Pulsed-field gel electrophoresis analysis of the genome of amino acid producing corynebacteria: chromosome sizes and diversity of restriction patterns

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A large number of species of corynebacteria are known to be amino acid producers, including members of the genera *Corynebacterium* and *Brevibacterium*. Pulsed-field gel electrophoresis (PFGE) of DNA fragments obtained by using endonucleases which recognize AT-rich hexanucleotide or octanucleotide sequences produces a discrete pattern of bands useful for fingerprinting and physical mapping of the chromosome. Using *PacI* and *SwaI* endonucleases the genome of *Brevibacterium lactofermentum* ATCC 13869 (genome size 3052 kb) was consistently cut into 26 and 20 bands, respectively, and the genome of *Corynebacterium glutamicum* ATCC 13032 (2987 kb) yielded 27 and 26 fragments, respectively. The pattern of restriction fragments was identical for related strains (*B. lactofermentum* ATCC 13869, *B. lactofermentum* BL0, *B. lactofermentum* R31) but different from the pattern of fragments of other soil isolates of the same species (*B. lactofermentum* DSM 20412) or from closely related organisms such as *C. glutamicum*; the different pattern of restriction fragments may be used to differentiate taxonomically related species. *Brevibacterium linens* showed a different behaviour, due to its high G + C content; its genome (3105 kb) was resolved into 8 or 15 fragments, respectively, by digestion with the hexanucleotide-recognizing endonucleases *DraI* and *Asel*. PFGE of DNA fragments obtained using these enzymes is a powerful technique for quick resolution of the corynebacteria genome into a small number of large fragments.

Keywords: *Corynebacterium*, *Brevibacterium*, PFGE

INTRODUCTION

Corynebacteria are pleomorphic, non-spore forming, Gram-positive bacteria (Jones & Collins, 1986). The group includes diverse taxa, some of which are plant or animal pathogens, and others, saprophytes. Among the non-pathogens there are strains of industrial relevance as amino acid producers, classified as members of the genera *Corynebacterium* and *Brevibacterium*.

Several closely related species are commonly referred to as 'glutamic-acid bacteria', including *Corynebacterium glutamicum* and *Brevibacterium lactofermentum*. The two species share many common characteristics, such as cellular fatty acid composition, the presence of mycolic acids and peptidoglycans containing meso-diaminopimelate in the

cell wall (Suzuki & Komagata, 1983), similar nucleotide sequence of genes encoding the same enzymes in both species, G + C content in the range 54–56% (typical of true members of the genus *Corynebacterium*) and a high degree of similarity by DNA–DNA hybridization studies. Based on these data, the transfer of *B. lactofermentum* to the genus *Corynebacterium* has been proposed (Liebl *et al.*, 1991). Differentiation of strains classified as *C. glutamicum* and *B. lactofermentum* has been made by analysis of rDNA restriction patterns (Liebl *et al.*, 1991) and by restriction fragment length polymorphisms around *hom* genes (Eikmanns *et al.*, 1991). *Brevibacterium linens*, the type species of genus *Brevibacterium* (usually isolated from cheese sources), has a higher G + C content than *B. lactofermentum* and *C. glutamicum*. One strain, *B. linens* ATCC 19391, is a patented L-lysine producer. This strain belongs to the DNA homology group of the type species, *B. linens* ATCC 9172 (Fiedler *et al.*, 1981).

Abbreviation: PFGE, pulsed-field gel electrophoresis.

Considerable progress has been made on the molecular genetics of amino-acid-producing corynebacteria, especially in relation to cloning vectors, transformation systems and the study of structure, expression and regulation of genes involved in amino acid biosynthetic pathways (Martín *et al.*, 1987; Martín, 1989), but little is known about their global genome organization.

Pulsed-field gel electrophoresis (PFGE) is a powerful technique for studying the size and organization of bacterial genomes. Physical maps can be generated in the absence of detailed genetic maps. Identification of species, or strains classified in the same species, has been attempted with the use of rare-cutting restriction endonucleases followed by separation of the fragments of PFGE. The procedure has been applied successfully to *Pseudomonas* species (Grothues & Tümmler, 1991), *Streptomyces ambofaciens* strains (Leblond *et al.*, 1990), *Rhizobiaceae* (Sobral *et al.*, 1991), *Lactococcus* (Tanskanen *et al.*, 1990) and *Staphylococcus aureus* strains (El-Adhami *et al.*, 1991).

In this paper we report the determination of chromosome sizes of strains classified as *B. lactofermentum*, *C. glutamicum* and *B. linens* by addition of the sizes of fragments obtained by digestion with two different enzymes that cut the genome infrequently. We also describe conditions for electrophoretic separation of large restriction fragments that allow comparison of strains on a single gel run. To our knowledge this is the first study of the restriction patterns and genome sizes of corynebacteria using PFGE.

Table 1. Strains used in this study and their corresponding G + C content

Data taken from Liebl *et al.* (1991).

Bacterial strains		% G + C
<i>B. lactofermentum</i>	ATCC 13869	54.3
<i>B. lactofermentum</i>	BLO (non-pigmented mutant)	
	R31 (restriction-deficient mutant)	
	ATCC 13869 derivatives	
<i>B. lactofermentum</i>	DSM 20412	54.8
<i>C. glutamicum</i>	ATCC 13032	54.6
<i>B. linens</i>	ATCC 19391	63.8

METHODS

Bacterial strains. Bacterial strains used in this study and their corresponding G + C content are listed in Table 1.

Preparation and digestion of high molecular mass DNA.

Bacteria were grown to an OD₆₀₀ of 0.8–1.0 in Trypticase Soy Broth containing 0.5% glucose and 1% (w/v) glycine. Cells were harvested, washed and resuspended in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and then mixed with 1 vol. 1% (w/v) low-gelling agarose at 42 °C. The mixture was allowed to solidify in 100 µl plastic moulds to form blocks that were subsequently treated for 24 h at 37 °C with lysis solution

Fig. 1. Restriction pattern of total DNA of *B. lactofermentum* ATCC 13869 with enzymes recognizing 6 bp AT-rich sequences (a) or 8 bp GC-rich sequences (b). (a) Lanes: 1, lambda partially digested with *KpnI*; 2, *DraI* digestion; 3, *SphI* digestion; 4, *XbaI* digestion. Separations were carried out in the LKB Pulsaphor at 170 V using pulses of 2.5, 5 and 7.5 s for 8 h each. (b) Lanes: 6, *HindIII*-digested lambda DNA; 7, lambda ladder; 8, *NotI* digestion; 9, *SfiI* digestion. Fragments were separated in the LKB Pulsaphor at 170 V using pulses of 6 s for 18 h, 12 s for 18 h and 25 s for 8 h. Sizes in kb are indicated on the left and right. Note that the enzymes recognizing 6 bp AT-rich sequences resulted in too many fragments of small size (a) whereas good resolution was obtained with enzymes that recognize 8 bp GC-rich sequences (b).

Table 2. Restriction fragments and sizes of the chromosomes of *B. lactofermentum*, *C. glutamicum* and *B. linens* strains

These estimates were based on the measurement of fragments generated from a number of PFGE experiments conducted utilizing various pulse times.

Band	<i>B. lactofermentum</i> ATCC 13869		<i>B. lactofermentum</i> DSM 20412		<i>C. glutamicum</i> ATCC 13032		<i>B. linens</i> ATCC 19391	
	<i>PacI</i>	<i>SwaI</i>	<i>PacI</i>	<i>SwaI</i>	<i>PacI</i>	<i>SwaI</i>	<i>DraI</i>	<i>AseI</i>
A	390	557	606	557	518	325	874	720
B	300	342	264	315	385	276	610	600
C	264	340	220	295	266	262	567	390
D	242	262	210	244	264	242	348	285
E	212	242	205	215	212	237	325	240
F	180	222	180	188	164	199	150	190
G	175*	215	164	176	157	179	115	170
H	168	154	162	160	138	168	76	115
I	148	128	150	146	135*	165		110
J	135	120	146	123	98	126		75
K	98	106	144	122	97	118		65*
L	94	70	108	116	84	110		45
M	84	51	100	78	62	90		20
N	62	50*	92	70	50	74		15
O	50	48	84	68	46*	70		
P	48	35	62	51	25	68		
Q	46*	21*	35	45	23	51		
R	37	18	28	35	18	45		
S	28		27	34	14	38		
T	13		23	21	13	30		
U	11		14	11	9	21		
V	8		13		8	18		
W	7		7		7*	15		
X	5				6	13*		
Y						5		
Genome size (kb)	3026	3052	3044	3070	2987	2958	3065	3105

* Indicates doublets.

[1 M NaCl, 10 mM Tris/HCl, 0.5% Sarkosyl, 0.2% sodium deoxycholate, 0.25 M EDTA and 1 mg lysozyme (Boehringer Mannheim) ml⁻¹], followed by a 24 h treatment at 50 °C with 1 µg proteinase K (Sigma) ml⁻¹ in 0.25 M EDTA, 1% Sarkosyl. Blocks were kept at 4 °C in the last solution without proteinase K.

For digestion with restriction endonucleases blocks were cut into pieces containing approximately 1 µg DNA and were washed for 1 h at room temperature in 1 ml TE with 1 mM PMSF added, followed by a 1 h wash in 10 mM Tris/HCl, and then equilibrated in the appropriate restriction buffer for 1 h. The digestions were carried out in a final volume of 100 µl in the presence of 10–20 units of enzyme. Restriction enzymes were purchased either from New England BioLabs or from Boehringer Mannheim.

PFGE. Separation of macrofragments of DNA was carried out either in a Pulsaphor apparatus (LKB) equipped with a hexagonal electrode array or in a Bio-Rad CHEF-DR II apparatus (Bio-Rad). Agarose gels were prepared at 1–1.2% concentration in 0.5 × TBE buffer (1 × TBE buffer: 90 mM

Tris/HCl, 90 mM boric acid, 2.5 mM Na₂EDTA, pH 8.2). Separation of fragments was done at 6 V cm⁻¹, at 8 °C, with pulse times varying with the size range. Chromosomes of *Saccharomyces cerevisiae* (Bio-Rad) and concatemers of lambda DNA were used as molecular size standards. The measurements were made on the negatives and then confirmed with measurements obtained from scanning densitometry and image analysis (BioImage, Millipore).

RESULTS

Selection of restriction endonucleases to obtain large DNA fragments

Due to the high G + C content of corynebacteria (Table 1) and taking into account the tri- and tetranucleotide frequencies obtained by analysis of corynebacterial DNA sequences deposited in GenBank (Malumbres *et al.*, 1993), we tested enzymes expected to recognize only a few sites in these genomes. These enzymes recognized (i) 6 bp AT-

Fig. 2. Comparison of the restriction pattern of *B. lactofermentum* strains ATCC 13869 and DSM 20412 and *C. glutamicum* ATCC 13032 after digestion with *PacI* or *SwaI*. Separations were carried out in the Bio-Rad CHEF at 200 V using increasing time pulses of 5–20 s for 16 h and then 20–70 s for 20 h (a) or 16 h (b). Lanes: 2, 6 and 10, DSM 20412; 3 and 7, ATCC 13869; 4 and 11, ATCC 13032. P, *PacI*; S, *SwaI*; λ K, lambda *KpnI*; λ L, lambda ladder; SC, *S. cerevisiae* chromosomes.

rich, or CTAG-containing sequences such as *DraI* (TTTAAA), *AseI* (ATTAAT), *SspI* (AATATT), *XbaI* (TCTAGA) and *SpeI* (ACTAGT) or (ii) 8 bp GC-rich or AT-rich recognition sequences such as *SfiI* (GGCCNGGCC where N stands for any of the four nucleotides), *NotI* (GCGGCCGC), *PacI* (TTAATTAA) and *SwaI* (ATTTAAAT).

In a first approach we digested the chromosome of *B. lactofermentum* with the enzymes referred to above. Endonucleases which recognized hexanucleotides were found to be of little interest for analysing the genomes of the typical glutamic acid bacteria, *B. lactofermentum* and *C. glutamicum*. As in most bacterial genomes, the sequence $^5\text{CTAG}^3$ is the rarest tetranucleotide present in the genome of corynebacteria, among those recognized by commercially available endonucleases, representing less than 0.02% of the total possible tetranucleotide combinations found in 43215 bp examined. Enzymes recognizing CTAG-containing sequences digested the chromosomal DNA in too many fragments to allow accurate resolution, with the largest being about 50 kb. Similar results were observed when enzymes which recognize 6 bp AT-rich sequences were used, despite the high G+C content of corynebacteria (Fig. 1a).

Endonucleases which recognize 8 bp GC-rich sequences gave too many fragments to be useful in routine restriction mapping of glutamic acid corynebacteria (Fig. 1b) whereas endonucleases recognizing 8 bp AT-rich sequences were adequate. Discrete patterns of fragments from the DNA of these bacteria were obtained with *PacI* and *SwaI*. The three strains analysed with these two enzymes showed between 20 and 27 restriction fragments, ranging from about 5 to 600 kb (Table 2), and with a distribution that allowed separation in a single gel run. Conventional electrophoresis and pulsed-field electro-

phoresis using short time pulses were used to estimate sizes of the lower molecular mass bands (data not shown). The restriction patterns were quite distinct for each strain; on visual comparison it was possible to find only a few possible common motifs in the DNAs of all three strains of *B. lactofermentum* and *C. glutamicum* using digestions with the same enzyme (Fig. 2). We tried to resolve all the fragments resulting from digestion with *NotI* and *SfiI* (recognizing GC-rich octanucleotides) by changing pulse times in order to get different windows of resolution. Nevertheless, we concluded that the sum of the *NotI* and *SfiI* restriction fragments of the chromosome of *B. lactofermentum* ATCC 13869 identified by this procedure was always smaller than the values obtained by summing the *PacI* or *SwaI* fragments, most probably due to the presence of several doublets or triplets that could not be resolved.

Restriction endonucleases which recognize 6 bp sequences proved to be useful for the analysis of the genome of *B. linens*. All the enzymes gave patterns that could be resolved by PFGE; *AseI* (not shown in the figure) and in particular *DraI* gave the smallest number of fragments (Fig. 3 and Table 2).

Genome sizes

A consistent pattern of restriction fragments was routinely obtained in experiments using either *PacI* or *SwaI* for cutting the DNA of either *B. lactofermentum* or *C. glutamicum*. Therefore, it was possible to establish the size of the genome by adding the size of different fragments. Similarly, using either *DraI* or *AseI*, it was possible to determine accurately the chromosome size of *B. linens* ATCC 19391.

The genome sizes were estimated to be 3052 kb for *B.*

Fig. 3. Resolution of the genome of *B. linens* with enzymes recognizing 6 bp AT-rich sequences. (a) Lanes: 1, lambda ladder; 2, *SpeI* digestion; 3, *SspI* digestion; 4, *XbaI* digestion. Fragments were separated in the LKB Pulsaphor at 170 V using pulses of 5, 10 and 15 s for 8 h each. (b) Lanes: 5, resolution into eight fragments (A–H) after digestion with *DraI*; 6, lambda ladder. Fragments were separated in the Bio-Rad CHEF at 200 V using ramped pulses from 10–30 s for 16 h and 40–80 s for 18 h. Note that except for *DraI*, the enzymes used resulted in too many fragments of small size to be accurately resolved. Sizes of the largest *DraI* fragments were calculated by comparison of their migration with that of *S. cerevisiae* chromosomes (not shown in the figure).

lactofermentum ATCC 13869, 3070 kb for *B. lactofermentum* DSM 20412, 2987 kb for *C. glutamicum* ATCC 13032 and 3105 for *B. linens* ATCC 19391 (Table 2).

Closely related strains show the same pattern of restriction fragments

It was of interest to study whether closely related strains showed differences in the restriction pattern. As shown in Fig. 4, strains *B. lactofermentum* BLO, a non-pigmented mutant, and *B. lactofermentum* R31 (an easily transformable strain which does not restrict exogenous DNA; L. M. Mateos, M. Malumbres and J. F. Martín, unpublished) which derived from ATCC 13869, showed the same restriction pattern as the parental *B. lactofermentum* strain. However *B. lactofermentum* ATCC 13869 and DSM 20412 showed some clearly different bands. Both of these soil isolates were initially classified as *B. lactofermentum*, although it has been suggested that strain DSM 20412 should be reclassified as *C. glutamicum* (Liebl *et al.*, 1991). These results indicate that separation of restriction fragments by PFGE can be used to identify derivatives obtained from a parental strain and to distinguish them from different soil isolates of the same species.

Fig. 4. Use of PFGE for identification of different strains of *B. lactofermentum* and discrimination of *C. glutamicum*. Total DNAs were digested with *NotI*. Lanes: 1, *B. lactofermentum* ATCC 13869; 2, *B. lactofermentum* BLO; 3, lambda ladder; 4, *B. lactofermentum* R31; 5, *C. glutamicum* ATCC 13032; 6, lambda ladder; 7, *B. lactofermentum* ATCC 13869. Both separations were carried out in the Bio-Rad-CHEF apparatus at 180 V using pulse times of 20 s for 8 h, 15 s for 16 h, 5 s for 12 h and 2.5 s for 8 h (left), and a pulse gradient from 2 to 40 s for 24 h (right).

DISCUSSION

The restriction patterns and genome sizes of three different corynebacterial species have been established by resolution of large DNA fragments using PFGE. Pulse conditions were adjusted so that all the restriction fragments could be resolved, as shown by the almost identical values of genome size obtained with two different enzymes. The chromosome of these bacteria is smaller than that of *Escherichia coli* (4700 kb) (Smith *et al.*, 1987) but it is similar to, or even larger than, the genome of related Gram-positive unicellular bacteria such as *Lactococcus lactis* (2500 kb), *Staphylococcus aureus* (2860 kb), *Streptococcus thermophilus* (1700 kb) (Bourgeois *et al.*, 1989; Jones & Collins, 1986; Krawiec & Riley, 1990), and *Leuconostoc oenos* (1100–1500 kb) (Daniel *et al.*, 1993). The genome size determined by this procedure agrees well with previous estimates (1.7×10^9 – 1.9×10^9 Da, approximately 2550–2850 kb) obtained by the renaturation rate method for strains belonging to the corynebacteria group (Bak *et al.*, 1970; Crombach, 1978).

The genomes of *B. lactofermentum* and *C. glutamicum* gave well-resolved restriction patterns when digested with endonucleases recognizing AT-rich octanucleotides. The same happens with other bacteria belonging to the 'glutamic acid group', such as *Brevibacterium flavum*, *Brevibacterium ammoniagenes* and *Corynebacterium callunae*;

the chromosome of *C. callunae* was about 500 kb smaller than those of the other strains (unpublished observations).

B. linens showed a distinct behaviour, perhaps because of its higher G+C content. The sequence 5'TTTAAA3' is rarer than predicted from the %G+C of this strain; assuming a value of 64 for the %G+C, the sequence should occur once every 30 kb, with about 100 fragments resulting from digestion with *Dra*I instead of the eight fragments observed. The 5'TTTAAA3' sequence is much rarer than the 5'CTAG3'-containing sequences recognized by *Xba*I and *Spe*I. In *E. coli* and other bacteria it was reported that the oligonucleotide frequency is not only dependent on G+C content but it is also strongly biased by codon usage (McClelland *et al.*, 1987; Phillips *et al.*, 1987). It is likely that the same occurs in *B. linens*, although due to the small extent of the chromosome of *B. linens* that has been sequenced it is not possible to confirm this possibility.

Some authors (Forbes *et al.*, 1991; Grothues & Tümmeler, 1991) have claimed that restriction fragment fingerprinting is useful for studying genetic relatedness of different strain isolates, but results with other species such as *Helicobacter pylori* (Taylor *et al.*, 1992) and *Bacillus cereus* (Carlson *et al.*, 1992), and also our own results, prove that, at least for groups of very heterogeneous bacteria, the criteria of grouping according to restriction fragment patterns has little taxonomic value. The two *B. lactofermentum* strains (and to a lesser degree the *C. glutamicum* strain) are very closely related from the point of view of biochemical and morphological characteristics, but they represent a heterogeneous family of isolates with different genome structures. Whether this diversity results from deep chromosome rearrangements will only be clarified by fine mapping and determination of relative gene positions on the genome of these strains.

This technique is, however, useful to establish the degree of relatedness among different strains of the same species. As shown in Fig. 4, closely related strains retain very similar restriction patterns, which are different from the restriction patterns of other soil isolates of the same species.

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