Restriction Endonucleases in *Clostridium pasteurianum* ATCC 6013 and *C. thermohydrosulfuricum* DSM 568

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A small collection of clostridia was surveyed for type II restriction endonucleases. Enzymes were detected in two organisms. *Clostridium pasteurianum* ATCC 6013 contains an isoschizomer of *Tha*I (*Fnu*DII) [5'-CGCG-3'] and preliminary evidence suggests that cleavage generates blunt-ended fragments. *Clostridium thermohydrosulfuricum* DSM 568 contains an isoschizomer of *Mbo*I (*Sau*3A) [5'-GATC-3'] that is inactive on *dam* methylated substrates. The DNA of this latter organism shows *dam* methylation.

INTRODUCTION

The clostridia are a heterogeneous collection of Gram-positive, anaerobic, spore-forming bacteria (Smith & Hobbs, 1974). The saccharolytic species are of interest because they may be used to effect biotransformations, especially reductions, to yield chiral products (Morris, 1983). Moreover, they may also be used to convert biopolymers that are major components of agricultural and certain industrial wastes into useful feedstocks for the chemical industry (Jones & Woods, 1986*a*). The development of procedures for genetic manipulation of saccharolytic clostridia would enhance prospects for their biotechnological exploitation (Young *et al.*, 1986; Jones & Woods, 1986*b*). In recent years there have been reports of protoplast fusion and transformation in *Clostridium acetobutylicum* (Reid *et al.*, 1983; Lin & Blaschek, 1984; Jones *et al.*, 1985), transformation of *C. thermohydrosulfuricum* (Soutschek-Bauer *et al.*, 1985) and conjugal plasmid transfer to *C. acetobutylicum* (Oultram & Young, 1985; Reyssset & Sebald, 1985; Yu & Pearce, 1986; Oultram *et al.*, 1987).

Restriction endonucleases present a potential barrier to genetic exchange. Recent compilations (Kessler & Hölte, 1986; Roberts, 1987) have included few enzymes of clostridial origin. Isoschizomers of *Mbo*I [5'-GATC-3'] have been found in strains of *C. perfringens* (*Cpf*I) and *C. pasteurianum* NRCC 33011 (*Cpa*I) and isoschizomers of *Bcl*I [5'-TGATCA-3'] and *Eco*RII [5'-CC(A/T)GG-3'] have been found in *C. thermocellum* ATCC 27405. *C. formicoaceticum* ATCC 23439 is the source of an enzyme, *Cfo*I [5'-GCGC-3'], that is produced commercially. Another enzyme, *Chi*I, of unknown specificity, has been detected in *C. histolyticum*. We have examined a small laboratory collection, mainly comprising saccharolytic clostridia, and have found type II restriction endonucleases in two strains.

METHODS

Organisms and their cultivation. *C. acetobutylicum* NCIB 8052, *C. butyricum* NCIB 6084 and NCIB 7423, *C. carbonei* NCIB 9359, *C. paraputrificum* ATCC 25780, *C. pasteurianum* ATCC 6013, *C. puniceum* NCIB 11596 and *C. tyrobutyricum* NCIB 10635 were grown anaerobically at 37 °C, in TYG broth (which contains, per litre:

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Tryptone, 30 g; yeast extract, 20 g; glucose, 5 g; sodium mercaptoacetate, 1 g). *C. thermohydrosulfuricum* DSM 568, DSM 2247 and NCIB 10956 were grown anaerobically, at 65 °C, in reinforced clostridial medium (RCM) modified as described by Soutschek-Bauer *et al.* (1985). Organisms were maintained on agar-solidified RCM (Difco) and routine manipulations were done in an anaerobic cabinet.

Bacillus subtilis 168 *trpC2* (pUB110, Km^R) was grown at 37 °C in Nutrient Broth (Difco) containing 10 µg kanamycin ml⁻¹.

Escherichia coli strain CA160 was used as host for plasmids pAT153, (Ap^RTc^R; Twigg & Sherratt, 1980) and pHV33 (Ap^RTc^RCm^R; Primrose & Ehrlich, 1981). It is a *r_Km⁻recBC* derivative of strain C600 F⁻ *thi-1 thr-1 leu-6 lacY ton azi supE44*. Strain SL2034, a Dam⁻ strain, the origin of which is obscure (Piggot *et al.*, 1984), was used as host for plasmid pJAB1 (Cm^R; Sargent & Bennett, 1985). Strain 699 *thy (λcI857 Sam7)* was used as the source of phage λ; it was kindly provided by M. G. Sargent (NIMR, Mill Hill, London, UK). Strains SL2034 and HB101 F⁻ *hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 (Sm^R) xyl-5 mtl-1 supE44* were used as recipients in transformations. All strains of *E. coli* were grown in LB medium (which contains, per litre: Tryptone, 10 g; yeast extract, 5 g; NaCl, 10 g). Antibiotics were added, as appropriate, at 20 µg ml⁻¹. Strain 699 was grown in the presence of 20 µg thymine ml⁻¹ at 30 °C; all other strains of *E. coli* were grown at 37 °C.

Preparation of DNA. A scaled-up version of the rapid boiling method of Holmes & Quigley (1981) was used to prepare plasmids from *E. coli*. The method of Gryczan *et al.* (1978), modified as described by Collins *et al.* (1985), was used to prepare plasmid pUB110 from *B. subtilis*.

Phage λ *cI857 Sam7* was prepared from *E. coli* 699 and DNA isolated from purified phages as described by Maniatis *et al.* (1982).

Chromosomal DNA was isolated from *C. thermohydrosulfuricum* essentially as described by Harris-Warwick *et al.* (1975), except that 1 g CsCl was added per ml of lysate before density gradient centrifugation.

Manipulation of DNA. The enzymes *DpnI* (NBL Enzymes Ltd), *HindIII* and *PstI* (Pharmacia), and *DraI*, *MboI*, *NruI*, *Sau3A* and *SalI* (BRL), were used according to the suppliers' instructions. Restriction fragments were purified from agarose gels using the method of Dretzen *et al.* (1981). T4 DNA ligase was obtained from BCL. Blunt-ended fragments were ligated in the presence of 0.04% Nonidet P40 (M. G. Sargent, personal communication).

Ligation mixtures were used to transform *E. coli* HB101; transformants were selected on LB agar containing 20 µg ml⁻¹ each of ampicillin and chloramphenicol. *E. coli* cells were permeabilized by treatment with CaCl₂ and a heat shock, as described by Mandel & Higa (1970).

Screening of clostridia for restriction endonucleases. The method of Whitehead & Brown (1985) was used to prepare extracts from strains of *C. thermohydrosulfuricum*. They were assayed by incubating samples (1–5 µl) with about 0.5 µg substrate DNA in a buffer containing 10 mM-Tris/HCl, pH 7.9, 50 mM-NaCl, 10 mM-MgCl₂, 10 mM-2-mercaptoethanol, for 2 h at 65 °C. The reaction products were analysed by electrophoresis (1.5–3 V cm⁻¹) through horizontal agarose gels (0.8–1.0%), staining with ethidium bromide and inspection on a UV transilluminator (UV Products Inc.). Extracts that converted the substrate into a ladder of discrete DNA fragments were retained and optimal reaction conditions with respect to pH, NaCl concentration and temperature were determined. The sizes of DNA fragments were estimated relative to the mobilities of standard samples of phage λ DNA digested with either *PstI* or *HindIII*.

The method of Schleif (1980) was used to prepare extracts from the remaining clostridial strains. They were assayed as described above, except that the reaction buffer contained 6 mM-Tris/HCl, pH 7.4, 6 mM-MgCl₂, 6 mM-2-mercaptoethanol, and incubation was at 37 °C for 2 h.

RESULTS

Screening for restriction endonucleases

Crude extracts prepared from *C. acetobutylicum* NCIB 8052, *C. butyricum* NCIB 7423 and NCIB 6084, *C. carbonei* NCIB 9359, *C. paraputrificum* ATCC 25780, *C. pasteurianum* ATCC 6013, *C. puniceum* NCIB 11596 and *C. tyrobutyricum* NCIB 10635 were screened for restriction endonuclease activity using, as substrate, phage λ DNA. A ladder of discrete DNA fragments was observed with the *C. pasteurianum* ATCC 6013 extract. The substrate was completely degraded by the extracts from *C. carbonei* and *C. puniceum*, whereas it remained apparently intact after incubation with the extracts from the strains of *C. butyricum*, *C. acetobutylicum*, *C. paraputrificum* and *C. tyrobutyricum*.

Crude extracts prepared from *C. thermohydrosulfuricum* DSM 568, DSM 2247 and NCIB 10956, were assayed using both phage λ DNA and plasmid pUB110. None of them showed activity with the former substrate. However, the *C. thermohydrosulfuricum* DSM 568 extract converted plasmid pUB110 DNA into a ladder of discrete fragments, whereas the extracts from the other two strains had little or no effect on this substrate.

Fig. 1. Determination of the site-specificity of *CpaAI*. Samples were run on a 1.5% agarose gel. Lane 1, λ *Pst*I; lane 2, λ *Hind*III; lane 3, λ *CpaAI*; lane 4, λ *CpaAI* + *Tha*I; lane 5, λ *Tha*I; lane 6, pHV33 *Tha*I; lane 7, pHV33 *CpaAI*; lane 8, λ *Pst*I; lane 9, λ *Hind*III.

Characterization of the endonuclease from *C. pasteurianum* ATCC 6013

The extract from *C. pasteurianum* ATCC 6013 was most active at pH 7.4 in the presence of 15 mM-NaCl. Substrates were completely degraded at NaCl concentrations above 30 mM, which suggested that the crude extract contained at least one additional nuclease. A restriction endonuclease, *CpaI*, that is an isoschizomer of *Mbo*I (*Sau*3A), has been reported from *C. pasteurianum* (see Roberts, 1987). The activity in the extract was therefore compared with that of *Sau*3A, using plasmid pAT153 as substrate. The sizes of the fragments produced did not correspond (data not shown). However, they were in approximate agreement with those expected after digestion with *Tha*I (*Fnu*DII), viz. 560 (581), 490 (493), 450 (452), 340 (355), 320 (332 + 330) and 210 (182) bp – predicted sizes in parentheses. Fragments smaller than about 200 bp were not detected in this experiment. The enzyme from *C. pasteurianum* ATCC 6013 was therefore denoted *CpaAI* to distinguish it from *CpaI*.

Confirmation that *CpaAI* is an isoschizomer of *ThaI* was obtained by comparing their activities using phage λ and plasmid pHV33 DNA as substrates. The mobilities of the fragments produced by these enzymes were indistinguishable (Fig. 1; lanes 3 and 5, 6 and 7). Moreover, double digestion of phage λ DNA with *CpaAI* and *ThaI* did not alter the digestion products from those obtained after digestion with each enzyme alone (Fig. 1, lanes 3–5).

Fig. 2. Determination of the site-specificity and *dam*-methylation-sensitivity of *CytI*. (a) 1% agarose gel. Lane 1, λ *Pst*I; lane 2, pJAB1 *Dam*⁺; lane 3, pJAB1 *Dam*⁺ *CytI*; lane 4, pJAB1 *Dam*⁺ *Sau*3A; lane 5, pJAB1 *Dam*⁻ *Sau*3A; lane 6, pJAB1 *Dam*⁻ *CytI*; lane 7, pJAB1 *Dam*⁻; lane 8, λ *Hind*III. The samples in lanes 4–6 are incompletely digested. (b) 0.8% agarose gel. Lane 1, λ *Hind*III; lane 2, λ *Dam*⁻ *Sau*3A; lane 3, λ *Dam*⁻ *Sau*3A + *CytI*; lane 4, λ *Dam*⁻ *CytI*; lane 5, λ *Dam*⁻; lane 6, λ *Pst*I.

Ligation of DNA fragments produced by *Cpa*AI was considerably enhanced by the addition of PEG 6000 (4%) or Nonidet P40 (0.04%) (data not shown). This provides circumstantial evidence (cf. Zimmerman & Pfeiffer, 1983) suggesting that *Cpa*AI, like *Tha*I, cleaves DNA within the sequence [5'-CG↓CG-3'] to generate blunt-ended fragments. A cloning experiment provided additional support. Cleavage of plasmid pJAB1 (Sargent & Bennett, 1985) with *Tha*I produces a 1099 bp fragment carrying the *Cm*^R gene, the termini of which have the sequence [5'-GCG....TCG-3']. Insertion of this fragment into the *Nru*I site of plasmid pAT153 will inactivate the *Tc*^R gene giving rise to one *Nru*I site [5'-TCG↓CGA-3']. To examine whether the 1.1 kbp *Cpa*AI fragment of pJAB1 behaves similarly it was purified from an agarose gel and then ligated, in the presence of 0.04% Nonidet P40, with a sample of *Nru*I-linearized pAT153. *E. coli* HB101 was transformed with the ligated DNA fragments. All eighteen of the Ap^R *Cm*^R transformants tested were *Tc*^S and they all harboured a plasmid of the expected size (3.6 + 1.1 = 4.7 kbp). Plasmids from four of the colonies were linearized by *Nru*I and they also gave the expected products after *Cpa*AI digestion. The 1.1 kbp insert has three asymmetrically positioned *Dra*I sites from which its orientation was determined. In all four cases the transcriptional orientation of the *Cm*^R gene was the same as that of the inactivated *Tc*^R gene. The remaining colonies contained plasmids insensitive to *Nru*I, although they had the predicted size and conferred the expected phenotype.

Characterization of the endonuclease from C. thermohydrosulfuricum DSM 568

The enzyme in *C. thermohydrosulfuricum* extracts was denoted CtyI and characterized using plasmid pJAB1 as substrate. Activity was greatest at 65–70 °C, and was negligible below 40 °C. It was relatively insensitive to pH over the range pH 7.0–8.0, but at suboptimal temperatures digestion was best between pH 7.4–7.9. CtyI was active throughout the entire range of salt concentrations tested, viz. 0–150 mM-NaCl.

The enzyme was assayed using phage λ DNA and plasmids pAT153, pJAB1 and pUB110 as substrates. The last two substrates only were cleaved (data not shown). This suggested that CtyI was affected by the activity of the *dam* methylase of *E. coli*. The λ and pAT153 DNA originated from Dam⁺ strains, whereas pJAB1 was from a Dam⁻ strain and pUB110 was from *B. subtilis*, which does not show *dam* methylation (Dreiseikermann & Wackernagel, 1981). The *dam*-methylation-sensitivity of CtyI was established by making a λ lysogen of the Dam⁻ strain *E. coli* SL2034. Phage DNA extracted from this strain was cleaved by CtyI (Fig. 2b, lane 4). Alternatively, when pJAB1 was introduced into a Dam⁺ strain, HB101, of *E. coli* and re-extracted, it was insensitive to CtyI (Fig. 2a; cf. lanes 3 and 6). CtyI appears to be an isoschizomer of *Mbo*I (*Sau*3A) [5'-GATC-3'], because it cleaves unmethylated plasmid pJAB1 to produce fragments of the following sizes: 2400 (2404), 750 (751), 510 (528), 380 (358) kb – predicted sizes are given in parentheses. Moreover, samples of unmethylated pJAB1 and phage λ DNA doubly digested with CtyI and *Sau*3A presented the same appearance as samples digested with each enzyme alone (Fig. 2a, lanes 4–6 and Fig. 2b, lanes 2–4, respectively).

As might be expected, *C. thermohydrosulfuricum* DNA appears to carry the Dam methylation pattern, since it is resistant to cleavage by CtyI and *Mbo*I, but sensitive to *Sau*3A and *Dpn*I (data not shown). *Mbo*I does not cleave *dam*-methylated DNA [5'-G*ATC-3'], whereas *Sau*3A is unaffected by *dam*-methylation, and *Dpn*I will only cleave *dam*-methylated DNA (Sussenbach *et al.*, 1976; Dreiseikermann *et al.*, 1979; Modrich, 1982; Lodwick *et al.*, 1986; Nelson & McClelland, 1987).

DISCUSSION

In this survey of eleven strains of *Clostridium*, type II restriction endonucleases were detected in two commonly used laboratory organisms, viz. *C. pasteurianum* ATCC 6013 and *C. thermohydrosulfuricum* DSM 568. The enzyme from the former organism was denoted *Cpa*AI to distinguish it from *Cpa*I which has been described previously from a different strain (see Roberts, 1987). *Cpa*AI is an isoschizomer of *Tha*I (*Fnu*DII). The site of cleavage within the recognition sequence, [5'-CGCG-3'], has not been established unequivocally, but circumstantial evidence suggested that like *Tha*I, cleavage generates blunt-ended fragments [5'-CG↓CG-3']. A cloning experiment designed to establish the cleavage position gave an indeterminate result. This may have arisen from the presence of a contaminating nuclease(s) in the crude extract. Additional experiments, using purified enzyme and direct sequencing (Brown & Smith, 1980), would enable the cleavage site of *Cpa*AI to be established unequivocally. However, since the enzyme does not have a novel specificity, this was not investigated further.

The enzyme CtyI from *C. thermohydrosulfuricum* has a specificity similar to that of *Mbo*I. It recognizes the sequence [5'-GATC-3'] and cleavage is prevented if the adenine is methylated. DNA extracted from *C. thermohydrosulfuricum* carries the Dam-methylation pattern.

Only eight type II restriction endonucleases have so far been reported from clostridia, but three of them (*Cpf*I, *Cpa*I and CtyI) are isoschizomers of *Mbo*I (*Sau*3A). Enzymes with this specificity may prove to be widely distributed in the genus *Clostridium*.

The presence of restriction endonucleases in both *C. pasteurianum* ATCC 6013 and *C. thermohydrosulfuricum* DSM 568 is relevant to the genetic manipulation of these organisms. Soutschek-Bauer *et al.* (1985) reported that plasmid pUB110, Km^R, and a derivative, pGS13, Km^R Cm^R, transformed PEG-treated, intact cells of *C. thermohydrosulfuricum* at a relatively low frequency (about 4×10^{-6} per viable cell). The DNA used in these experiments was extracted from *B. subtilis* 168; hence, it would not have carried the Dam-methylation pattern

(Dreiseikelmann & Wackernagel, 1981). If *CytI* does present a significant barrier to the establishment of incoming unmethylated plasmid molecules, higher transformation efficiencies should be obtained using suitably methylated DNA.

We are not aware of any published report of gene transfer to *C. pasteurianum*. However, in some of our unpublished experiments high frequency (10^{-3} per recipient) conjugal transfer of plasmid pAM β 1 from *Streptococcus lactis* to *C. pasteurianum* was observed. It would appear that for conjugal DNA transfer from *S. lactis*, at least, the *CpaAI* endonuclease does not present a serious barrier.

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