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Construction of a *Fibrobacter succinogenes* Genomic Map and Demonstration of Diversity at the Genomic Level

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Abstract. The genomic cleavage map of the type strain *Fibrobacter succinogenes* S85 was constructed. The restriction enzymes *Asc*I, *Avr*II, *Fse*I, *Not*I, and *Sfi*I generated DNA fragments of suitable size distribution that could be resolved by pulsed-field gel electrophoresis (PFGE). An average genome size of 3.6 Mb was obtained by summing the total fragment sizes. The linkages between the 15 *Asc*I fragments of the genome were determined by combining two approaches: isolation of linking clones and cross-hybridization of restriction fragments. The genome of *F. succinogenes* was found to be represented by the single circular DNA molecule. Southern hybridization with specific probes allowed the eight genetic markers to be located on the restriction map. The genome of this bacterium contains at least three rRNA operons. PFGE of the other three strains of *F. succinogenes* gave estimated genome sizes close to that of the type strain. However, RFLP patterns of these strains generated by *Asc*I digestion are completely different. Pairwise comparison of the genomic fragment distribution between the type strain and the three isolates showed a similarity level in the region of 14.3% to 31.3%. No fragment common to all of these *F. succinogenes* strains could be detected by PFGE. A marked degree of genomic heterogeneity among members of this species makes genomic RFLP a highly discriminatory and useful molecular typing tool for population studies.

Fibrobacter succinogenes is one of the major cellulolytic bacteria in the rumen [9, 18]. Interest in the development of genetic techniques for this and other ruminal fibrolytic bacteria has been aided, mainly, by the possibility to enhance their fiber-digesting potentials [6]. A number of genes coding the polymer-degrading enzymes have been successfully cloned from *F. succinogenes* and characterized [1, 3, 13, 15, 21, 22]. At the same time, other aspects of its genetic organization are scarcely represented in the current literature. One of these approaches, the physical mapping, has been improved methodologically to the point where the restriction map of the chromosome can be generated virtually from any prokaryotic organism. The use of pulsed-field gel electrophoresis (PFGE) allows overcoming of the problems associated with the absence of gene transfer systems or mutant strains. In recent years, this approach has been applied for physical mapping of more than 80 bacterial genomes [7]. In the particular field of ruminal microbiology, this methodology has been used to monitor the bacteriophage population variations [10, 19]. However, there have been no reports of physical mapping among rumen bacteria.

DNA fingerprints produced by PFGE are equivalent to the identification of restriction fragment length polymorphism (RFLP) at the genomic level among bacterial isolates. When the sequencing data establish phylogenetic relations for individual genes, using genomic PFGE may reveal the overall chromosome structure of bacterial isolates. Thus, it could detect the mosaic structure of the

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Table 1. Primer pairs used to generate the hybridization probes

Gene	Primer pair	Primer sequence	Product size (bp)	Primer position on original sequence	Accession no. or reference
cedA	cedA-1	GTG AAT TTG GGC GGA TGG	545	996	U07419
	cedA-2	GCG CTG TTC CAT TTG TTG		1523	
endB	EGB-1	AAA GAA TCG TAT GAA CTG	507	709	L14436
	EGB-2	CGA TTC CGT AGG CGT CAC		1198	
mgl	GLU-1	AGG TTC CGA GAG ATG ACG	495	929	M33679
	GLU-2	CCG ATA TTA CCG ATG AGC		1406	
cel3	cel3-1	CGG GTG CCG AAA ATC AGG	552	292	M29047
	cel3-2	CCA GCC ACC TTG AAC GAG		846	
gyrA-xynC	gyr-1	GTG GTA GCA AGG GTG TCC	471	224	U01037
	gyr-2	CGA TTA TCC GCC AAC AGC		677	
end-1	end-11	CTC CGG GCA ACA AGA ACG	529	217	X88561
	end-12	TCG AAG TCG GAA ATC ACG		745	
celG	celG-1	TCC AAC GGC GAA AGA AGT	587	203	U33887
	celG-2	CGC TTT TGA CCG ACT TGC		789	
rrs	10F	GAG TTT GAT CCT GGC TCA G	1486	1	This laboratory
	1500R	AGA AAG GAG GTG ATC CAG CC		1486	
rrl	11a	GGA ACT GAA ACA TCT AAG TA	2556	188	[4]
	97ar	CCC GCT TAG ATG CTT TCA GC		2744	

genome generated by recombination processes, horizontal transfers, and mobile elements. PFGE-generated patterns have been applied for study of genome, population, strain, and species structure of enteric, soil, and clinical isolates [7]. This approach is still to be tested for rumen bacterial isolates.

Here we present the first physical and genetic map of a rumen bacterium. The *Asc*I restriction map of the type strain *F. succinogenes* S85 with localization of the eight genetic markers was generated in this work. Genomic RFLP analysis with *Asc*I digestion and PFGE was applied to monitor the genetic diversity among *F. succinogenes* isolates.

Materials and Methods

Bacterial strains, isolation, and identification procedures, plasmid and growth media. The type strain of *F. succinogenes* subsp. *succinogenes* S85 (=ATCC 19169) was obtained from the ATCC and was cultivated in the chemically defined medium as described [16]. Other *F. succinogenes* strains used in this work were isolated by us from the rumen content of a fistulated cow. To minimize the strain heterogeneity introduced by geographical, seasonal, and diet variations, we sampled all strains from a single collection of the rumen content. Identification of the bacterial isolates was done by PCR amplification and sequence analysis of the 16S and 23S rDNA sequences as well as by the classical identification techniques. Competent *Escherichia coli* JM109 cells were purchased from Toyobo (Osaka, Japan) and plasmid pNEB193 from New England Biolabs (Beverly, USA). The *E. coli* strain was grown in LB medium supplemented, when necessary, by ampicillin (50 µg/ml).

Genomic DNA preparation and restriction digests. Agarose plugs containing *F. succinogenes* DNA were prepared according to a modified procedure of Birren and Lai [2]. The main modification involved the biomass collection and washing steps to be done in anaerobic conditions and with anaerobically prepared solutions. In brief, cells were harvested in the mid-log phase of growth, resuspended in TEN buffer (10 mM Tris-HCl, 5 mM EDTA, 0.9% NaCl) and embedded in low-melting-point agarose (Bio-Rad, Richmond). Blocks were removed from the mold and washed twice in ES buffer (0.5 M EDTA, 0.1% sarcosyl, pH 9.5) with gentle agitation. Cells then were digested with proteinase K (1 mg/ml) in ES buffer for 20 h at 55°C. After digestion, blocks were washed four times in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5) and stored at 4°C in the same buffer. Restriction enzymes were purchased from New England Biolabs and Takara (Kyoto, Japan) and used as specified by the manufacturers.

PFGE analysis. Electrophoresis was conducted with a CHEF-Mapper electrophoresis system (Bio-Rad) in 0.5× TBE buffer at 14°C. Optimal resolution of the restriction fragments was achieved with the Auto algorithm of a CHEF-Mapper power module. Lambda and 5-kb ladders (Bio-Rad) were used as molecular size markers.

Isolation of linking clones. The *F. succinogenes* chromosomal DNA in solution was prepared as described [2]. The DNA was cleaved with restriction enzyme *Sau*3AI or *Kpn*I, diluted to the concentration of 5 µg/ml, and self-ligated with T4 DNA ligase (Gibco-BRL) at 37°C for 4 h. The mix of the ligated fragments was then linearized with *Asc*I and ligated into the corresponding site of plasmid pNEB193 (New England Biolabs). This mix was transformed into *E. coli* JM109, and the recombinants were checked by restriction analysis and sequencing.

Preparation of genetic markers. Oligonucleotide primers for PCR amplification of genetic markers were designed on the basis of published sequences with the Oligo program for Macintosh (National Biosciences, Plymouth, UK). The primers were synthesized on an Applied Biosystems 394 DNA/RNA synthesizer. Information related to the primer sequences, PCR product sizes, and corresponding reference/GenBank accession numbers are summarized in Table 1. Digoxigenin-labeled probes were generated in a 100-µl PCR reaction that contained: (1) the PCR buffer and the enzyme mix of Expand™ High Fidelity PCR System (Boehringer, Mannheim, Germany) at the recommended concentrations, (2) 250 pmol of a primer couple, (3) one microgram of chromosomal DNA, and (4) digoxigenin labeling mixture (Boehringer Mannheim) as specified by the manufacturer. The first denaturation step

at 94°C for 2 min was followed by 25 cycles of 30 s of denaturation at 94°C, 30 s of annealing at 50°C, and 1 min of elongation at 72°C. The final elongation step was for 2 min. PCR products were purified from agarose gels with the GENECLEAN II kit (BIO 101 Inc., La Jolla, USA).

No information was available on the 23S rRNA sequence of *F. succinogenes*. The corresponding region was amplified by PCR with the primers targeted at the conserved regions of the gene as described [4]. The 16S rDNA sequences were retrieved by PCR amplification with the universal primer set (positions 10F and 1500R).

Southern hybridization analysis. After electrophoresis, DNA was transferred onto the NYTRAN membrane (Schleicher & Schuell) with a model 785 vacuum blotting system (Bio-Rad). Hybridization was done with a DNA labeling/detection kit from Boehringer Mannheim, under the standard conditions recommended by the manufacturer.

Results and Discussion

About 40 restriction enzymes recognizing the octanucleotide or hexanucleotide sequences were tested for possible use in mapping the *F. succinogenes* S85 chromosome. In addition, we also checked the three intron-encoded endonucleases, I-*Ceu*I, PI-*Tli*I and PI-*Sce*I. This strain has been shown to possess the type II restriction-modification system and has nonspecific endonuclease activity [12]. The overall GC content is 47–49% [11]. Restriction enzymes, therefore, were tested for their ability to digest the chromosomal DNA in solution. Some enzymes such as *Sse* 8387 I (CCT GCA GG) were found to be sensitive to the DNA methylation and were excluded from the mapping experiments. For unknown reason, the intron-encoded endonucleases did not cleave the DNA of *F. succinogenes* S85, either in solution or in the agarose-embedded form. Restriction enzymes that generated a manageable number of fragments were the four enzymes that recognize the 8-bp sequences containing exclusively C and G residues: *Asc*I (GGC GCG CC), *Fse*I (GGC CGG CC), *Sfi*I (GGC $C(N)_5G$ GCC), and *Not*I (GCG GCC GC). It is noteworthy that the hexanucleotide-recognizing enzyme, *Avr*II (CCT AGG), was also found to be useful in PFGE separation experiments.

Figure 1 shows the typical PFGE separation patterns of *F. succinogenes* genomic DNA digested by selected restriction enzymes. Series of PFGE experiments were conducted to improve the resolution of genomic fragments in overlapping regions. For example, in gels that were run with the pulse time of 0.5–35 s, the biggest *Asc*I fragment looks like a single band (Fig. 1A and B). The increase of the pulse time (40–70 s) led to its clear separation into two bands (Fig. 1C). Data from several experiments are summarized in Table 2. Genome size of this bacterium was estimated to be approximately 3.6 Mb; this is about 76.6% of the *E. coli* genome [17]. *Not*I and *Sfi*I digestions also produced the sums of fragments very close to this value (data not shown). According to

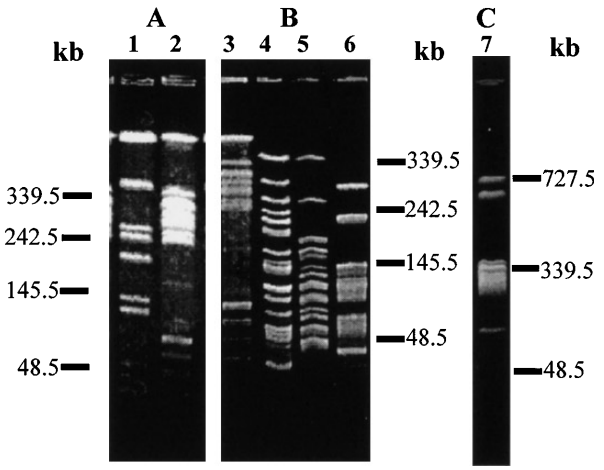

Fig. 1. PFGE separation of *F. succinogenes* genomic DNA cleaved with different restriction enzymes: lane 1, *Fse*I; lanes 2, 3, and 7, *Asc*I; lane 4, *Avr*II; lane 5, *Not*I; lane 6, *Sfi*I. These 1% agarose gels were subjected to electrophoresis for 20 h (A and C) and 15 h (B) at 200 V and at 14°C. The pulse time for A and B gels was 0.5 s to 35 s, and for C gel was 40 s to 70 s. The molecular size marker positions (Lambda ladder, Bio-Rad) are shown alongside the gels.

the bacterial genome size classification [5], the *F. succinogenes* genome belongs to the third group in the range of 3–4.5 Mb.

The *Asc*I restriction map of the chromosome was constructed by means of Southern hybridization analysis with probes derived from the linking clones (see Materials and Methods). We succeeded in establishing all linking pairs between the *Asc*I-produced fragments (Table 3). This linkage map was further verified in a series of additional experiments in which probes were generated from the genomic fragments produced by other enzymes. Particularly, the 240-kb *Avr*II fragment (Table 2) of the chromosome was used to demonstrate the linkage between the A5.2 and A8.2 fragments on the map (Fig. 2). The cross-hybridization experiments also approved the validity of localization of the two fragments, A8.1 and A8.2, that could not be resolved by PFGE. Finally, the complete circular form of the *F. succinogenes* chromosome was established (Fig. 2). Some bacteria have been demonstrated to have two chromosomes or the linear chromosome [5], but, in the case of *F. succinogenes*, the genome is usual in these respects: it is represented by the single circular chromosome.

In total, the eight genetic markers (Table 1) were localized on the *Asc*I restriction map of *F. succinogenes* S85 (Fig. 2). The 16S and 23S rDNA probes hybridized with the same chromosomal fragments, suggesting a close physical linkage. This was confirmed also by PCR amplification of the 16S–23S rDNA spacer region (unpublished data). It seems that *Campylobacter jejuni* and *C. coli* are exceptional in this regard, since these regions

Table 2. Restriction fragments generated by *Asc*I, *Fse*I, and *Avr*II digestions and the estimated size of the *F. succinogenes* S85 genome

Fragment no.	Fragment size (kb)		
	*Asc*I	*Fse*I	*Avr*II
1	(A1.1)[a] 715	850	370
2	(A1.2) 630	700	370
3	(A2) 360	540	280
4	(A3) 320	380	240
5	(A4) 290	270	220
6	(A5.1) 275	250	210
7	(A5.2) 270	230	210
8	(A6) 240	140	190
9	(A7) 225	130	190
10	(A8.1) 80	45	150
11	(A8.2) 80	30	130
12	(A9) 60		125
13	(A10) 45		125
14	(A11) 35		123
15	(A12) 30		95
16			95
17			80
18			62
19			50
20			45
21			40
22			38
23			36
24			35
25			33
Totals	3682	3565	3542

[a] Fragment designation on the *Asc*I restriction map (Fig. 2).

Table 3. Linkage of *Asc*I fragments determined by hybridization with linking clones

Linking clones	Linkage
pAS–8	A8.2–A12
pAS–108	A7–A9
pAS–122	A5.1–A12
pAS–219	A3–A10
pAS–232	A7–A11
pAS–237	A2–A4
pAS–301	A8.1–A11
pAS–307	A1.2–A6
pAS–328	A3–A5.1
pAS–504	A1.1–A4
pAS–510	A6–A9
pAS–512	A5.2–A8.2
pAS–609	A8.1–A10
pAS–908	A1.2–A2
pAS–922	A1.1–A5.2

have been found split on their chromosomes [20]. In most bacterial species the situation is standard: the 16S and 23S sequences are parts of the rRNA operon and transcribed as a single transcript. The 16S–23S spacer region

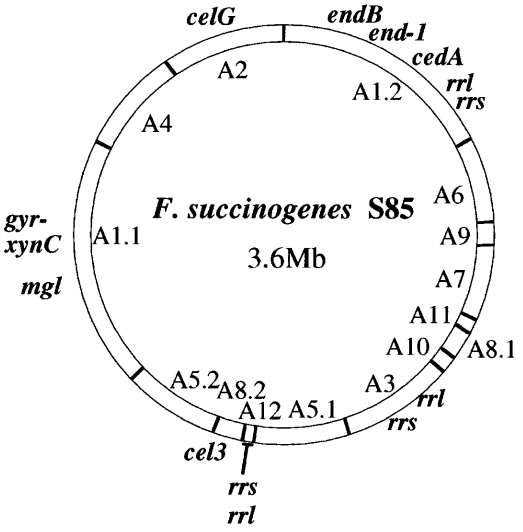

Fig. 2. *Asc*I restriction map of *F. succinogenes*. Genetic markers used for hybridization are listed in Table 1. Markers on A1.1 and A1.2 fragments are placed arbitrarily.

sequence has even been proposed as a typing and identification tool [8]. The ribosomal RNA genes of *F. succinogenes* have the operon structure, and there are at least three such operons on the chromosome.

The five genes, encoding the hydrolytic enzymes, were located on the biggest A1.1 and A1.2 fragments (Fig. 2). They were placed on the corresponding fragments arbitrarily, and the positioning does not reflect the true order of the genes in the chromosome. In an attempt to establish whether there is the physical linkage between the genes localized on the same (or adjoined) fragment(s), the series of PCR amplifications were performed with the primers (Table 1) in all possible combinations. In these experiments we used the LA-PCR v.2 kit from Takara (Japan), which is able to amplify the long sequences up to 20 kb long (Takara catalog). No amplification products were detected under the PCR conditions recommended by the manufacturer for amplification of long sequences. We concluded, therefore, that the genes residing on the same or linking fragments are separated by at least 20 kb intergenic sequences and certainly do not comprise any cistronic unit. Our data on the positioning of the cellulase/xylanase genes in the chromosome of *F. succinogenes* support the view that there is no *cis*-interaction between the genes, and these genes are randomly scattered in the genome.

The PFGE procedure optimized for the type strain of *F. succinogenes* allowed us to start the population studies with the other strains. At the 16S rDNA sequence level, the three isolated strains, FS3, FS5, and FS8, displayed the sequence variations in the range of 98.1–98.4% in relation to the type strain (unpublished data). Genomic

Table 4. *Asc*I restriction fragments of *F. succinogenes* strains

	Fragment size (kb)			
Fragment no.	Strain: S85	FS3	FS5	FS8
1	715	600	700	500
2	630	420	365	430
3	360	355	310	350
4	320	350	260	310
5	290	320	260	310
6	275	320	210	270
7	270	320	200	225
8	240	260	165	205
9	225	210	160	170
10	80	80	130	162
11	80	80	120	155
12	60	65	85	140
13	45	65	75	125
14	35	45	60	120
15	30	40	52	100
16		35	45	80
17			42	37
18			37	32
19			35	30
20			32	
21			27	
Totals	3682	3215	3370	3751

RFLP analysis, nonetheless, revealed considerably lower similarity (Table 4). Strains FS3, FS5, and FS8 shared, correspondingly, the 31.3%, 14.3%, and 21.1% of comigrating fragments with the type strain S85. There is no fragment common to all the studied strains. At the same time, the differences between their genome sizes are well in the limits of accuracy of PFGE, excluding the possibility of the artificial diversity of genome sizes owing to different DNA methylation patterns.

Bacterial isolates can be divided into two groups in relation to genome structure conservation [7]. At one extreme, including *E. coli* and *Salmonella*, *Streptomyces*, *Neisseria*, *Lactococcus*, *Clostridium*, and *Mycoplasma* spp., conserved gene order can be traced to different genera levels. At the other extreme, including *Bacillus*, *Rhodobacter* , *Helicobacter*, and *Leptospira* spp. and several cyanobacteria, the gene order is highly rearranged even for different strains of the same species [7]. Albeit there is no adequate biological explanation for this variance at the present time, the representatives of the second group have the natural transformability state in their life cycles [14]. Returning to the *F. succinogenes* strains, we may consider them as belonging to the second group with the high heterogeneity degree at the genomic level. A number of explanations can be postulated to account for genomic variability among *F. succinogenes* isolates. These include chromosome rearrangements, horizontal transfers, and mobile elements. Which factors have contributed to the heterogeneity in the restriction fragment patterns remains to be proven.

The macrorestriction map of *F. succinogenes* constructed in this report may serve as a basis for the development of physical and genetic maps of this bacterium. The finding of genomic heterogeneity among *F. succinogenes* strains in this study opens the potential application of PFGE as an identification tool. It can be useful in situations where the resolution of other techniques, such as hybridization, would not be sufficient for precise identification purposes.

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