Low-resolution sequencing of *Rhodobacter sphaeroides* 2.4.1^T: chromosome II is a true chromosome

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The photosynthetic bacterium *Rhodobacter sphaeroides* 2.4.1^T has two chromosomes, CI (~3.0 Mb) and CII (~0.9 Mb). In this study a low-redundancy sequencing strategy was adopted to analyse 23 out of 47 cosmids from an ordered CII library. The sum of the lengths of these 23 cosmid inserts was ~495 kb, which comprised ~417 kb of unique DNA. A total of 1145 sequencing runs was carried out, with each run generating 559±268 bases of sequence to give ~640 kb of total sequence. After editing, ~2.8% bases per run were estimated to be ambiguous. After the removal of vector and *Escherichia coli* sequences, the remaining ~565 kb of *R. sphaeroides* sequences were assembled, generating ~291 kb of unique sequences. BLASTX analysis of these unique sequences suggested that ~131 kb (45% of the unique sequence) had matches to either known genes, or database ORFs of hypothetical or unknown function (dORFs). A total of 144 strong matches to the database was found; 101 of these matches represented genes encoding a wide variety of functions, e.g. amino acid biosynthesis, photosynthesis, nutrient transport, and various regulatory functions. Two rRNA operons (*rnb* and *rnc*) and five tRNAs were also identified. The remaining 160 kb of DNA sequence which did not yield database matches was then analysed using CODONPREFERENCE from the GCG package. This analysis suggested that 122 kb (42% of the total unique DNA sequence) could encode putative ORFs (pORFs), with the remaining 38 kb (13%) possibly representing non-coding intergenic DNA. From the data so far obtained, CII does not appear to be specialized for encoding any particular metabolic function, physiological state or growth condition. These data suggest that CII contains genes which are functionally as diverse as those found on any other bacterial chromosome and also contains sequences (pORFs) which may prove to be unique to this organism.

Keywords: genome analysis, sequence skimming, complex genomes, genome evolution

INTRODUCTION

Rhodobacter sphaeroides 2.4.1^T is a facultative photoheterotroph belonging to the α -3 subdivision of the *Proteobacteria* (Woese *et al.*, 1984). The metabolic diversity of this group of organisms is unparalleled (Gest, 1972; Kiley & Kaplan, 1988). The structure and

function of the photosynthetic membrane protein complexes and their regulation, photolithotrophy, nitrogen fixation, hydrogen metabolism, carbon dioxide fixation, taxis and tetrapyrrole biosynthesis, when considered *in toto* in *R. sphaeroides*, are remarkable. Members of the α -3 *Proteobacteria* are also able to synthesize bacteriochlorophyll *a*, haem, sirohaem and vitamin B₁₂. Numerous studies (see reviews by Woese *et al.*, 1984; Yang *et al.*, 1985; Gest, 1972; Kiley & Kaplan, 1988) suggest a relationship between this group of organisms and the genesis of early mitochondrial system(s) and to the photosystem(s) of green plants. The ability of members

Abbreviations: CI, CII, chromosome I, II of *R. capsulatus*; dORF, database ORF (of hypothetical or unknown function); pORF, putative ORF.

The GenBank accession numbers for the sequences determined in this work are B07699–B07848.

of the α -3 *Proteobacteria* to undergo intracellular differentiation is a unique morphogenetic connection to eukaryotes. In addition, members of this group are able to detoxify high levels of a variety of metal oxides and oxyanions (Moore & Kaplan, 1992), which may make them useful organisms for bioremediation and some industrial processes. More recently *R. sphaeroides* 2.4.1^T has been shown to possess a genetic system for 'quorum sensing' (A. Puskas, E. P. Greenberg, S. Kaplan & A. L. Schaefer, unpublished).

Our finding that *R. sphaeroides* 2.4.1^T contains two different circular chromosomes, ~3.0 and ~0.9 Mbp (Suwanto & Kaplan, 1989, 1992), broke with the long-held dogma regarding our understanding of prokaryotic genome structure and organization. This discovery was rapidly followed by the finding that multiple chromosomes exist in at least four other bacterial genera, *Brucella* (Michaux *et al.*, 1993), *Leptospira* (Zuerner *et al.*, 1993), *Agrobacterium* (Allardet-Servent *et al.*, 1993) and *Pseudomonas* (*P. cepacia*) (Cheng & Lessie, 1994; *P. cepacia* was recently renamed *Burkholderia*: Rodley *et al.*, 1995). In the case of *Agrobacterium* one of these chromosomes is linear. Linear chromosomes had previously been shown to exist in members of the genus *Borrelia* (Ferdows & Barbour, 1989), again breaking with a long-held dogma that bacterial chromosomes are circular.

Analysis of the genomic structure of numerous independent isolates of *R. sphaeroides* reveals that most of these appear to have two chromosomes, although the chromosomes do vary in size and in the number of *rrn* operons (K. Nereng & S. Kaplan, unpublished) contained. A number of genes have been shown in *R. sphaeroides* 2.4.1^T to exist in duplicate copies between chromosomes I (CI) and II (CII), e.g. *hemA* and *hemT* (Neidle & Kaplan, 1993), *rdxA* and *rdxB* (Neidle & Kaplan, 1992), *cbbP*_I and *cbbP*_{II} (Hallenbeck *et al.*, 1990b; Tabita *et al.*, 1992), *cbbA*_I and *cbbA*_{II} (Tabita *et al.*, 1992; Hallenbeck *et al.*, 1990a), *rpoN*_I and *rpoN*_{II} (Meijer & Tabita, 1992; J. Zeilstra-Ryalls & S. Kaplan, unpublished) and others. The isozymes encoded by these duplicate genes are structurally similar but in most cases are differentially regulated. Additionally, the *rrn* operons and three tRNA genes (Dryden & Kaplan, 1990) are dispersed between these two circular chromosomes.

Although the concept that the prokaryotic genome can consist of more than a single chromosome has gained near-universal acceptance, the kinds of genes and their distribution within the second chromosome were previously unknown. Important questions leading to an understanding of the genome complexity of *R. sphaeroides* 2.4.1^T revolve around the actual genetic content, and its organization, present on each chromosome. For example, to what extent does CII contain a duplicate set of the genes on CI? Are the gene products from CII required for specialized functions, as for plasmids? Did CII originate endogenously or was it acquired exogenously by lateral transfer?

In search of the functional role(s) underlying this genomic diversification, a detailed, albeit low-resolution, genomic analysis of CII of *R. sphaeroides* 2.4.1^T was undertaken. Towards this goal, cosmid clones hybridizing to CII were ordered from a previously constructed pLA2917 recombinant genomic library (Dryden, 1992). A high-resolution physical map of CII was derived from a set of 47 overlapping cosmid clones that covered approximately 85% of CII (Choudhary *et al.*, 1994).

In this paper, we describe both partially and fully random sequencing strategies of 23 cosmids containing ~417 kb of unique insert DNA previously mapped and ordered to CII. This generated ~291 kb of unique DNA sequence. Analysis of these sequences suggested that approximately 87% of the total unique sequence was coding. That is, over ~253 kb of DNA either gave significant database BLASTX matches ($P \leq 10^{-3}$ and score ≥ 100) to known genes or database open reading frames (dORFs) or, if not giving a database match, gave putative open reading frames (pORFs) when analysed using CODONPREFERENCE. A total of 144 database matches was found, representing a wide variety of functions. The positions of two previously described rRNA operons (*rrnB* and *rrnC*) and three tRNAs, f-Met, Ala and Ile (Dryden & Kaplan, 1990) were confirmed. Two new tRNAs for Met and Val were also identified on this chromosome. The diverse range of metabolic functions encoded by CII suggests that CII does not appear to be specialized either for any particular metabolic role or growth condition.

METHODS

Bacterial strains, plasmids, cosmids, and growth conditions.

Escherichia coli cells were grown overnight in Luria broth (LB) on a rotary shaker at 37 °C, or on LB agar plates. To maintain plasmids or cosmids in *E. coli* cultures, ampicillin (100 µg ml⁻¹) or tetracycline (10 µg ml⁻¹), respectively, was added to media. Recombinant plasmid subclones were screened using blue/white colour selection on LB plates containing X-Gal (40 µg ml⁻¹).

Sequencing strategy. The general outline of the sequencing strategy is presented as a flowchart in Fig. 1.

Cosmid DNA preparation and the construction of libraries.

Cultures of *E. coli* S17-1 containing previously described cosmids (Choudhary *et al.*, 1994) were grown overnight from a single streak-purified colony. Cosmid DNA was isolated by using an alkali lysis method followed by CsCl gradient purification (Sambrook *et al.*, 1989). The identity of each cosmid clone was confirmed by comparison to the previously determined *SspI* pattern (Choudhary *et al.*, 1994).

Subcloning strategies. Our goal was to generate the maximum amount of sequence information for the minimum cost. We evaluated three subcloning strategies. (i) In the first case (cosmid 8536) we subcloned restriction fragments which were generated by four or five restriction enzymes which cut either rarely (fewer than three times) or not at all in the cosmid vector, but relatively frequently (5–10 times) in the cosmid insert. These restriction fragments were subcloned and then sequenced (Mackenzie *et al.*, 1997). (ii) For cosmid 8603 we

Fig. 1. Outline of the sequencing strategy.

generated a DNase I library of the total cosmid DNA, then screened the subclones by hybridization with the cosmid insert. A random sample of these insert subclones were sequenced. (iii) For cosmid 8621 we combined the strategies used for (i) and (ii) and screened a DNase I generated library of cosmid 8621 with *Bam*HI restriction fragments of 8621. The *Bam*HI restriction fragments were then subcloned and sequenced. In addition DNase I subclones which hybridized to two *Bam*HI fragments were considered to be linking subclones and were also sequenced.

Restriction enzyme sublibraries. Cosmid DNA was fragmented by enzymic digestion, and fragments were subcloned into the compatible sites in pBluescript SK(–) vector. The commonly used restriction enzymes employed to construct libraries were *Eco*RI, *Bam*HI, *Pst*I, *Eco*RV, *Bgl*II and *Not*I. For each sublibrary, 96 putative clones were grown in a 96-well microtitre plate. Plasmid DNA was purified using the alkaline lysis method (Sambrook *et al.*, 1989), and the insert clones were identified by appropriate restriction analysis. This method allowed us to rapidly screen hundreds of subclones, in order to identify those for subsequent sequencing.

DNase I sublibrary. DNase I libraries were made using the protocol of Demolis *et al.* (1995). Cosmid DNA was digested with RNase-free DNase I. The digest was then run on a 1% TAE low-melting-point agarose gel; fragments ~1 kb in size were isolated and cloned into the *Eco*RV site of the pBluescript SK(–) vector. About 1000 white transformants were picked into 96-well microtitre plates. The cultures were then pronged onto LB plates and the colonies screened by hybridization

using the cosmid insert as a probe. For method (iii), the *Bam*HI subclones were used to rescreen the cosmid insert-specific DNase I subclones. From the hybridization data, DNase I subclones which hybridized to two *Bam*HI fragments were considered to be linking clones. These linking clones, a number of non-linking clones and all the *Bam*HI subclones were sequenced. From the hybridization and sequence data, a restriction map and gene order was determined.

Of the three methods described above we finally settled on method (i) as it gave an even spread of sequence information which could then be used for mapping gene order. Method (ii) proved labour intensive but did not give us the even sequence coverage we had hoped for. In addition it proved more difficult to determine gene order without the advantage of restriction site markers which were present in method (i). Method (iii) gave the best coverage and most information in terms of order. However, these advantages were offset by the considerable amount of hands-on time which was required prior to sequencing.

Template purification. We used two different plasmid purification systems, the Prep-A-Gene (Bio-Rad) or QIAwell filter (Qiagen) for template purification.

Plasmid-containing strains were grown overnight in LB medium at 37 °C. Cells were harvested in a 96-well block by centrifugation for 5 min at 1500 g. The block was covered with an adhesive tape during centrifugation to avoid cross-contamination. Cell lysates were prepared by the alkaline lysis method (see details in REAL Prep 96 plasmid kit, Qiagen) and then transferred to the QIAfilter 96-well plate using an eight-channel pipette. The lysates were precipitated following the addition of 0.7 vol. 2-propanol. The DNA pellet was washed with cold 70% ethanol and air dried. Plasmid DNA was resuspended in 200 µl distilled water.

In the Prep-A-Gene protocol, each lysate was transferred to a clean tube containing 300 µl of the DNA mini-prep binding buffer provided in the kit. Then 60 µl of resuspended Prep-A-Gene matrix was added to each tube, and the contents were mixed by pipetting up and down a few times. The mixture was applied to a spin filter which was nested inside a 1.5 ml microfuge tube. The nested tubes were centrifuged for 30 s; 500 µl Prep-A-Gene wash buffer was then added to each spin filter unit and recentrifuged for 30 s. At this point each spin tube contained clean plasmid DNA. Plasmid DNA was eluted with 200 µl distilled water.

Automated sequencing. Templates were sequenced using dye-deoxy terminators purchased from ABI and used as recommended by the manufacturer. Primers annealing to the T3 and T7 sites of pBluescript SK(–) were used for sequencing restriction fragments. To sequence DNase I subclones, primers RVpBST3 (5'-GTGGATCCCCCGGCTGCAGG-3'; T_m 79 °C) and RVpBST7 (5'-CCCCCCTCGAGGTCGACG-GT-3'; T_m 78 °C), were used. Extra insert sequence was derived from each sequencing reaction using these primers since they are closer to the *Eco*RV site than conventional T3 and T7 primers (approximately 70 and 40 bases respectively).

The cycle sequencing programme consisted of a denaturation step at 98 °C for 30 s, an annealing step at 50 °C for 15 s, and an extension step at 60 °C for 4 min. Sequencing runs were performed using either an Applied Biosystems model 377 DNA sequencer as advised by the manufacturer or an ABI 373 (stretch-modified) machine. In the latter case, 5% (v/v) Long Ranger acrylamide gels (FMC Bio Products) were used with 48 cm plates. These were run for 14 h with 36 W constant power. The use of longer run times combined with the other

modifications effectively made the data obtained with the ABI 373 indistinguishable from those obtained with the ABI 377.

Sequence analysis. Computer analysis was performed using both the Genetics Computer Group (GCG) and Staden software packages. Sequence files were analysed by using the BLAST server at the National Center of Biotechnology Information (Bethesda, MD, USA). The BLASTX program translated each sequence in all six reading frames which were then used to search the non-redundant protein sequence database. Sequences were considered to have given a significant database match when the probability (*P*) value was $\leq 10^{-3}$ and score ≥ 100 .

ORF analysis was performed by running the assembled consensus sequences and their complements through the GCG CODONPREFERENCE program using an *R. sphaeroides* codon usage table (J. M. Eraso & S. Kaplan, unpublished). The codon usage table was derived using the *R. sphaeroides* sequences already available in the NCBI database but did not include sequence derived from the previously described ribosomal *rrn* operons (Dryden & Kaplan, 1990). The graphical output from the program was examined and the largest pORF found amongst the six possible frames was used to estimate the maximum coding capacity for each consensus sequence. The shortest length of sequence which we considered to be a pORF was 51 contiguous amino acids. Clearly, this decision was arbitrary and can be reconsidered as additional information becomes available. All consensus sequences were screened for the presence of tRNAs using the program TRNASCAN (Fichant & Burks, 1991).

RESULTS

Sequence 'skimming' of the small chromosome

Subclones were constructed from 23 cosmids containing a total of approximately 417 kb of unique insert DNA; a summary of the results is shown in Table 1. Sequencing

runs from cosmids 8536 and 8603 generated a lower read length of 300–400 nucleotides with 1% ambiguous bases. These formed part of our early sequencing efforts. Adjustment of conditions and an upgrade of the sequencing equipment resulted in ~650–700 nucleotides of sequence being the norm for the other cosmids. We have observed that >700 nucleotides of raw sequence often contained useful data for BLASTX searches, the determination of restriction sites, and fragment assembly.

A total of 944 sequence fragments containing CII DNA was assembled, and a total of 404 sequence contigs was generated. The average gap length was estimated to be ~500 nucleotides; therefore if required, a large number of gaps could be resolved by primer walking. The total unique DNA sequence was ~291 kb, representing approximately 70% of the total insert coverage. This coverage represents approximately one-third of the small chromosome of *R. sphaeroides*.

DNA composition and codon usage

The G+C composition of the *R. sphaeroides* genome was determined to be 66.0 mol%. This included 346 593 nucleotides of CI and CII sequence obtained from GenBank and this study. In one approach used to determine if CI and CII have different evolutionary origins we obtained from GenBank the sequences of protein-coding genes (i.e. no rRNAs or tRNAs) which have been mapped to CI and compared their overall mol% G+C content with those sequences which we had generated for CII. The G+C content was found to be 67.3 mol% and 65.7 mol% for CI and CII, respectively. For this analysis we excluded partial se-

Table 1. Summary of the CII sequencing project

Size of CII	910 kb
No. of cosmids sequenced	23
Total unique insert DNA	417 kb
Double-stranded templates	573
Average edited read length (% ambiguous bases)	559 ± 268 (2.8%)
Total DNA sequenced	640 kb
Total cosmid insert DNA sequence (includes multiple clones and overlaps)	565 kb
Coverage of the total cosmid inserts	1.3 ×
Total unique DNA sequence	291 kb
Unique sequence coverage	0.7 ×
Mol% G+C	65.7
DNA resulting in significant database matches	131 kb
DNA resulting in the designation of pORFs with no database matches	122 kb
DNA resulting in neither an ORF nor a database match	38 kb
No. of sequence fragments in random assembly	1145
No. of contigs	404
Average gap length	504 bp
No. of presumptive genes identified	144
No. of rRNA operons identified	2 (<i>rrnB</i> and <i>rrnC</i>)
No. of tRNA genes identified	5 (Ala, Ile, f-Met, Met and Val)

Table 2. Percentages of dinucleotide and trinucleotide repeats

	CI	CII
Dinucleotides		
CG	13.4	13.5
GC	12.7	12.0
GG	10.2	10.4
CC	9.4	9.9
GA	7.2	7.1
TC	7.1	7.0
CT	5.9	6.1
TG	5.8	5.2
CA	4.9	5.0
AG	4.6	4.1
AC	4.4	4.2
AT	4.0	3.9
GT	3.9	3.9
AA	2.9	3.3
TT	2.5	3.2
TA	1.1	1.2
Trinucleotides		
<i>Five most abundant</i>		
CGC	4.6	3.9
GCG	4.6	3.8
GGC	4.3	3.6
CGG	4.0	3.7
CCG	4.0	3.6
<i>Five rarest</i>		
TAG	0.1	0.2
TAA	0.1	0.1
TTA	0.1	0.1
GTA	0.2	0.2
ATA	0.2	0.3

quences obtained from three CII cosmids, 8737, 8503 and 8148, which contain the *rrn* operons whose sequence is near 50 mol % G + C (Dryden & Kaplan, 1990).

Partial sequence of nine of the twenty cosmids had a lower G + C content (60–62 mol %) than the average for CII. These cosmids did not overlap with those cosmids which contained the *rrn* operons: they were scattered around CII.

In addition, we examined the di- and trinucleotide frequencies of the sequences from both chromosomes; we found that the fraction of each di- or trinucleotide on each of the two chromosomes was approximately equivalent, as shown in Table 2. We also repeated this process with the 110 kb replicon for which we have 5790 bp of sequence (P. Sen & S. Kaplan, unpublished) and again found a similar pattern of rare and frequent di- and trinucleotides as those found on the chromosomes (unpublished observation).

We also examined the codon usage for genes mapping to CI and CII which had already been placed in GenBank. Here we looked at the relative fraction of redundant

Table 3. Selective codon usage

The fraction of each of the redundant codons of Arg, Leu, Ser and Lys found on CI and CII is shown. Sample size was 7501 bp and 6890 bp for CI and CII, respectively.

Amino acid	Codon	CI	CII
Arg	AGG	0.01	0.03
	AGA	0.01	0.01
	CGG	0.24	0.25
	CGA	0.33	0.29
	CGT	0.12	0.08
	CGC	0.29	0.34
Leu	TTG	0.02	0.04
	TTA	0.02	0.01
	CTG	0.31	0.35
	CTA	0.16	0.11
	CTT	0.18	0.17
	CTC	0.31	0.32
Ser	AGT	0.05	0.03
	AGC	0.19	0.22
	TCG	0.36	0.14
	TCA	0.10	0.16
	TCT	0.12	0.27
	TCC	0.17	0.18
Lys	AAG	0.86	0.81
	AAA	0.14	0.19
Cys	TGT	0.25	0.27
	TGC	0.75	0.73

codons for arginine, leucine and serine (see Table 3). We found that in general rare codons such as Arg-AGA and Leu-TTA were rare on both chromosomes, whereas relatively frequent codons such as Arg-CGA or Leu-CTG were frequent on both chromosomes. This was also true of amino acids specified by fewer redundant codons, such as lysine and cysteine. There were exceptions to this generality, however. For example, Ser-TCG occurs at a frequency of 0.36 on CI but only 0.14 on CII; likewise Ser-TCT occurs at a frequency of 0.12 on CI but 0.27 on CII. Whether these differences represent true differences between the codon usage of the two chromosomes or whether they reflect the relatively small sample sizes (7501 bp and 6890 bp for CI and CII, respectively) cannot be determined at this time. However, even when these exceptions are included, the overall picture is one of shared codon usage between the two chromosomes.

Analysis of the sequencing strategy

Libraries representing ~417 kb unique cosmid insert DNA were generated and used in 1145 sequencing runs. Of these, 944 runs represented cosmid insert DNA, the remainder either *E. coli* or cosmid vector sequences. After assembly these sequences represented ~291 kb of unique sequence. After analysis, the sequences could be placed into three categories. Of the total unique se-

Table 4. BLASTX results using the restriction fragment sequences

No.	Best match	Cosmid	Gene product or function	Organism showing best match	Sequence length*	% of gene covered†	Best hit sizes	% identity‡	% similarity§	Probability¶
1	<i>bioA</i>	8207	Diaminopimelic acid aminotransferase	<i>Rhizobium</i> sp.	467	48	84	40	60	3.1e-45
2	<i>redF</i>	8207	Integrase	<i>E. coli</i>	256	30	36	41	63	3.1e-45
3	<i>orf</i>	8207	Unknown	<i>Mycobacterium tuberculosis</i>	272	92	41	36	51	3.7e-07
4	<i>orf</i>	8207	Hypothetical protein	<i>E. coli</i>	142	75	57	43	52	1.0e-11
5	<i>bcsP31</i>	8207, 8737	Immunogenic cell surface protein	<i>Brucella abortus</i>	329	79	65	32	41	1.0e-11
6	<i>orf</i>	8207, 8737	Unknown	<i>Agrobacterium vitis</i>	324	75	39	35	58	5.7e-13
7	<i>bioD</i>	8737	Dethiobiotin synthetase 1	<i>H. influenzae</i>	242	37	41	31	56	5.7e-13
8	<i>orf</i>	8737	Hypothetical protein	<i>Coxiella burnetii</i>	311	61	111	42	60	4.7e-32
9	<i>orf</i>	8737	Unknown	<i>H. influenzae</i>	508	39	146	56	70	4.4e-69
10	<i>orf</i>	8737	Unknown	<i>Synechocystis</i> sp.	335	32	53	50	75	8.5e-25
11	<i>orf2</i>	8737	Downstream Orf of Fe-S fumarase	<i>Bacillus stearothermophilus</i>	265	31	37	51	83	8.5e-25
12	<i>rrnC</i>	8737, 8503	rRNA	<i>R. sphaeroides</i>	6163	21	88	43	63	1.3e-50
13	<i>proB</i>	8503	γ -Glutamyl kinase	<i>E. coli</i>	367	45	62	50	62	1.3e-50
14	<i>orf</i>	8503	Unknown	<i>Synechocystis</i> sp.	443	25	84	78	84	8.4e-97
15	<i>cpTaq</i>	8503, 8148	Carboxypeptidase	<i>Thermus aquaticus</i>	511	10	60	63	76	8.4e-97
16	<i>coxW</i>	8503, 8148	Cytochrome oxidase assembly factor	<i>Saccharomyces cerevisiae</i>	486	60	110	35	55	5.0e-16
17	<i>rpL21</i>	8503, 8148	50S ribosomal protein L21	<i>Bacillus subtilis</i>	102	100	82	30	58	2.5e-09
18	<i>orf</i>	8503, 8148	Hypothetical protein	<i>E. coli</i>	228	60	23	56	69	1.6e-31
19	<i>obg</i>	8503, 8148	GTP-binding protein	<i>H. influenzae</i>	390	77	23	56	69	1.6e-31
20	<i>proA</i>	8148	γ -Glutamyl phosphate reductase	<i>H. influenzae</i>	417	41	55	45	58	4.1e-10
21	<i>ech</i>	8148	Enoyl-CoA hydratase	<i>Clostridium difficile</i>	155	65	151	43	66	4.6e-64
22	<i>plsC</i>	8148	1-Acyl-sn-glycerol-3-phosphate acyltransferase	<i>Neisseria gonorrhoeae</i>	255	32	48	52	68	7.4e-16
23	<i>aepA</i>	8148	Regulatory protein	<i>Erwinia carotovora</i>	465	28	34	41	64	7.4e-16
24	<i>orf</i>	8148	Unknown	<i>E. coli</i>	219	42	61	36	54	1.3e-10
25	<i>rrnB</i>	8148, 8215	rRNA	<i>R. sphaeroides</i>	6565	10	34	35	44	1.3e-10
26	<i>orfT2</i>	8215	Transcriptional regulator in <i>rdxA</i> 3' region	<i>R. sphaeroides</i>	456	23	93	39	58	1.2e-35
27	<i>rdxA</i>	8215	Redox protein	<i>R. sphaeroides</i>	469	47	222	93	94	5.3e-161
28	<i>nmoB</i>	8215	Nitrilotriacetate monooxygenase	<i>Chelatobacter heintzii</i>	322	38	122	45	63	3.2e-30
29	<i>hemT</i>	8215	S-Aminolaevulinic synthase	<i>R. sphaeroides</i>	407	27	112	92	92	2.7e-74
30	<i>orf</i>	8215	Unknown	<i>Agrobacterium</i> (Ti plasmid)	322	40	72	88	93	2.1e-72
31	<i>potB</i>	8215	Putrescine transport	<i>E. coli</i>	226	55	78	46	71	3.4e-33
32	<i>orf</i>	8215	Unknown	<i>Agrobacterium</i> (Ti plasmid)	154	90	76	53	71	9.6e-36
33	<i>orf140</i>	8215	Unknown	<i>Rhizobium</i> sp.	140	73	103	39	56	7.5e-22
34	<i>potD</i>	8215, 8519	Putrescine transport	<i>E. coli</i>	348	40	82	41	52	4.4e-17
35	<i>oppD</i>	8215, 8519	Oligopeptide transport	<i>Rhizobium</i> sp.	335	52	144	49	61	2.9e-49
36	<i>dppB</i>	8519	Dipeptide transport	<i>E. coli</i>	306	19	59	32	54	2.6e-04
37	<i>oppF</i>	8519	Oligopeptide transport	<i>Rhizobium</i> sp.	353	34	70	52	67	1.1e-37
38	<i>dppA</i>	8519	Dipeptide transport	<i>E. coli</i>	512	17	85	34	56	2.4e-11
39	<i>potC</i>	8519	Putrescine transport	<i>H. influenzae</i>	247	40	56	37	67	2.3e-15
40	<i>potH</i>	8519	Putrescine transport	<i>E. coli</i>	317	49	34	50	76	2.3e-15
							77	51	71	1.2e-33

Table 4. (cont.)

No.	Best match	Cosmid	Gene product or function	Organism showing best match	Sequence length*	% of gene covered†	Best hit sizes	% identity‡	% similarity§	Probability¶
41	<i>orf</i>	8519	Hypothetical protein	<i>Xanthobacter</i> sp.	249	29	35	60	80	2.1e-18
							37	51	64	2.1e-18
42	<i>dmsA</i>	8519, 8508	DMSO reductase	<i>R. sphaeroides</i>	822	100	371	95	96	1.3e-127
43	<i>dmsB</i>	8519, 8508	DMSO reductase	<i>R. sphaeroides</i>	53	100	46	86	91	8.2e-22
44	<i>moaA</i>	8519, 8508	Molybdopterine synthesis	<i>E. coli</i>	411	53	94	32	55	2.6e-41
45	<i>moaA</i>	8519, 8508	Molybdopterine synthesis	<i>E. coli</i>	329	30	99	39	48	2.5e-11
46	<i>potA</i>	8508	Putrescine transporter	<i>E. coli</i>	378	78	143	56	68	7.4e-50
47	<i>torC</i>	8519, 8508	c-type cytochrome	<i>E. coli</i>	390	33	61	60	77	3.2e-58
							44	52	72	3.2e-58
48	<i>arcB</i>	8519, 8508	Aerobic control sensory kinase	<i>E. coli</i>	776	19	72	43	63	6.4e-11
49	<i>yexN</i>	8519, 8508	Transcriptional regulator	<i>Aeromonas salmonicida</i>	193	41	81	40	58	2.9e-12
50	<i>arcA</i>	8508, 8508	Aerobic respiration control protein	<i>E. coli</i>	238	47	57	42	61	1.2e-16
							33	45	62	1.2e-16
51	<i>orf</i>	8656	Hypothetical protein	<i>Synechocystis</i> sp.	332	53	131	35	58	2.2e-37
52	<i>qoxB</i>	8656	Quinol oxidase subunit I	<i>B. subtilis</i>	649	22	147	38	63	4.3e-37
53	<i>orf</i>	8656	Hypothetical protein	<i>E. coli</i>	98	35	35	54	85	4.5e-07
54	<i>mcpA</i>	8656	Methyl-accepting chemotaxis protein	<i>B. subtilis</i>	661	14	95	33	64	2.6e-13
55	<i>dadA</i>	8656, 8318	D-Amino acid dehydrogenase	<i>E. coli</i>	432	100	136	57	72	7.1e-50
56	<i>orf</i>	8318	Hypothetical protein	<i>Synechocystis</i> sp.	319	55	125	52	71	4.3e-60
57	<i>orf</i>	8318	Unknown	<i>E. coli</i>	255	50	85	45	67	1.2e-36
58	<i>orf</i>	8318	Hypothetical protein	<i>E. coli</i>	344	43	82	35	62	1.2e-20
59	<i>orf</i>	8318	Hypothetical transcriptional regulator	<i>Synechocystis</i> sp.	295	60	107	36	56	8.9e-32
60	<i>orf</i>	8318	Unknown	<i>Synechocystis</i> sp.	383	26	103	53	68	1.8e-29
61	<i>arsG</i>	8318, 8790	Arsenate reductase	<i>E. coli</i>	141	97	67	47	58	2.2e-14
62	<i>yjcG</i>	8790	Na ⁺ symport	<i>E. coli</i>	549	35	137	65	75	3.0e-72
63	<i>yjcH</i>	8790	Unknown	<i>E. coli</i>	104	79	53	35	52	1.9e-13
							30	56	73	1.9e-13
64	<i>orf</i>	8790	Unknown	<i>Sulfolobus acidocaldarius</i>	275	44	91	31	52	8.0e-19
65	<i>orf</i>	8790	Hypothetical protein	<i>E. coli</i>	534	98	156	55	72	5.8e-63
66	<i>orf</i>	8790	Transcriptional regulator	<i>H. influenzae</i>	288	61	103	35	53	1.5e-33
67	<i>orf</i>	8790	Unknown	<i>Mycobacterium tuberculosis</i>	498	32	68	36	51	3.9e-16
							24	50	70	3.9e-16
68	<i>orf</i>	8790	Unknown	<i>M. tuberculosis</i>	431	39	68	38	55	3.8e-13
							38	39	50	3.8e-13
69	<i>fabG</i>	8736	Ketoacyl-ACP reductase	<i>Vibrio harveyi</i>	244	47	89	42	60	5.5e-24
70	<i>xdhA</i>	8736	Hydroxysteroid dehydrogenase	<i>E. coli</i>	255	28	73	42	57	1.5e-12
71	<i>dapB</i>	8736	Dihydrodipicolinate reductase	<i>Pseudomonas aeruginosa</i>	216	35	75	32	49	8.0e-06
72	<i>dfp</i>	8736	Flavoprotein (DNA/pantothenate metabolism)	<i>H. influenzae</i>	400	50	120	46	67	9.8e-56
73	<i>cbiO</i>	8736	ABC transporter	<i>H. influenzae</i>	217	47	103	43	61	7.5e-21
74	<i>appC</i>	8736	Acid phosphatase	<i>E. coli</i>	514	22	38	36	63	4.9e-20
							41	34	53	4.9e-20
75	<i>piaA</i>	8736	Negative transcriptional regulator	<i>B. subtilis</i>	172	43	74	33	56	4.2e-09
76	<i>orf</i>	8736	Unknown	<i>Synechocystis</i> sp.	214	68	79	51	73	3.8e-25
77	<i>orf</i>	8736	Unknown	<i>E. coli</i>	732	25	99	43	59	2.0e-42
78	<i>cyoD</i>	8736	Cytochrome oxidase <i>d</i> subunit II	<i>E. coli</i>	182	41	74	32	56	1.2e-06
79	<i>cyoA</i>	8736, 8075	Cytochrome oxidase <i>d</i> subunit I	<i>Halobacterium salinarum</i>	160	91	88	48	71	8.5e-46
80	<i>sasA</i>	8075	Sensory kinase	<i>Synechococcus</i> sp.	380	49	62	37	59	1.6e-15
							87	25	43	1.6e-15
81	<i>fatD</i>	8075	Iron transporter	<i>B. subtilis</i>	316	100	140	40	74	1.7e-40
82	<i>mltB</i>	8075	Membrane-bound transglycosylase	<i>E. coli</i>	361	56	61	32	54	8.5e-16
							39	58	76	8.5e-16

Table 4. (cont.)

No.	Best match	Cosmid	Gene product or function	Organism showing best match	Sequence length*	% of gene covered†	Best hit sizes	% identity‡	% similarity§	Probability¶
83	<i>orf</i>	8075	Unknown	<i>Erwinia chrysanthemi</i>	132	57	76	43	56	6.5e-19
84	<i>bluB</i>	8075	BluB protein	<i>R. capsulatus</i>	206	82	112	37	47	4.8e-29
85	<i>orf</i>	8075	Transducer	<i>P. aeruginosa</i>	629	19	85	34	55	2.6e-08
86	<i>orf</i>	8075	Unknown	<i>E. coli</i>	279	57	55	61	72	7.0e-54
							44	65	75	7.0e-54
87	<i>dppA</i>	8075, 8025	Dipeptide transporter	<i>E. coli</i>	535	32	124	59	76	4.4e-63
88	<i>baeS</i>	8025	Sensory kinase	<i>E. coli</i>	466	22	83	27	45	5.8e-06
							20	35	65	5.8e-06
89	<i>copR</i>	8025	Transcriptional activator protein	<i>Pseudomonas syringae</i>	227	54	65	44	60	1.4e-32
90	<i>dppD</i>	8025	ATP-binding domain	<i>H. influenzae</i>	330	19	65	55	72	1.6e-17
91	<i>nikE</i>	8025	Nickel transport	<i>E. coli</i>	268	27	74	43	56	5.3e-13
92	<i>orf</i>	8025	Unknown	<i>E. coli</i>	370	41	106	62	65	3.6e-48
93	<i>orf</i>	8025	Unknown	<i>E. coli</i>	179	19	35	65	68	1.6e-07
94	<i>dap</i>	8025, 8219	D-Aminopeptidase	<i>Ochrobactrum anthropi</i>	520	35	115	53	68	6.0e-40
95	<i>dppB</i>	8025, 8219	Dipeptide permease	<i>E. coli</i>	339	95	89	69	83	1.2e-53
							113	46	69	1.6e-42
96	<i>dppF</i>	8025, 8219	Dipeptide transport	<i>Salmonella typhimurium</i>	334	50	136	61	81	3.0e-72
97	<i>cbpM_{II}</i>	8219	Ribulose bisphosphate carboxylase/oxygenase	<i>R. sphaeroides</i>	459	36	166	100	100	2.9e-140
98	<i>cbpA_{II}</i>	8219	Fructose bisphosphate aldolase	<i>R. sphaeroides</i>	354	79	281	99	99	1.1e-233
99	<i>cbpG_{II}</i>	8219	Glyceraldehyde-3-phosphate dehydrogenase	<i>R. sphaeroides</i>	333	62	209	96	98	1.2e-141
100	<i>cbpP_{II}</i>	8219	Phosphoribulokinase	<i>R. sphaeroides</i>	292	84	245	99	99	6.9e-177
101	<i>cbpT_{II}</i>	8219	Transketolase	<i>R. sphaeroides</i>	657	17	116	100	100	2.2e-74
102	<i>cbpF_{II}</i>	8219	Fructose bisphosphatase	<i>R. sphaeroides</i>	331	52	172	99	99	2.0e-134
103	<i>dppC</i>	8219	Dipeptide transport protein	<i>E. coli</i>	300	35	107	43	57	6.0e-25
104	<i>htrA</i>	8219	Heat-shock protein	<i>B. abortus</i>	513	14	73	45	63	2.9e-14
105	<i>amiC</i>	8219	Aliphatic amidase expression regulating protein	<i>Synechocystis</i> sp.	446	33	146	37	56	1.3e-30
106	<i>orf</i>	8219	Unknown	<i>R. sphaeroides</i>	38	100	38	100	100	6.2e-21
107	<i>act206</i>	8536	Acid inducible	<i>Rhizobium (Sinorhizobium) meliloti</i>	541	24	56	60	76	4.2e-31
108	<i>metK</i>	8536	S-Adenosylmethionine synthetase	<i>E. coli</i>	383	71	109	59	75	4.8e-41
109	<i>aroA</i>	8536	3-Phosphoshikimate 1-carboxyvinyltransferase	<i>Synechocystis</i> sp.	447	40	98	60	68	9.5e-98
110	<i>rpsA</i>	8536	30S ribosomal subunit proteins	<i>Rhizobium (Sinorhizobium) meliloti</i>	568	56	87	79	93	1.5e-52
111	<i>himD</i>	8536	Integration host factor b subunit	<i>R. capsulatus</i>	95	71	55	78	85	8.2e-32
112	<i>trpF</i>	8536	Anthranilate isomerase	<i>Caulobacter crescentus</i>	219	94	121	40	57	1.5e-27
113	<i>trpB</i>	8536	Tryptophan synthase	<i>C. crescentus</i>	406	49	122	78	84	6.2e-62
114	<i>gvpN</i>	8536	Gas vesicle protein N	<i>Anabaena flos-aquae</i>	335	30	100	38	50	2.4e-15
115	<i>hutH</i>	8536	Histidine ammonia lyase	Human	657	16	107	30	44	8.1e-09
116	<i>orf</i>	8536	Hypothetical protein	<i>Synechocystis</i> sp.	134	82	110	49	59	2.1e-30
117	<i>orf</i>	8536	Unknown	<i>M. tuberculosis</i>	406	10	39	56	66	9.1e-07
118	<i>def</i>	8536	Unknown	<i>Arabidopsis thaliana</i>	717	14	90	64	77	7.4e-37
119	<i>phoH</i>	8536, 8603	Phosphate-starvation-inducible protein	<i>Synechocystis</i> sp.	328	85	145	57	75	6.6e-92
120	<i>tlyC</i>	8536, 8603	Haemolysin	<i>H. influenzae</i>	299	48	100	41	60	4.8e-33
121	<i>orf</i>	8603	ABC transporter	<i>E. coli</i>	228	80	56	66	76	2.9e-55
122	<i>orf</i>	8603	Hypothetical protein	<i>H. influenzae</i>	474	57	61	62	77	8.1e-54
123	<i>orf</i>	8603	Hypothetical protein	<i>H. influenzae</i>	154	44	68	52	73	7.6e-23

Table 4. (cont.)

No.	Best match	Cosmid	Gene product or function	Organism showing best match	Sequence length*	% of gene covered†	Best hit sizes	% identity‡	% similarity§	Probability¶
124	<i>orf</i>	8603	Hypothetical protein	<i>H. influenzae</i>	246	40	65	32	52	6.6e-11
125	<i>orf</i>	8603	Hypothetical protein	<i>E. coli</i>	485	25	91	51	69	5.6e-37
126	<i>nifR4</i>	8603, 8621	σ^{54} RNA polymerase	<i>R. capsulatus</i>	426	80	104	39	54	7.6e-70
127	<i>orf1</i>	8621	Unknown	<i>R. (S.) meliloti</i>	180	72	113	30	52	5.6e-16
128	<i>groEL</i>	8621, 8064	GroEL	Thermophilic bacterium PS-3	538	10	52	53	76	3.2e-11
129	<i>ndvB</i>	8621, 8064	Cyclic β -(1,2)-glucan biosynthesis	<i>R. (S.) meliloti</i>	2870	90	181	63	77	1.7e-152
130	<i>cspB</i>	8064	Cold-shock-like protein	<i>E. coli</i>	71	88	63	44	60	2.3e-13
131	<i>dctP</i>	8591	C ₄ dicarboxylase binding protein	<i>R. capsulatus</i>	333	31	60	31	48	1.6e-06
132	<i>ISV-A2</i>	8591	Transposase	<i>Vibrio anguillarum</i>	306	22	34	52	70	5.1e-11
133	<i>lasR</i>	8591	Transcription factor	<i>P. aeruginosa</i>	239	43	45	40	60	5.6e-09
134	<i>orf</i>	8178	Unknown	<i>M. tuberculosis</i>	431	31	57	56	61	2.7e-26
135	<i>orf</i>	8324	Unknown	<i>E. coli</i>	114	81	62	48	67	1.6e-25
136	<i>exoIII</i>	8324	Exonuclease III	<i>Synechocystis</i> sp.	275	41	47	59	68	6.0e-27
137	<i>orf</i>	8324	Unknown	<i>Pseudomonas carboxydovorans</i>	295	64	83	36	49	1.3e-11
138	<i>gpc</i>	8324	Minor capsid protein-C	<i>Lambda phage</i>	439	23	101	41	56	3.5e-18
139	<i>gp5</i>	8324	Preconnector protein gp5	<i>Lambda phage</i>	501	19	97	44	57	1.3e-18
140	<i>XorIIIM</i>	8721	DNA methylase	<i>Xanthomonas oryzae</i>	424	50	122	66	74	3.1e-83
141	<i>vsr-dcm</i>	8780	Very-short patch repair	<i>Xanthomonas oryzae</i>	136	83	84	53	66	5.3e-38
142	<i>cepS</i>	8780	β -Lactamase	<i>Aeromonas sobria</i>	382	62	59	45	62	4.5e-55
143	<i>ampR</i>	8780	Amp regulator	<i>R. capsulatus</i>	289	26	52	76	86	1.1e-22
144	<i>rbsC</i>	8780	Ribose transport protein	<i>E. coli</i>	321	19	60	33	63	4.5e-05
145	<i>aldH</i>	8780	Aldehyde dehydrogenase	<i>E. coli</i>	519	49	54	61	79	5.2e-46
146	<i>sodH</i>	8780	L-Sorbose dehydrogenase	<i>Gluconobacter oxidans</i>	498	26	69	44	66	3.0e-26

* The length of the complete subject polypeptide or rRNA, expressed as amino acids or (in the case of rRNA) nucleotides, found in the BLAST search.

† This percentage is an estimate. It is derived from the amount of sequence we have for the proposed homologous *R. sphaeroides* gene as a proportion of the gene found in the database search. The assumption was made that the two genes were the same size.

‡ Percentage of *R. sphaeroides* amino acids which are identical to those within the region of homology of the protein, except the cases of *rrn* operons, where the identity is expressed as a percentage of the homologous nucleotide region.

§ Sum of *R. sphaeroides* amino acids which are identical and similar, expressed as a percentage of the total number of amino acids or nucleotides found within the region of homology.

¶ The probability computed by the BLASTX program that sequences found during the search matched by chance.

quence, ~131 kb DNA (45%) gave matches, $P \leq 10^{-3}$ and score ≥ 100 , to either genes or ORFs (dORFs) in the database. The remaining ~160 kb DNA (55%) of unique sequence did not give database matches. When this latter sequence was analysed using the CODONPREFERENCE program, 122 kb (42%) was estimated to consist of pORFs. These varied from ≥ 50 to 400 amino acid residues in length (the shortest stretch of sequence which we arbitrarily grouped as a pORF was 50 contiguous amino acids). Due to the fragmentary nature of these data, we have not made any attempt to estimate the number of pORFs. Rather we have presented only the fraction of the sequence which we consider may be coding, but which did not give a database match.

The remaining 38 kb (13%) of unique sequence, we believe, is made up of two components, intergenic non-coding sequence and sequence presently encoding puta-

tive products of less than 50 amino acid residues. The latter sequences were too short to fall into our definition of a pORF. However, we cannot rule out the possibility that smaller proteins, not showing similarities to known proteins from other organisms, have been missed in our analysis. Also these sequences may subsequently merge with existing gaps to generate recognizable ORFs. Therefore, in this respect we have probably overestimated the fraction of non-coding DNA; this reflects an intrinsic problem with the approach used here.

Considering these limitations we have estimated that CII of *R. sphaeroides* has a coding capacity of at least ~87% of its DNA, which is in keeping with the level described for other bacterial chromosomes. However, we did note that the number of gene matches was not equally distributed over CII and declined in regions where the mol% G + C was lower than the average.

A variety of gene functions map to CII

Analysis of the coding sequences resulted in the identification of 144 putative genes representing a wide variety of functions. A list of these genes, with their putative functions and levels of homology, is given in Table 4. The gene numbers in the table are cited in the text as ^(*nnn*). Possible roles have been assigned to 72 % of the total of the 144 presumptive genes identified on CII; the remaining 28 % of the genes were assigned as dORFs. In addition to putative protein-coding genes we have also identified tRNAs for valine (cosmid 8207) and methionine (cosmid 8603) and confirmed the position of tRNAs for formylmethionine, alanine and isoleucine and ribosomal RNAs *rrnB*⁽²⁵⁾ and *rrnC*⁽¹²⁾ to cosmid 8737.

Presumptive genes were placed into functional classes, for example: protein synthesis – ribosomal proteins S1⁽¹¹⁰⁾ (*rpsA*) and L21⁽¹⁷⁾ (*rpL21*); amino acid biosynthesis – S-adenosylmethionine synthetase⁽¹⁰⁸⁾ (*metK*), tryptophan synthetase⁽¹¹³⁾ (*trpB*), 3-phosphoshikimate 1-carboxyvinyltransferase⁽¹⁰⁹⁾ (*aroA*); fatty acid metabolism – 1-acyl-*sn*-glycerol-3-phosphate acyltransferase⁽²²⁾ (*plsC*), ketoacyl-ACP reductase⁽⁶⁹⁾ (*fabG*); transcriptional regulation – *piaA*⁽⁷⁵⁾, *copR*⁽⁸⁹⁾, *arcA*⁽⁵⁰⁾, *amiC*⁽¹⁰⁵⁾; energy metabolism – cytochrome oxidase assembly factor⁽¹⁶⁾ (*coxW*), *c*-type cytochrome⁽⁴⁷⁾ (*cytC* like), quinol oxidase⁽⁵²⁾ (*qoxB*); and structural components – murein hydrolase⁽⁸²⁾ (*mltB*). The genes *groEL* (K. Nereng & S. Kaplan, unpublished; Lee *et al.*, 1997) and *rpoN* or *nifR4* (Meijer & Tabita, 1992), which had previously been mapped to CI, were also found duplicated on CII^(128, 126).

It is beyond the scope of this paper to describe the function of each gene listed in Table 4, but some genes have attracted our attention. The gene tentatively identified as encoding ribosomal protein S1⁽¹¹⁰⁾ shows exceedingly high homology at the amino acid sequence level to other S1 proteins (Schnier *et al.*, 1988). Interestingly, the *rpsA* gene product (30S S1 ribosomal protein), which is a part of the translation apparatus, appears to be present as a single copy on CII when surveyed under low-stringency hybridization conditions (C. Mackenzie & S. Kaplan, unpublished).

Additionally, several genes seem to be important or even essential when considering the structure of the bacterial cell. For example, the gene *mltB*⁽⁸²⁾ encodes the enzyme murein hydrolase (Ehlert *et al.*, 1995), whose proposed role is as a pacemaker enzyme for murein enlargement or as a hydrolase enzyme during the cell division process involving cell septum formation. The involvement in murein metabolism is significant as it gives both mechanical stability and shape to the bacterium.

Regulatory genes represent ~12.5 % of the total genes presently localized on CII. These regulators, e.g. *aepA*⁽²³⁾, *yexN*⁽⁴⁹⁾, *piaA*⁽⁷⁵⁾, *amiC*⁽¹⁰⁵⁾ and *ampR*⁽¹⁴³⁾, seem to mediate various positive and negative transcriptional controls. Also, CII includes genes encoding a number of regulatory proteins involving two-component regulatory systems, namely sensory kinases

(*arcB*⁽⁴⁸⁾, *sasA*⁽⁸⁰⁾ and *baeS*⁽⁸⁸⁾) and response regulators (*arcA*⁽⁵⁰⁾ and *copR*⁽⁸⁹⁾).

In Gram-negative bacteria, small-molecule transport systems have been divided into several classes. One of these, constituting the osmotic shock-sensitive transport system, is responsible for the uptake of a wide range of small molecules including amino acids, sugars, vitamins and ions (Ames, 1986). Approximately 13 % of the total genes identified on CII of *R. sphaeroides* represent transport systems, i.e. dipeptide transport^(87, 95, 103, 90, 96) (*dppA,B,C,D,F*), oligopeptide transport^(35, 37) (*oppD,F*), putrescine/spermidine transport^(46, 31, 39, 34, 40) (*potA,B,C,D,H*), Na⁺ symport⁽⁶²⁾, iron transport⁽⁸¹⁾ (*fatD*), C₄ dicarboxylase transport⁽¹³¹⁾ (*dctP*) and the ribose transport protein⁽¹⁴⁴⁾ (*rbsC*).

Besides the expected metabolic and biosynthetic functions reported in Table 4, CII contains sequences which encode functions we consider unusual for this photosynthetic organism. We found database matches to *tlyC*⁽¹²⁰⁾, a gene encoding a haemolysin, and *ndvB*⁽¹²⁹⁾, a gene implicated in *Rhizobium* root nodule development in alfalfa. In the case of *ndvB*, we have now sequenced 90 % of this gene, which encodes a 2870 amino acid polypeptide, the largest protein revealed on CII to date. These findings were unanticipated, as *R. sphaeroides* has not been considered to be an animal pathogen nor a plant symbiont. Further analysis of these genes is currently under way.

Comparison to other bacterial genomes

We have compared the relative percentages of functional classes of genes on CII to the chromosomes of *E. coli* (Riley, 1993), *Haemophilus influenzae* Rd (Fleischmann *et al.*, 1995), *Methanococcus jannaschii* (Bult *et al.*, 1996), *Mycoplasma pneumoniae* (Himmelreich *et al.*, 1996), and *Mycoplasma genitalium* (Fraser *et al.*, 1995); see Table 5. CII of *R. sphaeroides* 2.4.1^T encodes a diverse set of functions. Some of these functions, i.e. cellular processes, biosynthesis of small molecules and intermediary metabolism, are found at approximately the same proportion as in other bacterial chromosomes. However, gene functions classified as involved in macromolecular synthesis (transcription and translation) and cell structure are under-represented, whereas regulatory functions are over-represented on CII when compared to other microbial genomes. Whether this is a true reflection of the composition of CII or an artifact of the regions which we have so far examined will require a complete analysis of the chromosome. Approximately 44 % of the total coding DNA (i.e. total unique DNA minus DNA presently classified as 'intergenic' DNA) remains as unassigned pORF DNA. This suggests that even with the large number of genome sequencing projects (Bult *et al.*, 1996; Fleischmann *et al.*, 1995) which have been deposited in the NCBI database there are still many sequences which are unique to CII of this organism and may reflect the ecological diversity of the members of the genus *Rhodobacter*.

In the absence of a single contiguous sequence, it was

Table 5. Comparison of gene-encoded functions in different bacterial species

A comparison of percentage gene content in *R. sphaeroides*, *E. coli*, *Haemophilus influenzae*, *Methanococcus jannaschii*, *Mycoplasma pneumoniae* and *Mycoplasma genitalium*, sorted by biological function. The sample size of *R. sphaeroides* genes is 144, compared with 1717 (*E. coli*), 1743 (*H. influenzae*), 1738 (*M. jannaschii*), 643 (*M. pneumoniae*) and 470 (*M. genitalium*). NA, Not applicable.

Metabolic pathway	<i>R. sphaeroides</i> (CII)	<i>E. coli</i>	<i>H. influenzae</i>	<i>M. jannaschii</i>	<i>M. pneumoniae</i>	<i>M. genitalium</i>
I. Intermediary metabolism	28.1	22.6	19.7	26.3	9.5	13.8
Central intermediary metabolism	9.4	13.8	3.0	5.2	1.1	1.9
Energy metabolism	6.2	6.2	10.4	20.0	7.0	9.7
Regulatory functions	12.5	2.6	6.3	1.1	1.4	2.2
II. Biosynthesis of small molecules	11.8	19.1	20.0	24.7	6.3	9.8
Amino acid biosynthesis	4.4	7.6	6.8	10.2	0.0	0.3
Biosynthesis of cofactors	3.7	4.9	5.4	7.4	1.5	1.6
Fatty acid and phospholipid metabolism	3.7	2.2	2.5	1.3	1.6	1.9
Purines, pyrimidines, nucleosides and nucleotides	0.0	4.4	5.3	5.8	3.2	6.0
III. Macromolecule metabolism	6.8	21.4	25.3	26.8	28.4	45.6
Replication	4.4	6.0	8.6	5.9	8.2	10.0
Transcription	0.6	1.4	2.7	2.9	2.3	3.8
Translation	1.8	14.0	14.0	18.0	17.9	31.8
IV. Cell structure	0.6	8.5	8.3	3.8	9.6	5.3
V. Cellular processes	18.0	18.3	17.5	14.7	11.4	17.3
Cellular processes	3.7	5.3	5.3	5.2	3.6	6.6
Transport and binding protein	14.3	13.0	12.2	9.5	7.8	10.7
VI. Other categories	28.0	9.8	9.2	3.6	34.2	8.2
Unassigned	44.0	NA	42.0	62.0	9.9	32.0

difficult to construct a precise genetic map from our database matches. Nonetheless, we found a number of genes on CII linked in a manner found in other bacterial species. For example, two genes, *proA*⁽²⁰⁾ (γ -glutamyl phosphate reductase) and *proB*⁽¹³⁾ (γ -glutamyl kinase) were linked on the overlapping region of cosmids 8503 and 8148. These two genes are also found linked in the distantly related genera *E. coli* (Deutch *et al.*, 1984), *Serratia marcescens* (Omori *et al.*, 1991), and *Thermus thermophilus* (Kosuge *et al.*, 1994). In addition *aroA*⁽¹⁰⁹⁾, *rpsA*⁽¹¹⁰⁾ and *himD*⁽¹¹¹⁾ are in this order on CII. This order is also found in *E. coli*. However, in *H. influenzae* (Fleischmann *et al.*, 1995) *rpsA*⁽¹¹⁰⁾ and *himD*⁽¹¹¹⁾ are linked in this way, but *aroA*⁽¹⁰⁹⁾ is located elsewhere on the genome. Also *rpL21*⁽¹⁷⁾ and *obg*⁽¹⁹⁾ were linked on CII of *R. sphaeroides*, and found to be conserved in that organization in *H. influenzae* (Fleischmann *et al.*, 1995). The genes *groEL*⁽¹²⁸⁾ and *rpoN* (*nifR4*)⁽¹²⁶⁾, which are linked in *Rhizobium* (*Sinorhizobium*) *meliloti* (Ogawa & Long, 1995) are also linked in CII of *R. sphaeroides*.

We have fully sequenced the DMSO reductase genes *dmsCBA*^(47,43,42) and have found them to be linked as in a close relative of *R. sphaeroides*, namely *Rhodobacter capsulatus* (A. McEwan, personal communication). However, the detailed organization of other genes is quite different in the same DMSO-reductase-linked region. For example, *R. sphaeroides* has only two genes involved in molybdenum cofactor biosynthesis (*moeA*⁽⁴⁴⁾ and *moaA*⁽⁴⁵⁾) compared to *R. capsulatus*, which contained at least four genes (*moaA*, *moaD*, *moeB* and *moaC*). Interestingly, five genes located in a 34 kb region of an *R. (S.) meliloti* replicon are also found on CII, i.e. *oppD*⁽³⁵⁾, *oppF*⁽³⁷⁾, *bioA*⁽¹⁾, *dct*⁽¹³¹⁾ and *fabG*⁽⁶⁹⁾; however, the positions of these genes are not in the same order as found in *R. (S.) meliloti* (Freiberg *et al.*, 1996). Thus we found some local conservation of linkage involving several genes found in various genera. However, when we examined fragments linked to the same regions, no conservation of linkage relationships at a more distant level between species was found.

At a more global level the genes encoding proteins involved in tryptophan metabolism appear to be dispersed to both C1 and C2 (Mackenzie *et al.*, 1995). These earlier results suggested that a subset of these genes was present on C2 (Choudhary *et al.*, 1994) and our analysis here indicates this to be the case.

DISCUSSION

Sequence strategies and genome analysis

Several bacterial genomes have been either completely sequenced (Fleischmann *et al.*, 1995; Fraser *et al.*, 1995; Bult *et al.*, 1996; Himmelreich *et al.*, 1996) or are currently in the process of being sequenced (Glaser *et al.*, 1993; Sensen *et al.*, 1996). Two main sequencing strategies have been used in these projects: the sequencing of large numbers of random subclones followed by computer assembly (*Haemophilus influenzae* and *Methanococcus jannaschii*), or a directed primer walking approach (*Mycobacterium tuberculosis*). However, both of these methods have high costs because they involve either severalfold (6 to 10 times) genome coverage, or the requirement for large numbers of primers and other materials to give finished 'base-perfect' sequence.

We have used a low-redundancy, semi-random approach to partially sequence C2 of *R. sphaeroides*, the aim being to generate the maximum amount of biological information for the lowest possible cost. Our aim was not to generate a base-by-base account of C2, but rather to generate an overview of the types of genes encoded by C2. We hoped that this approach might allow us to: (i) determine if this replicon encodes a specialized set of function(s), (ii) assess the relationship of this form of genome organization to that routinely observed in other bacteria, (iii) establish a paradigm with which to compare other bacteria having more than one chromosome, and (iv) ascertain if such a genomic organization served as a precursor to the diploid eukaryotes.

From a technical perspective we also wanted to determine how much information could actually be obtained by using a low-redundancy sequencing approach. Those few genomes which have been sequenced to 'perfection' have provided us with many insights into the bacterial and archaeobacterial kingdoms, but perhaps numerous bacterial genomes sequenced to low redundancy could be equally informative both to workers interested in these organisms and from a genetic diversity perspective.

We found that when restriction enzymes recognizing 6 bp were used to generate template subclones, for the most part, an even distribution of sequence data was obtained from each cosmid. These sequences when used in BLAST searches allowed us to determine which genes may be encoded in a particular cosmid, as shown in Table 4. However, these sequences also allowed us to deduce the potential genetic content of each cosmid by a process of extrapolation. For example, if 300 bp at one

end of a subclone matched a region of a gene which *in toto* encoded a 400 amino acid residue polypeptide, we could extrapolate the size of our partially sequenced gene to be 1200 bp. Therefore with only 300 bp of sequence we could account for 1200 bp of cosmid sequence. As the ends of restriction fragment which gave BLAST hits to the same gene could be abutted and assembled by computer, low-resolution genetic maps could be generated. These fragments of sequence information have allowed us to focus on particular genes of interest which we did not know existed in this organism until the cosmids were sequenced. In the case of the DMSO reductase, an initial 700 bp of sequence gave us an indication of the presence of these genes. This has led to the acquisition of their complete sequence and the functional study of their products (N. J. Mouncey, M. Choudhary & S. Kaplan, unpublished). Such genes of interest can be readily subcloned either by using the correct combination of restriction fragments, or by PCR. In both approaches the information required for subcloning had been generated by the low-redundancy approach.

Multiple chromosomes and gene duplication in prokaryotes

Genome sizes vary greatly among micro-organisms (Krawiec & Riley, 1990). Within a single taxonomic group or even within a single species, considerable levels of genome plasticity have been observed (Fonstein & Haselkorn, 1995). A number of reports have appeared describing several bacterial species possessing more than one chromosome (Michaux *et al.*, 1993; Zuerner *et al.*, 1993; Allardet-Servent *et al.*, 1993; Cheng & Lessie, 1994). In addition, linear rather than circular chromosomes have also been described in *Streptomyces* sp. (Lin *et al.*, 1993), *Borrelia* (Ferdows & Barbour, 1989), and *Agrobacterium* (Allardet-Servent *et al.*, 1993). Also some bacterial species of the genera *Pseudomonas* (Frantz & Chakrabarty, 1986), *Rhizobium* (Sobral *et al.*, 1991), and *Alcaligenes* (Rodley *et al.*, 1995) harbour very large accessory genetic elements (megaplasmids) which appear to encode 'housekeeping' functions (Finan *et al.*, 1986; Pardo *et al.*, 1994); however their 'essentiality' has not been demonstrated. These findings raise the question: what, if anything, defines a bacterial chromosome? Is it the possession of an *rrn* operon, is it size, topology, the possession of essential genes or any combination of these? Thus, we would propose for bacteria possessing complex genomes that the definition of a chromosome can only be considered in the context of the total genome, i.e. the distribution of genetic elements between the linkage groups.

We have shown previously that transposon insertions mapping to C2 can generate auxotrophic phenotypes (Choudhary *et al.*, 1994), suggesting that genes essential for the day-to-day survival of *R. sphaeroides* are carried on C2. We have also shown the presence on C2 of two rRNA operons and a number of tRNAs (Dryden & Kaplan, 1990). In addition, our sequencing has shown

that there are numerous genes carried on CII which have already been found on the single chromosomes of *E. coli*, *H. influenzae*, *M. tuberculosis* and others.

When considered *in toto*, the data reported here confirm the designation of each major linkage group in *R. sphaeroides* 2.4.1 as a chromosome. Although not yet completed, the low-resolution DNA sequencing of CII confirms the existence of all major groups of genes as applied to the analysis of those genomes sequenced in their entirety. Further, unlike the recently reported sequence of the rhizobial plasmid (Freiberg *et al.*, 1997) which in addition to lacking several of the major gene groupings, such as macromolecular biosynthesis, cell structure and intermediary metabolism reported here, contains sets of genes associated with a more dedicated lifestyle, i.e. nodulation, CII contains no discernible clusters of genes dedicated to a specific lifestyle or physiological state. In many respects, and granting the still limited level of analysis, CII is indistinguishable from the classical single, circular bacterial chromosome encoding a wide range of biological functions and as such must be considered essential in that broad context.

Here, for example, we have considered those genes encoding enzymes involved in tryptophan metabolism as essential, yet the same genes are absent from the *Mycoplasma pneumoniae* genome (Himmelreich *et al.*, 1996) and probably from the human genome. From this we could conclude that it is the amino acid L-tryptophan which is essential and not the genes. However, since *R. sphaeroides* or *E. coli* normally (the wild-type phenotype) make their own L-tryptophan, then in this narrow context, these genes are essential in our description of this organism. On the other hand the presence of a single *rpsA* gene on the small chromosome requires very little further definition of essentiality.

A growing number of gene homologues have been identified in *R. sphaeroides* with one member of each pair located on each chromosome. We have found additional pairs of homologues, such as *rpoN*, *groEL*, *hul* and *hip* duplicated either between the large and small chromosomes or between the large chromosome and a 110 kb replicon. Nonetheless, the physiological role(s) of many of these gene homologues have not yet been demonstrated (Krawiec & Riley, 1990; Labedan & Riley, 1995). Recent results (J. Zielstra-Ryalls & S. Kaplan, unpublished) involving the *hemA* and *hemT* gene pair of *R. sphaeroides* have now revealed that the *hemT* gene is likely to be transcribed by use of the *rpoE*-encoded sigma factor and *hemA* by RpoD. Thus, the presence of two genes encoding the same function has added an element of metabolic diversity and perhaps an increased level of adaptability to this organism.

Often when a gene is duplicated, one of the two copies may diverge in function to the point where these remain only remotely reminiscent of such a relationship or one copy may become nonfunctional. It is also possible, however, that these duplicated copies may retain their obvious identities but divergence might occur at the

level of gene regulation and expression, which could reflect an increased adaptability. Because of its genome structure and the clear presence of partial diploidy, *R. sphaeroides* may serve as a model system to study the origin and evolution of diploidy and of complex bacterial genomes.

This raises the question as to the evolutionary origin of CII. Did it arise by the disruption of a single large chromosome into two smaller chromosomes, was it acquired from some other organism, or was one of these two 'built' as the result of abnormal chromosomal segregation and gradual gene reassortment? In *Rhizobium* (*Sinorhizobium*) *meliloti* the 500 kb megaplasmid encodes a number of genes (Freiberg *et al.*, 1996) such as *bioA* and *fabG* which would normally be considered chromosomal. However, the lower G + C content of the genes on this megaplasmid versus the chromosome suggests that these genes, if not the megaplasmid itself, were acquired by horizontal transfer (Freiberg *et al.*, 1996, 1997).

The virtually identical G + C content, di- and trinucleotide frequencies and occurrence of rare codons existing between CI and CII suggest that CII was not derived exogenously. Therefore we do not have any evidence to support the hypothesis that CII was acquired by horizontal transfer from another organism. We found a similar situation when we examined the limited sequence which was available from the *R. sphaeroides* 110 kb replicon, which also appears to contain a growing list of essential genes (P. Sen, K. Nereng & S. Kaplan, unpublished). This suggests that CI, CII and perhaps the 110 kb replicon may have arisen either from a single larger replicon or have been constructed *de novo* by the reassortment of existing DNA sequence. If both CI and CII were derived from a larger 'mother' chromosome, then the genetic content of each chromosome would merely reflect the event which gave rise to the dissolution of the 'mother' chromosome. There is no reason to believe that such an event should have partitioned the various gene groupings in proportion to their overall occurrence on the 'mother' chromosome. Suppose that CII was constructed *de novo* from portions of the 'mother' chromosome and acquired, over time, its present display of genes. Then, there is also no reason to suggest that the acquisition of genetic elements should have occurred in proportion to their presence on the 'mother' chromosome. Therefore it is essential when considering the genetic content of those prokaryotes possessing more than a single chromosome, that the occurrence of various gene groupings reflects the genetic content of the total genome and not that each individual chromosome has to resemble the model bacterial chromosome. Such a concept would certainly be intuitive if we were discussing the genome of eukaryotic systems. Thus, detailed DNA sequence comparisons between *R. sphaeroides*, *R. capsulatus* and other members of the α -3 subgroup of the *Proteobacteria* should shed light on the origin of genome complexity in these bacteria.

Origin and evolution of the mitochondrial system

Although the data presented in this study have little direct bearing on the origin of the mitochondria, previous studies raised the likelihood that members of the α -3 subdivision of the *Proteobacteria* were the progenitors of the primitive mitochondrial system (Woese *et al.*, 1984; Yang *et al.*, 1985). It has also been postulated that the mitochondrial system originated through the reductive evolution of an early endosymbiont by reducing its genome size over time and subsequent loss of numerous genes from the 'evolving' mitochondrial genome. Many of these genes, however, may have become part of the host genome, where today they provide much of the observed nuclear/mitochondrial interaction. This original endosymbiont could have evolved in several different ways, ultimately existing as a pathogen (e.g. rickettsiae), or as a free-living symbiont (e.g. rhizobia), or as a benign mitochondrion. It can be envisioned that the presence of multiple chromosomes was significant in the development of these latter relationships. Thus, the endosymbiont could undergo genetic partitioning with its host organism without completely surrendering its genetic identity and autonomy through the assortment and reassortment of genetic information present on multiple chromosomes. Therefore, studies of genomic organization and gene structure and function among members of the *Proteobacteria* could further refine our knowledge and help to resolve the questions of both the ancestry and the mechanism of mitochondrial origin.

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