Wide diversity of genome size among different strains of *Clostridium acetobutylicum*

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The sizes of the genomes of five strains of *Clostridium acetobutylicum* were estimated by summing the sizes of macro-restriction fragments produced after cleavage of their DNA with *Sma*I and *Apa*I. The five *C. acetobutylicum* strains fell into three discrete groups. The NCIMB 8052 and NI-4081 strains have 6.5 Mbp genomes and restriction profiles of their DNA were indistinguishable. The sizes of the genomes of the ATCC 824 and DSM 1731 strains were about 4.0 and 3.5 Mbp (minimum values), respectively, and the restriction profiles of their DNA were very similar. One notable difference was the presence of an additional 532 kbp *Sma*I fragment in the DNA of strain ATCC 824. The NCP 262 strain of *C. acetobutylicum* has a genome size of 2.85 Mbp. Restriction fragment profiles of DNA from strains belonging to different groups were not obviously similar. Probes made from several cloned ATCC 824 genes hybridized with DNA fragments of different sizes from strains representative of each group. These data add further weight to the considerable body of evidence indicating that the species *C. acetobutylicum* comprises a heterogeneous collection of strains.

Introduction

Over the last few years there has been growing interest in the emergent field of clostridial genetics. Two species, *Clostridium acetobutylicum* and *Clostridium perfringens*, have assumed prominence as model saccharolytic and pathogenic representatives of the genus. Many clostridial genes have already been cloned and analysed in *Escherichia coli*, and a wide variety of cloning vectors based on clostridial replicons and antibiotic-resistance markers have been constructed. Routine methods have been developed for obtaining gene transfer to these model organisms and many species are amenable to transposon mutagenesis. The considerable progress that has already been made has recently been reviewed (Young *et al.*, 1989; Rood & Cole, 1991; Sebald, 1993; Woods, 1993; Young & Cole, 1993; Bennett & Petersen, 1993; Minton *et al.*, 1993). However, with the notable exception of *C. perfringens* (Canard & Cole, 1989, 1990; Garnier *et al.*, 1991; Canard *et al.*, 1992), very little is known about the organization of the bacterial chromosome.

The comparatively recent development of pulsed-field gel electrophoresis (PFGE) technology, permitting the separation of very large DNA fragments, has made it possible to analyse chromosome structure and gene organization in many prokaryotic organisms not yet amenable to genetic analysis (Krawiec & Riley, 1990). Genetic loci are assigned approximate positions on a physical map using appropriate homologous or heterologous genes as hybridization probes. As a first step towards the construction of a combined physical and genetic map of the chromosome of the model saccharolytic species, *C. acetobutylicum*, well-known for the acetone/butanol/ethanol (ABE) fermentation that it undertakes (Jones & Woods, 1986), the sizes of the genomes of five commonly used laboratory strains have been determined.

Methods

Bacterial strains and plasmids. These are listed in Table 1.

Culture media and growth conditions. Clostridial strains were grown anaerobically at 37 °C in clostridial basal medium (CBM) (O'Brien & Morris, 1971), solidified as appropriate with 1.5% (w/v) agar. Strains of *C. acetobutylicum* containing Tn1545 were selected on CBM supplemented with either erythromycin (10 µg ml⁻¹), kanamycin (80 µg ml⁻¹) or tetracycline (10 µg ml⁻¹). Strain DH5α of *Escherichia coli* containing recombinant plasmids was grown in Luria Bertani medium (Maniatis *et al.*, 1982) supplemented, as appropriate, with either kanamycin or ampicillin (both at 50 µg ml⁻¹).

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Abbreviation: PFGE, pulsed-field gel electrophoresis.

Table 1. *Bacterial strains and plasmids*

Strain	Genotype	Source/reference	
<i>Clostridium</i>			
<i>acetobutylicum</i>			
NCIMB 8052		Laboratory stock	
NI-4081	<i>pro rpoB lyt</i>	Reysset <i>et al.</i> (1988)	
ABKn8		M. Sebald, Institut Pasteur, Paris, France	
ATCC 824		Laboratory stock	
DSM 1731		P. Dürre, Göttingen, Germany	
NCP 262		D. R. Woods, Cape Town, South Africa	
AA029-AA039, AA180-AA209	<i>ermAM aphA-3 tetM</i>	Derivatives of NCIMB 8052 harbouring Tn1545 (this study)	
<i>Escherichia coli</i>			
DH5 α	F ⁻ λ^- <i>endA1 recA1 relA1</i> <i>hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44</i> (<i>argF-lacZYA</i>)U169 <i>thi-1</i> <i>gyrA96</i> (Nal ^R) [ϕ 80d <i>lacZ</i> Δ M15]	Hanahan (1983)	
<i>Enterococcus faecalis</i>			
BM4110::Tn1545		P. Courvalin, Institut Pasteur, Paris, France	
Plasmid	Relevant properties	Insert	Source/reference
pAT187	Km ^R <i>aphA-3</i>	0.8*	Trieu-Cuot <i>et al.</i> (1987)
pJC7†	Ap ^R <i>butK ptb</i>	4.4	Cary <i>et al.</i> (1988)
pDP253†	Ap ^R <i>'atoD atoA adc</i>	2.1	Petersen & Bennett (1990); Cary <i>et al.</i> (1990)
pBDH51†	Ap ^R <i>bdh</i> ‡	8.0	Petersen <i>et al.</i> (1991); Walter <i>et al.</i> (1992)
pThiol67†	Ap ^R <i>atoB</i>	0.6§	G. N. Bennett, Rice University, Houston, Texas, USA
pBTM100	Ap ^R <i>butK ptb</i>	8.3	N. P. Minton, PHLS, Porton, UK.
p7A12	Ap ^R <i>atoB</i>	9.3	N. P. Minton

* Approximate size of *aphA-3* coding sequence from the *Campylobacter coli* plasmid pIP1433 (Trieu-Cuot *et al.*, 1985).

† These plasmids contain DNA segments from the ATCC 824 strain.

‡ The cloned segment contains two distinct *bdh* genes.

§ Part of the insert in pTeco11 (Petersen & Bennett, 1991) containing the N-terminal region of the ATCC 824 thiolase structural gene.

|| These plasmids contain DNA segments from the NCIMB 8052 strain.

Filter matings. Tn1545 (Courvalin & Carrier, 1986) was transferred from *Enterococcus faecalis* BM4110::Tn1545 to *C. acetobutylicum* NCIMB 8052 as described by Woolley *et al.* (1989).

DNA preparation. Recombinant plasmids were isolated from *E. coli* DH5 α using the method of Del Sal *et al.* (1988).

A modified version of the technique described by Smith & Cantor (1987*a, b*) was developed for preparing 'intact', restrictable chromosomes from clostridia. Overnight cultures were diluted 1:50 in 250 ml prewarmed CBM and grown until early to mid-exponential phase (OD₆₈₀ 0.25–0.4). Bacteria were harvested by centrifugation, washed with 200 ml 50 mM-Tris/HCl, 1 mM-EDTA, pH 7.6, and suspended in STE buffer (50% sucrose, 50 mM-Tris/HCl, 1 mM-EDTA, pH 7.6) at a density of 6×10^8 c.f.u. ml⁻¹. An equal volume of 1.5% (w/v) low-temperature gelling agarose (Sigma, type VII) in STE buffer was mixed with the cell suspension and introduced into an insert former. Agarose-embedded bacteria in solidified plugs (approximately 250 μ l per plug) were placed in 4 vols lysis solution (10 mM-Tris/HCl, 1 M-NaCl, 100 mM-EDTA, 50% (w/v) sucrose, 0.5% sodium lauroylsarcosine, 10 mg lysozyme ml⁻¹, 20 μ g DNAase-free RNAase ml⁻¹, pH 7.6) and incubated overnight at 37 °C. All the above steps were carried out in an anaerobic cabinet.

After successful lysis, the plugs became translucent and more dense than the lysis solution. The plugs were then deproteinized for 60–72 h at 48 °C in 3 vols of a solution containing 0.5 M-EDTA, 1.0% (w/v) sodium lauroylsarcosine, 2 mg proteinase K ml⁻¹, pH 9.0. Before incubation with restriction endonucleases the plugs were washed with gentle agitation at room temperature, initially for 1 h in 4 vols 10 mM-Tris/HCl, 1 mM-EDTA, pH 8.0 (TE) containing 1.5 mM-phenylmethylsulphonyl fluoride and then six times for 1 h in 4 vols TE. Plugs were stored at 4 °C for up to one year in 50 mM-EDTA, pH 8.0.

Restriction enzyme digestion. Plug slices (50–70 μ l) were washed at room temperature for 1 h in 10 vols 10 mM-EDTA, pH 8.0, and then for 1 h in reaction buffer (Boehringer, buffer A; total volume of 300 μ l). Agarose-embedded DNA was digested at 30 °C overnight in 200 μ l fresh reaction buffer with 40 U *Sma*I (Northumbria Biologicals) or *Apa*I (Boehringer). Electrophoretograms contained approximately 340 ng DNA per lane.

PFGE. DNA fragments were separated using a contour-clamped homogeneous electric field apparatus [CHEF DRII, BioRad] (Chu *et al.*, 1986; Chu, 1989). For most experiments, the following conditions were used. Agarose gels (1.0%, w/v; BioRad, ultrapure

DNA grade) were prepared in TAE buffer (Maniatis *et al.*, 1982). Plug slices were fixed into place using molten agarose (1% w/v). DNA fragments in the size range 50–500 kbp were separated (200 V) using a linear ramp of 1–60 s over a 20 h period, whereas larger DNA fragments, ranging in size from 500 to 2100 kbp, were separated (200 V) using a linear ramp of 50–120 s over a 20 h period. The buffer was cooled to 14 °C throughout the electrophoresis. DNA fragments were visualized after staining with ethidium bromide (0.5 µg ml⁻¹) using a UV transilluminator.

Intact chromosomes from *Saccharomyces cerevisiae* YNN295 and *Schizosaccharomyces pombe* (BioRad), and bacteriophage λ concatemers, were used as size standards. The last were prepared from CsCl-purified phage λ c1857 Sam7 DNA. After embedding in agarose, as indicated above, at a concentration of 75 µg DNA ml⁻¹, plugs were stored at 4 °C in 50 mM-EDTA. Concatemerization occurred spontaneously during storage and samples were suitable for use after about 5 d. Plugs were discarded after about three months, when the lower concatemers were no longer visible after gel electrophoresis.

Southern hybridization. DNA fragments in agarose gels were depurinated and transferred to Hybond-N (Amersham) by capillary blotting using standard procedures (Southern, 1975; Maniatis *et al.*, 1982). Hybridization probes were prepared using a digoxigenin-based nonradioactive DNA labelling kit (Boehringer) and hybridizations were carried out as indicated by the manufacturer. Hybridization experiments with heterologous probes were carried out without stringent washing and membranes were re-used up to four times, after stripping off bound probes according to the method described by Gebeyehu *et al.* (1987).

Results and Discussion

Optimization of DNA extraction

The procedure described in Methods was optimized for extracting DNA from the NCIMB 8052 strain of *C. acetobutylicum*. An important modification was the inclusion of hypertonic sucrose in the lysis buffer, without which extensive DNA degradation occurred. However, even with this modification, a slight background fluorescence was noted in all samples after gel electrophoresis, indicating the occurrence of some residual DNA degradation during extraction. The extensive proteinase K treatment and repeated washing did not suffice to abolish contaminating nuclease(s). The residual nuclease activity was most pronounced in low ionic strength buffers (Wilkinson, 1992) and therefore only those enzymes active in high ionic strength buffers could be employed to generate macro-restriction fragments. The optimized method permitted extraction of restrictable DNA from most, but not all, of the clostridia listed in Table 1. It will require further modification to produce high-quality DNA from the ABKn8 strain of *C. acetobutylicum*.

DNA of sufficient quality for macro-restriction analysis was only extractable from exponentially growing bacteria containing (presumably) actively replicating chromosomes. The chromosomes finally obtained did not therefore present the appearance of intact circular molecules; undigested DNA samples penetrated the gel

during PFGE. Under conditions which permitted the separation of fragments of up to about 600 kbp, undigested DNA samples migrated as a discrete band beyond the upper limit of resolution (cf. Leblond *et al.*, 1990). Under different conditions, that separated the three *Sch. pombe* chromosomes, the undigested DNA no longer migrated as a discrete band, but had a smeared appearance up to a maximum size of about 3.5 Mbp (data not shown). The presence of partially replicated chromosomes would also be expected to contribute to the background fluorescence noted above. It therefore seems likely that the discrete band appearing in undigested DNA samples results from extraction of actively replicating chromosomes, superimposed upon which there was probably some slight DNA degradation during extraction.

Screening for suitable restriction endonucleases

The DNA of the mesophilic clostridia has a very low d(G+C) content (< 30 mol%). Restriction enzymes with recognition sequences containing mainly G and C nucleotides would be expected to generate a limited number of fragments, ranging in size from 100 to 2000 kbp (McClelland *et al.*, 1987). Samples of *C. acetobutylicum* NCIMB 8052 DNA were digested with a range of enzymes including *ApaI* (GGGCC↓C), *BamHI* (G↓GATCC), *BssHII* (G↓CGCGC), *EclXI* (C↓GGCCG), *FspI* (TGC↓GCA), *MluI* (A↓CGCGT), *NaeI* (GCC↓GGC), *NarI* (GG↓CGCC), *NotI* (GC↓GGCCGC), *SalI* (G↓TCGAC), *SmaI* (CCC↓GGG) and *StuI* (AGG↓CCT). Restriction enzymes such as *SacII* (CCGC↓GG), requiring buffers of low or medium ionic strength for optimal activity, were not employed, since there was substantial DNA degradation in these buffers (see above). None of these enzymes gave a small number (15 or less) of macro-restriction fragments. *ApaI*, *BssHII*, *EclXI*, *MluI*, *NarI*, *NaeI*, and *SmaI* each gave 25 or more fragments, whereas *NotI* did not, apparently, cleave the d(A+T)-rich *C. acetobutylicum* NCIMB 8052 DNA (Wilkinson, 1992). Further analysis was undertaken using *ApaI* and *SmaI*.

Estimation of genome sizes

Linear ramping was employed to resolve fragments over a large size range (Carle *et al.*, 1986); representative data are shown in Figs 1 and 2. Visual inspection (confirmed by densitometry) indicated that some bands in the gel electrophoretograms were comparatively intense. Initially, it was anticipated that these might represent fragments lying close to the chromosome replication origin. Other bands were rather diffuse.

The diffuse uppermost band in *SmaI*-digested DNA

Fig. 1. *SmaI*-digested DNA from various strains of *C. acetobutylicum*. Lanes: 1, λ concatemers; 2, NI-4081; 3, ATCC 824; 4, NCP 262; 5, DSM 1731; 6, NCIMB 8052.

samples from the NCIMB 8052 strain was clearly resolved into two discrete bands under conditions that gave maximum resolution of fragments in the 600 kbp range (Wilkinson, 1992). All the other diffuse or intense bands in DNA samples from this strain resulted from co-migration of two or more macro-restriction fragments. This was demonstrated using Tn1545, a conjugative transposon that is readily transferred from *Ent. faecalis* to *C. acetobutylicum* NCIMB 8052 (Woolley *et al.*, 1989). This large (25.3 kbp) element lacks restriction sites for both *SmaI* and *ApaI* (Caillaud *et al.*, 1987a) and therefore, DNA fragments into which it had inserted increased in size by about 25 kbp. Forty transconjugants, each harbouring one or more Tn1545 insertions, were obtained from mating experiments using *Ent. faecalis* BM4110::Tn1545 as donor. DNA was isolated from them and analysed after digestion with *ApaI* and *SmaI*. It was often possible to identify insertions in particular

Fig. 2. *ApaI*-digested DNA from various strains of *C. acetobutylicum*. Lanes: 1, λ concatemers; 2, NCIMB 8052; 3, NI-4081; 4, NCP 262; 5, DSM 1731; 6, ATCC 824.

restriction fragments by visual inspection of gel electrophoretograms (Fig. 3). All samples were hybridized with plasmid pAT187 as probe. This plasmid contains the *aphA-3* gene of the *Campylobacter coli* plasmid, pIP1433, the sequence of which is identical to that of the *aphA-3* gene of Tn1545 (Trieu-Cuot & Courvalin, 1983; Trieu-Cuot *et al.*, 1985, 1987; Caillaud *et al.*, 1987b). Tn1545 insertions were unambiguously assigned to all bands indicated as doublets in Tables 2 and 3. These data provided evidence for the presence of three different *ApaI* fragments in the diffusely staining region of gel electrophoretograms corresponding to a size of about 165 kbp (see Figs 2 and 3). The presence of at least three more *ApaI* fragments within this region was inferred from the results of hybridization experiments using cloned chromosomal DNA fragments (Wilkinson, 1992). Although some fluctuation in relative band intensity might be expected from the fact that chromosomes actively engaged in replication were analysed, it has tentatively been assumed that similarly intense or diffuse

Fig. 3. Analysis of DNA from strains harbouring Tn1545. The electrophoretogram is shown in (a) and the corresponding hybridization patterns in (b). The positions of fragments enlarged as a result of Tn1545 insertion are indicated with arrows in the electrophoretogram. The strains used were AA198 (lanes 1–3), AA199 (lanes 5–7) and AA200 (lanes 8–10). DNA samples were digested with *SmaI* (lanes 1, 5 and 8), *ApaI* (lanes 3, 7 and 10) and *SmaI* + *ApaI* (lanes 2, 6 and 9). Lane 4 contains λ concatemers. Strain AA200 harbours two copies of Tn1545.

Table 2. Sizes of *SmaI* fragments of the chromosomes of different *C. acetobutylicum* strains

Values in the body of the table are means ($\pm$ standard deviation) of five independent determinations.

Band	NCIMB 8052	NI- 4081	NCP 262	DSM 1731	ATCC 824
SmA	625 $\pm$ 5	625 $\pm$ 9	619 $\pm$ 7	638 $\pm$ 5	638 $\pm$ 5
SmB	596 $\pm$ 15	602 $\pm$ 13	605 $\pm$ 9	469 $\pm$ 9	532 $\pm$ 8
SmC	573 $\pm$ 15	578 $\pm$ 11	529 $\pm$ 2	322 $\pm$ 3	469 $\pm$ 9
SmD	362 $\pm$ 6	359 $\pm$ 4	195 $\pm$ 2	280 $\pm$ 7	319 $\pm$ 2
SmE	357 $\pm$ 6	354 $\pm$ 4	175 $\pm$ 5	246 $\pm$ 6†	280 $\pm$ 6
SmF	334 $\pm$ 8	328 $\pm$ 3	157 $\pm$ 2	219 $\pm$ 2	247 $\pm$ 8†
SmG	327 $\pm$ 11	322 $\pm$ 3	119 $\pm$ 4	202 $\pm$ 5	222 $\pm$ 6
SmH	302 $\pm$ 2	299 $\pm$ 3	108 $\pm$ 3†	172 $\pm$ 6	203 $\pm$ 8
SmI	292 $\pm$ 3	284 $\pm$ 7	91 $\pm$ 3	135 $\pm$ 3	173 $\pm$ 8
SmJ	278 $\pm$ 3	272 $\pm$ 7	72 $\pm$ 2†	127 $\pm$ 2	138 $\pm$ 3
SmK	250 $\pm$ 7*	248 $\pm$ 6†	51 $\pm$ 3	112 $\pm$ 5	131 $\pm$ 4
SmL	230 $\pm$ 8	233 $\pm$ 6		106 $\pm$ 4	115 $\pm$ 4
SmM	221 $\pm$ 4	221 $\pm$ 5		96 $\pm$ 2	105 $\pm$ 4
SmN	178 $\pm$ 6	176 $\pm$ 4		55 $\pm$ 4†	96 $\pm$ 6
SmO	166 $\pm$ 5	166 $\pm$ 4			55 $\pm$ 3†
SmP	144 $\pm$ 3*	142 $\pm$ 5†			
SmQ	103 $\pm$ 5	104 $\pm$ 4			
SmR	80 $\pm$ 4*	80 $\pm$ 4†			
SmS	63 $\pm$ 4	62 $\pm$ 3			
SmT	54 $\pm$ 3	53 $\pm$ 3			
SmU	47 $\pm$ 3	47 $\pm$ 3			
Genome size (kbp)	6056	6025	2901	3480	4025

* Denotes presence of two co-migrating fragments.

† Denotes presence of two or more co-migrating fragments.

bands in the electrophoretograms of DNA samples from the other strains also reflect the presence of co-migrating DNA fragments.

The sizes of DNA fragments were determined by comparison with λ concatemers and/or yeast chromosome size standards. A minimum of five independent experiments were carried out with DNA from each strain, from which mean values were obtained for the sizes of each restriction fragment (Tables 2 and 3). Minimum values for the size of the chromosome of each strain were then obtained by summing these data. The values obtained for each strain using *ApaI* and *SmaI* were within 5% of each other. The NCP 262 strain has the smallest genome, with a size of about 2.85 Mbp. The chromosomes of the ATCC 824 and DSM 1731 strains are of intermediate complexity, with sizes of about 3.9 and 3.45 Mbp, respectively. The chromosomes of the NCIMB 8052 and NI-4081 strains are considerably larger than those of the other strains. The value derived from the data in Tables 2 and 3 is about 5.9 Mbp. This is known to be an underestimate since there also exist a substantial number of small (< 50 kbp) *SmaI* and *ApaI* fragments, many of which are detected using probes specific for 16S and 23S rDNA (Wilkinson, 1992). Digestion of strain NCIMB 8052 DNA with *RsrII* produces six fragments, of approximate sizes 2.1, 1.7, 1.3,

Table 3. Sizes of *ApaI* fragments of the chromosomes of different *C. acetobutylicum* strains

Values in the body of the table are means ($\pm$ standard deviation) of five independent determinations.

Band	NCIMB 8052	NI- 4081	NCP 262	DSM 1731	ATCC 824
ApA	886 $\pm$ 5	882 $\pm$ 8	1031 $\pm$ 11	443 $\pm$ 5	499 $\pm$ 9
ApB	414 $\pm$ 8*	420 $\pm$ 8†	510 $\pm$ 8	404 $\pm$ 6	405 $\pm$ 5
ApC	326 $\pm$ 8*	329 $\pm$ 6†	460 $\pm$ 6	301 $\pm$ 4	299 $\pm$ 7
ApD	289 $\pm$ 10	291 $\pm$ 9	309 $\pm$ 4	266 $\pm$ 2	264 $\pm$ 4
ApE	253 $\pm$ 8	260 $\pm$ 5	148 $\pm$ 6	253 $\pm$ 3†	255 $\pm$ 4
ApF	231 $\pm$ 8*	238 $\pm$ 7†	111 $\pm$ 6	216 $\pm$ 2	218 $\pm$ 3†
ApG	219 $\pm$ 4	222 $\pm$ 6	84 $\pm$ 5	197 $\pm$ 5	204 $\pm$ 4
ApH	189 $\pm$ 5*	197 $\pm$ 4†	75 $\pm$ 4†	179 $\pm$ 2	182 $\pm$ 3
ApI	185 $\pm$ 8	183 $\pm$ 7	15 $\pm$ 1	154 $\pm$ 2†	154 $\pm$ 2†
ApJ	171 $\pm$ 5†	170 $\pm$ 5†		144 $\pm$ 2	145 $\pm$ 4
ApK	159 $\pm$ 2†	156 $\pm$ 4†		137 $\pm$ 3	136 $\pm$ 4
ApL	152 $\pm$ 3	150 $\pm$ 4		120 $\pm$ 1	125 $\pm$ 7
ApM	99 $\pm$ 4	99 $\pm$ 4		111 $\pm$ 6	114 $\pm$ 4
ApN	85 $\pm$ 5*	80 $\pm$ 4†		92 $\pm$ 8	98 $\pm$ 6
ApO	58 $\pm$ 2*	60 $\pm$ 3†		73 $\pm$ 5†	80 $\pm$ 4†
ApP	50 $\pm$ 2*	56 $\pm$ 3†		64 $\pm$ 2	66 $\pm$ 3†
ApQ				61 $\pm$ 1	48 $\pm$ 2
ApR				49 $\pm$ 1	
Genome size (kbp)	5779	5825	2818	3418	3810

* Denotes presence of two co-migrating fragments.

† Denotes presence of two or more co-migrating fragments.

‡ Denotes presence of three co-migrating fragments.

0.7, 0.5 and 0.25 Mbp, suggesting that the size of the genome is, in fact, about 6.5 Mbp.

The five strains of *C. acetobutylicum* fell into three distinct groups on the basis of the *SmaI* and *ApaI* fragment profiles of their DNA (Figs 1 and 2). The profiles of the NCIMB 8052 and NI-4081 strains were indistinguishable, those of the ATCC 824 and DSM 1731 strains were very similar to each other, but different from those of the former two strains, and that of the NCP 262 strain was quite different from those of all the other strains. The most obvious difference between *SmaI*-digested DNA from the ATCC 824 and DSM 1731 strains is the presence of an additional 532 kbp fragment in the former. These data, considered in conjunction with results obtained previously from comparative studies of physiology, biochemistry, cell wall lytic enzymes, susceptibility to the *Ent. faecalis* conjugative transposon Tn916, and nucleic acid hybridization (Woolley, 1988; Woolley *et al.*, 1989; Woolley & Morris, 1990; Croux & García, 1991), indicate that the various strains denoted *C. acetobutylicum* do not form a homogeneous species.

Comparative hybridization using cloned genes concerned with solventogenic metabolism

Although not obviously related on the basis of their genome sizes and their *ApaI* and *SmaI* macro-restriction

Table 4. *ApaI* and *SmaI* restriction fragments detected by genes cloned from the ATCC 824 strain

Probe*	Enzyme	ATCC 824	NCIMB 8052	NCP 262
<i>butK+ptb</i>	<i>SmaI</i>	247 (SmF)	230 (SmL)	195 (SmD)
	<i>ApaI</i>	136 (ApK)	99 (ApM)	309 (ApD)
	<i>SmaI</i> + <i>ApaI</i>	45†	30†	195‡
<i>bdh</i>	<i>SmaI</i>	222 (SmG)	MB	ND
	<i>ApaI</i>	218 (ApF)	MB	ND
	<i>SmaI</i> + <i>ApaI</i>	150†	MB	ND
<i>atoDA+adc</i>	<i>SmaI</i>	203 (SmH)	625 (SmA)	ND
	<i>ApaI</i>	204 (ApG)	189 (ApH)	ND
	<i>SmaI</i> + <i>ApaI</i>	110†	189‡	ND
<i>atoB</i>	<i>SmaI</i>	247 + 222 (SmF + G)§	625 (SmA)	ND
	<i>ApaI</i>	218 ± 80 (ApF + O)§	289 (ApD)	ND
	<i>SmaI</i> + <i>ApaI</i>	160† + 80‡	289‡	ND

MB, Multiple bands detected; ND, no hybridization detected.

* *butK*, butyrate kinase; *ptb*, phosphate transbutyrylase; *bdh*, butanol dehydrogenase (two genes); *atoDA*, CoA transferase; *adc*, acetoacetate decarboxylase; *atoB*, thiolase.

† *SmaI*-*ApaI* fragment.

‡ Internal *SmaI* or *ApaI* fragment.

§ Two fragments detected.

|| These fragments were also detected by the corresponding genes cloned from the NCIMB 8052 strain.

fragment profiles, *C. acetobutylicum* strains belonging to the three distinct groups all share the ability to produce acetone, butanol and ethanol when grown on sugary substrates. Probes were prepared from four cloned DNA segments from the ATCC 824 strain, representing eight genes whose products are implicated in the solventogenic fermentation. They were employed in hybridizations with DNA from the ATCC 824, NCIMB 8052 and NCP 262 strains.

As was expected from the lack of similarity between restriction fragment profiles (Figs 1 and 2), the probes detected DNA segments of different sizes in the DNA from different strains. The results obtained (Table 4) indicate that three of the probes, representing the ATCC 824 *atoDA+adc*, *butK+ptb* and *atoB* genes, detected sequences presumed to represent the corresponding genes in the NCIMB 8052 strain. This presumption was confirmed in two cases (*butK+ptb* and *atoB*), since probes made from the corresponding genes isolated from the NCIMB 8052 strain detected the same macro-restriction fragments from NCIMB 8052 as did the heterologous probes (Table 4). A probe encompassing two *bdh* genes in the ATCC 824 strain gave a multiple banding pattern with DNA of strain NCIMB 8052.

Given the similar fermentation characteristics of the NCP 262 and NCIMB 8052 strains (Woolley & Morris, 1990), it might have been expected that similar results would have been obtained in hybridizations with DNA from strain NCP 262. However, only one probe, representing the *butK+ptb* genes, successfully detected its counterpart in the NCP 262 strain. It would be of

interest to determine whether cloned genes involved in the acetone/butanol/ethanol fermentation from the NCIMB 8052 and NCP 262 strains have sufficient similarity to cross-hybridize.

The hybridization results obtained with the *atoB* probe (using low-stringency conditions) suggest that there might be two distinct thiolase genes in the ATCC 824 strain of *C. acetobutylicum*. However, Petersen & Bennett (1991), using high-stringency nucleic acid hybridization, found no evidence for the existence of two thiolases in this organism.

The observed hybridization of genes concerned with the solventogenic fermentation from one strain to DNA from another contrasts with results previously obtained using randomly cloned DNA segments (Woolley, 1988). These particular genes may possibly have been more highly conserved than most other genes in these organisms. Alternatively, they may have been horizontally transferred to the divergent chromosomal types. Nucleotide sequence data will be required to shed light on the evolutionary relationships between these genes and also between the various strains currently known as *C. acetobutylicum*. Their heterogeneity would seem to indicate that they are not correctly viewed as a single species.

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