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Multiple Chromosomes in Bacteria: Structure and Function of Chromosome II of *Rhodobacter sphaeroides* 2.4.1^T

MADHUSUDAN CHOUDHARY,[1] CHRISTOPHER MACKENZIE,[2] KIRSTEN S. NERENG,[1] ERICA SODERGREN,[2] GEORGE M. WEINSTOCK,[2] AND SAMUEL KAPLAN[1]*

Department of Microbiology and Molecular Genetics[1] and Department of Biochemistry and Molecular Biology,[2] The University of Texas at Houston Medical School, Houston, Texas 77225

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Although multiple chromosomes occur in bacteria, much remains to be learned about their structural and functional interrelationships. To study the structure-function relationships of chromosomes I and II of the facultative photosynthetic bacterium *Rhodobacter sphaeroides* 2.4.1^T, auxotrophic mutants were isolated. Five strains having transposon insertions in chromosome II showed requirements for *p*-aminobenzoic acid (*p*ABA)-dihydroxybenzoic acid (dHBA), serine, thymine, uracil, or histidine. The His, Thy, and *p*ABA-dHBA mutants reverted to prototrophy at low frequency and concordantly lost their transposon insertions from the genome. The Ser, Ura, and *p*ABA-dHBA mutants were complemented by cosmids that carried the region of chromosome II where the transposon insertions were located. The cosmids used for complementation analysis were selected, on the basis of map position, from a set of overlapping clones that had been ordered by a combination of hybridization and restriction endonuclease mapping. These experiments provide the basis for detailed studies of the structure, function, and interaction between each chromosome, and they demonstrate at this early stage of investigation that no fundamental differences exist between each chromosome.

Until recently, a major criterion which defined the prokaryotic cell type was the possession of a single circular chromosome (24). However, two circular chromosomes were found by Suwanto and Kaplan in the photosynthetic bacterium *Rhodobacter sphaeroides* 2.4.1^T (42, 44). Since then, the presence of multiple chromosomes has been demonstrated in four additional *Proteobacteria* species: *Brucella melitensis* 16M (28), *Leptospira interrogans* (54), *Agrobacterium tumefaciens* C58 (1), and, very recently, *Pseudomonas cepacia* 17616 (7). In the case of *A. tumefaciens* C58, the smaller chromosome has been shown to be linear rather than circular. Linear chromosomes have also been found in *Borrelia burgdorferi* B31 (14), *Rhodococcus fascians* (8), and *Streptomyces lividans* 66 (25), as well as six other *Streptomyces* species (25). In light of these investigations, the classical dogma that bacteria (and perhaps prokaryotes in general) possess a single circular chromosome has been rendered obsolete.

This diversity of prokaryotic genome structures raises numerous issues of whether or not special functions, size restrictions, modes of interactions, etc., are associated with or encoded by multiple chromosomes. For example, in the case of bacteria possessing more than one chromosome, are biosynthetic pathways relegated to one of the chromosomes, is there substantial duplication of DNA sequences, or is a haploid set of genes distributed between the replicons? Related to this are questions about the mechanism of replication, partitioning, and stabilization of multiple chromosomes and the selection required to maintain this configuration. The aim of the present study is to begin to address these issues.

The genome of the facultative photoheterotrophic bacterium *R. sphaeroides* 2.4.1^T is contained in two circular chromosomes of 3.0 and 0.9 Mbp (43–45) and five endogenous plasmids (17). The small chromosome, designated chromosome II (CII), is present in 1:1 stoichiometry with the larger chromosome (CI) and is as stably maintained as the larger replicon, and attempts to cure either CI or CII have been unsuccessful (44). The small chromosome encodes two of the three rRNA operons, *rrnB* and *rrnC*, the former having a promoter that is substantially stronger than the promoter for *rrnA*, on CI (12). The smaller linkage group also encodes several tRNA genes (11) and has been shown to be involved in complex expression of the duplicated set of genes encoding Calvin cycle enzymes (20, 21).

To better define the functional roles of both chromosomes of *R. sphaeroides* 2.4.1^T and to assess the physiological basis for the observed genetic diversification, we have constructed numerous transposon insertions throughout the *R. sphaeroides* 2.4.1^T genome. A number of these map to the small chromosome and have coincidentally given rise to strains with auxotrophic phenotypes (i.e., requirements for *p*-aminobenzoic acid [*p*ABA] plus dihydroxybenzoic acid [dHBA], serine, uracil, thymine, or histidine). In addition, and as a prerequisite to an even more detailed study of CII (Fig. 1), we also describe the ordering and high-resolution mapping of 46 cosmid clones into four contigs that cover approximately 85% of the small chromosome as well as the construction of a higher-resolution restriction endonuclease cleavage map of CII. These have provided us with the basis for a comprehensive genome analysis of *R. sphaeroides* 2.4.1^T.

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MATERIALS AND METHODS

Materials, strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise stated, the bacterial strains were grown as follows. *R. sphaeroides* 2.4.1^T and its derivatives were grown at 30°C, either in Luria-Bertani medium (LB), Sistrom's minimal medium A (SMM) (lacking glutamate and

* Corresponding author. Electronic mail address: skaplan@utmmg.med.uth.tmc.edu.

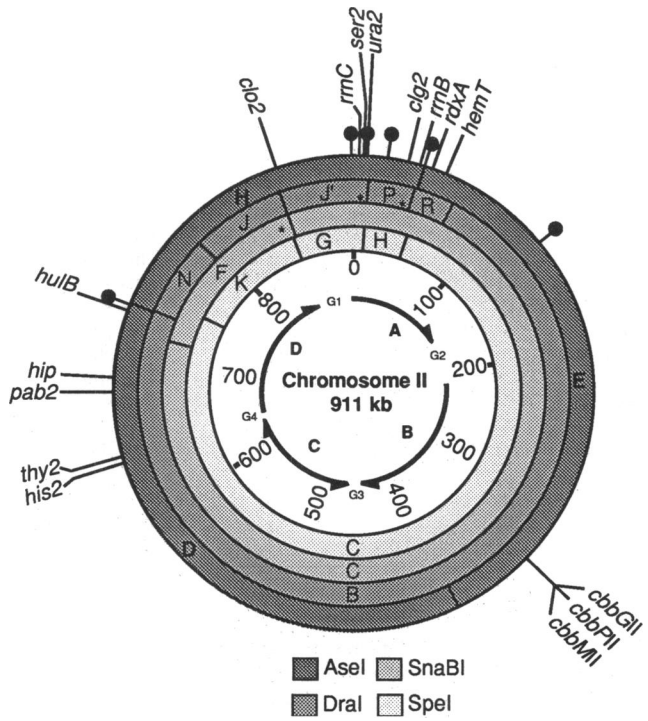

FIG. 1. Physical and genetic map of chromosome II of *R. sphaeroides* 2.4.1^T. The physical map was constructed by using the four infrequently cutting enzymes, with the corresponding sizes of the fragments (in kilobases) as follows: *Ase*I, D = 360, E = 340, and H = 214; *Dra*I, B = 675, J = 65, J′ = 65, N = 55, P = 31, and R = 25; *Sna*BI, C = 784 and F = 130; and *Spe*I, C = 710, G = 65, H = 32, and K = 105. Lettering designations were given as part of a larger genomic map. Asterisks refer to the restriction fragments (<10 kb) which were only detected by cosmid DNA analysis and not detected on the pulsed-field gel. The physical map origin has been placed 42 kb from the end of *Ase*I fragment H within the *rrnC* region. The locations of the four cosmid clone contigs (A, B, C, and D) are shown as arrowed arcs with gaps G1 to G4. The arrows represent the orientation of the cosmid contigs with respect to the chromosome map. Contig C has not been oriented in this manner. The following loci were placed by Tn*5* mutagenesis in this work: auxotrophs, *ser2*, *ura2*, *his2*, *thy2*, and *pab2* (*pABA-dHBA*); and color mutants, *clo2* (orange mutant) and *clg2* (green mutant). Black lollipops define Tn*5* insertions with wild-type phenotypes. The suffix *2* signifies that the insertion is in CII. All other loci were placed by Southern hybridization as follows: *rrnC* and *rrnB*, rRNA genes (11); *rdxA*, a membrane protein involved in a redox process (31); *hemT*, 5-aminolevulinate synthetase (32); $cbbG_{II}$, glyceraldehyde 3-phosphate dehydrogenase (21, 47); $cbbM_{II}$, ribulose-1,5-bisphosphate carboxylase/oxygenase form II (21, 47); $cbbP_{II}$, phosphoribulokinase (20, 47); *hip*, integration host factor β-subunit (39); and *hulB*, histonelike (*Hu*) binding protein (39).

aspartate), or SMM supplemented with 10% (vol/vol) LB. Where appropriate, $K_2TeO_3^{-2}$ (Te) and trimethoprim (TMP) were added to final concentrations of 10 and 50 μg/ml, respectively.

Escherichia coli S17-1 (40) was grown at 37°C. When harboring the plasmid pSUPTn*5*TpMCS, it was grown in LB containing 50 μg of TMP per ml, 60 μg of ampicillin per ml, and 34 μg of chloramphenicol per ml. S17-1 cells containing pLA2917-based cosmids were grown in M63 medium supplemented with 10% (vol/vol) LB. Tetracycline (TET) and TMP were added to final concentrations of 10 and 50 μg/ml, respectively. Antibiotics and other quality-grade reagents were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Transposon mutagenesis and screening for auxotrophs. The mobilizable suicide plasmid pSUP5Tp (38) harbors a Tn*5*-derived transposon which carries TMP resistance (Tp^r). The plasmid was modified to give pSUPTn*5*TpMCS by the introduction of a polylinker (MCS1 and MCS2) at a unique *Spe*I site within the transposon. The sequences of the oligonucleotides used to generate the polylinker are given below. The oligonucleotides were mixed together in equimolar ratios and hybridized to make double-stranded DNA before insertion into the transposon. This resulted in a cluster of restriction sites—*Spe*I-*Sna*BI-*Ase*I-*Ssp*I-*Dra*I—which are cut infrequently within the *R. sphaeroides* genome (43).

R. sphaeroides 2.4.1ΔS was mutagenized with Tn*5*TpMCS by a method essentially described previously (29, 38) and spread on LB-TMP-Te plates. Tellurite selects against S17-1 but not *R. sphaeroides* 2.4.1ΔS (30). The plates were incubated for 3 days at 30°C and then were replica plated onto SMM-TMP-Te plates. After 7 days of incubation, the replicas were compared with the master plates and auxotrophic candidates were picked and purified. Color mutants and colonies having unusual morphology were also selected.

The fidelity of the auxotrophs was checked by streaking purified cultures on LB-TMP and SMM-TMP plates. The LB-TMP master plate was then replicated onto a series of SMM-TMP auxonographic pools to determine the nutritional requirements of the auxotrophs as described previously (9). To verify these results, 0.1 ml of a 300-Klett-unit culture of each auxotroph was spread onto an LB-TMP plate and an SMM-TMP plate. A few grains of the nutrient required by the auxotroph were placed in the center of the SMM-TMP plate. After several days of incubation, a lawn around the nutrient grains was considered a positive indication of the nutrient requirement.

Reversion analysis. Reversion analysis was carried out according to the fluctuation analysis protocol of Luria and Delbrück (26). Auxotrophs were grown in LB-TMP for 24 h, and then 0.1 ml was removed and spread onto an SMM plate. Each culture was diluted 10^6-fold and divided to give 18 cultures of 0.3 ml (each containing 250 to 750 cells) which were then added to the wells of a microtiter dish. The titer of a portion of the remaining diluate was determined to analyze the number of cells in the initial inoculum of each small culture. After 56 h of shaking, 50 μl of each culture was removed, and the aliquots were pooled and their titers were determined to analyze the mean number of cells present in each small culture. The mean number of generations for each culture could then be determined. The remainder of each culture was plated onto an SMM plate and incubated at 30°C for 7 days. The number of plates with revertants and the number of revertants per plate were then determined. The plates were incubated until the colonies were clearly visible. Each revertant was restreaked onto SMM-TMP and SMM plates to determine if TMP resistance had been lost with the return to prototrophy.

The reversion rate was determined with the equation $P_o = e^{-\mu(n_t - n_0)}$, where μ is the mutation rate per generation, P_o is the proportion of plates without any revertants, and $(n_t - n_0)$ is the number of generations for each culture between initial inoculation and plating $[(t = 56) - (t = 0)]$.

Mapping transposon insertions. Isolates were grown to 300 Klett units in LB-TMP. The intact genomic DNA and gel inserts were prepared as described previously (43). Before loading, the plugs were melted at 70°C, and the molten agarose containing the digested DNA was loaded into the wells of a 1× Tris-borate-EDTA–1.2% SeaPlaque GTG agarose (FMC Co.,

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Source or reference
Strains		
R. sphaeroides		
2.4.1	Wild type, 5 endogenous plasmids, prototroph	W. Sistrom (49)
2.4.1ΔS	2.4.1(Δ42-kb plasmid)	A. Suwanto (46)
GR0229	2.4.1ΔS *his*::Tn*5*TpMCS:Tp^r (His, CI)	This study
GR0293	2.4.1ΔS *his*::Tn*5*TpMCS:Tp^r (His, CII)	This study
GR0192	2.4.1ΔS *thy*::Tn*5*TpMCS:Tp^r (Thy, CI)	This study
GR0268	2.4.1ΔS *thy*::Tn*5*TpMCS:Tp^r (Thy, CII)	This study
GR0292	2.4.1ΔS *pABA*::Tn*5*TpMCS:TpMCS:Tp^r (*p*ABA, CI)	This study
GR0295	2.4.1ΔS *pABA-dHBA*::Tn*5*TpMCS:Tp^r (*p*ABA CII)	This study
GR0251	2.4.1ΔS *ura*::Tn*5*TpMCS:Tp^r (Ura, CI)	This study
GR0154	2.4.1ΔS *ura*::Tn*5*TpMCS:Tp^r (Ura, CII)	This study
GR0117	2.4.1ΔS *leu*::Tn*5*TpMCS:Tp^r (Leu, CI)	This study
GR0125	2.4.1ΔS *cys*::Tn*5*TpMCS:Tp^r (Cys, CI)	This study
GR0185	2.4.1ΔS *ser*::Tn*5*TpMCS:Tp^r (Ser, CII)	This study
E. coli S17-1	Pro$^-$ *hsdR hsdM*$^+$ *recA* integrated plasmid RP4-Tc::Mu-Km::Tn*7*	A. Puhler (40)
Plasmids		
pSUP5Tp	Tn*5*-derived transposon carrying Tp^r	Sasakawa (38)
pSUPTn*5*TpMCS	Insertion of a polylinker at a unique *Spe*I site within pSUP5Tp	This study

Rockland, Maine) gel. Pulsed-field gels were run in a CHEF DRII tank (Bio-Rad, Hercules, Calif.) in 1× Tris-borate-EDTA at 200 V. Pulse times were increased linearly from 5 to 75 s over 24 h. The gel was then stained, the results were recorded, and the gel was run as before, but the pulse times varied from 90 to 160 s for a further 18 h.

Cosmid libraries. The mobilizable cosmid vectors pLA2917 (2) and pJRD215 (22) were used to construct two independent *R. sphaeroides* 2.4.1^T genomic DNA libraries (10). The pLA 2917 library comprised 800 clones with an average insert size of 23 kb and was used for the initial ordering of the cosmid clones into contigs. Cosmids from the pJRD215 library were then used to narrow gaps between the ordered contigs.

Complementation. Cosmids mapping to the region of transposon insertion were mated from S17-1 to the auxotrophs as described for the transposon mutagenesis above (29, 38). The exconjugants were plated on LB-TMP-TET (to determine the mating efficiency) and SMM-TMP-TET plates (to determine if complementation had occurred). The exconjugants were plated at dilutions which should give a minimal number of revertants. Complementing cosmid DNA obtained from the auxotrophs was then reintroduced into *E. coli* S17-1 and mated into the same auxotrophic strains from which it had been isolated. This ensured that complementation rather than reversion within the auxotrophic strain had resulted in the restoration of prototrophy.

Hybridization of cosmid library. Individual clones were picked, grown in wells of 96-well microtiter plates, and stored at −70°C. A prong device was used to transfer overnight *E. coli* cultures from each microtiter plate onto a 150-mm-diameter LB plate containing 10 μg of TET per ml. After overnight growth on LB plates, colonies were transferred onto a Qiabrane nylon membrane (Qiagen, Inc., Chatsworth, Calif.). The membranes were processed as described previously (27). After briefly drying the membranes, the DNA was cross-linked to the membrane by using an energy mode of 120,000 μJ/cm^2 in a UV cross-linker from Hoefer Scientific Instruments. Colony hybridization was performed as described previously (27). The library was initially screened with pulsed-field gel electrophoresis-isolated CII-specific *Ase*I fragments D, E, and H of *R. sphaeroides* 2.4.1^T (Fig. 1). This sublibrary of 92 CII-specific cosmid clones was then used for further hybridization analysis. The membranes were prehybridized for at least 15 min at 42°C in a 10-ml solution containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), 1× deionized formamide, and 0.1 ml of salmon sperm DNA (10 μg/ml). Hybridizations were carried out overnight at 42°C in a hybridization incubator (Robbins Scientific Corporation, Sunnyvale, Calif.). The membranes were washed once in a 10-ml solution of 2× SSC–0.1% SDS for 15 min at room temperature and once in 0.1× SSC–0.1% SDS for 15 min at 65°C. Autoradiographic signals were obtained by exposing X-ray films (Kodak XAR-5) to the membranes for 4 to 48 h.

Oligonucleotides. The oligonucleotides were synthesized by an Applied Biosystems (model 394) DNA/RNA synthesizer by using the phosphoramidite method. They were then purified with an oligonucleotide purification cartridge, also supplied by Applied Biosystems. The primers were synthesized at the Core Facility of the Department of Microbiology and Molecular Genetics at the University of Texas Health Science Center in Houston.

The following primers were used in this study: pLA1, 5′-GG CGCAGGGGATCAAGATC-3′; pLA2, 5′-CCTGTCTCTT GATCAGATC-3′; pJRD1, 5′-CTGCAGGTCGACGGATC-3′; pJRD2, 5′-CTTATGGTACCCGGGGATC-3′; MCS polylinker-1, 5′-CTAGTACGTATTAATATTTAAAG-3′; and MCS polylinker-2, 5′-CTAGCTTTAAATATTAATACGTA-3′.

DNA probes. The site-specific probe was generated from individual cosmid clones by primer extension in a linear PCR with primers (pLA1 or pLA2). Priming sites were located in the cosmid arms at the arm-insert junction. Primer extension reactions were performed in a total volume of 10 μl with 500 ng of cosmid DNA, 2 pmol of primers, 5 U of *Taq* polymerase (Promega Corp., Madison, Wis.), 10 μCi of [α-^{32}P]dCTP (3,000 Ci/ml), buffer, and deoxynucleoside triphosphate-dideoxynucleoside triphosphate mix (as recommended by the supplier). After initial heating at 92°C for 5 min, reactions were run through 40 cycles of denaturation for 60 s at 92°C, an annealing cycle for 60 s at 55°C, and extension for 30 s at 72°C in a PCR thermal cycler (M. J. Research, Inc., Watertown, Mass.). The probe was purified on a Nuctrap push column (Stratagene Co., La Jolla, Calif.).

TABLE 2. Characteristics of auxotrophs used in this study

Strain (chromosome)[a]	Phenotype (required nutrient)	Reversion (rate/cell/generation)	Tp^s (no. Tp^s/no. of revertants found)[b]
GR0229 (I)	Histidine	10^{-9}	184/188
GR0293 (II)	Histidine	10^{-9}	177/177
GR0192 (I)	Thymine	10^{-10}	23/23
GR0268 (II)	Thymine	10^{-10}	3/3
GR0292 (I)	*p*ABA	10^{-9}	104/104
GR0295 (II)	*p*ABA-dHBA	10^{-11}	2/2
GR0251 (I)	Uracil	10^{-8}	295/295
GR0154 (II)	Uracil	10^{-4}	0/480
GR0117 (I)	Leucine	10^{-9}	30/30
GR0125 (I)	Cysteine	10^{-9}	34/34
GR0185 (II)	Serine	10^{-5}	0/480

[a] The chromosome number (CI or CII) to which the transposon insertions have been mapped.

[b] All of the auxotroph revertants which grew on SMM plates were restreaked onto SMM and SMM-TMP plates to determine Tp^s.

Enzymatic manipulation of DNA and Southern hybridization. DNA was prepared as described previously (27). The cosmids were digested with *Eco*RI, *Bam*HI, and *Ssp*I and then were separated on an 0.8 to 1.0% agarose gel. Restriction digests were performed under conditions recommended by the manufacturers (New England Biolabs, Beverly, Mass.; Promega Corp.). Oligonucleotides were end labelled (37) with terminal deoxynucleotidyl transferase (Life Technologies, Inc., Gaithersburg, Md.), and Southern hybridization was performed as described previously (27).

RESULTS

Isolation of auxotrophs. After Tn*5* mutagenesis, we screened approximately 10,000 mutants for the ability to grow on minimal media. We recovered 33 auxotrophic strains, each strain having a transposon insertion at a different position within the genome. These strains fell into 18 classes, each class requiring a different nutritional supplement for growth. Five auxotrophic strains were found to have transposon insertions which mapped to CII. Auxonography indicated that these had the following phenotypes: His$^-$, *p*ABA-dHBA$^-$, Ser$^-$, Ura$^-$, and Thy$^-$ (Table 2). The other auxotrophic classes were due to the insertions mapped to CI (unpublished data). The auxotrophs retained their phenotypes when grown under a variety of growth conditions; i.e., they would grow under anaerobic light or anaerobic dark conditions only when the medium was supplemented with the appropriate nutrient. With the exception of Ser$^-$, different auxotrophs having the same phenotype were also found to map to CI. This suggested a randomization of essential housekeeping information between the two chromosomes, possibly even when the intermediate products are part of the same metabolic pathway. This is an extremely significant observation because it directly illustrates the apparent lack of selectivity regarding the distribution of metabolic markers to each chromosome.

Two different approaches were used to demonstrate that the transposon insertions were responsible for the observed phenotypes. (i) We generated revertants and determined whether they were Tp^r or Tp^s, and (ii) we carried out complementation analysis with cosmids specific to the regions of the chromosome containing the transposon.

Reversion analysis. We carried out reversion analysis of 11 different strains (Table 2): 4 different pairs of auxotrophs, with each member of a pair having a similar phenotype but containing a transposon on different chromosomes, 2 additional auxotrophs mapping to CI, and a final auxotroph mapping to CII. In nine strains, reversion to prototrophy occurred at a low frequency ($\leq 10^{-8}$ per cell per generation), which nearly always resulted in the simultaneous loss of the nonselected Tp^r marker. Two of the auxotrophs, GR0154 (Ura$^-$, CII) and GR0185 (Ser$^-$, CII), showed high reversion rates (10^{-4} to 10^{-5}), and all of the revertants remained Tp^r, indicating that the transposon was still present. Auxotrophs with similar reversion characteristics, i.e., high reversion rates and Tp^r, were also observed in two strains (GR0283 and GR0280 [data not shown]) auxotrophic for adenosine and glutamate, respectively, with transposon insertions in CI. In all strains in which the reversion rate was high and Tp^r was maintained, the revertants failed to grow at rates comparable to that of the prototrophic parent, suggesting that the revertants were pseudorevertants (e.g., second-site suppressors).

Where possible, reversion analysis was carried out with strains showing the same auxotrophic phenotype but having transposon insertions on different chromosomes. We found that for the Thy$^-$ and His$^-$ auxotrophs, reversion rates were comparable irrespective of the chromosome into which the transposon had inserted. In the case of Ura$^-$, the reversion rate for the CII insertion was much higher (10^4-fold) than that for CI. The *p*ABA-dHBA$^-$ CII insertion was the most stable insertion of those examined and reverted at a rate 20-fold lower than that of a CI insertion which had a *p*ABA$^-$ phenotype. These results suggested that for those auxotrophs examined, reversion rates of transposon insertions are chromosome independent.

We also determined whether the reversion rates of other CI auxotrophic markers were comparable to those of markers seen on CII. We chose Leu$^-$ and Cys$^-$ auxotrophs as representatives of CI markers because no cognate markers have yet been found on CII. Both Cys$^-$ and Leu$^-$ auxotrophs reverted at rates (Table 2) comparable to those found for the His$^-$, Thy$^-$, and *p*ABA-dHBA$^-$ auxotrophs as described above. Thus, it appears that markers on the two chromosomes generally behave in a similar manner with respect to reversion.

Complementation analyses. To further confirm that the transposon insertions had generated the observed phenotypes, cosmids mapping precisely to the region of transposon insertion were used to complement the auxotrophic phenotypes. To minimize the risk of detecting revertants rather than complemented colonies, the number of cells spread on each plate was at least 10-fold lower than their reversion frequency. Cosmids were not available for complementation of either the His$^-$ or Thy$^-$ auxotrophs because transposon insertions generating these phenotypes lay within a gap in the cosmid map. Complementation was successful for the Ser$^-$ (pUI8503), Ura$^-$ (pUI8148 and pUI8503), and *p*ABA-dHBA$^-$ (pUI8536) auxotrophs. On the basis of hybridization analysis and limited DNA sequence, the pUI8536 cosmid also contains the *hip* gene for one subunit of the integration host factor (39). In all cases, the Tp^r marker was present. To confirm these results, the cosmids were isolated from the complemented auxotrophs, transformed into *E. coli* S17-1, and then reintroduced into the auxotrophic strains. In all cases, the cosmids complemented as before, showing that complementation and not reversion restored the cells to prototrophy. Additionally, the cosmids were introduced into the auxotrophs and the exconjugants were plated on LB agar (supplemented with the relevant nutrient) containing TMP-TET. In this way, we were selecting only for the presence of the cosmid, rather than prototrophy, thus reducing the selective pressure for reversion. The results prove that cosmid complementation, rather than reversion, had

restored prototrophy. These results suggest that there are genes on CII that are essential for survival of this organism and cannot be complemented by the presence of the larger linkage group.

Ordering cosmid clones in CII. Probes generated from CII-specific *Ase*I fragments hybridized to 92 clones from a genomic cosmid clone bank. These cosmid clones contained an average insert of 23 kb of DNA. The largest DNA insert was approximately 35 kb, and the smallest was slightly over 1 kb (data not shown). However, only a total of eight clones were represented by these two categories. Although the total CII coverage was approximately 2.5- to 3.0-fold, the genomic coverage was in excess of 6.0-fold.

Our strategy for mapping was first to assign each DNA insert, by hybridization, to one of the three *Ase*I restriction fragments making up the small chromosome. Each cosmid was subsequently linked within its cognate larger *Ase*I fragment, followed by linkage of the three *Ase*I fragments themselves. After screening 92 cosmid clones with site-specific DNA probes from each end of the insert, three different hybridization patterns were observed: (i) no hybridization, (ii) hybridization with both probes, and (iii) hybridization with one probe (i.e., one of the two ends). Clones hybridizing with one probe were analyzed further by *Eco*RI and *Bam*HI restriction analysis. This showed the extent of overlap of the cosmid with its neighbor (data not shown) and determined the insert size (Table 3). Hybridizing clones having the smallest overlap and the largest insert were selected and used for the identification of the next overlapping cosmid, and so on. These cosmids are shown ordered in Fig. 2. To confirm the order, most neighboring cosmids were shown to overlap by reciprocal hybridization with the corresponding insert ends.

This generated 46 clones ordered into four contigs that covered approximately 85% of the chromosome as follows: contig A, 187 kb, gap G2, 80 kb; contig B, 183 kb, gap G3, 10 kb; contig C, 211 kb, gap G4, 10 kb; and contig D, 176 kb, gap G1, 20 kb (Table 3 and Fig. 2). We also observed that nine cosmids gave a positive hybridization signal when probed independently with radiolabelled *Ase*I fragments D and E. On the basis of restriction pattern, these cosmids were not overlapping, suggesting that repetitive elements or duplicated genes may be encoded within these fragments of the small chromosome.

High-resolution *Ssp*I mapping. *Ssp*I was used to digest the cosmids, and on average, this enzyme cut once per insert. Radiolabelled oligonucleotides pLA1 and pLA2 were used to probe *Ssp*I-generated vector-insert hybrid fragments. Thus, the exact location of the *Ssp*I site was determined within the insert and with respect to the vector arms (Fig. 2). Over the 800-kb ordered cosmid map there were 40 *Ssp*I sites (i.e., one site per 20 kb). In addition, the contigs were oriented with respect to the rare cutting sites on the physical map of the small chromosome generated by Suwanto and Kaplan (43).

TABLE 3. Clones of the ordered cosmid collection

Contig	Clone	Map to *Ase*I fragment	Insert size (kb)	No. of *Ssp*I sites
A (187 kb)	pUI8207	H	19.9	2
	pUI8737	H	24.0	2
	pUI8503	H	26.9	1
	pUI8148	H + E	29.1	2
	pUI8215	H + E	31.0	2
	pUI8519	E	27.8	1
	pUI8508	E	23.7	3
	pUI8656	E	24.2	1
	pUI8318	E	21.1	1
	pUI8790	E	23.4	1
B (183 kb)	pUI8736	E	28.1	1
	pUI8075	E	24.3	1
	pUI8025	E	16.6	0
	pUI8219	E	23.3	2
	pUI8127	E	27.1	0
	pUI8463	E	22.0	0
	pUI8093	E + D	22.0	0
	pUI8590	E + D	22.4	1
	pUI8465	D	31.1	1
	pUI8520	D	30.7	0
	pUI8531	D	25.2	1
C (211 kb)	pUI8483	D	20.6	1
	pUI8327	D	26.6	1
	pUI8758	D	25.4	2
	pUI8027	D	24.9	2
	pUI8430	D	24.0	1
	pUI8682	D	12.5	1
	pUI8183	D	24.5	2
	pUI8468	D	22.8	3
	pUI8059	D	22.0	0
	pUI8143	D	27.1	0
	pUI8569	D	30.5	1
	pUI8750	D	32.1	2
	pUI8275	D	20.7	1
	pUI8575	D	21.0	2
D (176 kb)	pUI8536	D	24.2	1
	pUI8603	D	29.1	2
	pUI8621	D	22.0	0
	pUI8064	D	22.5	1
	pUI9490	D	12.0	0
	pUI8591	D	19.2	0
	pUI8178	D + H	13.4	1
	pUI8324	H	30.7	0
	pUI8721	H	17.5	1
	pUI9678	H	7.5	0
	pUI8780	H	7.2	0

DISCUSSION

Essential functions on CII of *R. sphaeroides*. We have demonstrated that transposon insertions in the small chromosome of *R. sphaeroides* 2.4.1^T result in auxotrophic phenotypes. These phenotypes are observed under a variety of growth conditions, (e.g., aerobic and anaerobic growth in light and dark). Thus, CII provides functions that are essential for growth in minimal media under a variety of environmental conditions. It has been suggested that two distinguishing features of chromosomes are that they are nonexpendable and nonautonomous (6). Nonexpendable means that the genetic element is absolutely required for the growth of the organism in its natural environment, and nonautonomous means that the linkage group in question cannot be uncoupled from the genome and a chromosome must be considered in the context of the entire complement. The results presented in this report show that CII fulfills both of these criteria.

To demonstrate that the transposon insertions were responsible for the observed phenotypes, we employed two approaches, reversion analysis and complementation with cosmids localized to the sites of insertion. For the *p*ABA-dHBA auxotroph, all prototrophic revertants had simultaneously lost the transposon (become Tp^s), thus fulfilling the expectation of the reversion test. The *p*ABA-dHBA mutant also satisfied the second criterion, namely, being complemented by a cosmid

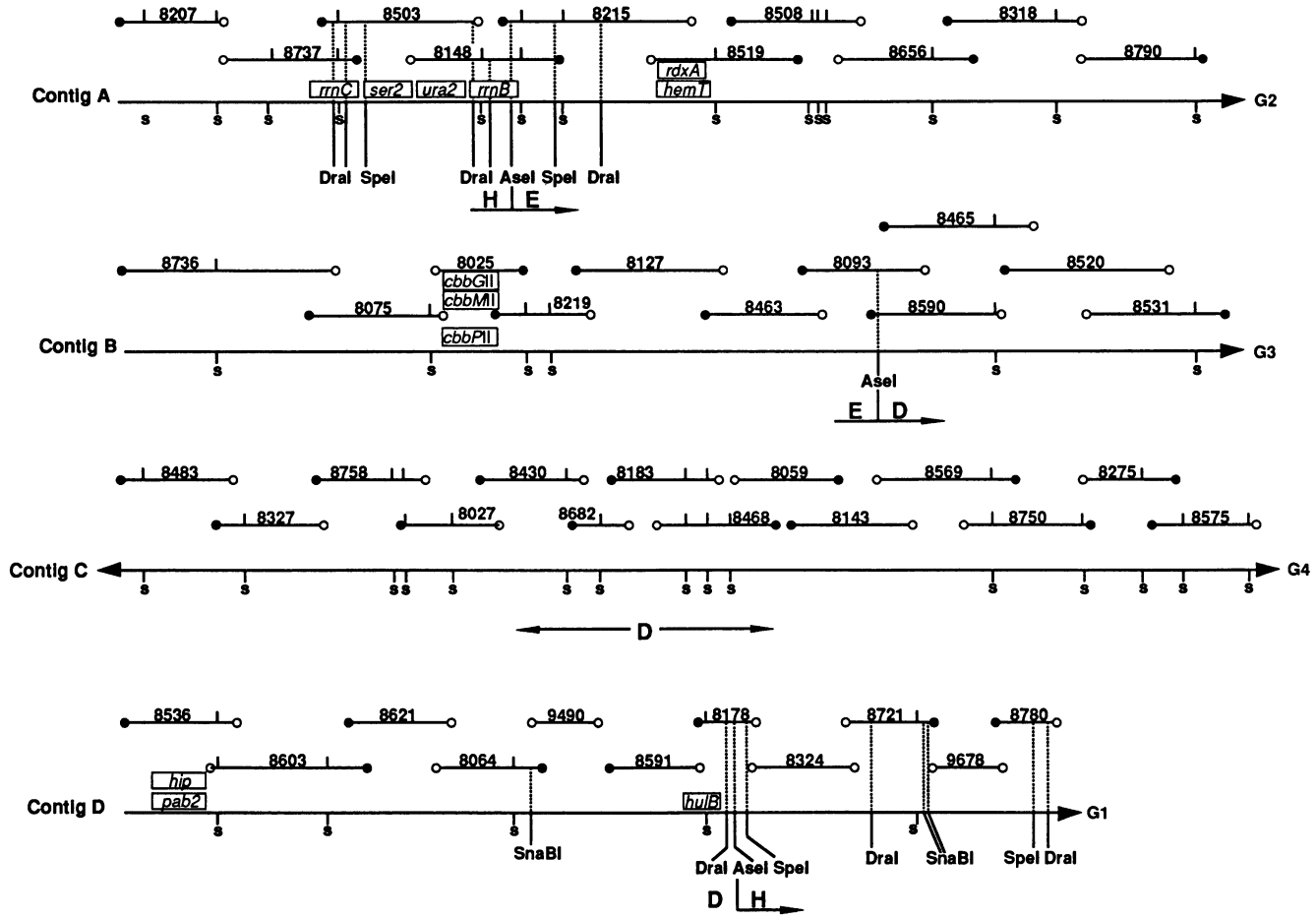

FIG. 2. High-resolution physical and genetic map of the small chromosome. This map represents four ordered cosmid contigs, A (187 kb), B (183 kb), C (211 kb), and D (176 kb), and four gaps, G1 to G4. With the exception of contig C, the contigs have been arranged with respect to the physical and genetic map presented in Fig. 1. The *R. sphaeroides* 2.4.1^T DNA inserts within individual cosmids are represented by horizontal lines. On each cosmid, the pLA1 and pLA2 primer sites are shown by open and closed circles, respectively. The orientations of the inserts in cosmids, pUI8324, pUI9490, and pUI9678 have not been determined, so both ends of the inserts have been given open circles. On contig D, gaps were filled with cosmids pUI9490 and pUI9678 from a pJRD215 library. All other cosmids are of pLA2917 origin. Mapped genes are shown by boxes above each cosmid. The letter "s" specifies an *Ssp*I cleavage site. Arrowed letters beneath the contigs refer to the *Ase*I fragment to which they have been localized. Other rare sites (see text) are also given beneath each contig, with dotted vertical lines showing their positions on the relevant cosmids.

that contained an insert from the immediate region of the transposon insertion.

For two mutants, the His and Thy auxotrophs, no cosmids were available for the complementation test. However, both behaved in the expected manner in the reversion test, supporting the hypothesis that the insertions into CII were responsible for the observed auxotrophies. For the other two mutants, requiring serine and uracil, reversion produced a high frequency of prototrophs that still contained the transposon. However, because both of these auxotrophs could be complemented to prototrophy by cosmids with inserts overlapping the sites of the transposon insertions, the simplest explanation is that the location of the mutation must be in this region of CII and is most likely the insertion itself. The reversion behavior of these strains could be due to second-site mutations that suppress the defect in the insertion mutant. Alternatively, if the insertion causes the defect indirectly (e.g., by a polar effect on expression in an operon or by inactivation of a positive regulator of expression of the biosynthetic genes), mutations that suppress this effect would also give rise to the observed phenotype. The observation that the Tp^r revertants from these strains did not grow as well as either the prototrophic parent or the complemented strains is consistent with suppression involving a second alteration.

The fact that CII mutations can result in requirements for amino acids, pyrimidines, or vitamins indicates that a range of central metabolic processes are dependent on genes of this chromosome. Thus, there may not be strict partitioning of functions required for certain types of growth. Rather, these results are more consistent with a distribution of numerous overlapping functions between the two chromosomes, as in multichromosomal eukaryotic genomes. This constitutes a significant initial characterization of the two chromosomes in *R. sphaeroides* 2.4.1^T.

The serine auxotrophy has, to date, only been found on CII. Serine biosynthesis from 3-phosphoglycerate requires few enzymes (36, 48), making it possible that these could be localized on this chromosome. However, it is also possible for a nutritional requirement to be created by excess degradation, and in this connection, the well-known serine dehy-

dratase activity (19) found in other bacteria is a possibility. The other auxotrophic phenotypes can be seen as resulting from insertions in either chromosome. The biosyntheses of both pyrimidines and histidine are multistep processes. Numerous unlinked *ura* genes are found in other bacteria (34), and this genetic arrangement appears to be the case in *R. sphaeroides* 2.4.1^T, because a number of uracil-requiring mutations have been mapped to different locations on CI (unpublished data).

The requirement for both *p*ABA and dHBA in one auxotroph could reflect a defect in a step early in the biosynthesis of these compounds, e.g., after shikimate but before a branch point in the pathway. The CI mutation, requiring only *p*ABA, would be predicted to occur later in the pathway and after the branch point that leads to *p*ABA. The insertion in CII occurred in a sequence similar to the tryptophan synthase β-subunit (unpublished results), while *p*ABA synthase is similar to anthranilate synthase. Thus, this region may contain a cluster of genes encoding enzymes related to those for aromatic biosynthesis. Because there is no aromatic amino acid requirement in this mutant, these genes are not essential for its synthesis. Perhaps they have diverged for the synthesis of other compounds. We do not know if the insertion inactivates *p*ABA synthesis by disrupting the *trpB*-like gene or is polar on downstream functions. Answers to these questions await extensive DNA sequence information.

The thymine-requiring phenotype is interesting since it may occur by mutations in either chromosome. The biosynthesis of thymine (as dTMP) generally occurs in a single step performed by thymidylate synthetase. Because we have only limited DNA sequence data at this time, it is impossible to arrive at a firm conclusion. In this connection, it is noteworthy that these mutants grow on LB which contains only low levels of thymine compounds, often not enough to support the growth of a thymidylate synthetase mutant. However, several significant conclusions are possible from the limited data made available through these initial studies. There appears to be a clear division of labor residing within the chromosome complement of *R. sphaeroides*. New and as yet uncharacterized genetic interactions appear to be involved. Structural gene information encoding well-established biosynthetic pathways appears to have shown greater diversity of informational content than anticipated.

Ordered cosmid map. The number of cosmids used to provide the ordered array of CII was statistically sufficient to provide greater than 99% coverage of the genome, yet only 85% of CII was covered. Similar observations of gaps were made by other groups involving genomic ordering such as *Mycobacterium leprae* (13), *Bacillus subtilis* (3), *Mycoplasma pneumoniae* (50, 51), *E. coli* (23), and *Helicobacter pylori* (5). In all, 46 cosmids, 58 restriction endonuclease cleavage sites, and approximately 20 genes and insertions have now been mapped to CII and together provide a relatively high density of landmarks on this 900-kb replicon.

Complex genome structure and bacterial classification. So far, the existence of more than one chromosome has been reported only in the five genera of bacteria described above: *R. sphaeroides* 2.4.1^T, *B. melitensis* 16M, *A. tumefaciens* C58, *P. cepacia* 17616, and *L. interrogans*. The first four organisms belong to the *Proteobacteria* class (52, 53). *L. interrogans*, which also has two chromosomes, belongs to the family *Spirochaetaceae*. The metabolic richness, genome plasticity, and diversity of species evolving from purple photosynthetic ancestry in this subdivision are remarkable. In addition to these cases, some bacterial species of the genera *Pseudomonas*, *Rhizobium*, *Agrobacterium*, and *Alcaligenes* contain large accessory genetic elements (also called megaplasmids) which encode housekeeping functions and express species-specific phenotypic characteristics. For example, *Pseudomonas* spp. harbor megaplasmids carrying genes to degrade aromatic and other organic compounds (18). *Rhizobium meliloti* harbors two incurable megaplasmids, pSym-a and pSym-b (41), which are involved in nodulation and symbiotic nitrogen fixation (4). *Rhizobium tropici* harbors a megaplasmid that encodes a key enzyme in the tricarboxylic acid cycle, citrate synthase, and genes responsible for effective symbiotic nodulation (35). In addition to two chromosomes, *R. sphaeroides* 2.4.1^T also harbors five endogenous plasmids (17). In separate hybridization experiments using an amplified *fnr*-like sequence (39) and *R. meliloti ftsZ*, required for septum formation, as probes (33), positive signals were given by the 110-kb plasmid (pRS241a) of *R. sphaeroides* 2.4.1. Copies of both of these genes are also found on CI. The occurrence of all of these important phenotypic traits on accessory genetic elements raises the possibility that these linkage groups are either incipient, transient, regressing, or established chromosomes. Thus, the distinction between chromosome and plasmid takes on increased interest as the diversity of microbial systems under study increases.

A closely related bacterium, *Rhodobacter capsulatus* SB1003, has a single circular chromosome of 3.7 Mb (15, 16) that encodes four ribosomal operons and a single *hemA* gene that encodes 5-aminolevulinate synthase (15). In contrast, *R. sphaeroides* contains two homologs, *hemA* and *hemT*, with one located on each chromosome (32). Furthermore, *R. sphaeroides* 2.4.1^T also contains $cbbP_I$ and $cbbP_{II}$ (20, 47), $cbbG_I$ and $cbbG_{II}$ (21, 47), $cbbA_I$ and $cbbA_{II}$ (21, 47), and *rdxA* and *rdxB* (31) duplicate genes, with one copy of these homologous loci on each chromosome. Also, in these two organisms, the photosynthetic gene clusters are differently organized. This makes *R. sphaeroides* 2.4.1^T unmistakably different from *R. capsulatus* and thus a model system to study the origin and evolution of gene duplication and diploidy. Thus, the apparent presence of partial diploidy existing between two differently sized chromosomes makes such organisms neither haploid, nor diploid, nor polyploid. They appear to occupy some middle ground, and thus we suggest that they be designated mesoploid.

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