Genomic Relationship of Five Species of the Genus *Chromatium* by Analysis of Large Restriction Fragments (Macrorestriction Analysis) Using Pulsed-Field Gel Electrophoresis

V. PAVÓN* AND N. GAJU

*Institute for Fundamental Biology and Department of Genetics and Microbiology,
Autonomous University of Barcelona, 08193 Bellaterra, Spain*

Large restriction fragment (macrorestriction) patterns resolved by pulsed-field gel electrophoresis (PFGE) were used to calculate the genomic similarity within and among five members of the genus *Chromatium* with closely related phenotypes: *C. minutissimum*, *C. vinosum*, *C. gracile*, *C. salexigens*, and *C. tepidum*. PFGE allowed the study of the genomic organizations of these organisms. The results reveal a high level of homogeneity among the strains of *C. vinosum* analyzed. Moreover, there seems to be a close genetic relationship between *C. vinosum* and *C. minutissimum*.

Chromatiaceae are anaerobic phototrophic organisms able to grow with sulfide or elemental sulfur as the sole photosynthetic electron donor. The arrangement of genera and species of the family *Chromatiaceae* is entirely based on simple phenotypic characteristics (13). Phylogenetic relationships showing that this family constitutes a phylogenetically coherent group, with S_{AB} values of more than 0.66, have been established (3).

The type genus of the family, the genus *Chromatium*, is distinguished from other motile genera of purple sulfur bacteria with intracellular sulfur globules by its rod-shaped morphology and the lack of gas vesicles. The DNA ratios of the genus (G+C content, 45.5 to 70.4 mol%) indicate that genetically distantly related species are grouped together. This idea has been corroborated by 16S rRNA analysis, which shows that the species of *Chromatium* analyzed do not form a coherent genus (3).

The genus *Chromatium* consists of three distinct groups based on their predominant carotenoid. We have focused our work on the group that contains carotenoids of the spirilloxanthin series (*C. vinosum*, *C. minutissimum*, *C. gracile*, *C. salexigens*, and *C. tepidum*). The phenotypic traits used to differentiate between these species are cell volume, requirements for NaCl and cyanocobalamin, type of carotenoid, optimum growth temperature, and moles percent G+C (13). In some cases species are differentiated on the basis of only one characteristic; e.g., *C. vinosum* and *C. minutissimum* differ only in their cell sizes. Since this characteristic and other phenotypic features are strongly influenced by the culture conditions and the methodology used to determine them (4), it is necessary to search for genetic relationships rather than for merely phenotypic resemblances among strains.

Recently, pulsed-field gel electrophoresis (PFGE) has been used to examine chromosomal polymorphisms existing within and among bacterial species (7, 10). Chromosomal polymorphisms are related to differences in the number and sizes of specific fragments of genomic DNA that are generated by restriction enzymes. The number of different polymorphisms found in each species is linked to that species' genetic variability.

A total DNA restriction pattern (macrorestriction pattern) permits reliable identification of a single strain among very similar isolates, and it can also be used to generate dendrograms based on genome similarities (7).

In this study, *AseI* and *SpeI* digestion and PFGE were used to examine the chromosomal polymorphisms of five species of *Chromatiaceae* while, at the same time, the suitability of this technique was assessed as a tool for the identification of strains within each species.

Strains and microbiological methods. Strains belonging to five species of the genus *Chromatium* were used (Table 1). Cultures of these strains were grown photolithoautotrophically in rubber-capped bottles at 23°C and under continuous illumination of 50 microeinstein $\text{cm}^{-2} \text{s}^{-1}$. The thermophilic bacterium *C. tepidum* ATCC 43061^T was grown at 45°C (9). Cultures were grown in Pfennig minimal medium at pH 7.0 to 7.2 (18) to about 10^8 cells ml^{-1} . For strains with saline requirements, the medium was supplemented with 2.5% (*C. gracile* strains) or 10% (*C. salexigens*) NaCl. *Chromatium* cell sizes were measured by phase-contrast light microscopy as described previously (4) with an Olympus BH2 microscope.

PFGE methods. Cells were embedded in low-melting-point agarose to prepare the DNA inserts as described previously (16). Culture of *C. minutissimum* DSM 1376^T showed an extremely high level of exonuclease activity, and DNA was prepared according to Römmling et al. (14). Following restriction with endonucleases, agarose blocks were subjected to PFGE (150 s of pulse time, 330 V, 36 h) in order to remove proteinase K and cell debris.

Restriction enzymes, purchased from New England Biolabs (Beverly, Mass.), were selected for their specific cleavage sites. Some of them were chosen because they contain potentially rare nucleotide combinations in their recognition sequences. Restriction assays were carried out as previously described (10). A variety of restriction endonucleases were tested in *C. vinosum* DSM 180 (5). The majority of enzymes screened were inappropriate, since they resulted in either too few or too many fragments. The best results were obtained with *AseI* (AT'TA AT) and *SpeI* (A'CTAGT).

PFGE (15) was performed in a Pharmacia LKB apparatus. Gels were made of 1% agarose (SeaKem LE Agarose; FMC) and run at 15°C in modified TBE buffer (100 mM Tris, 100 mM boric acid, 0.2 mM EDTA; final pH, 8.4). Different resolution windows were obtained by varying the pulse time between 1 and 100 s for 36 h at a field strength of 10 V cm^{-1} in each

* Corresponding author. Mailing address: Institute for Fundamental Biology and Department of Genetics and Microbiology, Autonomous University of Barcelona, 08193 Bellaterra, Spain. Phone: 34-3-581 12 78. Fax: 34-3-581 20 11. E-mail: ibfn0@cc.uab.es.

TABLE 1. Species, strain numbers, cell sizes, chromosome sizes, and extrachromosomal material of *Chromatium* strains analyzed in this study

Strain	Cell size (length by width [μ m])		Mean chromosome size $\pm$ SD (kb) ^b	Extrachromosomal DNA ^c of:	
	Data from literature ^a	This work		High molecular wt	Low molecular wt
<i>C. vinosum</i> DSM 180	2.5–6 by 2	3.6 by 2.2	3,674 $\pm$ 130	+	+
<i>C. vinosum</i> DSM 185		3.5 by 2.4	3,674 $\pm$ 130	+	+
<i>C. vinosum</i> ATCC 17899 ^T	2.5–6 by 2	5.9 by 3.3	3,674 $\pm$ 130	+	+
<i>C. minutissimum</i> DSM 1376 ^T	2 by 1–1.2	3.6 by 2.4	3,329 $\pm$ 116	ND	ND
<i>C. gracile</i> DSM 203 ^T	2–6 by 1–1.3	3.6 by 2.1	3,777 $\pm$ 99	–	ND
<i>C. salexigens</i> DSM 4395 ^T	4–7.5 by 2–2.5	3 by 2	3,590 $\pm$ 115	ND	ND
<i>C. tepidum</i> ATCC 43061 ^{Tc}	2.8–3.2 by 1–2	3.1 by 2.2	3,695 $\pm$ 170	ND	ND
<i>C. gracile</i> BF 7200 ^d		3 by 2.1	3,777 $\pm$ 99	ND	ND
<i>C. gracile</i> EP 2201 ^e		3.1 by 2.2	3,918 $\pm$ 151	ND	ND
<i>C. gracile</i> EP 2203 ^f		3.4 by 2.2	3,777 $\pm$ 99	ND	+

^a References 1, 9, and 13.^b Chromosome sizes were estimated from *AseI* and *SpeI* macrorestriction patterns.^c Symbols: +, present; –, absent; ND, not detected.^d Isolated and provided by M. Madigan, Southern Illinois University.^e Isolated by B. Macarrón, Universitat Autònoma de Barcelona, Barcelona, Spain, from microbial mat samples from Ebro Delta, Spain.^f Isolated and provided by P. Caumette, Université de Bordeaux, Bordeaux, France.

restriction endonuclease assay. For each restriction analysis of a strain, the whole range of fragment sizes was subdivided into several regions and band positions were determined from the gel with optimum resolution. Chromosomes of *Saccharomyces cerevisiae* YPH80, Lambda concatemers (48.5-kb monomer), and Lambda *HindIII* fragments (New England Biolabs) were used as size standards.

Cluster analysis. Each fragment from the PFGE restriction patterns was treated as a unit character and scored as 1 (present) or 0 (absent). A tolerance value was applied to each band to compensate for misalignment of homologous bands due to technical imperfections (7): 2% for fragments of more than 135 kb and 5% for bands of up to this size. The similarity of fragment length patterns between two strains was calculated with Jaccard and Dice coefficients (17). The distance matrices were thus used as data for hierarchical clustering by an unweighted pair group method based on Euclidean distances.

Chromosomal restriction patterns. Genomic DNAs of *Chromatium* strains generated 18 to 36 fragments of 10.8 to 660 kb when they were digested with the restriction endonuclease *AseI* and 8 to 30 bands of 8 to 953.3 kb when they were digested with *SpeI*. Figure 1 is a semilogarithmic representation comparing the restriction patterns created after digestion with both enzymes for all the *Chromatium* strains analyzed.

The genomic patterns of *C. gracile* strains were highly conserved with *AseI*, and only one strain, EP 2201, had a different *SpeI* pattern. Interestingly, a high level of conservation was also observed in *C. vinosum* collection strains (5) and in several strains of *C. vinosum* isolated from Catalanian lakes and microbial mats (data not shown). In some groups of eubacteria, an important degree of polymorphism of PFGE patterns has been found at the intraspecific level (6, 11), but in some species one or two prevalent PFGE types have been detected (8). In *C. vinosum* strains we have observed a single specific PFGE pattern with two enzymes. The same result has been observed in *C. gracile* digests with endonuclease *AseI*. In both cases, however, we cannot reject the existence of intraspecific polymorphism since the number of strains analyzed was too small. This could be particularly important for *C. vinosum* strains where the reported variation of the G+C ratio (61 to 66%) (13) shows that these species represent a heterogeneous group of strains.

Genomic heterogeneity. To analyze the genomic heterogeneity of all the strains, their whole chromosomal restriction patterns were compared. A total of 87 *AseI* and 63 *SpeI* fragments were considered for the cluster analysis. *Chromatium*

strains were clustered according to *AseI* restriction fragments, *SpeI* restriction fragments, or the sum of fragments generated by both enzymes. From all of these data, Jaccard and Dice similarity coefficients were calculated (Table 2). The dendro-

FIG. 1. Schematic representation of total macrorestriction patterns with the endonucleases *AseI* (lane numbers with an A suffix) and *SpeI* (lane numbers with a B suffix) of several *Chromatium* species: *C. vinosum* strains DSM 180, DSM 185, and ATCC 17899^T (lanes 1); *C. minutissimum* DSM 1376^T (lanes 2); *C. gracile* strains DSM 203^T, BF 7200, and EP 2203 (lanes 3); *C. gracile* EP 2201 (lanes 4); *C. salexigens* DSM 4395^T (lanes 5); and *C. tepidum* ATCC 43061^T (lanes 6). Sizes are arranged according to a logarithmic scale and expressed in kilobase pairs. Lanes were assigned to more than one organism when different strains displayed identical patterns.

TABLE 2. Binary comparison of macrorestriction patterns of *Chromatium* strains with Dice and Jaccard coefficients^a

Strain	<i>C. vinosum</i> DSM 180	<i>C. vinosum</i> DSM 185	<i>C. vinosum</i> ATCC 17899 ^T	<i>C. minutissimum</i> DSM 1376 ^T	<i>C. gracile</i> DSM 203 ^T	<i>C. gracile</i> BF 7200	<i>C. gracile</i> EP 2203	<i>C. gracile</i> EP 2201	<i>C. salexigens</i> DSM 4395 ^T	<i>C. tepidum</i> ATCC 43061 ^T
<i>C. vinosum</i> DSM 180		1	1	0.222	0.138	0.138	0.138	0.151	0.124	0.103
<i>C. vinosum</i> DSM 185	1		1	0.222	0.138	0.138	0.138	0.151	0.124	0.103
<i>C. vinosum</i> ATCC 17899 ^T	1	1		0.222	0.138	0.138	0.138	0.151	0.124	0.103
<i>C. minutissimum</i> DSM 1376 ^T	0.457	0.457	0.457		0.113	0.113	0.113	0.160	0.212	0.083
<i>C. gracile</i> DSM 203 ^T	0.129	0.129	0.129	0.226		1	1	0.407	0.108	0.081
<i>C. gracile</i> BF 7200	0.129	0.129	0.129	0.226	1		1	0.407	0.108	0.081
<i>C. gracile</i> EP 2203	0.129	0.129	0.129	0.226	1	1		0.407	0.108	0.081
<i>C. gracile</i> EP 2201	0.184	0.184	0.184	0.316	0.529	0.529	0.529		0.236	0.133
<i>C. salexigens</i> DSM 4395 ^T	0.135	0.135	0.135	0.375	0.182	0.182	0.182	0.353		0.110
<i>C. tepidum</i> ATCC 43061 ^T	0.141	0.141	0.141	0.169	0.159	0.159	0.159	0.202	0.144	

^a Dice coefficients were configured at the left, and Jaccard coefficients were configured on the upper axis.

gram shown in Fig. 2 is based on Dice coefficients. Jaccard coefficients yielded remarkably similar dendrograms (figure not shown).

Cluster analysis of restriction patterns shows that *C. tepidum* differs from all other species studied. *C. vinosum* and *C. minutissimum* are the most closely related species of this group, as was suggested by phenotypical data. The genomic relationships shown in Fig. 2 suggest that the distance between *C. minutissimum* and *C. vinosum* is not large enough to consider them two different species. Furthermore, *C. gracile* does not cluster with *C. vinosum* and *C. minutissimum* in spite of their phenotypic similarities. This is consistent with their different G+C contents, with those of *C. gracile* strains being 69 to 70% higher than those of *C. vinosum* and *C. minutissimum* (13). The NaCl requirement was the single phenotypic characteristic found to differ among these strains, since our cell size results were not

discriminatory (Table 1) (13). On the other hand, *C. gracile* is related to *C. salexigens*, which also has saline requirements.

Genomic data. PFGE provides an alternative tool for the study of the relationships among strains by facilitating the study of their genome structures. The chromosome sizes were estimated by addition of the individual fragment lengths represented in each restriction endonuclease digest (Table 1). All of the strains had circular chromosomes with sizes that scored in group 3 of bacterial species (3 to 4.5 Mb) (2). Extrachromosomal material has been detected in several strains (Table 1), and it is remarkable that in all the strains of *C. vinosum*, we detected the same extrachromosomal elements of high molecular weight. These results reaffirm the high genetic homogeneity among the strains of *C. vinosum* analyzed.

Concluding remarks. The results described here reveal the existence of a high degree of genomic homogeneity among the

FIG. 2. Dendrogram of similarity values of macrorestriction fragment patterns for the *Chromatium* strains and comparison of phenotypic characteristics (data are from references 1, 9, and 13). The cluster analysis was calculated from the similarity of the cumulative *Ase*I and *Spe*I fingerprints (Dice coefficient [S_D]) by the unweighted pair group method. ND, not determined.

strains of *C. vinosum* analyzed. On the other hand, the results obtained with *C. vinosum* and *C. minutissimum* suggested that there are not sufficient reasons for differentiating these strains into two species, as they are phenotypically identical. The availability of a small number of strains is a disadvantage in taxonomic studies of *Chromatiaceae*. Nevertheless, the methodology used constitutes a simple and reliable tool to analyze the genetic heterogeneity among different strains and species. Further application of this methodology to a broader number of species of purple sulfur bacteria and comparison of data obtained by other techniques will improve our understanding of the taxonomic structure of this group of bacteria.

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