosome). PS, photosynthetic growth; Aux, auxotroph.

compare or contrast the strains under study physically. Thus, as stated above, the *Ase*I schizotype of strain ΔS is identical to that of strain 2.4.1.

puhA is located ca. 31 kb from *pufBA* (35), so that the presence of the Km^r insertion containing an *Ase*I site in PUHA1 resulted in digestion of the 73-kb *Ase*I fragment present in ΔS into 31- and 42-kb *Ase*I fragments in PUHA1ΔS (35). The Sp/Sm^r cartridge has two *Ase*I sites flanking the gene for Sp/Sm^r (25), so insertion of *oriT*$_S$-Sp/Sm^r into *pufM*, depending on the orientation, should yield a predictable *Ase*I schizotype for strains HΔSA and HΔSB, as depicted in Fig. 1B. *Ase*I schizotyping of strains ΔS, HΔSA, and HΔSB, in conjunction with Southern hybridization analysis using a 1.3-kb *Sal*I fragment of pAS354 (Table 1) containing *oriT*$_S$, unambiguously demonstrated that HΔSA and HΔSB contained the recombined DNA fragment (*oriT*$_S$-Sp/Sm^r) in opposite orientations (Fig. 2). The hybridization signals at 19.5 (HΔSA) and 5.6 (HΔSB) kb revealed the presence of *oriT*$_S$ in opposite orientations in these recombinant strains. The hybridization signal at 31 kb was due to the similarity between DNA sequences in the S factor (including the *oriT*$_S$ region) and plasmid D (10, 37). The 31- and 63-kb *Ase*I fragments are derived from plasmid D (see plasmid fingerprinting in reference 37). A weak hybridization signal also detected at the 110-kb *Ase*I fragment corresponds to pRS241a (37).

Recipient strain construction. A 5.5-kb *Eco*RI fragment containing the Tc^r gene from pMH1701 (37) was inserted into the *Eco*RV site in the *rrnB* operon (8), which is located on chromosome II. The entire DNA fragment containing *rrnB*::Tc^r (ca. 11.2 kb) was excised as an *Eco*RI fragment and inserted into the *Eco*RI site of pSUPssΩ to yield pAS404A, and the altered *rrnB* operon was introduced into the small chromosome of strains ΔS (Crt$^+$) and 48ΔS (Crt$^-$) as previously described (37; Table 1) to yield strains ΔSR and 48ΔSR, respectively (Table 1).

Chromosome transfer from strain HΔSA or HΔSB into strain 48ΔSR. The relevant characteristics of these donor and recipient strains are indicated in Table 1, and the structures are shown in Fig. 1A. Since HΔSA and HΔSB were not able to grow photosynthetically, mating was performed under LB-DMSO conditions as described in Materials and Methods. The results are summarized in Table 2.

Mating between HΔSB and 48ΔSR consistently yielded at least 10-fold more exconjugants than that of HΔSA and 48ΔSR. In fact, under short-term mating conditions (8 h), we were unable to detect any Km^r exconjugants from HΔSA × 48ΔSR. From these results, the orientation of *oriT*$_S$ can be deduced such that Km^r in HΔSB will be one of the earliest markers (ca. 30 kb from *oriT*$_S$) and Km^r in HΔSA will be one of the last markers (ca. 3,000 kb from *oriT*$_S$) to be transferred into the recipient (Fig. 1A).

HΔSB × 48ΔSR matings yielded 70% green and 30% red exconjugants. All of the green exconjugants were Sp/Sm^s, and 90% of the red exconjugants were Sp/Sm^r. These genetic results suggested that the *crt* marker was more closely linked to Sp/Sm^r (inserted into the *puf* operon) than to *puhA*. These genetic results were supported by the actual physical distances of the *crt* marker, which are approximately 11 kb from *puf* and 20 kb from *puhA* (38).

HΔSA × 48ΔSR mating yielded 99% red, Sp/Sm^r exconjugants and was the result of the orientation of *oriT*$_S$ in this donor. The Km^r marker in this strain would be one of the last markers to enter the recipient (ca. 31 kb before the point of chromosome recircularization), so that the exconjugants were more likely generated from the transfer and stabilization of all of chromosome I, as was the case in 27% of the exconjugants of HΔSB × 48ΔSR. This result also helps to explain why, in the short period of mating (LB-DMSO for 8 h), we were unable to observe any exconjugants from the HΔSA × 48ΔSR mating, since in this mating we demanded the transfer of nearly all of chromosome I. The generation time of *R. sphaeroides* grown anaerobically in the dark in LB-DMSO is approximately 9 to 12 h or three times longer than when it is grown photosynthetically at 10 W/m^2, so that the overall metabolic activity, including chromosome transfer, would be expected to be slower than that observed during optimum photosynthetic mating conditions (see below).

To determine whether introduction of *oriT*$_S$ from HΔSA and HΔSB into strain 48ΔSR could induce plasmid cointegration or rearrangement mediated by plasmid D (37), representative colonies among the exconjugants from HΔSA or HΔSB matings with 48ΔSR were subjected to plasmid fingerprinting analysis (37). The results showed that all of the representative exconjugants had normal plasmid profiles. Despite the homology between plasmid D and *oriT*$_S$, no recombination or rearrangements were observed in these exconjugants. These representative exconjugants were further examined by schizotyping as described previously (35). The results showed the expected schizotypes. Similarly, the exconjugants displayed the anticipated phenotypes.

Construction of a suicide vector carrying Km^r–*oriT*$_S$–*sacR-sacB* (KTS). Since we were able to demonstrate polarized chromosome transfer directed by *oriT*$_S$, we sought to develop a mechanism by which we could readily construct

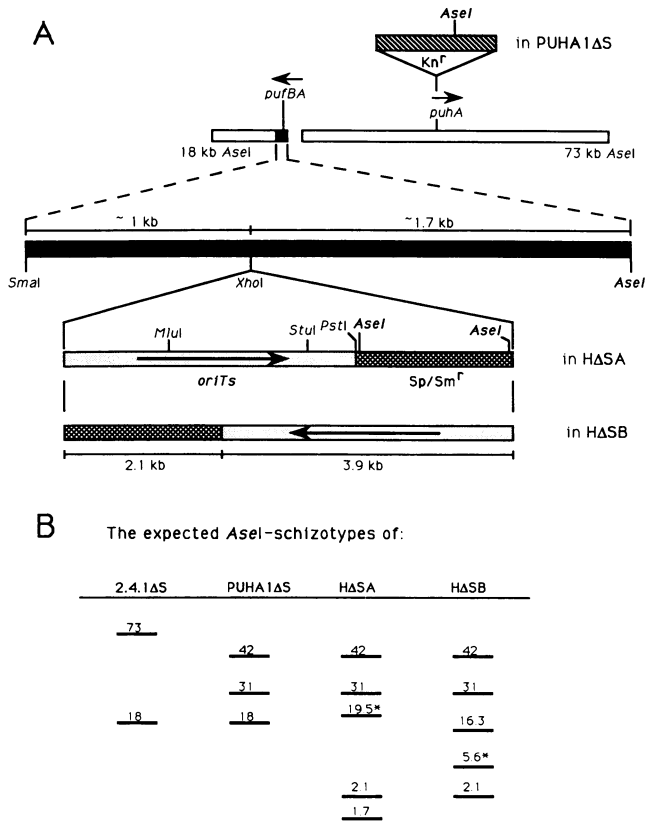

FIG. 1. Construction of donor strains containing $oriT_S$ inserted in two different orientations. (A) A 6-kb *Eco*RI DNA cassette consisting of $oriT_S$ (3.9 kb) and the gene for Sp/Sm^r (2.1 kb) was excised from pAS341A. This fragment was inserted into a unique *Xho*I site in pAS403 by blunt-end ligation, yielding recombinant suicide plasmids each with a 6-kb $oriT_S$-Sp/Sm^r gene cassette in different orientations (pAS348A and pAS348B). Introduction of each of these plasmids into strain PUHA1ΔS generated two kinds of Hfr donor strains (HΔSA and HΔSB) with respect to the relative orientation of the DNA fragment containing $oriT_S$. (B) Diagram of the expected *Ase*I schizotypes of HΔSA and HΔSB in comparison with those of ΔS and PUHA1ΔS. Other *Ase*I fragments larger than 73 kb in these four strains are identical and are not depicted. A Km^r cartridge in strain PUHA1ΔS results in digestion of the 73-kb *Ase*I fragment into 42- and a 31-kb *Ase*I fragments upon digestion with *Ase*I (35). These 42- and 31-kb *Ase*I fragments should be conserved in strains HΔSA and HΔSB. The Sp^r Sm^r cassette has two *Ase*I sites (25); therefore, insertion of a 6-kb DNA cassette containing $oriT_S$-Sp/Sm^r in the 18-kb *Ase*I fragment generates three new *Ase*I fragments with molecular sizes corresponding to the relative orientation of $oriT_S$-Sp/Sm^r in the chromosome. *Ase*I fragments which carry $oriT_S$ sequences are indicated by asterisks (see also Fig. 2).

additional Hfr-like strains by random insertion of $oriT_S$ into the *R. sphaeroides* 2.4.1 genome. Moreover, the $oriT_S$-containing DNA fragment which we used was only 600 bp, while that used to construct HΔSA and HΔSB was 3.9 kb.

Plasmid pUTmini-Tn*5* Km1 (14, 22) was chosen as a vehicle for construction of an $oriT_S$ delivery system. Modification of this plasmid by insertion of $oriT_S$, *sacR-sacB*, and the Tc^r gene yielded recombinant suicide plasmid pAS380 (Fig. 3A). This plasmid (Fig. 3B) has a γ origin of replication from plasmid R6K; therefore, it can replicate only in a host providing a Pir protein, such as SM10λ*pir* or S17-1λ*pir* (14). Transfer into the recipient was achieved by utilizing RP4

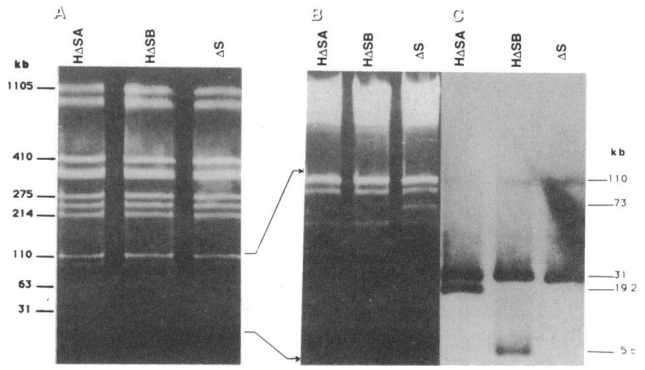

FIG. 2. Genome analysis of strains HΔSA and HΔSB. (A) The *Ase*I schizotypes of DNA fragments larger than 73 kb from strains HΔSA and HΔSB are identical to that of the reference strain (ΔS). (B) The *Ase*I schizotypes of the smaller-molecular-size fragments (≤73 kb) of strains HΔSA and HΔSB showed the expected results, as depicted in Fig. 1B. (C) Confirmation of the restriction patterns in panel B by Southern hybridization analysis using a 1.3-kb *Sal*I fragment containing $oriT_S$ from pAS354 as a probe. The strong signal at the 31-kb *Ase*I fragment and the weak signal at the 110-kb *Ase*I fragment are due to cross-hybridization of the probe with sequences from *Ase*I-digested plasmids D and pRS241a, respectively (37). The TAFE conditions for panel A were as follows: stage 2, 50-s pulse, 6 h; stage 3, 23-s pulse, 8 h; stage 4, 7-s pulse, 4 h; stage 5, 4-s pulse, 2 h. Those for panel B were as follows: stage 2, 8-s pulse, 8 h; stage 3, 4-s pulse, 6 h; stage 4, 2-s pulse, 3 h.

mob, which can be driven by the products of the *tra* genes of plasmid RP4, which are provided in *trans*, such as in *E. coli* S17-1 (30). Transposition, as opposed to plasmid cointegration, was indirectly ascertained by replica patching on Tc-containing medium. The gene for Km^r (from Tn*903*) has a unique *Ase*I site, while the *sacB* gene contains *Sna*BI and *Dra*I sites. These restriction enzyme sites facilitate precise localization of $oriT_S$ (or KTS in its entirety) in the *R. sphaeroides* genome (35).

In addition, we also constructed a derivative of pAS378 which can replicate independently of the Pir protein by inserting a 3.4-kb *Dra*I-*Ssp*I fragment from pBR322 containing both *oriV* and the Tc^r gene into the *Sca*I site of pAS378. This chimeric plasmid (pAS384) is essentially identical to

TABLE 2. Analysis of exconjugants from HΔSA × 48ΔSR and HΔSB × 48ΔSR matings

Mating[a]	Frequency[b]	Description[c]
PUHA1ΔS × 48ΔSR	$<10^{-9}$	Chromosomes not able of transfer by themselves
HΔSB × 48ΔSR	10^{-6}–10^{-7}	70% green Sp/Sm^s, 27% red Sp/Sm^r, 3% red Sp/Sm^s
HΔSA × 48ΔSR	10^{-7}–10^{-8}	99% red Sp/Sm^r, 1% green Sp/Sm^s

[a] Mating was performed in the dark in liquid LB-DMSO medium at 32°C for 24 h.

[b] Calculated as the number of exconjugants (Tc^r Km^r) per donor (Km^r Sp/Sm^r) obtained from the average of three separate experiments.

[c] Sp/Sm^r and Sp/Sm^s phenotypes were calculated from replica patches of 100 randomly picked colonies of the HΔSB × 48ΔSR mating and from all of the colonies which appeared from the HΔSA × 48ΔSR mating (20 to 50 colonies). All exconjugants were PS$^-$, since selection for Km^r concomitantly generated a strain with inactive *puhA* (see the text for details).

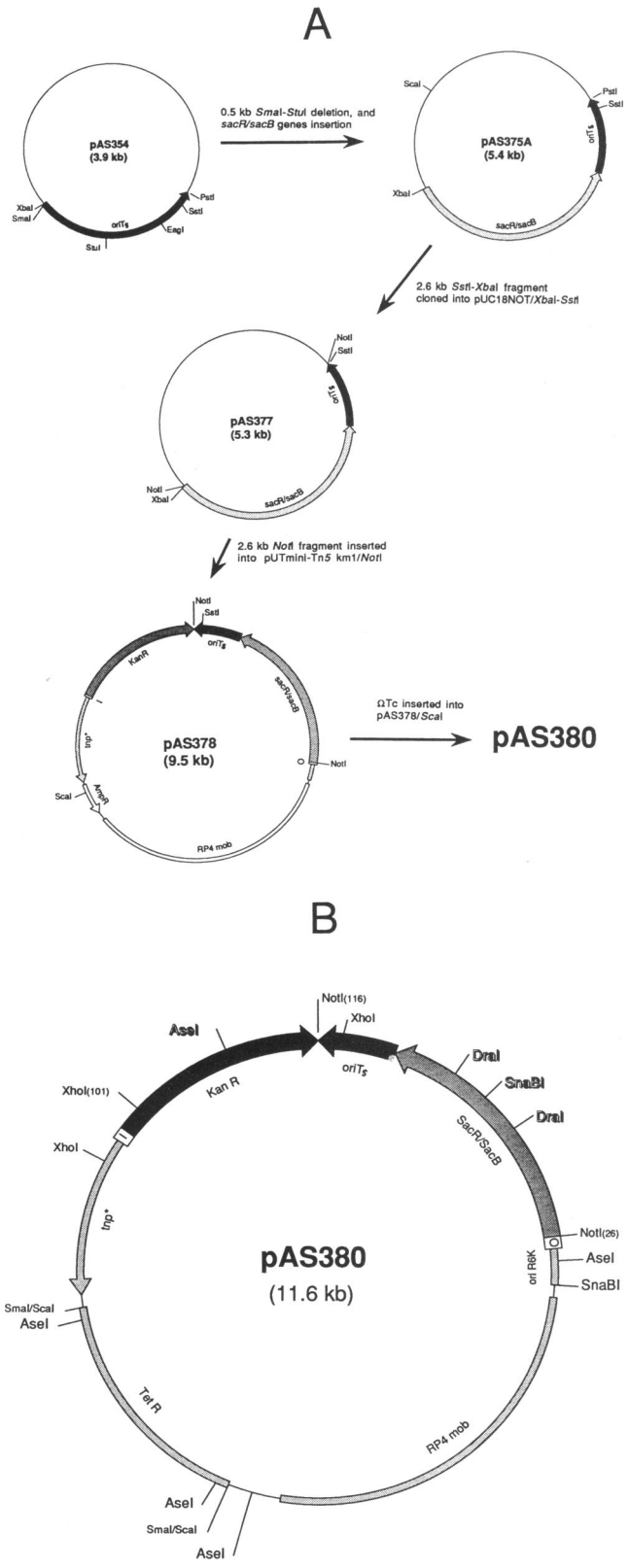

FIG. 3. Construction and physical structure of pAS380. (A) Construction of pAS380. A 0.5-kb *Sma*I-*Stu*I fragment was deleted from plasmid pAS354 (Table 1) and replaced with a 2-kb *Bam*HI fragment containing the *sacR* and *sacB* genes from plasmid Ia which was made blunt ended by Klenow DNA polymerase, to yield pAS380, except that it carries a pBR322 (pMB1) origin of replication (Table 1).

Random KTS insertion into the *R. sphaeroides* 2.4.1 genome. S17-1(pAS380) was mated with strains LΔS, 48ΔS, and ΔS (Table 1). The exconjugants were selected on LB–Sp-Sm–Km for LΔS and on Sis-Km for 48ΔS and ΔS. S17-1(pAS380) cannot grow on Sis minimal medium, since S17-1 is a proline auxotroph. Km^r exconjugants were obtained at a low frequency (10^{-7} to 10^{-8} per donor), despite several repeated matings. This may have been due to the inherent properties of this engineered transposon, in which *tnp** has been displaced outside the I and O ends (7), since similar transposition utilizing S17-1(pMH1701) (37) routinely yields high-frequency transposition (10^{-5} to 10^{-6} per donor) (38).

Km^r exconjugants were replica patched onto Tc-containing medium to screen for exconjugants generated from cointegration of all of pAS380 with *R. sphaeroides* genomic DNA. From 80 to 90% of the Km^r exconjugants were Tc^s. Some of these colonies were further analyzed to determine the location and relative orientation of $oriT_S$. The precise location of KTS in the exconjugants was determined by digestion of total genomic DNA with restriction endonucleases *Ase*I, *Sna*BI, and *Dra*I, and then each of the schizotypes was compared with those of the parental strains (i.e., ΔS, 48ΔS, and LΔS). The relative orientation of $oriT_S$ in each strain was determined by Southern hybridization analysis using an $oriT_S$-containing DNA fragment as a probe. This approach generated Hfr-like donors with different points of origin within the *R. sphaeroides* 2.4.1 genome, including the four remaining endogenous plasmids. Several representative Hfr strains with $oriT_S$ located in the chromosomes are described in Fig. 4 and Table 1.

A gradient of chromosomal marker transfer directed by $oriT_S$. Strains L1 and L9 possess a KTS insertion at about 11 and 3 o'clock, respectively, in chromosome I (Fig. 4). Each of these strains also has an Sp/Sm^r marker in *rrnA* (at 5 o'clock) and dominant normal Crt^+ (red) pigmentation as an additional genetic marker located at 12 o'clock (36). The orientation of $oriT_S$ in L9 is counterclockwise (ccw), while in L1 it is clockwise (cw).

To demonstrate the gradient of transfer of the Sp/Sm^r-red-Km^r markers in both L1 and L9, we performed an interrupted-mating experiment using strain 48ΔSR as the recipient. Strain 48ΔSR, as already described (Table 1), has a Tc^r marker in chromosome II and a recessive Crt^- (green) pigmentation.

The conditions and results of L1 × 48ΔSR and L9 × 48ΔSR matings are described in Fig. 5. As a control, LΔS

plasmid pAS375A. A 2.6-kb *Sst*I-*Xba*I fragment of pAS375A was cloned into the *Sst*I-*Xba*I sites in pUC18NOT (Table 1) and yielded pAS377. A *Not*I fragment containing *sacR-sacB* and $oriT_S$ from pAS377 was excised and inserted into a unique *Not*I site in pUT-mini-Tn*5* Km-1. A 2-kb *Sma*I fragment containing an ΩTc cassette from pHP45ΩTc was inserted into the *Sca*I site of pAS378 to generate pAS380. (B) Physical structure and some relevant genetic loci on pAS380. The arrowheads in $oriT_S$, the gene for Km^r, *tnp**, and *sacR-sacB* indicate their relative orientations. *tnp**, *ori* R6K, the gene for Km^r, and RP4 *mob* were described previously by Herrero et al. (14). I and O denote the I and O ends of IS50, which also indicate the border of the transposed elements, i.e., KTS. The *Ase*I, *Dra*I, and *Sna*BI restriction sites in KTS, used to localize the KTS insertions, are highlighted (shadowed). Other *Dra*I sites may be present in the vector DNA.

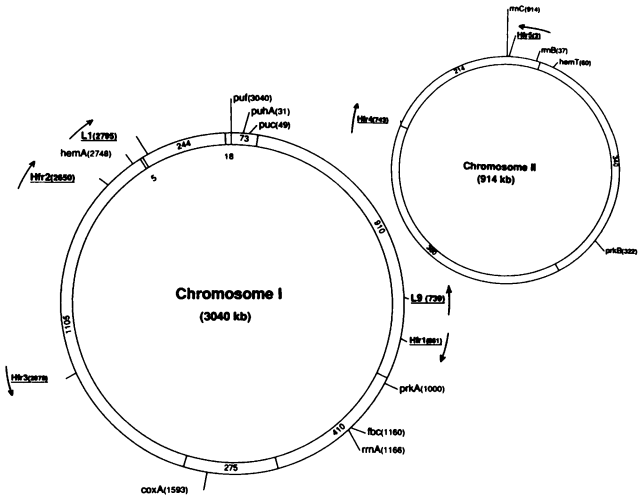

FIG. 4. Location and orientation of $oriT_S$ in some representative Hfr donor strains generated by KTS insertion. The size of each *Ase*I fragment (36) is shown inside or below the fragment. *pufBA* and *rrnC* are arbitrarily assigned the 12 o'clock position on the physical map of chromosomes I and II, respectively. The number in parentheses following a gene or Hfr designation indicates the relative distance (in kilobases) of that particular gene or $oriT_S$ in each Hfr strain clockwise to either *pufBA* or *rrnC*. The arrow by each Hfr strain indicates the $oriT_S$ orientation in that Hfr strain. For example, $oriT_S$ in L9 was located ca. 739 kb from *pufBA* (or at 3 o'clock) in a ccw direction from *pufBA*.

(the parent strain of L1 and L9) was mated with 48ΔSR and no exconjugants ($<10^{-9}$ per donor) were detected after 7, 9, and 20 h of mating. These results are completely consistent with the physical distances of Sp/Sm^r from the origin of transfer, which are 400 kb in L9 and 1,600 kb in L1.

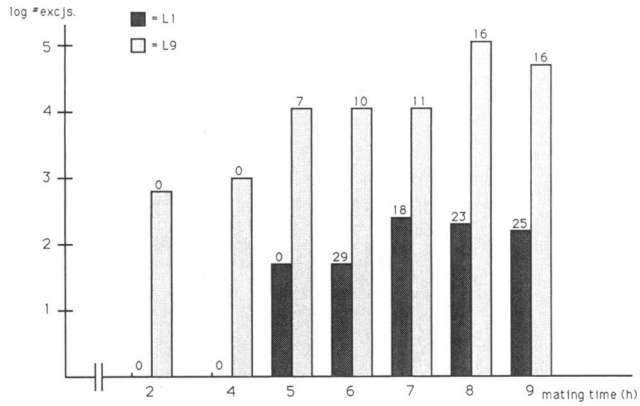

FIG. 5. Histogram of interrupted-mating experiments of L1 × 48ΔSR and L9 × 48ΔSR. Donors and recipients were pregrown photosynthetically in Sis–10% LB until the concentration was approximately 2.0×10^9 viable cells per ml, which corresponds to a 16- to 18-h incubation time. One milliliter each of the donor and recipient were mixed in 5-ml Wheaton glass vials filled with LB and incubated photosynthetically. At the end of each mating period, 200 to 500 μl of the mating mixture was withdrawn and agitated vigorously in a Vortex mixer. The exconjugants (excjs.) were calculated from the colonies which appeared on LB–Sp-Sm–Tc plates. The frequencies of total exconjugants after 7 h of mating for L1 and L9 were 2×10^{-5} and 3×10^{-7} per donor, respectively. The numbers above the bars are percentages of red colonies in the total number of colonies appearing on LB–Sp-Sm–Tc plates.

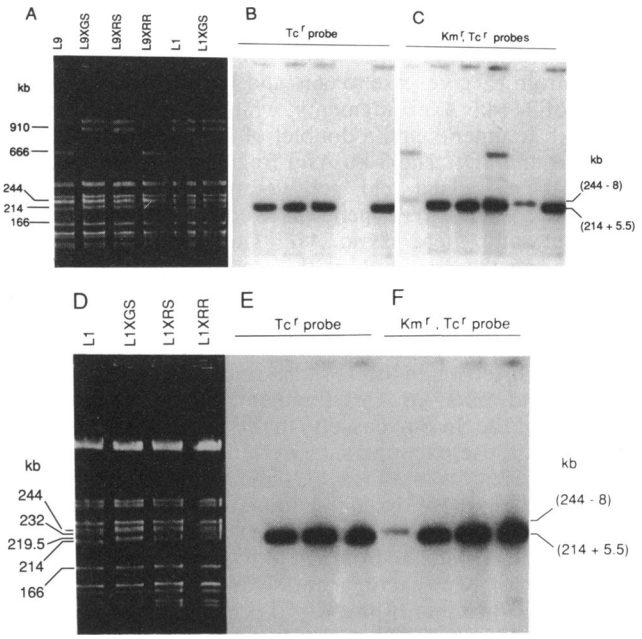

FIG. 6. Genome analysis of L1 × 48ΔSR and L9 × 48ΔSR exconjugants. (A) *Ase*I schizotypes of the following strains: L9, L9XGS (L9 exconjugants, green, Km^s), L9XRS (L9 exconjugants, red, Km^s), L9XRR (L9 exconjugants, red, Km^r), L1, and L1×GS (L1 exconjugants, green, Km^s). (B) Autoradiogram of panel A with the gene for Tc^r as a probe. (C) Autoradiogram of panel A with the genes for Tc^r and Km^r as probes. (D) *Ase*I schizotypes of the following strains: L1, L1XGS, L1XRS (L1 exconjugants, red, Km^s), and L1XRR (L1 exconjugants, red, Km^r). Panels E and F are autoradiograms of panel D with the genes for Tc^r and Tc^r Km^r, respectively, as probes. The Tc^r gene probe was a 5.5-kb *Eco*RI fragment containing the Tc^r gene from pMH1701 (37). The Km^r gene probe was a *Bam*HI fragment containing the Km^r gene from pUC4K. Hybridization signals appearing near the top edges of panels B, C, E, and F are due to the remnants of DNA which stayed in the wells of TAFE gels. The TAFE conditions for panel A were as follows: stage 2, 48-s pulse, 8 h; stage 3, 23-s pulse, 7 h; stage 4, 7-s pulse, 3 h. The TAFE conditions for panel D were as follows: stage 2, 25-s pulse, 9 h; stage 3, 20-s pulse, 5 h; stage 4, 10-s pulse, 4 h.

Transfer of the red (*crt*) marker was detected after 5 h of mating and continued to increase until 9 h of mating. Because of the distance of the *crt* marker relative to the origin of transfer, the frequency of inheritance of red pigmentation was lower than that of Sp/Sm^r. Further analysis of the exconjugants indicated that all of the green Tc^r Sp/Sm^r exconjugants were Km^s, while the red exconjugants from L9 and L1 donors were 89 and 97% Km^r, respectively. The genetic status of the exconjugants was further examined at the DNA level following schizotyping and Southern hybridization analysis as described in the legend to Fig. 6. The green Km^s, red Km^s, and red Km^r exconjugants from L1 and L9 donors exhibited the expected *Ase*I schizotypes. Strains L9 and L1 are both derived from strain LΔS, which has an Sp/Sm^r cassette inserted in *rrnA* (Table 1 and Fig. 4), so that *Ase*I digests the 410-kb fragment normally present in strain ΔS (wild type) into new 244- and 166-kb *Ase*I fragments because of the presence of *Ase*I sites in the Sp/Sm^r cassette (25). In addition, KTS insertion in L9 results in cleavage of the 910-kb *Ase*I fragment into new 244- and 666-kb *Ase*I fragments because of the presence of *Ase*I sites in the Km^r

gene, while KTS insertion in strain L1 generates new ca. 236- and 8-kb *Ase*I fragments from the 244-kb *Ase*I fragment present in the wild type. Therefore, upon digestion with *Ase*I, strain L9 gives rise to 666- and 166-kb fragments and a triplet of 244-kb *Ase*I fragments, while strain L1 shows 236- and 8-kb fragments and a doublet of 244-kb *Ase*I fragments (Fig. 6A and 6D). The 8-kb *Ase*I fragment was not detected under these pulsed-field gel electrophoresis conditions. Strain 48ΔSR has a Tc^r gene inserted into the 214-kb *Ase*I fragment, and there is no *Ase*I site present in this Tc^r cassette, so the presence of this cassette can be detected because of the increased molecular size of the particular fragment into which Tc^r was inserted.

The L9 green Km^s and red Km^s exconjugants showed a 5.5-kb Tc^r insertion in the 214-kb *Ase*I fragment, a doublet of 244 kb, and a 166-kb *Ase*I fragment, which confirmed the presence of the Sp/Sm^r cassette in the 410-kb *Ase*I fragment. Therefore, these exconjugants were new strains with genotypes different from those of their parental strains. The genotypes of these exconjugants are *rrnA*::Sp/Sm^r and *rrnB*::Tc^r, with Crt$^-$ (green) or Crt$^+$ (red) resulting from chromosome I transfer from L9 to 48ΔSR.

The L9 red Km^r exconjugants exhibited an *Ase*I schizotype identical to that of the Km^s L9 exconjugants, with the only exception being that the 910-kb *Ase*I fragment was digested into 244- and 666-kb *Ase*I fragments. Digestion of the 910-kb *Ase*I fragment in this strain indicated the presence of KTS. Thus, the genotype of these exconjugants is *rrnA*::Sp/Sm^r *rrnB*::Tc^r Crt$^+$ KTS insertion at 9 o'clock on chromosome I in a ccw orientation. These results suggested that the Km^r exconjugants from this mating should be the result of transfer and recircularization of all of chromosome I into the recipient cells.

Similarly, the L1 exconjugants showed an *Ase*I schizotype and Southern hybridization analysis results which were entirely consistent with the interpretation of the conjugation data (Fig. 6D, E, and F). For example, Km^r exconjugants exhibited a KTS insertion in the 244-kb *Ase*I fragment and an Sp/Sm^r insertion into the 410-kb *Ase*I fragment, as in the donor strain (L1). However, the donor strain was unambiguously distinguished from the exconjugants by its lack of Tc^r (Fig. 6E and F). Schizotyping in conjunction with Southern hybridization analysis in this experiment clearly demonstrated that the recipients acquired either an Sp/Sm^r gene or a Km^r gene from the donors during mating to yield various exconjugants, as stated above. These combined data suggested that *oriT*$_S$ is able to direct a gradient transfer of chromosomal markers, and all of chromosome I may recircularize into the recipient cells.

Genetic evidence of two unique circular chromosomes. Mating between strains Hfr5 (Table 1; Fig. 4) and LΔS was performed as described in Materials and Methods. The exconjugants were selected on LB–Km–Sp-Sm. The results showed that chromosome II was transferred into the recipient strain, since acquisition of Km^r by LΔS was achieved only after transfer and recircularization of chromosome II from the donor (Table 3).

LΔSf5 is an exconjugant from the mating of LΔS and Hfr5. The genotype of this strain is *rrnA*::Sp/Sm^r KTS (II, 3, ccw) (Table 1; Fig. 4). Schizotyping verified the genotype of this exconjugant (Fig. 7). To examine the donor property of LΔSf5, it was mated with strain 48ΔSR. Selection was made on LB containing Km-Nx, Km-Tc, or Km–Sp-Sm. As shown in Table 3, selection on Km-Nx yielded the highest frequency of exconjugants. Although the donor (LΔSf5) itself can spontaneously mutate to Nx^r, it is easily distinguished from 48ΔSR because of its red pigmentation. The exconjugants of this mating (green Nx^r Km^r) were designated 48ΔSf5. Replica patches of 48ΔSf5 indicated that all of these exconjugants (from 100 representative colonies) were Tc^s. Furthermore, pulsed-field gel electrophoretic analysis of 48ΔSf5 showed a schizotype identical to that of Hfr5. Therefore, chromosome II::Km^r from donor strain LΔSf5 was transferred into recipient strain 48ΔSR and concomitantly replaced the resident chromosome II::Tc^r in 48ΔSR.

TABLE 3. Transfer of chromosome II

Mating[a]	Selection	Frequency[b]	Description[c]
LΔS × 48ΔSR	Sp-Sm–Tc	$<10^{-9}$	No exconjugants
LΔS × Hfr5	Sp-Sm–Km	2.0×10^{-4}	100% red
LΔSf5 × 48ΔSR	Km-Nx	2.0×10^{-5}	Tc^s, 99% green[d]
LΔSf5 × 48ΔSR	Km-Tc	1.0×10^{-7}	100% green
LΔSf5 × 48ΔSR	Sp-Sm–Tc	1.0×10^{-7}	50% green, 50% red
48ΔSf5 × LΔS	Km–Sp-Sm	2.0×10^{-6}	100% red

[a] Strain 48ΔSR or LΔS was the recipient in these Hfr matings.
[b] Calculated as the number of exconjugants divided by the number of donors. The results are averages from two separate experiments.
[c] Relevant phenotypes of exconjugants.
[d] The red colonies (1%) in this mating were spontaneous Nx^r derivatives of strain LΔSf5 (see the text for details).

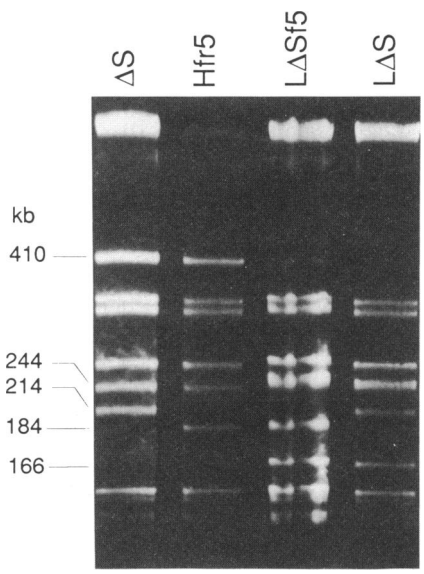

FIG. 7. Schizotype verification of strain LΔSf5. Strain LΔS has an Sp/Sm^r insertion in the 410-kb *Ase*I fragment such that the *Ase*I schizotype of this strain yielded new 244- and 166-kb *Ase*I fragments. Thus, the 244-kb *Ase*I fragment in LΔS is a doublet. Strain Hfr5 has a KTS insertion in the 214-kb *Ase*I fragment such that the 214-kb *Ase*I fragment in this strain was digested into 184- and 30-kb *Ase*I fragments. LΔSf5 is strain LΔS which has undergone chromosome II displacement with chromosome II::Km^r from strain Hfr5. The *Ase*I schizotype of this strain yielded a hybrid pattern inherited from its parental strains; i.e., both the 410- and 214-kb *Ase*I fragments in the wild type (ΔS) were replaced by four new *Ase*I fragments with molecular sizes of 244, 184, 166, and 30 kb. The 30-kb *Ase*I fragment was not clearly visible with this TAFE condition. The TAFE conditions for this experiment were as follows: stage 2, 30-s pulse, 8 h; stage 3, 23-s pulse, 9 h.

Consequently, 48ΔSf5 should be a third-generation donor strain generated from three successive matings (i.e., Hfr5 > LΔSf5 > 48ΔSf5) as shown in Table 3.

Although chromosome II::Km^r from LΔSf5 successfully replaced the resident chromosome II::Tc^r in 48ΔSR, the pigmentation of 48ΔSf5 was still green, as was that of the parental recipient strain (48ΔSR). This result suggested that although all of chromosome II had been transferred, the dominant *crt* marker (i.e., red) was not inherited by the recipient. Therefore, the *crt* marker should be located on another replicon which is different from chromosome II. In fact, from our physical mapping analysis (38) we knew that the *crt* marker is located between *puf* and *puhA* on chromosome I. These data also suggested that the Nx^r marker is not located on chromosome II, since chromosome II::Km^r replaced chromosome II::Tc^r at a high frequency in the Nx^r recipient (48ΔSR).

Selection of an LΔSf5 × 48ΔSR mating mixture on Km-Tc yielded fewer exconjugants (10^{-7} per donor) than when selection was made on Km-Nx. Since both the Km^r and Tc^r markers are located on chromosome II, for inheritance of Km^r Tc^r, chromosome II::Km^r from the donor must not just replace the resident chromosome II::Tc^r from the recipient but must subsequently recombine with the resident chromosome II::Tc^r, yielding a recombinant chromosome II with the Km^r and Tc^r markers in the same replicon. Since the distance between Tc^r (in *rrnB*) and KTS of 48ΔSf5 (near *rrnC*) is relatively short (~30 kb), we anticipate that this recombination event would be rather infrequent, thereby resulting in the low apparent transfer frequency upon simultaneous Km-Tc selection.

Selection of an LΔSf5 × 48ΔSR mating mixture on Sp-Sm–Km yielded a low frequency of exconjugants (Table 3). Since the Sp/Sm^r marker is located on chromosome I, we did not anticipate finding a high frequency of exconjugants from this selection. Half of these exconjugants were red. This probably resulted from transfer of chromosome II::Km^r into 48ΔSR following recombination with the resident chromosome II::Tc^r to gain Tc^r, and then this chromosome II::Km^r Tc^r was retransferred into LΔSf5, yielding Km^r Tc^r Sp/Sm^r exconjugants. The other exconjugants, which were green, indicated that both the Km^r and Sp/Sm^r markers were transferred into the recipient.

Putting all the data together, we have been able to demonstrate genetically that *R. sphaeroides* 2.4.1 contains two separate linkage groups (previously designated chromosomes I and II (36)). Unidirectional chromosome transfer from the Hfr donor strains was mediated by *oriT*$_S$ inserted into either chromosome I or chromosome II. The exconjugants generated by either chromosomal displacement or KTS inheritance will behave as new Hfr donor strains.

DISCUSSION

This study demonstrated that a 600-bp *oriT*$_S$-containing DNA fragment was able to promote chromosome transfer in an oriented manner when provided in *cis*. Thus far, outside of enteric bacteria, chromosome transfer mediated by endogenous plasmids in gram-negative bacteria has been reported only in *Pseudomonas* sp. (16), *Agrobacterium tumefaciens* (5), and this study. Under optimal mating conditions, a chromosomal marker located at a distance of 400 kb from the origin of transfer was mobilized into the recipient at a frequency of 10^{-5} to 10^{-4} exconjugants per donor in a 2- to 5-h mating. The Tra functions required for conjugal transfer in this system appear to be provided by plasmid D, another transmissible plasmid of *R. sphaeroides* 2.4.1 (37), although definitive proof of this conclusion is lacking. The stable presence of plasmid D provides a simple approach to the construction of Hfr donors. However, since plasmid D is also present in recipient cells derived from strain 2.4.1 and if it is analogous to F or the F-like plasmids of *E. coli*, then surface exclusion mechanisms (47) and repression-derepression of plasmid transfer mediated by plasmid D may be involved in determining the ultimate transfer frequency. Further work will be required to verify this.

KTS insertion mutagenesis, in conjunction with schizotyping, has been used to locate *oriT*$_S$ precisely within the genome of *R. sphaeroides*. These insertions appear to be stable, since the transposase gene (*tnp**) is not part of the transposed element (14). All of the resulting *oriT*$_S$-containing strains have been demonstrated to behave like Hfr donors, and some of these Hfr-like strains have been used to demonstrate a gradient of chromosomal transfer. By using a genetic approach, the presence of two unique circular chromosomes was revealed. This analysis supports the results of physical mapping (36) and the use of γ irradiation (46).

Although circular chromosomes are the most common chromosomal topology in bacteria (20), linear chromosomes have also been described (9, 20). Similarly, although bacterial plasmids are predominantly double-stranded circular DNA molecules, some linear DNA plasmids have been reported (9, 19). It is perhaps not a surprise that continued investigation of diverse groups of bacteria has laid to ruin the initial dogma surrounding the bacterial genome. Another element of that dogma has been the belief that bacteria have only a single chromosome, although in certain stages of growth or in certain bacteria multiple copies of the same chromosome can be present (27).

Several gram-negative bacteria which are phylogenetically related to *R. sphaeroides* (49), such as *Rhizobium*, *Agrobacterium*, *Alcaligenes*, *Pseudomonas*, and *Paracoccus* spp., harbor very large extrachromosomal replicons termed megaplasmids (11, 15, 33, 44). The sizes of some of these megaplasmids are much larger than the chromosomes of certain bacteria (20, 46). However, many of these very large replicons have been shown to be self-transmissible (1, 3, 5, 12, 26) and/or curable without an overall deleterious effect on the growth of the organism under all physiological conditions; i.e., loss of the replicon appears to affect only a single growth mode characteristic of the organism. Thus, self-transmissibility and size appear to be two of the criteria used to distinguish plasmids from chromosomes in bacteria. Invariably, when chromosome transfer is recognized it has been found to be mediated by acquisition of functions normally found associated with plasmids and transposons (4, 16, 17, 28, 43). The concept of essentiality has been used to distinguish chromosomes from plasmids. In many instances, this distinction is straightforward; i.e., plasmid loss results in loss of only a limited growth characteristic or ability, with the derivative being able to grow optimally under other growth conditions. Although many plasmids remain cryptic and difficult to cure, isolates of the same species or strain often show a high degree of variability as to plasmid content but not chromosomal profile.

From the standpoint of DNA size, either the 3,050-kb or the 914-kb circular DNA molecule of *R. sphaeroides* 2.4.1 is considered large enough to be a chromosomal DNA element, as described above, and neither is capable of spontaneous transfer. Additionally, these replicons have the following properties which make them more like bacterial chromosomes than plasmids. (i) Each carries critical housekeeping

genes, such as *rrn* (one on the large chromosome and two on the small chromosome) and tRNA genes, including f-*met* tRNA (8), *hemA* or *hemT* (41), *cobA* (36), *gapB* (39), and the transketolase gene (40), which have been found only in the "chromosome" of procaryotic organisms. Insertional inactivation of *rrnB* and *rrnC* or *rrnA* and *rrnC* genes resulted in an extremely slow growth rate of the mutants under all of the growth conditions attempted (8a). Inactivation of any one of the *rrn* operons yielded only a slightly diminished growth response under all of the growth conditions tested (8a). Thus, the presence of these genes appears to be important, at least to maintain normal growth in the laboratory under all of the conditions attempted. (ii) Each chromosome is present in a fixed stoichiometry relative to the others. (iii) All of the strains of *R. sphaeroides* examined by us have two chromosomes apparently similar in size to those observed for 2.4.1. (iv) On the other hand, all of the strains examined displayed extreme plasmid profile variability. As revealed here, each chromosome can behave genetically independently of the others. Thus, we are led to define a chromosome of a procaryotic organism as a DNA molecule of some "minimal" size which is non-self-transmissible and is a member of the genomic complement of an organism consisting of independent replicons, all of which are collectively necessary to provide optimal cell growth under all environmental conditions.

Here we have demonstrated how a combination of physical mapping techniques and pseudogenetics can be exploited to advance knowledge of the genomic structure of bacteria which are not yet considered in the mainstream of scientific development. As revealed here, schizotyping of recombinant organisms provides the necessary physical dimension to the development of a genetic system.

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REFERENCES

1. **Bender, C. L., D. K. Malvick, and R. E. Mitchell.** 1989. Plasmid-mediated production of the phytotoxin coronatine in *Pseudomonas syringae* pv. tomato. J. Bacteriol. **171**:807–812.
2. **Blanco, G., F. Ramos, J. R. Medina, and M. Tortolero.** 1990. A chromosomal linkage map of *Azotobacter vinelandii*. Mol. Gen. Genet. **224**:241–247.
3. **Bodman, S. B., J. E. McCutchan, and S. K. Farrand.** 1989. Characterization of conjugal transfer functions of *Agrobacterium tumefaciens* Ti plasmid pTiC58. J. Bacteriol. **171**:5281–5289.
4. **Clewell, D. B., and M. C. Gawron-Burke.** 1986. Conjugative transposons and the dissemination of antibiotic resistance in streptococci. Annu. Rev. Microbiol. **40**:635–659.
5. **Dessaux, Y., A. Petit, J. G. Ellis, C. Legrain, M. Demarez, J.-M. Wiame, M. Popoff, and J. Tempe.** 1989. Ti plasmid-controlled chromosome transfer in *Agrobacterium tumefaciens*. J. Bacteriol. **171**:6363–6366.
6. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for gram negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77**:7347–7351.
7. **Dodson, K. W., and D. E. Berg.** 1991. A sequence at the inside end of IS50 down regulates transposition. Plasmid **25**:145–148.
8. **Dryden, S. C., and S. Kaplan.** 1990. Localization and structural analysis of the ribosomal RNA operons of *Rhodobacter sphaeroides*. Nucleic Acids Res. **18**:7267–7277.

8a. **Dryden, S. C., and S. Kaplan.** Unpublished data.

9. **Ferdows, M. S., and A. G. Barbour.** 1989. Megabase-sized linear DNA in the bacterium *Borrelia burgdorferi*, the lyme disease agent. Proc. Natl. Acad. Sci. USA **86**:5969–5973.
10. **Fornari, C. S., M. Watkins, and S. Kaplan.** 1984. Plasmid distribution and analysis in *Rhodopseudomonas sphaeroides*. Plasmid **11**:39–47.
11. **Frantz, B., and A. M. Chakrabarty.** 1986. Degradative plasmids in *Pseudomonas*, p. 295–323. *In* J. R. Sokatch and L. N. Ornston (ed.), The bacteria, vol. 10. Academic Press, Inc., New York.
12. **Friedrich, B., C. Hogrefe, and H. G. Schlegel.** 1981. Naturally occurring genetic transfer of hydrogen-oxidizing ability between strains of *Alcaligenes eutrophus*. J. Bacteriol. **147**:198–205.
13. **Guiney, D. G., and E. Lanka.** 1989. Conjugative transfer of IncP plasmids, p. 27–56. *In* C. M. Thomas (ed.), Promiscuous plasmids of gram negative bacteria. Academic Press, Inc., New York.
14. **Herrero, M., V. de Lorenzo, and K. N. Timmis.** 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J. Bacteriol. **172**:6557–6567.
15. **Hogrefe, C., and B. Friedrich.** 1984. Isolation and characterization of megaplasmid DNA from lithoautotrophic bacteria. Plasmid **12**:161–169.
16. **Holloway, B. W.** 1979. Plasmids that mobilize bacterial chromosome. Plasmid **2**:1–19.
17. **Jacob, F., and E. L. Wollman.** 1961. Sexuality and the genetics of bacteria. Academic Press, Inc., New York.
18. **Johnson, D. A.** 1988. Construction of transposons carrying the transfer functions of RP4. Plasmid **20**:249–258.
19. **Kinashi, H., and M. Shimaji.** 1987. Detection of giant linear plasmids in antibiotic producing strains of *Streptomyces* by the OFAGE technique. J. Antibiot. **40**:913–916.
20. **Krawiec, S., and M. Riley.** 1990. Organization of bacterial chromosome. Microbiol. Rev. **54**:502–539.
21. **Lederberg, J., and E. Tatum.** 1946. Gene recombination in *E. coli*. Nature (London) **216**:343–346.
22. **Lorenzo, V., M. Herrero, U. Jakubzik, and K. N. Timmis.** 1990. Mini-Tn*5* transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. J. Bacteriol. **172**:6568–6572.
23. **Lueking, D. R., R. T. Fraley, and S. Kaplan.** 1978. Intracytoplasmic membrane synthesis in synchronous cell populations of *Rhodopseudomonas sphaeroides*. J. Biol. Chem. **253**:451–457.
24. **Pemberton, J. M., and A. R. St. G. Bowen.** 1981. High-frequency chromosome transfer in *Rhodopseudomonas sphaeroides* promoted by broad-host-range plasmid RP1 carrying mercury transposon Tn*501*. J. Bacteriol. **147**:110–117.
25. **Prentki, P., and H. M. Krisch.** 1984. In vitro insertional mutagenesis with a selectable DNA fragment. Gene **29**:303–313.
26. **Pretorius-Güth, I.-M., A. Pühler, and R. Simon.** 1990. Conjugal transfer of megaplasmid 2 between *Rhizobium meliloti* strains in alfalfa nodules. Appl. Environ. Microbiol. **56**:2354–2359.
27. **Punita, S. J., M. A. Reddy, and H. K. Das.** 1989. Multiple chromosomes of *Azotobacter vinelandii*. J. Bacteriol. **171**:3133–3138.
28. **Salyers, A. A.** 1987. Recent advances in *Bacteroides* genetics. Crit. Rev. Microbiol. **14**:49–71.
29. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
30. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1**:37–45.
31. **Sistrom, W. R.** 1977. Transfer of chromosomal genes mediated by plasmid R68.45 in *Rhodopseudomonas sphaeroides*. J. Bacteriol. **131**:526–532.

32. **Smith, C. L., J. Econome, A. Schutt, S. Klco, and C. R. Cantor.** 1987. A physical map of the *E. coli* K12 genome. Science **236**:4481–4490.
33. **Sobral, B. W. S., R. J. Honeycutt, A. G. Atherly, and M. McClelland.** 1991. Electrophoretic separation of the three *Rhizobium meliloti* replicons. J. Bacteriol. **173**:5173–5180.
34. **Sockett, R. E., T. J. Donohue, A. R. Varga, and S. Kaplan.** 1989. Control of photosynthetic membrane assembly in *Rhodobacter sphaeroides* mediated by *puhA* and flanking sequences. J. Bacteriol. **171**:436–446.
35. **Suwanto, A., and S. Kaplan.** 1989. Physical and genetic mapping of the *Rhodobacter sphaeroides* 2.4.1 genome: genome size, fragment identification, and gene localization. J. Bacteriol. **171**:5840–5849.
36. **Suwanto, A., and S. Kaplan.** 1989. Physical and genetic mapping of the *Rhodobacter sphaeroides* 2.4.1 genome: presence of two unique circular chromosomes. J. Bacteriol. **171**:5850–5859.
37. **Suwanto, A., and S. Kaplan.** 1992. A self-transmissible, narrow-host-range endogenous plasmid of *Rhodobacter sphaeroides* 2.4.1: physical structure, incompatibility determinants, origin of replication, and transfer functions. J. Bacteriol. **174**:1124–1134.
38. **Suwanto, A., and S. Kaplan.** Unpublished data.
39. **Tabita, F. R.** 1988. Molecular and cellular regulation of autotrophic carbon dioxide fixation in microorganisms. Microbiol. Rev. **52**:155–189.
40. **Tabita, F. R.** 1991. Regulation and biochemistry of CO_2 fixation. VII International Symposium on Photosynthetic Procaryotes, Amherst, Mass. Abstract no. 93B.
41. **Tai, T.-N., M. D. Moore, and S. Kaplan.** 1988. Cloning and characterization of the 5-amino levulinate synthase gene(s) from *Rhodobacter sphaeroides*. Gene **70**:139–151.
42. **Tomcsanyi, C. M. B., S. H. Phadnis, and D. E. Berg.** 1990. Intermolecular transposition by a synthetic IS50 (Tn*5*) derivative. J. Bacteriol. **172**:6348–6354.
43. **Torres, O. R., R. Z. Korman, S. A. Zahler, and G. M. Dunny.** 1991. The conjugative transposon Tn*925*: enhancement of conjugal transfer by tetracycline in *Enterococcus faecalis* and mobilization of chromosomal genes in *Bacillus subtilis* and *E. faecalis*. Mol. Gen. Genet. **225**:395–400.
44. **Van Montagu, M., and J. Schell.** 1979. The plasmids of *Agrobacterium tumefaciens*, p. 71–95. *In* K. N. Timmis and A. Puhler (ed.), Plasmids of medical, environmental and commercial importance. Elsevier, North-Holland Biomedical Press, New York.
45. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an m13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **9**:259–268.
46. **Walker, E. M., J. K. Arnett, J. D. Heath, and S. J. Norris.** 1991. *Treponema pallidum* subsp. *pallidum* has a single, circular chromosome with a size of ~900 kilobase pairs. Infect. Immun. **59**:2476–2479.
47. **Willetts, N., and R. Skurray.** 1987. Structure and function of the F factor and mechanism of conjugation, p. 1110–1133. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 2. American Society for Microbiology, Washington, D.C.
48. **Willison, J. C., G. Ahombo, J. Chabert, J.-P. Magnin, and P. M. Vignais.** 1985. Genetic mapping of the *Rhodopseudomonas capsulata* chromosome shows non-clustering of genes involved in nitrogen fixation. J. Gen. Microbiol. **131**:3001–3015.
49. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51**:221–271.