Pulsed-Field Gel Electrophoresis Study of the Genome of *Caldocellum saccharolyticum*

Kim M. Borges and Peter L. Bergquist

Centre for Gene Technology, Department of Cellular and Molecular Biology, University of Auckland, Auckland, New Zealand

Abstract. The genome of the anaerobic thermophilic eubacterium *Caldocellum saccharolyticum* was analyzed by pulsed field gel electrophoresis. The restriction endonucleases *NheI* and *SstII* cleaved the genome into 23 and 21 fragments, respectively, and the sums of the fragments gave a genome size of approximately 2.78 Mbp. Hybridization of probes for several cellulolytic and hemicellulolytic genes, as well as a 16S ribosomal RNA gene, gave complex signals on PFGE-separated restriction fragments. Mapping of the genome by use of linking clone hybridizations had been initiated.

*Caldocellum saccharolyticum* is an anaerobic thermophilic eubacterium that was isolated from a New Zealand hot spring [9]. The organism has an optimum growth temperature of 68°C. Although *Ca. saccharolyticum* degrades a variety of cellulose materials, as does the anaerobic thermophile *Clostridium thermocellum*, they are only distantly related [9]. Phylogenetic analysis of 16S ribosomal RNA gene sequences has established that *Ca. saccharolyticum* is one of the most ancient anaerobic eubacteria yet discovered, coming off the eubacterial phylogenetic tree alongside *Thermus* and other thermophilic species [23].

No genetic studies have been done with this organism, although several genes involved in cellulose and hemicellulose degradation have been cloned and expressed in *Escherichia coli* [5]. In this study, a pulsed-field gel electrophoresis (PFGE) examination of the genome of *Ca. saccharolyticum* was initiated to facilitate analysis of the genetics and chromosome structure of this novel bacterium.

Hybridization of cloned genes to PFGE-separated restriction fragments. PFGE-separated restriction fragments were exposed to 254 nm ultraviolet irradiation to fragment the DNA; then were denatured with 1.5 M NaCl, 0.5 M NaOH for 60 min; neutralized with 1.5 M NaCl, 0.5 M Tris-HCl (pH 7.2); 1 mM EDTA for 60 min; and transferred to positively charged nylon membranes (Boehringer Mannheim) by capillary transfer with 15 × SSC buffer (20 × SSC stock solution is 3 M NaCl, 0.3 M trisodium citrate). Membranes were baked at 120°C for 30 min to fix the DNA to the filter.

Twenty-five to 50 ng of gel-purified PCR product or plasmid DNA (linearized with an appropriate restriction endonuclease) was radiolabeled with 20 μCi [α-32P]-dCTP (3000 Ci/mmol, NEN-Dupont, Boston, Massachusetts) by use of the Gigaprime DNA labeling kit (Bresatec Limited, Adelaide, Australia). Membranes were prehybridized at 60°C for at least 1 h in 0.5 M sodium phosphate (pH 7.2), 1 mM EDTA, 5% SDS; then the probe was added and the membranes were hybridized overnight. Membranes were washed twice with 2 × SSC, 0.1% SDS at room temperature for 5 min, followed by two washes with 0.1 × SSC, 0.1% SDS at 60°C for 10 min. Autoradiography was performed for 16–72 h with Hyperfilm MP X-ray film (Amersham Australia, Auckland, New Zealand).

Preparation of linking clones. Genomic DNA isolated from *Ca. saccharolyticum* [21] was partially digested with *Sau3A* endonuclease, fractionated on a salt gradient [21], and 6–7 kb fragments were ligated into *BamHI*-digested, alkaline phosphatase-treated pUC19 plasmid DNA. The ligation mixture was transferred into competent *Escherichia coli* DH5-α cells [12], and ampicillin (25 μg/ml)-resistant transformants were selected. Plasmid DNA was prepared from pooled colonies by alkaline lysis followed by banding on a cesium chloride gradient [21]. Plasmid DNA was digested with either *NheI* or *SstII* endonuclease and was ligated to a *NheI* or *SstII*-digested kanamycin cassette derived from pUC4K [4] to select plasmids containing *Ca. saccharolyticum* DNA with the rare restriction endonuclease site. The ligation mixture was transformed into DH5-α cells, and transformants containing *Ca.*

Materials and Methods

DNA preparation, restriction endonuclease digestion, and PFGE. Chromosomal DNA gel plugs were prepared from *Ca. saccharolyticum* cells as described [6]. DNA gel plugs were stored in TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) at 4°C. Restriction endonuclease digestions and PFGE were performed as described [6]. Specific electrophoresis conditions are described in the figure legends.

Address reprint requests to: Dr. Peter L. Bergquist, Centre for Gene Technology, Department of Cellular and Molecular Biology, University of Auckland, Auckland, New Zealand.
Ca. saccharolyticum DNA with a rare restriction endonuclease site were selected by resistance to both ampicillin and kanamycin (each 25 μg/ml). Linking clones were linearized, radiolabeled, and hybridized to the appropriate PFGE-separated restriction fragments as described above.

Results and Discussion

To study the genome of Ca. saccharolyticum by PFGE, the chromosome must be cleaved into a small number of fragments with a restriction endonuclease. The restriction fragments must differ in size sufficiently to be readily separable by PFGE. Endonucleases which cleave at G + C-rich recognition sites were tested initially, since the genome of Ca. saccharolyticum has a low G + C content (34%) [9]. Forty-two restriction endonucleases were screened for their ability to cleave Ca. saccharolyticum DNA into a suitable number of resolvable restriction fragments (data not shown).

Most endonucleases cleaved the chromosome into too many small fragments (less than 150 kb) to be useful, while others consistently gave only partial genome digestion or did not cleave the genome at all (data not shown). Eleven enzymes produced some DNA fragments greater than 250 kb and cleaved the genome into an estimated 25 or fewer fragments. In most cases, however, some of the bands were spaced too closely for adequate resolution. The endonucleases NheI and SstII were chosen for genome analysis because they generated the smallest numbers of DNA bands (19 and 20, respectively), and the bands could be resolved by PFGE.

Figure 1 shows typical PFGE banding patterns produced by cleavage of Ca. saccharolyticum DNA with NheI and SstII endonucleases. Cleavage by NheI enzyme generated fragments ranging in size from 376 kb to 4 kb, while SstII cleavage yielded fragments ranging from 341 kb to 20 kb (Fig. 1 and Table 1). Figure 1A shows the separation of NheI bands 3–19 and SstII bands 3–20. NheI bands 7, 9, and 11 appeared to consist of two comigrating fragments, as did SstII band 13. When the largest two bands of each restriction pattern were resolved, NheI band 2 was found to consist of two comigrating fragments (Fig. 1B). Figure 1C shows that the NheI band 15 was separable into two bands (15 and 16). Conventional fixed-field electrophoresis was also used to attempt to detect smaller bands, and three more NheI bands (17–19) were detected under these conditions (data not shown). However, these small bands (less than 9 kb) were not detected consistently; it is possible that low-molecular-weight fragments diffused from the gel plugs during digestion or storage, thus making their electrophoretic detection unreliable. Significant quantities of DNA fragments 50 kb or smaller have been shown to elute from gel plugs during restriction endonuclease digestion and washing [10]. In total, cleavage of the Ca. saccharolyticum genome resulted in at least 23 NheI fragments (contained on 19 bands) and 21 SstII fragments (on 20 bands).

The Ca. saccharolyticum genome size (determined by adding the lengths of the restriction fragments from the NheI and SstII digestions) was calculated to be 3.0 and 2.5 Mbp respectively, for an average chromosome size of 2.78 Mbp. The chromosome is comparable in size to some of the smaller eubacterial genomes, such as Lactococcus sp. (2–2.7 Mbp) [13, 26] and Staphylococcus aureus (2.75 Mbp) [19]. The genome is also similar in size to the 3-Mbp genome of Sulfolobus acidocaldarius [28], a thermophilic archaeabacterium. However, it is larger than those of other thermophilic microorganisms measured by PFGE: the genome of Thermoplasma acidophilum is 1.75 Mbp [13], the Thermus thermophilus HB8 genome is estimated to be 1.74 Mbp [7], and the genome of the archaeabacterium Thermococcus celer is 1.89 Mbp [18]. The chromosome is significantly smaller than the 3.6-Mbp genome of Clostridium perfringens [8], another anaerobic eubacterium that has been studied by PFGE. Thus, the genome of this anaerobic extreme thermo-