Genomic Analysis of Different *Chlorobium* Strains by Pulsed-Field Gel Electrophoresis and Ribotyping

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Pulsed-field gel electrophoresis was used to characterize the genomes of 15 *Chlorobium* strains. An analysis of chromosomal macrorestriction patterns allowed us to determine the chromosome sizes, which ranged from 1,435 to 3,342 kb depending on the strain. Moreover, this analysis revealed that, even though the genus *Chlorobium* is a phylogenetically coherent taxon, there is a great deal of genomic heterogeneity within it. This heterogeneity was corroborated by performing a ribotyping analysis, which showed that the rRNA hybridization patterns of most strains are unique.

The green sulfur bacteria are anaerobic, photolithotrophic organisms that require light as an energy source and suitable electron donors, such as hydrogen sulfide or elemental sulfur (11). They are commonly found in anaerobic zones of karstic-sulfated lakes and in very thin layers of stratified microbial communities (4). The genus *Chlorobium*, which includes six species, is the most representative taxon of this group of bacteria. The different *Chlorobium* species are distinguished on the basis of their morphological, biochemical, and ultrastructural properties (10). Consequently, the genus is heterogeneous genetically since the G+C contents of the species range from 49 to 58.1 mol%. Moreover, comparisons of 16S rRNAs have been performed with only some strains of this genus (3). The heterogeneity of the genus has been recently demonstrated with the species *Chlorobium limicola* (8), in which we described chromosomal and plasmidic differences between strains. Thus, a genotypically determined classification of *Chlorobium* strains, both above and below the species level, is needed. Nucleic acid studies should provide greater objectivity and a unifying taxonomic concept based on genetic relatedness. Nevertheless, even though genetic techniques have been developed in the last few years (1, 5, 7, 21), genomic analysis of the green sulfur bacteria has been carried out only with the species *C. limicola* (9) and *Chlorobium tepidum* (10).

According to many studies, pulsed-field gel electrophoresis (PFGE) is an especially useful technique for analyzing bacterial genomes. Moreover, combining PFGE with restriction enzymes that cut bacterial chromosomes infrequently makes it possible to obtain chromosome restriction patterns that provide a trustworthy fingerprint of each microorganism. Comparative analysis of these patterns makes it feasible to detect the genomic heterogeneity within a group of microorganisms (14). On the other hand, other molecular techniques, such as ribotyping, are currently available for strain typing, and these techniques can be used to confirm the results derived from analyses of chromosomal restriction patterns obtained by PFGE (9). These two approaches were used in this study to analyze the genetic heterogeneity of 15 *Chlorobium* strains belonging to five species.

Materials. The bacterial strains studied are listed in Table 1. New isolates were identified on the basis of their biochemical, morphological, and ultrastructural properties (11, 12). *Chlorobium chlorovibrioides* UdG6026 and *C. limicola* UdG6038,

UdG6041, and UdG6042 were characterized at the Department of Microbiology, University of Girona, Girona, Spain (13). Most cultures were grown photolithoautotrophically in rubber-capped bottles at 25°C with 50 microeinsteins of continuous illumination $\cdot \text{m}^{-2} \cdot \text{s}^{-1}$. *C. tepidum* TLS^T (T = type strain) was grown at 45°C (22). Cultures were grown in Pfennig minimal medium at pH 6.6 to 6.9 (20) to concentrations of about 10^8 cells per ml.

DNA preparation and restriction enzyme digestion for PFGE. Intact genomic DNAs were prepared in agarose plugs as described by Smith and Cantor (18). Restriction assays were carried out as described previously (6, 15). Restriction enzymes and DNA standards were purchased from New England Biolabs, Beverly, Mass., and Boehringer Mannheim, Mannheim, Germany.

PFGE. PFGE (17, 19) was performed in an apparatus obtained from Pharmacia-LKB, Uppsala, Sweden. Gels were made of 1% agarose (SeaKem LE Agarose; FMC, Rockland, Maine) and were electrophoresed at 15°C in modified TBE buffer (100 mM Tris, 100 mM boric acid, 0.2 mM EDTA; final pH 8 to 8.4).

Ribotyping. Heterologous rRNA probes from *Escherichia coli* were used for ribotyping. DNA extraction, digestion, and hybridization for ribotyping were performed as described in the *Technical Handbook of Acetylaminofluorene Ribosomal RNA* (Eurogentec, Seraing, Belgium).

For ribotyping, *EcoRI* DNA fragments were separated by low-voltage ($1.5 \text{ V} \cdot \text{cm}^{-1}$) electrophoresis overnight on 0.5% agarose gels in $1 \times \text{TAE}$ buffer. Digoxigenin-labeled *Citrobacter* sp. strain 32 DNA digested with *MluI* was used as the size marker.

Plasmid analysis. Plasmids smaller than 50 kb were detected by conventional electrophoresis. DNA isolated by the alkaline method (15) was electrophoresed through a 0.7% agarose gel for 6 h with a constant electric field of $5 \text{ V} \cdot \text{cm}^{-1}$. Different *Chlorobium* strains contained different plasmids smaller than 50 kb (Table 1). Interestingly, the previously reported 14-kb plasmid which confers the ability to utilize thiosulfate and had been detected in *C. limicola* f. sp. *thiosulfatophilum* DSM 249 and *C. limicola* f. sp. *thiosulfatophilum* BF8000 was detected in *C. limicola* f. sp. *thiosulfatophilum* UdG6042. The presence of plasmids larger than 50 kb was studied by using samples subjected to PFGE at a field strength of $10 \text{ V} \cdot \text{cm}^{-1}$ and different pulse times (100 to 150 s) without restriction cleavage. Large extrachromosomal elements were detected in *C. limicola* DSM 245^T, *C. limicola* f. sp. *thiosulfatophilum* DSM 249, BF8000, and UdG6038, and *C. vibrioforme* f. sp. *thiosulfatophi-*

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TABLE 1. Bacterial strains

Strain	Source ^a	Thiosulfate utilization	G+C content (mol %)	Presence of plasmids smaller than 50 kb (size)
<i>C. limicola</i> DSM 245 ^T	DSM	— ^b	51.0	—
<i>C. limicola</i> f. sp. <i>thiosulfatophilum</i> DSM 249	DSM	+	58.1	+ (14 kb)
<i>C. limicola</i> BF8000 ^c	UAB	+	ND ^d	+ (14 kb)
<i>C. limicola</i> BF8010 ^c	UAB	—	ND	—
<i>C. limicola</i> UdG6038 ^c	UdG	+	56.4	+ (ND)
<i>C. limicola</i> UdG6041 ^c	UdG	—	ND	—
<i>C. limicola</i> UdG6042 ^c	UdG	+	ND	+ (14 kb)
<i>C. phaeobacteroides</i> DSM 266 ^T	DSM	—	49.0	—
<i>C. phaeobacteroides</i> BF8600 ^f	UAB	—	ND	—
<i>C. phaeobacteroides</i> BF8610 ^f	UAB	—	ND	—
<i>C. vibrioforme</i> f. sp. <i>thiosulfatophilum</i> DSM 263	UAB	+	53.5	+ (ND)
<i>C. vibrioforme</i> BF8200 ^f	UAB	+	ND	+ (ND)
<i>C. vibrioforme</i> BF8210 ^g	UAB	—	ND	—
<i>C. chlorovibrioides</i> UdG6026 ^c	UdG	—	ND	—
<i>C. tepidum</i> TLS Th	UAB	+	56.5	+ (ND)

^a DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen; UAB, Universitat Autònoma de Barcelona; UdG, Universitat de Girona.

^b —, negative; +, positive.

^c Strain isolated by M. Algueró, Universitat Autònoma de Barcelona.

^d ND, not determined.

^e Strain isolated by Imma Pibernat, Universitat de Girona.

^f Strain isolated by Balbina Nogales, Universitat Autònoma de Barcelona.

^g Strain isolated by Sebastián Méndez-Alvarez, Universitat Autònoma de Barcelona.

^h Strain provided by Michael Madigan, Southern Illinois University, Carbondale.

lum DSM 263 and BF8200. The sizes of these large extrachromosomal elements were not estimated.

Chromosome restriction patterns. To analyze the restriction patterns of the chromosomes from different *Chlorobium* strains, the following restriction endonucleases were tested: *AseI*, *BclI*, *DraI*, *EcoRV*, *PacI*, *SfiI*, and *SwaI*. We decided to utilize two of these restriction endonucleases, *PacI* (data not shown) and *SwaI*, because they provided the best resolution in PFGE. However, *PacI* could be used only with some strains of *C. limicola* (8) because it has a high exonuclease activity, which makes it difficult to obtain restriction patterns for most strains. For the different strains the numbers of restriction fragments generated by *SwaI* varied from 6 to 23, and the fragment sizes ranged from 10 to 900 kb (Table 2).

The PFGE analysis of chromosomal DNAs digested with *SwaI* showed that there were clear differences in the restriction patterns of the various *Chlorobium* strains. The restriction pattern for *C. limicola* f. sp. *thiosulfatophilum* DSM 249 was identical to the pattern for *C. limicola* f. sp. *thiosulfatophilum* BF8000 but different from the pattern for *C. limicola* f. sp. *thiosulfatophilum* UdG6042. Interestingly, *Chlorobium thiosulfatophilum* UdG6038, which was assigned to the species *C. limicola* on the basis of phenotypic characteristics, had a restriction pattern identical to the pattern obtained for *C. vibrioforme* f. sp. *thiosulfatophilum* DSM 263 and BF8200. Finally, all of the other strains had unique restriction patterns, and the big differences observed between strains assigned to the same species were remarkable.

Chromosome sizes for *Chlorobium* strains. The sizes of the chromosomes were estimated by adding the sizes of the individual fragment lengths in each restriction endonuclease digest. For each restriction analysis of a strain, the range of fragment sizes was subdivided into several classes, and band positions were determined from the gel with optimum resolution in each molecular weight class. The size of each DNA fragment was determined by calibration with linear DNA. Thus, the numbers of bands and chromosome sizes for all of the strains were compared when the enzyme *SwaI* was used. On the basis of the results obtained with *SwaI* digests, the

estimated sizes of the genomes of 15 *Chlorobium* strains ranged from 1,435.5 kb for *C. chlorovibrioides* UdG6026 to 3,342 kb for *C. limicola* BF 8010 (Table 2).

Chromosome sizes have been used successfully to differentiate bacterial strains (16). The sizes of the genomes of bacteria vary from 600 kb for *Mycoplasma genitalium* to 9,450 kb for *Myxococcus xanthus* (2). The values obtained in this study place the chromosomes of *Chlorobium* strains around the intermediate size group of bacterial genomes (2). The size of the

TABLE 2. Sizes of chromosome *SwaI* restriction fragments of *Chlorobium* strains^a

Strain	Sizes of restriction fragments (kb)	Total (kb)
DSM 266 ^T	385, 335, 270, 242, 195, 155, 140, 120, 105, 90, 85, 75, 70, 40	2,307
BF8600	400, 330, 270, 245, 200, 160, 145, 97, 80, 70, 60, 50, 40, 20	2,172
BF8610	480, 440, 340, 291, 275, 224, 195, 180, 137, 123, 75, 55, 48, 30, 20	2,913
DSM 249	804, 556, 271, 224, 218, 194, 176, 88, 40, 20, 15	2,616
BF8000	804, 556, 271, 224, 218, 194, 176, 88, 40, 20, 15	2,616
UdG6042	375, 300, 275, 230, 220, 150, 145, 120, 75, 70, 65, 55, 55, 48, 45, 35, 30, 25, 20, 17	2,355
DSM 245 ^T	400, 400, 291, 291, 242, 200, 170, 115, 73, 65, 60, 50, 45, 40, 17, 15	2,414
BF8010	436, 388, 380, 340, 271, 240, 224, 206, 190, 170, 145, 135, 120, 97	3,342
UdG60401	390, 291, 265, 230, 205, 200, 180, 170, 160, 145, 121, 116, 97, 48, 48, 40, 35, 30, 30, 25, 20, 17, 15	2,831
UdG6038	600, 375, 245, 170, 140, 135, 97, 10	2,112
DSM 263	600, 375, 245, 170, 140, 135, 97, 10	2,112
BF8200	600, 375, 245, 170, 140, 135, 97, 10	2,112
BF8210	915, 450, 388, 295, 280, 215	3,128
UdG6026	700, 230, 225, 97, 48.5, 35, 30	1,435.5
TLS ^T	436.5, 339.5, 280, 260, 200, 160, 145, 75, 17, 12	1,925

^a For complete names see Table 1.

TABLE 3. Sizes of the rRNA hybridization bands of *Chlorobium* strains^a

Strain	Sizes of rRNA hybridization bands (bp)
DSM 266 ^T	11,000, 9,500
BF8600	17,000, 14,000, 11,000, 7,500, 6,000, 1,500
DSM 249	13,000, 12,500, 11,500, 7,500, 7,000, 3,500, 1,500
BF8000	13,000, 12,500, 11,500, 7,500, 7,000, 1,500
UdG6042	12,750, 12,000, 6,900, 6,000
DSM 245 ^T	11,500, 8,000
BF8010	16,500, 13,000, 7,500, 6,900, 6,000, 1,500
UdG6041	12,500, 11,500, 6,900, 6,000, 1,500
UdG6038	12,750, 12,000, 7,500, 6,900, 3,500
DSM 263	13,000, 12,500, 9,000, 8,000
BF8200	13,000, 12,500, 9,000, 8,000
BF8210	13,000, 7,500, 6,000, 2,700, 2,500, 2,200
UdG6026	17,000, 13,000, 7,500, 2,800, 1,500
TLS ^T	12,500, 12,000, 10,500

^a For complete names see Table 1.

chromosome of *C. tepidum* was estimated to be 2,150 kb (10) when the enzymes *PacI* and *XbaI* were used, and this value is within the accepted range of size divergence with our estimate of 1,925 kb (around 10% divergence).

rRNA restriction patterns. A total of 14 *Chlorobium* strains were analyzed by ribotyping, and 13 restriction patterns were observed, although some of these patterns were nearly identical. The number of fragments that hybridized with the rRNA probe varied from two to seven for the different strains (Table 3). Ribotyping allowed us to distinguish some strains with identical PFGE macrorestriction patterns.

Concluding remarks. The results described above reveal that there is great genomic heterogeneity within the genus *Chlorobium*, both in chromosome material and in plasmid material. Thus, different strains contain different extrachromosomal elements, exhibit different chromosome macrorestriction patterns, and have different chromosome sizes. Moreover, ribotyping increased the level of discrimination among strains. In conclusion, it will be necessary to perform more molecular studies to clarify the real genetic relationships within this group of microorganisms.

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