Physical and genetic map of the *Clostridium saccharobutylicum* (formerly *Clostridium acetobutylicum*) NCP 262 chromosome

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A physical and genetic map of the *Clostridium saccharobutylicum* NCP 262 chromosome was constructed. The order of macrorestriction fragments was determined by analysing fragments generated after single and double digestion with the restriction enzymes *Bss*HII, *I*-*Ceu*I, *Sse*8387I, *Rsr*II and *Sfi*I and separation by PFGE. The *I*-*Ceu*I backbone of *C. saccharobutylicum* was constructed by indirect end-labelling with *rrs*- and 3' *rri*-specific probes located on either side of the *I*-*Ceu*I site in the *rrn* operon, and reciprocal separation of *Bss*HII and *I*-*Ceu*I digestion products by two-dimensional PFGE. The positions of *Bss*HII fragments on the physical map were determined using a library of linking clones containing *Bss*HII cleavage sites. The size of the circular genome was estimated to be 5.3 Mb with a mean resolution of approximately 140 kb. The chromosome of *C. saccharobutylicum* contains 12 *rrn* operons, located on 46% of the chromosome, which are transcribed divergently from the deduced origin of replication. The genetic map was constructed by determining the location of 28 genes involved in house-keeping, heat-shock response, sporulation, electron transfer and acid- and solvent-formation. Comparison of the *C. saccharobutylicum* genetic map with those of the spore-forming bacteria *Bacillus subtilis*, *Clostridium acetobutylicum*, *Clostridium perfringens* and *Clostridium beijerinckii* indicated *C. saccharobutylicum* to be most similar to the latter two *Clostridium* species, with the order of the genes within the *gyrAB* and *recA* loci being conserved.

Keywords: solvent-producing clostridia, PFGE, 2D-PFGE, linking library, genome organization

INTRODUCTION

Clostridium saccharobutylicum (formerly *Clostridium acetobutylicum*) NCP 262 is an obligately anaerobic spore-forming bacterium and one of four distinct species of solvent-producing clostridia capable of fermenting various carbohydrates to produce acetone and butanol (Johnson *et al.*, 1997; Keis *et al.*, 1995). Solvent-producing clostridia were used extensively during the early part of the twentieth century for the commercial production of solvents (Jones & Woods, 1986). Both the industrial acetone-butanol fermentation process and the solvent-producing clostridia have continued as an active

focus of research due to their potential applications in biotechnology.

The first successful industrial solvent-producing strains to be isolated, patented and used for the large-scale production of solvents from starch-based substrates were classified as *C. acetobutylicum*. Following the switch to molasses as the preferred fermentation substrate for commercial fermentation during the mid-1930s, numerous new saccharolytic, solvent-producing clostridial strains were isolated that performed more efficiently on these sugar-based substrates. Many of these new industrial strains were patented under novel species names but none of these were recognized as legitimate species. Once the acetone-butanol fermentation process declined during the latter part of the twentieth century these names fell into disuse. Sub-

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Abbreviation: 2D, two-dimensional.

sequently, the majority of the industrial solvent-producing clostridial strains became designated as either *C. acetobutylicum* or *Clostridium beijerinckii*. Recent studies have, however, revealed that the various strains of industrial solvent-producing clostridia belong to four distinct species: *C. acetobutylicum*, *C. beijerinckii*, *C. saccharobutylicum* and '*Clostridium saccharoperbutyl-aceticum*' (Johnson *et al.*, 1997; Keis *et al.*, 1995), with the latter two species in the process of being formally classified (S. Keis, R. Shaheen & D. T. Jones, unpublished results).

Most of the studies undertaken over the last two decades have focused on strains belonging to the species *C. acetobutylicum* and *C. beijerinckii*, which are phylogenetically only distantly related (Johnson *et al.*, 1997; Keis *et al.*, 1995). Physical and genetic maps of the chromosomes of these two species have been published (Cornillot *et al.*, 1997; Wilkinson & Young, 1995), and the entire *C. acetobutylicum* genome sequence has now been determined (http://www.genomecorp.com/sequence_centre/bacterial_genomes/).

Industrial strains belonging to the recently named *C. saccharobutylicum* species were amongst the most successful saccharolytic, solvent-producing clostridia utilized for the commercial production of solvents from molasses (Jones & Keis, 1995; Keis *et al.*, 1995; Shaheen *et al.*, 2000). Nevertheless, *C. saccharobutylicum* has been less intensively studied than the other two solvent-producing species and currently little is known about the genomic structure and organization of this organism.

In this study, we have used one- and two-dimensional (2D) PFGE, *Bss*HIII linking clones, and Southern hybridization analysis to construct a physical and genetic map of the *C. saccharobutylicum* type strain, NCP 262. The size of the circular chromosome was estimated to be 5.3 Mb and it contains 12 *rrn* operons that are transcribed divergently from the origin of replication. The genetic map of this species was compared with those of the spore-forming bacteria *Bacillus subtilis*, *C. acetobutylicum*, *C. beijerinckii* and *Clostridium perfringens*.

METHODS

Bacterial strains, plasmids and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *C. saccharobutylicum* NCP 262 was grown anaerobically in *Clostridium* Basal Medium (CBM) (O'Brien & Morris, 1971). *Escherichia coli* was grown aerobically at 37 °C in 2 × YT broth or Luria-Bertani (LB) agar (Sambrook *et al.*, 1989) supplemented as required with 100 µg ampicillin ml⁻¹, 50 µg kanamycin ml⁻¹, 24 µg IPTG ml⁻¹ and 40 µg X-Gal ml⁻¹.

Chromosomal DNA preparation and restriction enzyme digestions. Chromosomal DNA embedded in agarose plugs was prepared as described previously (Keis *et al.*, 1995). Restriction enzymes were purchased from New England Biolabs and Roche Molecular Biochemicals, except for *Sse*8387I, which was obtained from Amersham. Slices of DNA plugs (200 µl) were equilibrated three times at room tem-

perature for 15 min in 100 µl of the restriction enzyme buffer recommended by the manufacturer, with the exception of *Bss*HIII digests, which were equilibrated and digested in 1 × KGB buffer (100 mM potassium glutamate, 25 mM Tris/acetate pH 7.6, 10 mM magnesium acetate, 50 µg bovine serum albumin ml⁻¹, 0.5 mM 2-mercaptoethanol) (McClelland *et al.*, 1988). Plugs were then digested with 10 U restriction enzyme at the optimum temperature for 3 h. Double digestions of DNA plugs were carried out consecutively. For reproducibility, agarose plugs digested with *I-CeuI* were treated after digestion with 0.5 mg proteinase K ml⁻¹ in ES buffer (0.5 M EDTA pH 9.0, 1%, w/v, *N*-lauroyl-sarcosine) for 30 min at 56 °C and washed twice with TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) for 10 min at room temperature.

For 2D digestions, a 4-mm-wide slice of the lane, containing the first digestion, was equilibrated in a 15 ml polypropylene tube for 30 min at 4 °C with 2 ml of the second restriction enzyme buffer. The DNA slice was then preincubated overnight at 4 °C in 2 ml restriction enzyme buffer containing 100 U enzyme to allow the enzyme to diffuse into the agarose slice. This was followed by incubation in a Hybaid oven at the appropriate temperature with slow rotation for 3 h.

Pulsed-field and agarose gel electrophoresis. PFGE was performed in a contour-clamped homogeneous electric field (CHEF) electrophoresis system (CHEF-DRIII, Bio-Rad). Agarose plugs were equilibrated three times in 0.5 × TBE buffer (45 mM Tris/borate, 1 mM EDTA) for 15 min prior to loading agarose (Molecular Biology Grade; Bio-Rad) gels. Gels were routinely subjected to electrophoresis at 6 V cm⁻¹, 14 °C, at an included angle of 120°. Agarose concentrations, pulse times and total running times varied according to the size range of fragments to be separated; gel electrophoresis conditions for individual experiments are described in the figure legends. Lambda DNA concatemers with lambda *Hind*III-digested fragments (Low Range PFG Marker; New England Biolabs), lambda concatemers (Lambda Ladder PFG Marker; New England Biolabs), and chromosomes of *Saccharomyces cerevisiae* YNN295 (Bio-Rad) were used as molecular size standards. Conventional agarose gels were subjected to electrophoresis at 1.4 V cm⁻¹ for 18 h and lambda DNA digested with *Hind*III was used as a size standard.

Southern hybridization. DNA fragments separated by PFGE were transferred onto Hybond-N⁺ nylon membranes (Amersham) using a VacuGene (Pharmacia-LKB) vacuum blotting system. The DNA was depurinated with 0.25 M HCl for 20 min, denatured (in 1.5 M NaCl, 0.5 M NaOH) for 1 h, and transferred in 20 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 2 h. DNA in conventional agarose gels was depurinated, denatured and transferred for 10 min, 10 min and 1 h, respectively.

Plasmid DNA used for probing was isolated with the High Pure plasmid isolation kit (Roche Molecular Biochemicals). PCR products used as probes were extracted from 1% (w/v) SeaPlaque low-melting-point agarose (FMC BioProducts) gels using the QIAEXII gel extraction kit (Qiagen).

DNA was labelled with [α -³²P]dCTP (Amersham) by random priming using the RTS RadPrime DNA labelling kit (GibcoBRL). Prehybridization and hybridization were carried out in the same hybridization buffer (Church & Gilbert, 1984) at 65 °C for 18 to 24 h. After hybridization, membranes were washed sequentially for 5 min in 2 × SSC/0.1% SDS (37 °C), 20 min in 1 × SSC/0.1% SDS (65 °C), and 20 min in 0.1 × SSC/0.1% SDS (65 °C) before exposure to X-ray film (Cronex, DuPont). For heterologous probes, membranes were washed

Table 1. Bacterial strains, plasmids and PCR product used in this study

Strain or plasmid	Relevant characteristics	Source	Reference
<i>C. saccharobutylicum</i> NCP 262	Wild-type		
<i>E. coli</i> DH10B	F ⁻ <i>mcrA</i> Δ (<i>mrr-bsdRMS-mcrBC</i>) ϕ 80 <i>dlacZ</i> Δ M15 <i>ΔlacX74 deoR recA1 endA1 araD139 Δ(ara,</i> <i>leu)7697 galU galK λ⁻ rpsL nupG</i>		Hanahan <i>et al.</i> (1991)
Plasmids			
pUC8	Cloning and sequencing vector		Yanisch-Perron <i>et al.</i> (1985)
pNEB193	pUC19-derived vector		New England Biolabs
pUC4K	Contains kanamycin cassette		Vieira & Messing (1982)
pJP3	<i>glnA</i> (glutamine synthetase)	<i>C. saccharobutylicum</i>	Janssen <i>et al.</i> (1988)
pAT-1	<i>spoIID</i> (sporulation)	<i>C. saccharobutylicum</i>	S. Reid*
pCar1	<i>regA</i> (<i>ccpA</i> homologue)	<i>C. saccharobutylicum</i>	Davison <i>et al.</i> (1995)
pHS92	<i>metF</i> (methionine synthase)	<i>C. saccharobutylicum</i>	Stutz (2000)
pWT1	<i>sum</i> (metronidazole susceptibility)	<i>C. saccharobutylicum</i>	Collett <i>et al.</i> (1997)
pCADH-463	<i>adh1</i> (alcohol dehydrogenase)	<i>C. saccharobutylicum</i>	Youngleson <i>et al.</i> (1989)
pF2	<i>aad</i> (alcohol/aldehyde dehydrogenase)	<i>C. saccharobutylicum</i>	This study
pHZ301	<i>xylA</i> (xylanase)	<i>C. saccharobutylicum</i>	Zappe <i>et al.</i> (1987)
pHZ100	<i>eglA</i> (endoglucanase)	<i>C. saccharobutylicum</i>	Zappe <i>et al.</i> (1986)
pCBS2	<i>scrB</i> (sucrase operon)	<i>C. beijerinckii</i>	Reid <i>et al.</i> (1999)
pEK30	ISCb1 (partial IS element)	<i>C. beijerinckii</i>	Liyanage <i>et al.</i> (2000)
pSU1	<i>recF</i> ; <i>gyrA gyrB</i> (homologous recombination, DNA gyrases)	<i>C. acetobutylicum</i>	Ullmann & Dürre (1996)
prec1	<i>recA</i> (recombination)	<i>C. acetobutylicum</i>	Cornillot <i>et al.</i> (1997)
pKG3	<i>dnaKJ</i> (hsp70 cluster)	<i>C. acetobutylicum</i>	Narberhaus <i>et al.</i> (1992)
pMFH1	<i>hydA</i> (complete locus)	<i>C. acetobutylicum</i>	Gorwa <i>et al.</i> (1996)
pJC7	<i>butK</i> , <i>ptb</i> (butyrate operon)	<i>C. acetobutylicum</i>	Walter <i>et al.</i> (1993)
pK42	<i>hsp18 trna</i> _{ACG}	<i>C. acetobutylicum</i>	Sauer & Dürre (1993)
pFN4	<i>groESL</i> (hsp10–60 operon)	<i>C. acetobutylicum</i>	Narberhaus & Bahl (1992)
pb37	<i>hbd</i> (BCS operon)	<i>C. acetobutylicum</i>	Boynton <i>et al.</i> (1996)
pCoAT4	<i>ctfAB</i> (<i>sol</i> operon)	<i>C. acetobutylicum</i>	Cary <i>et al.</i> (1990)
pEco14	<i>bdbAB</i> (butanol dehydrogenases)	<i>C. acetobutylicum</i>	Walter <i>et al.</i> (1992)
pEC10	<i>rrl</i> (3' end of 23S rRNA gene)	<i>C. perfringens</i>	Cornillot <i>et al.</i> (1997)
PCR probe 16SrRNA	<i>rrs</i> (position 830–1383, <i>E. coli</i> numbering)	<i>C. saccharobutylicum</i>	Keis <i>et al.</i> (1995)

* Personal communication.

less stringently by carrying out the initial wash as above, followed by 20 min in 2 × SSC/0.1% SDS at 50 °C.

Genomic library construction and cloning of small *Bss*HII fragments. Genomic DNA was extracted from agarose plugs using the QIAEXII gel extraction kit (Qiagen). The DNA was sheared by nebulization and end-repaired with T4 DNA polymerase (Roche Molecular Biochemicals) and Klenow (Roche Molecular Biochemicals) (Andersson *et al.*, 1996). The end-repaired DNA was separated on a 1% (w/v) SeaPlaque low-melting-point agarose (FMC BioProducts) gel. DNA ranging from 4 to 23 kb was excised from the gel, and the DNA was extracted using the QIAEXII gel extraction kit, followed by ligation into dephosphorylated, *Sma*I-digested pUC8. For the cloning of small (< 5 kb) *Bss*HII fragments, DNA was extracted from *Bss*HII-digested genomic DNA plugs and ligated into dephosphorylated *Bss*HII-digested pNEB193. Electrocompetent *E. coli* cells were transformed by electroporation and white ampicillin-resistant transformants were selected on media containing X-Gal, IPTG and ampicillin.

Preparation of linking clones. The kanamycin cassette from pUC4K was amplified using the PCR conditions described below and the primers KANFWD (5'-TCGACTACGCGT-AGCTTCACGCTGCCGCAAGC-3') and KANREV (5'-AGTACGACGCGTGGGGTGGGCGAAGAACTCCA-3'), each containing *Mlu*I recognition sites (shown in bold) near their 5' ends. This cassette was digested with *Mlu*I to create a kanamycin cassette with *Bss*HII-compatible ends.

Plasmid DNA was isolated from the library of pooled recombinant NCP 262 random pUC8 clones using a Qiagen maxi-prep kit. The DNA was digested with *Bss*HII, treated with alkaline phosphatase (Roche Molecular Biochemicals) and ligated with the *Mlu*I-digested kanamycin cassette. Clones with *C. saccharobutylicum* NCP 262 DNA containing the rare *Bss*HII restriction enzyme site were obtained by selection on media containing ampicillin and kanamycin. These linking clones were radiolabelled and hybridized to Southern blots of PFGE-separated restriction digests as described above.

Sequence analysis of linking clones. Plasmid DNA from linking clones was prepared for sequencing using a Quantum

Prep plasmid miniprep kit (Bio-Rad). *Bss*HII linking clones were sequenced with the following specifically designed primers, SEQKANLEFT (5'-CCAGTAGCTGACATTCAT-CCG-3') and SEQKANRIGHT (5'-AGGTTGGGCTTCGG-AATCGT-3'), which were complementary to the DNA sequences at either end of the kanamycin cassette. Sequencing was carried out using a BigDye terminator cycle sequencing ready reaction sequencing kit (Applied Biosystems) and a model ABI377 automated DNA sequencer (Applied Biosystems).

Homology searches of non-redundant databases at the National Centre for Biotechnology Information, using BLASTX and BLASTN (Altschul *et al.*, 1997), were done through the WWW BLAST Server (www.ncbi.nlm.nih.gov).

Confirmation of *Bss*HII-linked fragments by PCR. Synthetic primers, based on sequences obtained from linking clones, were designed to amplify products of approximately 400 bp which spanned NCP 262 genomic *Bss*HII sites. PCR was carried out using the Expand High Fidelity PCR system (Roche Molecular Biochemicals) and NCP 262 genomic DNA embedded in agarose plugs was used as the template. The PCR programme consisted of an initial cycle of denaturation (94 °C for 2 min), annealing (50 °C for 2 min) and extension (72 °C for 5 min). This was followed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min, and a final cycle of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 5 min. PCR products were purified, radiolabelled and hybridized to PFGE-separated restriction digests as described above. Purified PCR products were also sequenced as described above with their respective PCR primers.

RESULTS

Restriction fragment analysis and estimation of genome size

The G+C content of the genomes of mesophilic *Clostridium* species is less than 30 mol%; therefore restriction enzymes with GC-rich recognition sites were screened to select those with a limited number of cleavage sites on the genome of NCP 262. *Nae*I, *Nar*I, *Sma*I, *Eag*I, *Ksp*I and *Bgl*I all produced too many fragments (> 20) to be suitable for mapping. By contrast, *Not*I did not cleave the NCP 262 genome. Enzymes chosen for mapping were *Bss*HII and *Sse*8387I and the intron-encoded endonuclease *I-Ceu*I, which cleaves within the *rrl* (23S) genes of *rrn* operons. These enzymes produced 16, 8 and 12 fragments, respectively (Table 2, Fig. 1).

A low-resolution physical map of *C. beijerinckii* NCIMB 8052 was constructed by Wilkinson & Young (1995) using the enzymes *Rsr*II and *Sfi*I. We found the genome of NCP 262 to be cleaved twice by *Rsr*II and once by *Sfi*I, as determined after double digestion with *Bss*HII and *I-Ceu*I. Hence, we included the *Rsr*II and *Sfi*I sites on the physical map.

The enzyme *Apa*I was also found to yield a suitable number of fragments (12–20), several of which co-migrated with those of *I-Ceu*I. This is due to the presence of an *Apa*I site in the *rrs* gene of the *rrn* operon, as confirmed by analysis of the 16S rRNA gene sequence of NCP 262 (GenBank accession number U16147; Keis

Table 2. Sizes of restriction fragments generated by cleavage of the *C. saccharobutylicum* NCP 262 chromosome

Fragment	Fragment size (kb)* obtained with:				
	<i>Bss</i> HII	<i>I-Ceu</i> I	<i>Sse</i> 8387I	<i>Rsr</i> II	<i>Sfi</i> I
A	1151 ± 29	2908†	1794†	4774†	5337‡
B	631 ± 8	1039 ± 14	1555†	563 ± 8	
C	598 ± 9	675 ± 6	855 ± 6		
D	417 ± 5	316 ± 7	497 ± 11		
E	395 ± 5	159 ± 3	362 ± 6		
F	372 ± 5	112 ± 1	194 ± 3		
G	320 ± 5	74 ± 2	35 ± 2§		
H	306 ± 7	17 ± 1			
I	238 ± 3	9·6			
J	219 ± 5	8·5			
K	205 ± 5	6·5			
L	192 ± 6	6·1			
M	145 ± 2				
N	69 ± 3§				
O	5				
Total	5332	5331	5327	5337	5337

* The size indicated is the mean ± standard deviation for at least five independent values, except where otherwise noted.

† The sizes of bands *Ceu*A, *Sse*A and *Sse*B were obtained by summing the sizes of fragments related to them from double digestions with *Bss*HII (*Ceu*A = 1151 + 417 + 395 + 339 + 238 + 192 + 145 + 26 + 5 = 2908; *Sse*A = 565 + 347 + 320 + 219 + 205 + 69 + 69 = 1794; *Sse*B = 417 + 395 + 306 + 236 + 145 + 51 + 5 = 1555). The size of *Rsr*A was obtained by subtracting *Rsr*B from the mean total genome size of 5337 kb.

‡ Single recognition site.

§ Fragment is a doublet.

et al., 1995). Since *Apa*I sites would lie in close proximity to *I-Ceu*I sites in most instances, *Apa*I was not used for mapping.

The size of the *C. saccharobutylicum* genome was estimated to be 5·3 Mb by summation of the fragments obtained from individual *Bss*HII, *I-Ceu*I, *Sse*8387I and *Rsr*II digestions (Table 2) and from *Bss*HII/*I-Ceu*I, *I-Ceu*I/*Sse*8387I and *Bss*HII/*Sse*8387I double digestions (Table 3, Fig. 1). The fragment sizes were determined from at least five different gels using different PFGE parameters. Attempts to resolve the *Ceu*A and *Rsr*A fragments using PFGE conditions recommended by Bio-Rad for the separation of *Hansenula wingei* and *Schizosaccharomyces pombe* chromosomes were not successful. Hence, the sizes of *Ceu*A and *Rsr*A were obtained by analysis of double digests. Fragments *Sse*A and *Sse*B could not be separated (Fig. 1c), and as a consequence their sizes were also determined more accurately from double digestions. The smallest *Bss*HII fragment (*Bss*O) was not observed in pulsed-field gels nor in conventional agarose gels but was detected by hybridization (see Fig. 3).

Fig. 1. PFGE of restriction enzyme digests of *C. saccharobutylicum* NCP 262 DNA. (a) DNA was digested with *Bss*HII (lane 2), *Bss*HII/*Sse*8387I (lane 3), *Sse*8387I (lane 4), *Sse*8387I/*I-Ceu*I (lane 5) and *I-Ceu*I (lane 6). The Low Range PFG Marker (New England Biolabs) was used as a molecular size marker (lanes 1 and 7). Electrophoresis was performed in 1.2% agarose and the pulse time was ramped from 5 to 30 s for 24 h. (b) DNA was digested with *Bss*HII (lane 2), *Bss*HII/*I-Ceu*I (lane 3) and *I-Ceu*I (lane 4). The Lambda Ladder PFG Marker (New England Biolabs) was used as a molecular size marker (lane 1). Electrophoresis was performed in 1.1% agarose and the pulse time was ramped from 20 to 50 s for 20 h. (c) DNA was digested with *I-Ceu*I (lane 2), *Sse*8387I/*I-Ceu*I (lane 3) and *Sse*8387I (lane 4). The *Sse*8387I fragments are indicated. Electrophoresis was performed in 1% agarose and the pulse time was ramped from 60 to 120 s for 22 h. The yeast chromosomes of *S. cerevisiae* YNN 295 (Bio-Rad) were used as a molecular marker (lane 1). All sizes are in kilobases.

Construction of the *I-Ceu*I physical map

The intron-encoded endonuclease *I-Ceu*I has been found to cleave exclusively within *rrl* genes, allowing the enumeration of *rrn* operons of a given bacterium. Digestion of the NCP 262 genome with *I-Ceu*I generated 12 fragments, ranging in size from 5 kb to 2900 kb (Table 2), indicating that this species contains 12 *rrn* operons. The circular genomic backbone of NCP 262, as defined by the position of the *rrn* operons and their orientation with respect to the origin of replication, was constructed using a strategy similar to that devised by Cornillot *et al.* (1997). This involved probing Southern blots of single and double *Bss*HII and *I-Ceu*I digestions with a labelled PCR product specific to the *rrs* (16S) gene located on one side of the *I-Ceu*I site, and a probe (pEC10) containing the 3' end of the *rrl* gene located on the other side of the *I-Ceu*I site. All resolvable *I-Ceu*I fragments hybridized with both the *rrs* and 3' *rrl* probes, except for *Ceu*G, which hybridized only with the *rrs* probe at twice the intensity of the other fragments (data not shown), indicating that the *rrn* operons are transcribed divergently from within this fragment. All the *Bss*HII fragments that were cleaved by *I-Ceu*I, and no other *Bss*HII fragments, hybridized with both the *rrs* and 3' *rrl* probes, with the *Bss*H fragment hybridizing at high intensity (data not shown).

This indirect end-labelling approach was then combined with 2D-PFGE. The NCP 262 genome was digested with *I-Ceu*I and separated by PFGE in the first dimension, followed by digestion with *Bss*HII and separation in the

second dimension (or vice versa) (Fig. 2a). This allowed the arrangement of the *I-Ceu*I restriction sites to be determined by identifying the *Bss*HII fragments that overlapped each *I-Ceu*I site. *Ceu*A, *Ceu*B, *Ceu*C, *Ceu*D and *Ceu*E were the only *I-Ceu*I fragments cleaved by *Bss*HII (Table 4). Inversely, *Bss*B, *Bss*C, *Bss*F, *Bss*H and *Bss*J were the only fragments cut by *I-Ceu*I (Fig. 2). *Bss*B was cut by *I-Ceu*I yielding two fragments of 577 kb (BC1) and 53 kb (BC7), which hybridized with the 3' *rrl* and *rrs* probe, respectively (Fig. 2b). The 577 kb fragment was also found on a 2D pulsed-field gel when *Ceu*C was cleaved by *Bss*HII in the second digestion, whereas the 53 kb fragment was obtained after *Bss*HII digestion of *Ceu*D (Table 4). Therefore, *Bss*B spans the *I-Ceu*I site that joins *Ceu*C and *Ceu*D. *Ceu*D produced a second fragment of 262 kb (BC3) after *Bss*HII digestion (Table 4) and this fragment, which hybridized with the 3' *rrl* probe, was also released from *Bss*F after *I-Ceu*I digestion (Fig. 2b). *Bss*F also yielded a *rrs*-specific 114 kb (BC6) fragment after *I-Ceu*I digestion (Fig. 2b) and this fragment was also obtained after *Bss*HII digestion of *Ceu*E (Table 4); hence, *Bss*F overlaps *Ceu*D and *Ceu*E. *Ceu*E produced a 3' *rrl*-specific 53 kb (BC8) end-fragment when digested with *Bss*HII (Table 4) and this fragment was also found when *Bss*H was digested by *I-Ceu*I and separated in the second dimension (Fig. 2b). Besides this 53 kb end-fragment, the *Bss*H fragment not only contained the seven *I-Ceu*I fragments *Ceu*F to *Ceu*L, it produced another end-fragment of 26 kb (BC10) after *I-Ceu*I digestion which also hybridized only with the 3' *rrl* probe (Fig. 2b). This indicated that the *rrn*

Table 3. Fragment sizes resulting from double digestions of the *C. saccharobutylicum* NCP 262 chromosome

Name* and fragment size (kb)† obtained with:									
<i>Bss</i> HII/I- <i>Ceu</i> I		I- <i>Ceu</i> I/ <i>Sse</i> 8387I		<i>Bss</i> HII/ <i>Sse</i> 8387I		<i>Rsr</i> II/I- <i>Ceu</i> I		<i>Rsr</i> II/ <i>Sse</i> 8387I	
Fragment	Size	Fragment	Size	Fragment	Size	Fragment	Size	Fragment	Size
BssA	1151 ± 29	CS1	1100 ± 32	SseC	855 ± 6	RC1	1619‡	SseA	1794
BC1	577 ± 3	CeuB	1039 ± 14	SB1	565 ± 7	CeuB	1039 ± 14	RS1	1252 ± 37
BssD	417 ± 5	SseC	855 ± 6	BssD	417 ± 5	RC2	726 ± 12	SseC	855 ± 6
BssE	395 ± 5	SseD	497 ± 11	BssE	395 ± 5	CeuC	675 ± 6	SseE	362 ± 6
BC2	339 ± 7	CS2	430 ± 10	SB2	347 ± 4	RsrB	563 ± 8	RS2	330 ± 7
BssG	320 ± 5	CS3	306 ± 8	BssG	320 ± 5	CeuD	316 ± 7	RS3	260 ± 5
BC3	262 ± 5	SseF	194 ± 3	BssH	306 ± 7	CeuE	159 ± 3	RS4	224 ± 5
BC4	260 ± 4	CS4	186 ± 4	SB3	236 ± 5	CeuF	112 ± 1	SseF	194 ± 3
BssI	238 ± 3	CS5	176 ± 6	SB4	219 ± 5	CeuG	74 ± 2	SseG	35 ± 2§
BssK	205 ± 5	CeuE	159 ± 3	BssJ	219 ± 5	CeuH	17 ± 1		
BssL	192 ± 6	CS6	131 ± 8	BssK	205 ± 5	CeuI	9·6		
BC5	175 ± 3	CeuF	112 ± 1	BssL	192 ± 6	CeuJ	8·5		
BssM	145 ± 2	CeuG	74 ± 2	SB5	190 ± 4	CeuK	6·5		
BC6	114 ± 1	SseG	35 ± 2§	SB6	168 ± 4	CeuL	6·1		
CeuF	112 ± 1	CeuH	17 ± 1	BssM	145 ± 2				
CeuG	74 ± 2	CeuI	9·6	SB7	138 ± 2				
BssN	69 ± 3§	CeuJ	8·5	SB8	110 ± 3				
BC7	53 ± 3	CeuK	6·5	BssN	69 ± 3§				
BC8	53 ± 3	CeuL	6·1	SB9	51 ± 3				
BC9	37 ± 2			SseG	35 ± 2§				
BC10	26 ± 1			SB10	31 ± 2				
CeuH	17 ± 1			BssO	5				
CeuI	9·6								
CeuJ	8·5								
CeuK	6·5								
CeuL	6·1								
BssO	5								
Total	5336		5337		5322		5331		5341

* Fragments resulting from double digestions are designated by a two-letter code followed by a number; the higher the number the smaller the fragment.

† The size indicated is the mean for at least five independent values, except where otherwise noted.

‡ The size of RC1 was obtained by subtracting the sizes of fragments related to it ($RC1 = 2908 - 726 - 563 = 1619$).

§ Fragment is a doublet.

operons contained within this BssH fragment are confluent transcribed from CeuG, which hybridized only with the *rrs* probe and is contained within BssH. The order of the I-*Ceu*I fragments within BssH was not resolved.

CeuC (linked to CeuD as described above) also produced a 37 kb (BC9) *rrs*-specific end-fragment after BssHII digestion (Table 4), and this fragment was also obtained when BssJ was digested with I-*Ceu*I (Fig. 2b). The other fragment that BssJ generated after I-*Ceu*I digestion was a 175 kb (BC5) fragment, which hybridized with the *rrs* probe (Fig. 2b), and a fragment of this size was produced from CeuB after BssHII digestion (Table 4). Consequently, BssJ links CeuC to CeuB. The other end-fragment that CeuB produced after BssHII digestion in the second dimension was a 260 kb (BC4) *rrs*-specific

fragment (Table 4) which was also obtained when BssC was digested with I-*Ceu*I (Fig. 2b). Besides this fragment, BssC also generated a 339 kb (BC2) 3' *rrl*-specific end-fragment (Table 4). At this stage the following I-*Ceu*I fragments had been linked with their 3' *rrl* end-fragments as follows: BC2-CeuB-CeuC-CeuD-CeuE-(CeuF to CeuL)-BC10, with the order of CeuF to CeuL unresolved. This left CeuA as the only fragment not accounted for and it was concluded that this large fragment closed the map into a circular genome via the 3' *rrl*-specific end-fragments BC2 and BC10.

Alignment of the Sse8387I fragments

The construction of the I-*Ceu*I backbone of the genome of *C. saccharobutylicum* NCP 262 and the placement of a number of the BssHII fragments enabled the Sse8387I

Fig. 2. Two-dimensional PFGE of complete *BssHII*/I-*CeuI* double digestions. (a) DNA was digested with *BssHII* and subjected to electrophoresis in the first dimension (1% agarose, ramped pulse time from 5 to 50 s for 24 h), followed by digestion with I-*CeuI* in the second dimension (1.2% agarose, pulse time of 25 s for 13 h and then 50 s for 6 h). (b) Hybridization diagram summarizing the results with both ribosomal probes (3' *rrl* and *rrs*). The small I-*CeuI* fragments (CeuH to CeuL) were not visible on the gel after ethidium bromide staining; however, these fragments gave strong signals when they hybridized with either ribosomal probe. Crossed circles indicate fragments that hybridized with the 3' *rrl* probe only. Open circles indicate fragments that hybridized with the *rrs* probe only, and black circles are I-*CeuI* fragments that hybridized with both ribosomal probes. The plus symbol depicts *BssHII* fragments not digested by I-*CeuI*. The *BssHII* fragments cleaved by I-*CeuI* are shown below the arrows. See Table 3 for the sizes of the fragments. *BssA* was not resolved under these electrophoresis conditions.

Table 4. Sizes of fragments obtained after digestion of *C. saccharobutylicum* NCP 262 with I-*CeuI* in the first dimension and *BssHII* in the second dimension

	Fragment size (kb)				
	CeuA	CeuB	CeuC	CeuD	CeuE
	339 (BC2)*	260 (BC4)†	577 (BC1)*	262 (BC3)*	114 (BC6)†
	26 (BC10)*	175 (BC5)*	69 (BssN)	53 (BC7)†	53 (BC8)*
	()‡	()‡	37 (BC9)†		
Total	2908	1029	683	315	167

* 3' *rrl*-specific end-fragment.

† *rrs*-specific end-fragment.

‡ The sizes of the remaining fragments are not given.

fragments to be located on the physical map. Double digestions of I-*CeuI* and *Sse8387I* generated six fragments (CS1 to CS6) (Table 3). *SseA*, *SseB* and *SseE* were cleaved by I-*CeuI*, and *CeuA*, *CeuC* and *CeuD* were cleaved by *Sse8387I* (Fig. 1a, c). Since *CeuC* and *CeuD* were linked and both were cleaved by *Sse8387I*, a *Sse8387I* fragment had to overlap this *CeuC*-*CeuD* linkage. The only *Sse8387I* fragment of the appropriate size that was cleaved by I-*CeuI* was *SseE* (362 kb), which

produced the fragments CS4 (186 kb) and CS5 (176 kb). Subtracting the 186 kb fragment from *CeuD* (316 kb) left a 130 kb fragment and this accounted for the new CS6 fragment. Hence, it could be concluded that the CS5 fragment was situated within *CeuC* (675 kb). The remaining size difference of 499 kb for *CeuC* did not account for any of the new fragment sizes that resulted from a I-*CeuI*/*Sse8387I* double digestion. Partial *Sse8387I* digestions produced fragments of about 395 kb

Table 5. *Bss*HII linking clones and their sequence homologies to genes or proteins from the genebank databases

Linking clone	Linkage	Description of similar sequence*	Source of similar sequence	Probability score†	Accession no.
25	BssN-BssJ	Transcriptional regulator AraC/XylS family homologue YdeE	<i>Bacillus subtilis</i>	2e-56	G69777
26	BssO-BssI	ND			
27	BssE-BssO	ND			
A3	BssM-BssE	Hypothetical 47.0 kDa protein in <i>glnQ-ansR</i> intergenic region	<i>Bacillus subtilis</i>	2e-53	P54545
A10	BssF-BssH	Spore cortex-lytic enzyme (<i>sleC</i>)	<i>Clostridium perfringens</i>	3e-13	I40824
B7	BssB-BssN	Insertion element (ISCb1)	<i>Clostridium beijerinckii</i>	1e-165	AJ250468
	BssN'-BssG				
B10	BssD-BssM	ND			
B12	BssK-BssC	ND			
C2	BssH-BssD	Prolyl-tRNA	<i>Micrococcus luteus</i>	5e-13	X55109
C4	BssL-BssA	ND			
D4	BssG-BssK	ND			
F7	BssC-BssA	Hypothetical ABC transporter permease protein	<i>Escherichia coli</i>	8e-58	P77651

* ND, No homology determined; linkage was confirmed by PCR.

† BLASTX (or BLASTN for linking clone C2) probability scores based on approximately 1200 bp of linked DNA sequence spanning *Bss*HII sites.

Fig. 3. Identification of the 5 kb *Bss*O fragment as detected by Southern hybridization of digested NCP 262 genomic DNA probed with a PCR product spanning the *Bss*HII site of linking clone 26. DNA was digested with *Bss*HII and separated in a conventional 1% agarose gel (lane 1). Lambda DNA digested with *Hind*III was used as a size marker (lane 2). The compression zone of fragments is indicated by 'C'. All sizes are in kilobases.

and 430 kb (data not shown), and the only combination of linked *Sse*8387I fragments that would result in these sizes would be *Sse*E-*Sse*G and *Sse*E-*Sse*G-*Sse*G', respectively. Therefore, *Sse*G and *Sse*G' were located within *Ceu*C, and the new 430 kb (CS2) fragment accounted for the remaining size difference. Since *Ceu*C was linked to *Ceu*B, and the latter was not cleaved by

*Sse*8387I, *Sse*A had to bridge *Ceu*B to produce a new fragment (CS3) of around 300 kb. Similarly, the eight *I-Ceu*I fragments *Ceu*E to *Ceu*L did not contain *Sse*8387I sites, and the only *Sse*8387I fragment cleaved by *I-Ceu*I large enough to produce the remaining new 1100 kb fragment (CS1) was *Sse*B. *Sse*F, *Sse*C and *Sse*D were not cleaved by *I-Ceu*I and were therefore contained within *Ceu*A. *Sse*F (194 kb) was cleaved by *Bss*HII producing fragments of about 168 kb (SB6) and 31 kb (SB10), which placed it adjacent to *Sse*A. The orientation of *Sse*C and *Sse*D could not be resolved until the position of the *Rsr*B fragment had been determined.

Alignment of *Rsr*II fragments

Double digestions of the NCP 262 genome with *Rsr*II and *I-Ceu*I produced two new fragments of 1619 kb (RC1) and 726 kb (RC2), with all of the resolvable *I-Ceu*I fragments remaining intact as well as the *Rsr*B fragment (Table 3). Therefore, *Rsr*B had to be located within *Ceu*A. *Rsr*B was placed more accurately on the physical map by analysis of a *Rsr*II/*Sse*8387I double digestion. This digest generated four new fragments (Table 3), with *Sse*B, *Sse*D and *Rsr*B being cleaved. *Rsr*B (563 kb) was cleaved by *Sse*8387I to produce the fragments RS2 (330 kb) and RS4 (224 kb), with *Sse*D overlapping the *Rsr*B fragment by 224 kb and producing the third 260 kb fragment (RS3). The fourth 1252 kb fragment (RS1) was the result of *Sse*B containing the second *Rsr*II site.

Positioning of the single *Sfi*I site on the physical map

The unique *Sfi*I site was located on the physical map from the analysis of a *Bss*HII/*Sfi*I double digestion,

Fig. 4. Copy number of an IS element as detected by Southern hybridization of digested NCP 262 genomic DNA probed with a 378 bp PCR product. (a) DNA separated by PFGE in a 1.1% agarose gel with pulse times ramped from 5 to 50 s for 24 h. Lane 1, *Bss*HII fragments; lane 2, lambda DNA concatemers. (b) DNA separated in a conventional 1% agarose gel and digested with *Hind*III (lane 2), *Eco*RV (lane 3) and *Xba*I (lane 4). Lanes 1 and 5, lambda DNA digested with *Hind*III. All sizes are in kilobases.

which resulted in *Bss*J (219 kb) being cleaved into two new fragments of about 195 kb and 25 kb (data not shown). The *Sfi*I site was placed more accurately by the analysis of a *Sfi*I/*I-Ceu*I double digestion, which generated two fragments of approximately 160 kb and 880 kb, resulting from the cleavage of *Ceu*B (data not shown).

Ordering of the *Bss*HII fragments on the physical map

The construction of the *I-Ceu*I backbone of the NCP 262 genome resulted in the positioning of five *Bss*HII fragments which were cleaved by *I-Ceu*I, namely *Bss*C, *Bss*J, *Bss*B, *Bss*F and *Bss*H, with the latter three being linked (see Fig. 5). Another strategy was employed to determine the order and position of the remaining *Bss*HII fragments. This involved the preparation of a library of *Bss*HII linking clones. To construct linking clones, pooled recombinant NCP 262 random pUC8 clones were digested with *Bss*HII and ligated to a kanamycin cassette which had *Bss*HII-compatible ends as described in Methods. These *Bss*HII linking clones were used as probes to determine which *Bss*HII fragments they hybridized with. Sequence was obtained from NCP 262 DNA flanking the kanamycin cassette in linking clones, and was used to carry out BLASTX and BLASTN searches. Linkages were confirmed when sequences flanking a *Bss*HII site showed similarity to the same gene or protein, indicating that it spanned the site. Five linkages were established using this strategy (Table 5), with linking clone A10 reconfirming the linkage of *Bss*F to *Bss*H as already determined during the construction of the *I-Ceu*I backbone. Sequences obtained from linking clones 26, 27, B10, B12, C4 and D4 (Table 5) showed no significant similarity to genes or proteins in the genebank databases. Therefore, linkages obtained using each of these clones were confirmed by PCR and Southern hybridization. Primers were designed from sequences obtained from each linking clone to amplify approximately 400 bp regions spanning *Bss*HII sites from genomic DNA. The resulting PCR products were

then used as probes to confirm linkages of the *Bss*HII fragments.

Linking clones 26 and 27 (Table 5) were unexpectedly found to hybridize to single fragments (*Bss*I and *Bss*E, respectively), and PCR products were obtained from genomic DNA using primers designed from sequence flanking the *Bss*HII sites contained in these clones. This suggested that a small *Bss*HII fragment could be located between *Bss*I and *Bss*E, which was not resolved by PFGE. Consequently, these PCR products were labelled and used to probe Southern blots of *Bss*HII-digested NCP 262 DNA separated by conventional agarose gel electrophoresis. Both were found to hybridize to a previously undetected 5 kb fragment which was designated *Bss*O (Fig. 3).

In a number of cases, linking clones hybridized to more than two *Bss*HII fragments due to the presence of repetitive DNA in the genome. Probes derived from short PCR products which spanned *Bss*HII sites were therefore used to determine actual linkages. For example, linking clone B10 hybridized with *Bss*D, *Bss*H and *Bss*M, whereas a PCR product obtained from genomic template DNA using primers designed from the sequence flanking the *Bss*HII site of B10 hybridized only with *Bss*D and *Bss*M. However, linking clone B7 hybridized with fragments *Bss*B, *Bss*F, *Bss*G, *Bss*J and *Bss*N, and a 378 bp PCR product spanning the *Bss*HII site in linking clone B7 hybridized with *Bss*B, *Bss*G and *Bss*N, with the latter fragment appearing at higher intensity (Fig. 4a). A BLAST search of the sequences flanking the *Bss*HII site from linking clone B7 revealed strong similarity to the IS element ISCb1 from *C. beijerinckii* (Table 5). Analysis of the ISCb1 sequence showed that it contained a *Bss*HII site (Liyanage *et al.*, 2000). We found that clone pEK30, containing a region of ISCb1 spanning the *Bss*HII site, hybridized with *Bss*B, *Bss*G and *Bss*N, with *Bss*N again hybridizing at higher intensity (data not shown). To determine the copy number of the ISCb1-like IS element found on NCP 262, the PCR product (378 bp) obtained from B7 was used to

Fig. 5. Physical and genetic map of the *C. saccharobutylicum* NCP 262 genome. The arrows indicate the orientation of the ribosomal operons. The orientation of the *rrn* operons lying within the BssH fragment were determined; however, the order of the I-CeuI fragments bearing these operons has not been resolved. The genes are positioned on the map to the minimum region located by hybridization. The order of genes and/or operons given in a particular region is arbitrary. The BssHII linking clones are given on the inside of the innermost circle. The positions of the following genes are indicated: *adh1*, alcohol dehydrogenase; *aad*, alcohol/aldehyde dehydrogenase; BCS, *crt*, *bcd*, *etfAB*, *hbd* genes in the BCS operon; *butK*, *ptb*, butyrate operon; *dnaKJ*, HSP70 coding gene cluster; *eglA*, endoglucanase; *glnA*, glutamine synthetase; *hydA*, hydrogenase; *hsp18*, *trna_{ACG}*, small heat-shock gene cluster; *metF*, methionine synthase; *regA*, *ccpA* homologue; *recA*, multifunctional protein in homologous recombination; *recF*, *gyrAB*, homologous recombination protein, DNA gyrase subunits and putative origin of replication; *scrB*, sucrose operon; *spoIID*, sporulation; *sum*, metronidazole susceptibility; *xylA*, xylanase.

probe genomic digestion products obtained using the frequent-cutting enzymes *EcoRV*, *HindIII* and *XbaI*. The recognition sites for these enzymes were not present in the PCR product. Two fragments from each digest hybridized to the probe, indicating that two copies of

the IS element are present on the NCP 262 genome (Fig. 4b). Hence, it was concluded that one copy of the IS element linked BssN to BssB and a second copy linked BssG to a second fragment, the same size as BssN, designated BssN' (Fig. 5).

Only two linkages remained to be resolved, namely the linkage of BssI to BssL and of BssJ to BssN' (Fig. 5). No linking clones were isolated which confirmed these links. To rule out the possibility of the presence of additional small BssHII fragments (< 5 kb) lying between these BssHII fragments, BssHII-digested genomic NCP 262 DNA was ligated into the cloning vector pNEB193. Only clones containing the 5 kb BssO fragment were obtained and no other small BssHII fragments were cloned (data not shown). This indicated that the presence of further small BssHII fragments was unlikely, and analysis of both double digestions and Southern hybridizations linked BssJ to BssN', and BssI to BssL (Fig. 5).

Wilkinson & Young (1995) reported that some macrorestriction fragments in electrophoretograms from *C. beijerinckii* were overrepresented when compared with fragments of similar size. They suggested that fragments flanking the origin of replication are overrepresented due to the presence of multiple replication forks in genomic DNA extracted from cells during the exponential growth phase. The genomic DNA from NCP 262 was also isolated from cells in the exponential growth phase and the fragments BssB, BssD, BssF and BssH, along with SseE, were found to be overrepresented in electrophoretograms, whereas the fragments BssC and SseF were underrepresented (Fig. 1). This suggested that the former fragments were near the origin of replication, whereas the latter two fragments were close to the replication terminus, and this was confirmed by the mapping data presented here.

Location of genetic markers on the chromosome

Cloned genes of homologous and heterologous origin (Table 1) were used to probe Southern blots of single and double digestions to determine gene positions on the physical map of the *C. saccharobutylicum* NCP 262 genome, and further confirmed the order, position and relatedness of macrorestriction fragments (Fig. 5). Genes identified from the linking clones (Table 5) do not appear on the map. Their positions are precisely known, in contrast to those shown, whose approximate positions have been determined by hybridization. The order of genes and/or operons assigned to the same fragment are arbitrary.

The positions of house-keeping genes, and genes involved in the heat-shock response and sporulation were determined, as was the location of some of the genes involved in electron transport and solvent production (Fig. 5). The *C. acetobutylicum* butanol dehydrogenase isozyme genes (*bdhAB*) and coenzyme A (CoA)-transferase genes (*ctfAB*) did not hybridize to the genome of NCP 262.

DISCUSSION

A physical map of *C. saccharobutylicum* NCP 262 was constructed using PFGE and hybridization techniques. The size of the circular genome was estimated to be

5.3 Mb, and a total of 39 restriction sites were positioned on the map using five restriction enzymes resulting in a mean resolution of approximately 140 kb. The positions of 28 genes were determined, which represents a first step towards the construction of a genetic map of this bacterium.

The map was assembled using a number of complementary methods including PFGE, analysis of single and double digestion products, along with Southern hybridization, PCR and DNA sequence analysis of linking clones. The I-CeuI backbone was assembled using *rrs*- and 3' *rrl*-specific probes located on either side of the I-CeuI site and reciprocal separation of BssHII and I-CeuI digests by 2D-PFGE. *C. saccharobutylicum* NCP 262 has 12 *rrn* operons located on 46% of the chromosome transcribed divergently from *CeuG*. At present, this is the second-highest number of *rrn* operons reported, with *C. beijerinckii* NCIMB 8052 containing 14, the most reported to date for a eubacterium (Wilkinson & Young, 1998). Multiple copies of *rrn* operons are often found in bacterial species capable of rapid growth (Krawiec & Riley, 1990). *C. saccharobutylicum* has a mean generation time of about 30 min under optimal conditions, a characteristic which favoured its use in industrial solvent production (McCutchan & Hickey, 1954).

The B subunit of DNA gyrase (*gyrB*) is located near the origin of replication in bacterial chromosomes (Salazar *et al.*, 1996), and the *C. acetobutylicum* *gyrB* probe (Table 1) hybridized to *CeuG*, which also hybridized only with the *rrs*-specific probe and not the 3' *rrl*-specific probe. Hence, the 12 *rrn* operons of *C. saccharobutylicum* are transcribed confluent with the direction of chromosomal replication, which proceeds bidirectionally from an origin of replication located within *CeuG* to a terminus located within *CeuA*, and multiple copies of *rrn* operons are clustered close to the replication origin.

The juxtaposition of the majority of the BssHII fragments was determined using a library of BssHII linking clones and Southern hybridization. Linkages were also confirmed by analysis of sequence obtained from regions flanking the BssHII sites present in linking clones. This analysis also allowed the mapping of a number of genes not previously reported for NCP 262 as listed in Table 5. Many of the linking clones detected more than two BssHII fragments when used as probes, indicating the presence of repetitive DNA on the NCP 262 chromosome. Analysis of one such linking clone led to the discovery of two copies of an insertion sequence very similar to *ISCb1*, an insertion sequence of the IS4 family isolated from *C. beijerinckii* NCIMB 8052 which contains a BssHII site (Liyanage *et al.*, 2000).

The genes involved in controlling electron flow play an important role during the complex biphasic fermentation of carbohydrates to solvents in solvent-producing clostridia. Two of these genes, the iron-only hydroxylase (*hydA*) and the metronidazole susceptibility (*sum*) gene, were found to be near the replication origin

Fig. 6. Genome comparisons, as adapted from Cornillot *et al.* (1997), of the spore-forming bacteria *B. subtilis* (Anagnostopoulos *et al.*, 1993), *C. acetobutylicum* (Cornillot *et al.*, 1997), *C. perfringens* (Cole *et al.*, 1998), *C. beijerinckii* (Wilkinson & Young, 1998) and *C. saccharobutylicum*. To facilitate comparisons, the circular chromosomes were opened and aligned at the putative origin of replication, corresponding to the *gyrAB* genes. The arrows indicate *rrn* operons. Open arrows correspond to *rrn* operons whose orientations have not been confirmed.

in *C. saccharobutylicum*, with the former gene also reported to be close to the origin of replication in *C. acetobutylicum* (Cornillot *et al.*, 1997).

Some of the genes involved in the acid- and solvent-forming pathway were found to be clustered in a small region, representing only 2.5 % of the NCP 262 genome. This included the butyryl-CoA synthesis (BCS) operon genes, *crt*, *bcd*, *etfAB* and *hbd*, which reduce acetoacetyl-CoA to butyryl-CoA in three consecutive reactions, and the butyrate operon genes, *ptb* and *butK*, involved in the conversion of butyryl-CoA to butyrate. The BCS operon was found to be in close proximity to the *groESL* heat-shock operon, as is the case for both *C. acetobutylicum* (Cornillot *et al.*, 1997) and *C. beijerinckii* (Wilkinson & Young, 1995). The *adh1* gene isolated from *C. saccharobutylicum* NCP 262 was reported to be downstream from the *hbd* gene (Youngleson *et al.*, 1989); hence it was located on the same region as the BCS operon. This NADPH-dependent alcohol dehydrogenase was shown to have activity using butanol and ethanol as substrates (Youngleson *et al.*, 1989). The genes involved in the final steps of solvent production are the aldehyde/alcohol dehydrogenase (*aad*) and CoA-transferase (*ctfAB*) genes, which form the *sol* operon (Dürre *et al.*, 1995), along with the acetoacetate decarboxylase (*adc*) and butanol dehydrogenase (*bdhAB*) genes. Toth *et al.* (1999) reported that a probe derived from the *C. acetobutylicum* *aad* gene did not hybridize to three species of solvent-producing clostridia, including *C. saccharobutylicum* NCP 262. However, we have recently isolated a gene from *C. saccharobutylicum* NCP 262 with significant similarity to the *C. acetobutylicum* *aad* gene (unpublished results). It is located on the genome

together with the other genes concerned with solvent and acid production (Fig. 5). The *bdhAB* genes and the bifunctional *aad* gene are involved in butanol formation, and the *ctfAB* and *adc* genes convert acetoacetyl-CoA to acetone, and are therefore very likely to be found on the NCP 262 genome. However, the *C. acetobutylicum* probes for the genes *ctfAB* and *bdhAB* did not hybridize with the NCP 262 genome under the conditions we used. Taken together, these results suggest that there is considerable sequence divergence between certain solvent-producing genes found in the solvent-producing clostridia. Further studies are under way to identify and sequence the remaining *sol* cluster genes of NCP 262.

The genome size and organization of *C. saccharobutylicum* were compared with those of the spore-forming bacteria *B. subtilis*, *C. acetobutylicum*, *C. perfringens* and *C. beijerinckii* (Fig. 6). The genome of *C. saccharobutylicum* was smaller than that of *C. beijerinckii* but larger than the genomes of the other three spore-forming bacteria. On the basis of genome organization, *C. saccharobutylicum* is most similar to *C. beijerinckii* and *C. perfringens*, with the order of the genes *gyrAB*, *groESL*, *spoIID*, *dnaKJ* and *recA* being conserved. In addition, several *rrn* operons in *C. beijerinckii*, *C. perfringens* and *C. saccharobutylicum* are transcribed divergently from the origin of replication, as opposed to only one *rrn* operon being transcribed divergently to the remaining *rrn* operons in *C. acetobutylicum* and *B. subtilis*.

The construction of a physical map and the initiation of a genetic map of NCP 262 has provided preliminary insight into the genome structure of this industrially

important bacterium. Only one other industrial solvent-producing *Clostridium* species, '*C. saccharoperbutyl-aceticum*', remains to be mapped. It would be of interest to compare its genome organization to those of the other three solvent-producing *Clostridium* species, to gain further insight into the relationships that exist between their genome size and structure, and the location of the sporulation and solventogenesis genes.

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