Appl Microbiol Biotechnol (1996) 45:127–131

ORIGINAL PAPER

T. R. Klapatch · A. L. Demain · L. R. Lynd

Restriction endonuclease activity in *Clostridium thermocellum* and *Clostridium thermosaccharolyticum*

Received: 10 March 1995/Received revision: 4 September 1995/Accepted: 13 September 1995

Abstract *Clostridium thermocellum* cell extracts exhibit specific endonuclease activity with very little non-specific exonuclease activity at 55°C. The Dam methylation system of *Escherichia coli* offers complete protection from digestion by *C. thermocellum* ATCC 27405 cell extracts for all DNA tested (totaling > 100 kb, insuring that most potential restriction sequences have been exposed). Based on both the Dam recognition sequence and the similarity of cell extract and *Mbo*I DNA digests, the *C. thermocellum* restriction enzyme recognition sequence appears to be 5′ GATC 3′. Cell extracts made from a second thermophile, *C. thermosaccharolyticum* ATCC 31960 do not exhibit specific endonuclease activity under the conditions tested. Genomic DNA from *C. thermocellum* exhibits a Dam^+ phenotype while genomic DNA from *C. thermosaccharolyticum* exhibits a Dam^- phenotype.

Introduction

Clostridium thermocellum is an obligately anaerobic, cellulolytic, thermophilic bacterium that has often been considered for use in ethanol production from cellulosic biomass (Slapack et al. 1987). *C. thermosaccharolyticum*, an anaerobic, non-cellulolytic, pentose-utilizing thermophile, has been considered for use as a partner in co-culture with *C. thermocellum* in biomass fermentation (Saddler and Chan 1983; Venkateswaran and Demain 1985). Molecular manipulation with the aim of either increasing ethanol selectivity of both organisms or improving the cellulase system of *C. thermocellum* would be of great interest. A prerequisite for any such manipulation is the ability to introduce foreign DNA into these bacteria.

Restriction/methylation (RM) systems are usually composed of an endonuclease that digests DNA at a specific base sequence and a methylase that protects the sequence from digestion by its action at a specific position on one or more bases in that same sequence (Modrich and Roberts, 1982). RM systems have been hypothesized to have evolved as protective mechanisms to guard bacteria from foreign DNA (e.g. phage infection) (Klaenhammer 1987). RM systems can also be obstacles to transformation, as they digest incoming DNA that is not properly methylated. The action of endonucleases has been shown to be the primary barrier to achieving efficient transformation of *C. acetobutylicum* using DNA that is not protected by appropriate methylation (Azeddoug et al. 1989; Mermelstein et al. 1992). The understanding of restriction/modification systems in organisms on the receiving end of molecular genetics may often be the first step to developing successful transformation protocols.

This communication investigates whether endonuclease activity is present in *C. thermocellum* ATCC 27405 and *C. thermosaccharolyticum* ATCC 31960, as well as associated recognition sequences and methylase-mediated protection from endonuclease attack.

T. R. Klapatch · L. R. Lynd
Department of Biological Sciences, Dartmouth College, Hanover, N.H., USA

A. L. Demain
Department of Biology, Massachusetts Institute of Technology, Cambridge, Mass., USA

L. R. Lynd (✉)
Thayer School of Engineering, Dartmouth College, Hanover, N.H., USA

Materials and methods

Organisms, plasmids, phages and enzymes

C. thermocellum ATCC 27405 and *C. thermosaccharolyticum* HG8 (ATCC 31960) were used in this study. *Escherichia coli* strains were a generous gift from New England Biolabs (Beverly, Mass., USA)

and are: GM271 (Dam$^+$/Dcm$^-$), ED8767 (Dam$^+$/Dcm$^+$), GM2163 (Dam$^-$/Dcm$^-$), GM475 (Dam$^-$/Dcm$^-$). Plasmid and phage DNAs were purchased from Sigma, (St. Louis, Mo., USA) and Stratagene (La Jolla, Calif., USA), except for plasmid pCTC1 (Williams et al. 1990) which was a kind gift from M. Young. Restriction enzymes were from Stratagene or New England Biolabs.

Cell and protoplast extract preparations

Cell extracts of *C. thermocellum* strain ATCC 27405 or *C. thermosaccharolyticum* strain ATCC 31960 were prepared from stationary phase cells grown as described previously (Hogsett et al. 1992; Lynd et al. 1991). Two methods were employed which yielded equivalent results. The first method involved 100 ml of culture passed once through a Microfluidics (Newton, Mass., USA) cell disrupter that was packed in ice. Cell extracts were also prepared by passage of cells once through a French press cell disrupter (20 000 psi or 137.9 MPa) refrigerated in a cold room at 4°C. Extracts were collected, divided into smaller volumes and stored at −20°C. Protoplast extracts were prepared by the method of Mermelstein et al. (1992) and stored at −70°C.

Examination of restriction endonuclease activity

DNA digests were prepared by combining approximately 0.4 µg DNA (as specified in the text), 1 µl of cell extract (or 1 µl protoplast extract) and 1 µl of 10x high salt buffer (USB, Cleveland, Ohio, USA) in a final volume of 10 µl. The mixture was incubated at 55°C unless otherwise indicated for periods of time specified in the text. Agarose gel electrophoresis (1.2%) separated the resultant fragments.

Isolation of genomic DNA from *C. thermocellum* and C. *thermosaccharolyticum*.

Total genomic DNA was isolated by centrifuging 5 ml of culture (Optical density at 600 nm, $OD_{600} - 0.7$) and resuspending the pellet in 500 ml TE (i.e. Tris/EDTA buffer 100/10 mM) with 30 mg/ml lysozyme plus 1000 U mutanolysin per millilitre and incubating at 37°C for 15 min. Sodium dodecyl sulfate (SDS) was added at 1% and the mixture was incubated at 65°C for 10 min. Proteinase K was then added to a concentration of 200 µg/ml and incubated for 1h at 65°C. The mixture was extracted twice with phenol/chloroform and once with chloroform. The DNA was precipitated with 2.5 volumes ethanol and air dried. The pellet was dissolved in 200 ml Tris-HCl, pH 7.5 and RNase was added to a concentration of 100 mg/ml. This mixture was incubated at 37 C for 30 min followed by one extraction with phenol and one with phenol/chloroform. The DNA was again precipitated with ethanol, dissolved in 25 ml distilled water and digested with restriction endonucleases.

Results

Cell extracts of *C. thermocellum* exhibit endonuclease activity

The *Staphylococcus* plasmid pUB110, prepared from *Bacillus subtilis*, is digested by both cell and protoplast extracts of *C. thermocellum* into discrete fragments (Fig. 1), indicative of specific endonuclease activity predominating over non-specific exonuclease activity. Incubation of the plasmid DNA for 1h with the cell extract resulted in incomplete digestion (Fig.1, lane 2) but 24 h of incubation produced nearly complete digestion of the plasmid (Fig. 1, lane 5). Non-specific exonuclease digestion of fragments was not observed with 24 h of incubation at 55°C. We did observe complete, non-specific degradation of linear DNA (used to eliminate the effects of topoisomerase-like activity seen with intact plasmids) incubated at 63°C (but not at 55° or 60°C) for 1h in the presence of either *C. thermocellum* or *C. thermosaccharolyticum* cell extracts (data not shown). The *C. thermocellum* cell extract restriction enzyme activity is very stable, retaining activity after four freeze/thaw cycles (data not shown). Comparison of restriction recognition between a protoplast extract of a recently acquired *C. thermocellum* ATCC 27405 strain and cell extract made from the same strain maintained in our laboratory for 7 years resulted in similar patterns of digestion (Fig. 1, lanes 6 and 2 respectively). Protoplast extracts show somewhat less endonuclease activity than cell extracts (Fig. 1, lanes 3 and 6). After

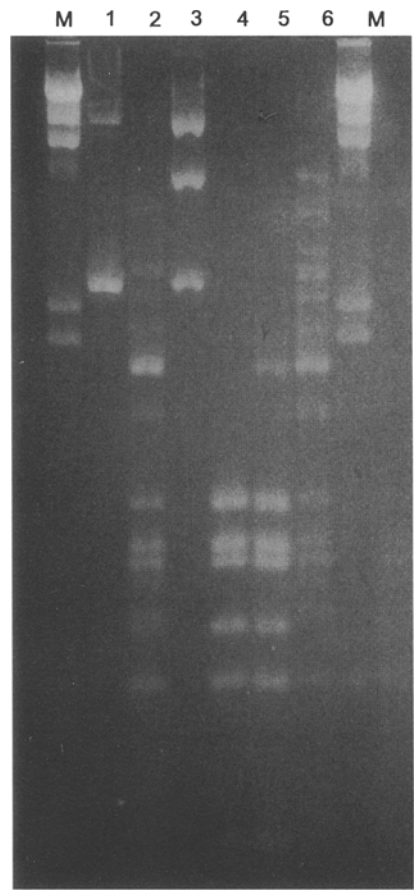

Fig. 1 Agarose gel1.2% of pUB110 plasmid DNA incubated with *Clostridium thermocellum* cell or protoplast extract as indicated. Marker (*M*) λ DNA *Hin*dIII digest; *lane 1*, uncut; Lane 2, cell extract, 1-h incubation; *lane 3*, protoplast extract, 1-h incubation; *lane 4*, *Mbo*I digest; *lane 5*, cell extract, 24-h incubation; *lane 6* protoplast extract, 24-h incubation; *M*, λ DNA *Hin*dIII digest

a 48-h incubation, the pattern of protoplast and cell extract digestion became less distinct presumably due to low-level non-specific nuclease activity of the cell extract (data not shown). Although digestion of DNA was observed with both low, medium and high salt buffers, it was determined that incubation with a commercially available high salt buffer (USB, Cleveland, Ohio, USA) provided the best activity (data not shown).

The Dam methylation system of *E. coli* protects *C. thermocellum* DNA from digestion

The following *E. coli* strains were used to investigate Dam (G^mATC) and Dcm [($C^mC(A/T)GG$] methylation with respect to protection of linearized pSK^- (*Eco*RI digested): GM271 (Dam^+/Dcm^-), ED8767 (Dam^+/Dcm^+), GM2163 (Dam^-/Dcm^-), GM475 (Dam^-/Dcm^+). *C. thermocellum* cell extract digests of plasmids prepared from either *B. subtilis* (Fig. 1) or from *E. coli* strains GM2163 and GM475 (Fig. 2), all lacking Dam methylation systems, resulted in discrete DNA fragments after a 1-h incubation. Dam methylation protected linearized pSK^- from digestion by the extract cocktail (Fig. 2, lanes 1 and 2). Dcm-methylation did not protect (Fig. 2, compare lanes 3 and 4), as Dcm-methylated pSK^- was completely digested in the presence of the cell extract. Other DNAs totaling approximately 60000 base pairs were investigated (pBR322, pBR325, pCTC1, λ DNA) with similar results, showing that Dam methylation protected DNA from digestion. These results were consistent with the restriction sequence recognized by the *C. thermocellum* system being the same as the Dam methylation sequence: GATC.

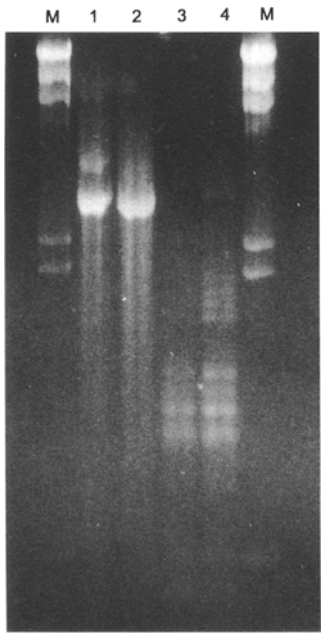

Fig. 2 Agarose gel 1.2% gel of *Xho* linearized pSK^- plasmid DNA prepared from the indicated *E. coli* strains and incubated with *C. thermocellum* cell extract for 1 h. *M* λ DNA *Hin*dIII digest; *lane 1*, GM271; *lane 2*, ED8767; *lane 3* GM2163; *lane 4*, GM475; *M*, λ DNA *Hin*dIII digest

The restriction sequence of *C. thermocellum* is GATC

The observed Dam methylation protection prompted us to search the sequence of pUB110 in the GenBank database for the sequence of GATC. The location of GATC sites on pUB110 was consistent with the pattern on the gel and a search for nGATC or GATCn sequences produced no common pattern. To test the hypothesis that GATC is the restriction site recognized by the *C. thermocellum* cell extract, the restriction enzyme *Mbo*I that recognizes and cuts at GATC was used to digest pUB110 (Fig. 1, lane 4). Comparison of the fragments generated by *Mbo*I digestion and the cell extract (24-h incubation, lane 5) supports the hypothesis that the recognition sequence is the same for both. A band in the 24-h lane that does not correspond to the *Mbo*I pattern is taken to be the result of incomplete digestion by the *C. thermocellum* cell extract (Fig. 1, lane 5).

All observed *C. thermocellum* restriction activity is associated with the sequence GATC

Digestion of T7 phage (40 kb) and λ phage DNA (48kb) was undertaken in order to search for any restriction sequences other than GATC. Figure 3 shows identical digestion patterns between T7 phage digested with the *C. thermocellum* extract overnight at 55°C (lane 8) and the *Mbo*I digest for 1-h at 37°C (lane 9). A 24-h digestion of λ phage DNA by the *C. thermocellum* cell extract (lane 3) results in similar bands to those observed for *Mbo*I-digested λ DNA (lane 4), except that the cell extract lane also contains a higher molecular weight band. We interpret this band to be the result of incomplete digestion since the double digest of *C. thermocellum* cell extract followed by *Mbo*I results in a pattern indistinguishable from that of *Mbo*I alone. All cutting observed in pBR322, pSK-, pUB110 T7 and λ phage DNA (total of > 100 kb) can be explained by cutting at GATC.

No endonuclease activity was observed for *C. thermosaccharolyticum*

When cell extracts prepared from *C. thermosaccharolyticum* were incubated with DNA from phages

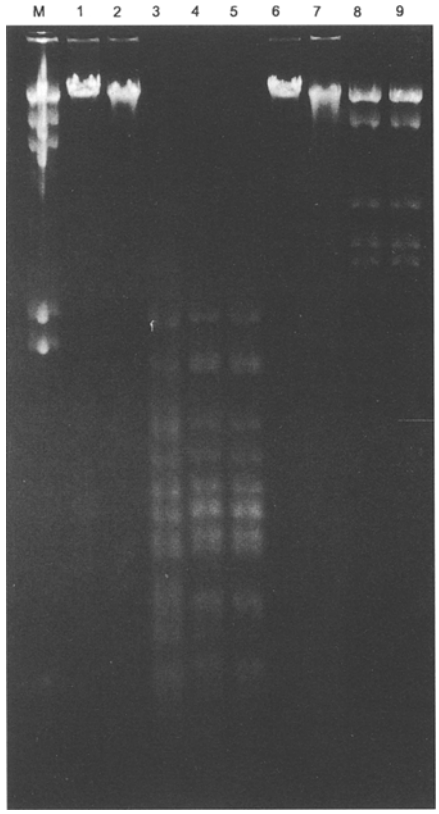

Fig. 3 Agarose gel 1.2% gel of unmethylated λ phage DNA or of linear T7 λ DNA. Marker, λ DNA *Hin*dIII digest; *lane 1* uncut λ; *lane 2* λ plus *C. thermosaccharolyticum* cell extract, 24-h digest at 55°C; *lane 3* λ plus *C. thermocellum* cell extract, 24-h digest at 55 C; *lane 4 Mbo*I 1-h digest at 37 C *lane 5* λ plus *C. thermocellum* cell extract, 24-h digest at 55 C followed by *Mbo*I 1-h digest at 37 C; *lane 6* uncut T7; *lane 7* T7 plus *C. thermosaccharolyticum* cell extract, 24- h digest at 55°C; *lane 8* T7 plus *C. thermocellum* cell extract, 24-h digest at 55 C; *lane 9* T7 plus *Mbo*I 1 h digest at 37°C

λ (Fig. 3, lane 2) and T7 (Fig. 3, lane 7), the banding pattern was indistinguishable from the uncut plasmids except for some slight non-specific degradation in the case of T7. pBR325 and pCTC1 were also exposed to the *C. thermosaccharolyticum* extracts, with similar results (data not shown). Although a total of > 100 kb was examined, no significant digestion was observed. The presence of *C. thermosaccharolyticum* cell extract does not affect digestion by *Mbo*I or by the *C. thermocellum* extract (data not shown), indicating that the extract does not contain factors inhibitory to digestion.

The genome of C. thermocellum is phenotypically Dam$^+$, while that of *C. thermosaccharolyticum* is phenotypically Dam$^-$

To protect the genome of *C. thermocellum*, methylation would be expected to inhibit the endogenous restriction system that is active at the site GATC. Figure 4 shows an agarose gel of genomic DNA isolated from either *C.*

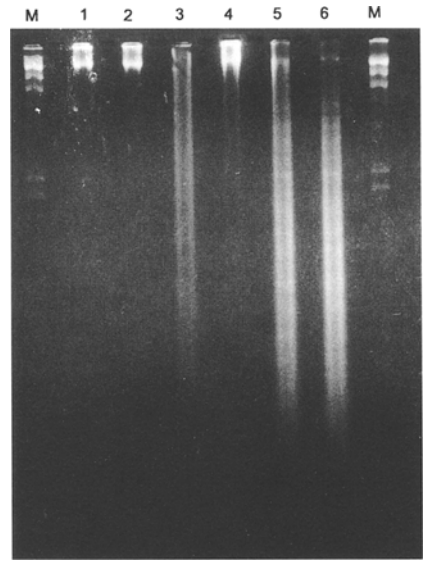

Fig. 4 Agarose gel 1.2% of genomic DNA. *M*, λ DNA *Hin*dIII digest; *lanes 1–3*, *C. thermocellum* genomic DNA uncut, digested with *Mbo*I, digested with Sau 3A I; *lanes 4–6 C. thermosaccharolyticum* genomic DNA uncut, digested with *Mbo*I, digested with *Sau*3AI; *M*, DNA *Hin*dIII digest

thermocellum or *C. thermosaccharolyticum* and digested with *Mbo*I (digests unmethylated GATC) or its isoschizomer *Sau*3A I that digests GATC regardless of the methylation pattern. The genome of *C. thermocellum* is not digested by *Mbo*I (Lane 2) but is completely digested by *Sau*3A I (Lane 3). The genome of *C. thermosaccharolyticum* is digested equally well by either enzyme (Lanes 5 and 6).

Discussion

Whole cell extracts of *C. thermocellum* prepared by high pressure disruption or protoplast extraction exhibit sequence-specific restriction endonuclease activity with little non-specific exonuclease activity. This is contrary to certain strains of C. *acetobutylicum*. (Azeddoug et al. 1989, Mermelstein et al 1992) where active cell wall-associated exonucleases made whole cell extracts impossible to use when studying restriction endonuclease activity. It is interesting to note that in studies with *C. acetobutylicum*, high ionic strength buffers, which also promote maximal restriction activity with *C. thermocellum* cell extract digestions, have been shown to promote maximum restriction activity (Lee et al. 1992) while limiting DNA degradation by DNase (Williams et al. 1990). Based on comparison with *Mbo*I digestions, the restriction sequence recognized by *C. thermocellum* was determined to be GATC. Protection from digestion was seen if plasmids were prepared from a Dam$^+$ strain of *E. coli*. Plasmids prepared from Dam$^-$ *E. coli* strains

or *B. subtilis* which do not exhibit Dam methylation (Dreiseikelmann and Wackernagel 1981) were digested by the cell extract cocktail. The genome of *C. thermocellum* exhibits a Dam^+ phenotype. These results suggest that *E. coli* shuttle plasmids used in attempts to transform *C. thermocellum* should be prepared from a Dam^+ *E. coli* host to escape digestion at this commonly occurring (on average, once every 266 base pairs) site.

Clostridium thermosaccharolyticum does not appear to exhibit restriction endonuclease activity using two independently prepared cell extracts that were exposed to > 100 kb DNA. In contrast with *C. thermocellum*, the genome of *C. thermosaccharolyticum* is digested by MboI indicating a Dam^- phenotype.

It is worthy of note that a second thermophile, *C. thermohydrosulfuricum*, also exhibits restriction activity at GATC which is prevented by Dam methylation found in this organism (Richards et al. 1988). In *C. acetobutylicum*, *Mbo*I-like activity has also been reported (Azeddoug et al. 1989) but the type of restriction system has been shown to be strain-specific (Azeddoug et al. and Reysett 1991) in this species. In *C. thermocellum* ATCC 27405, isoschizomers of *Bcl*I (TGATCA) and *Eco*RII [(CC(A/T)GG)] have been reported (Young et al. 1989), but we have not observed digestion by cell extracts at these sequences, although they were present in the DNA studied.

Acknowledgments The authors gratefully acknowledge support from the NSF (Grant Numbers BCS-9215130 and BCS-9058392) and the John Merck Fund. We thank S. Pal for protoplast preparation and M. L. Guerinot, E. T. Papoutsakis, and L. Mermelstein for helpful comments.

References

Azeddoug H, Reysset G (1991) Recognition sequence of a new methyl-specific restriction system from Clostridium acetobutylicum strain ABKn8. FEMS Microbiol Lett 78:153–156

Azeddoug H, Hubert J, Reysset G (1989) Characterization of a methyl-specific restriction system in *Clostridium acetobutylicum* strain N1-4081. FEMS Microbiol Lett 65:323–326

Dreiseikelmann B, Wackernagel W (1981) Absence in *Bacillus subtilis* and *Staphylococcus aureus* of the sequence specific deoxyribonucleic acid methylation that is conferred in *E. coli* K-12 by the dam and dcm enzymes. J Bacteriol 147:259–261

Hogsett DA, Ahn H-J, Bernardez TD, South CR, Lynd LR (1992) Direct microbial conversion: progress, prospects, and obstacles. Appl Biochem Biotechnol 34/35:527–541

Klaenhammer TR (1987) Plasmid-directed mechanisms for bacteriophage defense in lactic streptococci. FEMS Microbiol Rev 46:313 325

Lee SY, Mermelstein LD, Bennett GN, Papoutsakis ET (1992) Vector construction, transformation, and gene amplification in *Clostridium acetobutylicum* ATCC 824. N Y Acad of Scie Biochemical Engineering VII. 665:39–51

Lynd LR, Ahn H-J, Anderson G, Hill P, Kersey DS, Klapatch T (1991) Thermophilic ethanol production: investigation of ethanol yield and tolerance in continuous culture. Appl Biochem Biotechnol 28/29:549–570

Mermelstein LD, Welker NE, Bennett GN, Papoutsakis ET (1992) Expression of cloned homologous fermentative genes in *Clostridium acetobutylicum* ATCC 824. Biotechnology 10:190 195

Modrich P, Roberts RJ (1982) In: Linn S, Roberts RJ (eds) Nucleases. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., pp 109–154

Richards DF, Linnett PE, Oultram JD, Young M (1988) Restriction endonucleases in *Clostridium pasteurianum* ATCC 6013 and *C. thermohydrosulfuricum* DSM 568. J Gen Microbiol 134: 3151 3157

Saddler JN, Chan MK-H (1983) Conversion of pretreated lignocellulosic substrates to ethanol by *Clostridium thermocellum* in mono- and co-culture with *Clostridium thermosaccharolyticum* and *Clostridium thermohydrosulfuricum*. Can J Microbiol 30:212–220

Slapack GE, Russell I, Stewart GG (1987) Thermophilic microbes in ethanol production. CRC, Boca Raton, Fla.

Venkateswaran S, Demain AL (1985) The *Clostridium thermocellum-Clostridium thermosaccharolyticum* ethanol production process: nutritional studies and scale-down. Chem Eng Commun 45:53 60

Wilkinson SR, Young M (1993) Wide diversity of genome size among different strains of *Clostridium acetobutylicum*. J Gen Microbiol 139:1069–1076

Williams DR, Young DI, Young M (1990) Conjugative plasmid transfer from *Escherichia coli* to *Clostridium acetobutylicum*. J Gen Microbiol 136:819–826

Young M, Minton NP, Staudenbauer WL (1989) Recent advances in the genetics of the clostridia. FEMS Microbiol Rev 63:301–326