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Physical Map of the *Clostridium beijerinckii* (Formerly *Clostridium acetobutylicum*) NCIMB 8052 Chromosome

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A combined physical and genetic map of the single, circular, 6.7-Mbp chromosome of the NCIMB 8052 strain of *Clostridium beijerinckii* (formerly *Clostridium acetobutylicum*) has been constructed by using a combination of cloned DNA fragments as hybridization probes and a bank of strains harboring insertions of the conjugative transposon Tn*1545*. The positions of 81 restriction endonuclease cleavage sites and 32 genes have been determined. Eight genes concerned with solventogenic fermentation are found at three different locations. The chromosome contains at least 13 *rrn* operons, 11 of which have been located on the map. Their transcriptional orientation diverges from the presumed location of the replication origin.

Clostridium beijerinckii (formerly *Clostridium acetobutylicum*) is an obligately anaerobic spore-forming bacterium that produces acetone, butanol, and small amounts of ethanol when grown on sugary substrates. This process, known as ABE fermentation, has been used extensively for the industrial scale production of acetone and butanol (28). Several different strains are currently under investigation in various laboratories worldwide. They have been separated into three distinct groups on the basis of their biochemical and physiological characteristics, the extent of hybridization between their DNAs, and macrorestriction fragment profiles of their genomes (54, 57). The NCIMB 8052 strain, which belongs to the group that has the largest chromosome, is amenable to genetic manipulation (59, 60). It lacks the active restriction system that is found in the ATCC 824 strain (38). The NCIMB 8052 strain has no indigenous plasmids, but it supports replication of a variety of vectors based on plasmids from *Clostridium butyricum*, *Enterococcus faecalis*, and *Streptococcus cremoris* (41). They may be introduced by either electroporation (46) or intergeneric conjugation with *Escherichia coli* donors (56). This strain is also susceptible to Tn*1545* mutagenesis (58). Homologous recombination between incoming sequences and their counterparts in the bacterial chromosome has recently been demonstrated (55).

As an additional aid to genetic analysis of this organism, we have constructed a physical map of the 6.7-Mbp chromosome using pulsed-field gel electrophoresis (PFGE) (50). The application of PFGE to the analysis of bacterial genomes has revealed that the majority of bacteria have single circular chromosomes. However, several exceptions to this general rule have been described. Some bacteria, such as *Borrelia burgdorferi* and *Rhodococcus fascians*, have linear chromosomes (4, 20, 24), whereas *Streptomyces lividans* and *Streptomyces coelicolor* have linear chromosomes that may become circular as a result of deletions spanning both telomeres (34, 35). Still others, such as *Brucella melitensis* and *Leptospira interrogans*, have segmented genomes (40, 63). The segmented genome of *Agrobacterium tumefaciens* has one circular and one linear component (1). The results reported here indicate that the NCIMB 8052 strain of *C. beijerinckii*, like most other bacteria, has a single circular chromosome.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Tables 1 and 2.

Culture media. *C. beijerinckii* NCIMB 8052 was grown anaerobically at 37°C in clostridial basal medium (CBM) (45) solidified, as appropriate, with 1.5% (wt/vol) agar. Strains harboring Tn*1545* or integrational plasmids were selected on CBM plates supplemented with erythromycin (10 μg ml^{-1}). Strains of *E. coli* containing recombinant plasmids were routinely grown in Luria-Bertani medium (36) supplemented, as appropriate, with kanamycin or ampicillin (both at 50 μg ml^{-1}).

Conjugative transfer of Tn*1545*. Strains of *C. beijerinckii* harboring Tn*1545* were isolated after the wild-type NCIMB 8052 strain had been conjugated with an *Enterococcus faecalis* BM4110::Tn*1545* donor, as previously described (58).

DNA manipulations. Recombinant plasmids were extracted from *E. coli* strains by the method of Del Sal et al. (21). The technique of Smith and Cantor (51), as modified by Wilkinson and Young (54); was employed to generate agarose plugs containing intact restrictable DNA from *C. beijerinckii*. DNA was digested in agarose plugs as described previously (54). Buffer A (Boehringer Mannheim) was employed for all digestions, and restriction enzymes were purchased from Boehringer Mannheim, New England Biolabs, and Northumbria Biologicals, U.K. A small number of *Apa*I linking clones were isolated essentially as described by Bancroft et al. (3). DNA samples were digested with *Hin*dIII, self-ligated at a low DNA concentration (<20 μg ml^{-1}) to form predominantly circular products, and then digested with *Apa*I, and the resulting linear *Apa*I fragments were cloned in the *Apa*I site of the pBluescript polylinker. Three further linking clones with sites for both *Apa*I and *Sma*I were identified for *E. coli* strains harboring recombinant plasmids containing randomly cloned *Eco*RI or *Sau*3A (partial) fragments.

PFGE. Macrorestriction fragments were size fractionated by using the Bio-Rad CHEF DrII contour-clamped homogeneous electric field apparatus (16). Plug slices containing about 340 ng of digested DNA were fixed into wells of a 1.0% (wt/vol) agarose gel (ultrapure DNA grade dissolved in TAE buffer [36]; Bio-Rad) by using molten 1.0% (wt/vol) agarose. Fragments with sizes between 50 and 500 kbp were routinely separated over a 20-h period by using a linear ramp from 1 to 60 s and a constant field strength of 6 V cm^{-1}. By increasing the linear ramp to 60 to 180 s and keeping the other conditions the same, DNA fragments from 200 to 2,000 kbp could be separated. Fragments of between 4 and 400 kbp were separated by electrophoresis for 15 h by using a linear ramp from 1 to 30 s. The buffer was maintained at 14°C throughout electrophoresis. DNA bands in agarose gels were visualized after the gel was stained with ethidium bromide (0.5 μg ml^{-1}) by using a UV transilluminator (UV Products Inc.). Chromosomes from *Saccharomyces cerevisiae* YNN295 (Bio-Rad) were employed as size standards when bands up to 2,000 kbp were fractionated; phage λ concatemers were generated as described previously (54) and used as size standards for separating fragments up to 500 kbp. Phage λ DNA digested with *Hin*dIII was also incorporated, as required, for determining the sizes of fragments below 50 kbp.

DNA hybridization. Standard procedures (36, 52) were employed for depurination and transfer of DNA from agarose gels to Hybond-N membranes (Amersham). Digoxigenin-labelled probes were prepared by using a random-primed

TABLE 1. Bacterial strains used in this study

Strain	Genotype	Source or reference
C. beijerinckii		
NCIMB 8052		Laboratory stock
AA029–AA039	NCIMB 8052::Tn*1545*	56
AA180–AA209	NCIMB 8052::Tn*1545*	53
AA210	NCIMB 8052::pSRW35	54
AA219	NCIMB 8052::pSRW44	54
AA252	*met*::Tn*1545*	This study
AA256	*cysK*::Tn*1545*	This study
A10	*deg*::Tn*1545*	29
E. coli DH5α	F^- λ^- *endA1 recA1 hsdR17* (r_K^- m_K^+) (*argF-lacZYA*) U169 *relA1 supE44 thi-1 gyrA96* (Nal^r) [Φ80d*lacZ* ΔM15]	26

DNA labelling kit, and hybridizations were performed as described by the manufacturer (Boehringer Mannheim). Since Tn*1545* lacks sites for *Sma*I, *Apa*I, *Sfi*I, and *Rsr*II, DNA fragments containing Tn*1545* insertions increased in size by about 25 kbp (8, 55a); correction was made for this in deducing the sizes of macrorestriction fragments containing Tn*1545*.

RESULTS

A low-resolution map for *Sfi*I and *Rsr*II. The enzymes *Sfi*I and *Rsr*II cleave the chromosome of *C. beijerinckii* NCIMB 8052 to yield three and six fragments, respectively (Fig. 1A and Table 3). Nine fragments would be expected after double digestion with *Sfi*I and *Rsr*II, but usually, only seven electrophoretically distinct bands were produced (Fig. 1A). The bands at about 1,360 and 730 kbp each contain two comigrating DNA fragments. They were resolved in strains that contained a copy of Tn*1545* (which is not cleaved by either *Sfi*I or *Rsr*II) in one of the fragments. The two fragments within the 1,360-kbp band (referred to as 1,380- and 1,345-kbp fragments in Table 3) were resolved to form a doublet in DNA from strains AA030, AA033, AA199, and AA203, which harbor a single copy of Tn*1545* in one or the other fragment. Similarly, the two discrete DNA fragments in the 730-kbp band were resolved after electrophoresis of DNA from strains AA029 and AA031.

The relationships between the *Sfi*I and *Rsr*II fragments shown in Fig. 1B and 2 were established by Southern hybridization using cloned genes and random genomic fragments as probes, as well as a variety of strains harboring Tn*1545* (Table 2, columns 10 to 12). For seven of the nine map intervals defined by *Sfi*I and *Rsr*II sites, *Sfi*I-*Rsr*II hybridization profiles were confirmed by using two or more different probes. Fragment RsF (which lies within SfB) and the 90-kbp overlap between fragments RsD and SfC were each detected by a single probe. The latter was detected as a weak secondary signal obtained with probe p239. These data are not included in Table 2, since there was no apparent secondary signal amongst the *Sma*I and *Apa*I macrorestriction fragments of greater than 50 kbp, the lower resolution limit in this experiment. The hybridization probes detected all possible overlaps between *Sfi*I and *Rsr*II fragments, indicating that the NCIMB 8052 strain of *C. beijerinckii* has a single, circular, 6.7-Mbp chromosome (Fig. 2). The presence of a 770-kbp fragment in partially digested DNA samples (data not shown) provided independent confirmation that the two smallest *Rsr*II fragments, RsE (519 kbp) and RsF (252 kbp), lie adjacent to each other. Since they lie entirely within SfB, their order relative to the flanking *Rsr*II fragments could not be ascertained from these data alone. This was determined subsequently (see below).

The *Sfi*I site within RsD was assigned coordinate zero in Fig. 2, since there is circumstantial evidence suggesting that the replication origin of the bacterial chromosome lies nearby. Visual inspection of electrophoretograms indicated that the bands at about 1,430 kbp (SfC) and 720 kbp (RsD) were overrepresented. Similarly, in DNA samples digested with *Sfi*I and *Rsr*II the bands at 640 and 90 kbp (derived from RsD) were overrepresented in comparison with other bands of similar sizes, including one (730 kbp) which is a doublet (Fig. 1B). The largest *Sfi*I and *Rsr*II fragments were underrepresented in electrophoretograms. This was because DNA (containing replication forks) was isolated from exponentially growing bacteria, and some slight nonspecific DNA degradation probably also occurred during extraction (cf. reference 54). As a result, there was always a smear in each lane. The relative amounts of the smaller fragments, from which the tentative location of the replication origin was deduced, should be less susceptible to these interfering factors than that of the larger DNA fragments, since they offer a smaller target. Other evidence consistent with the location of the replication origin close to coordinate zero is considered below.

Higher-resolution mapping by using *Sma*I and *Apa*I. Preliminary analysis of *Sma*I- and *Apa*I-digested DNA samples from the NCIMB 8052 strain of *C. beijerinckii* yielded a partial catalogue of 27 *Apa*I and 24 *Sma*I macrorestriction fragments (54). It was recognized that the total number of fragments generated by each of these enzymes was likely to be considerably higher than these values, because they accounted for only 5.8 to 6.0 Mbp of the bacterial chromosome and there was evidence suggesting the presence of a substantial number of additional *Apa*I and *Sma*I fragments smaller than about 50 kbp. Overlapping *Sma*I and *Apa*I fragments were identified by their hybridization with cloned genes from *C. beijerinckii* (or heterologous genes from other organisms), or with randomly cloned DNA fragments. A bank of strains harboring insertions of Tn*1545* (8, 19) was also employed for mapping. Fragments containing this element increase in size by about 25 kbp and are detected by an *aphA3*-specific probe (9). By using a combination of cloned DNA fragments and strains harboring Tn*1545* insertions, 34 different hybridization patterns were detected with 48 DNA probes and 33 strains containing Tn*1545* insertions (Table 2, columns 1 to 7). After careful analysis, these data were collated and the *Apa*I and *Sma*I fragments were assembled into 14 contigs. These data accounted for all of the *Sma*I and *Apa*I fragments formerly catalogued (54), with the exception of three *Sma*I fragments (SmQ, 103 kbp; SmR′, 80 kbp; and SmS, 63 kbp) and three *Apa*I fragments (ApJ‴, 171 kbp; ApN′, 85 kbp; and ApP′, 50 kbp). Significant further progress was unlikely with this mapping strategy because the great majority of new probes or transposon-containing strains detected previously documented hybridization profiles. Many contigs have *Apa*I and *Sma*I sites lying close to both extremities, making it improbable that probes extending them could be isolated without an inordinate amount of additional work. Since most instances of *Apa*I and *Sma*I sites lying in close proximity correspond to the locations of *rrn* operons (see below), an alternative mapping strategy will have to be adopted to link contigs.

Assignment of *Sma*I-*Apa*I contigs to the low-resolution map. Contigs containing sites for either of the rarely cutting enzymes, *Rsr*II and *Sfi*I, were identified in experiments in which DNA samples digested with *Apa*I or *Sma*I in conjunction with either *Rsr*II or *Sfi*I were hybridized with appropriate probes. These data are summarized in Table 2, columns 8 to 12. They

TABLE 2. Hybridization profiles obtained using cloned DNA fragments and strains containing insertions of Tn*1545*

Strain[a] or plasmid[b]	Gene(s)	Reference no. or source[c]	Hybridizing fragment (size in kbp) with restriction enzyme:									Contig
			*Sma*I	*Sma*I + *Apa*I	*Apa*I	*Sma*I-*Apa*I profile	*Sma*I + *Rsr*II or *Sfi*I[d]	*Apa*I + *Rsr*II or *Sfi*I[d]	*Rsr*II	*Rsr*II + *Sfi*I	*Sfi*I	
AA035/AA183 AA196[e]												
pThiol67[f]	*atoB*	A										
p7A12[g]	*atoB*	B	SmA (625)	290	ApD (289)	1	260^R	170^R	RsE (519)	520	SfB (2,505)	1
pHZ302[h]	*xyn*	62										
A10	*deg*											
p258[g]/**p522**[g]		C										
AA037			SmA (625)	60	ApO (58)	2						1
AA193[e]/AA194												
AA195/AA200[e]			SmA (625)	190	ApH (189)	3	360^R	190^R	RsA (2,065)	730	SfB (2,505)	1
pRNA23[i]	*rrl*	D										
pDP253[f]	*adc ctfAB*	47										
p259[g]		C										
AA031[e]			SmB (596)	75	ApL (152)	4			RsB (1,755)	1,000	SfB (2,505)	2
AA039[e]AA201												
pBUD62[g]	*bdh*	B	SmB (596)	410	ApB (414)	5			RsB (1,755)	1,000	SfB (2,505)	2
AA182												
p261[g]		C										
pRNA23[i]	*rrl*	D	SmJ (278)	280	ApA (886)	6			RsA (2,750)	1,345	SfC (1,430)	3
AA202[j]												
p243[g]/**p262**[g]		C	SmC (573)	570	ApA (886)	7			RsA (2,750)	1,345	SfC (1,430)	3
AA204[j]												
AA252	*met*::Tn*1545*											
pVE1057[g]	*recA*	22	SmD (362)	230	ApF (231)	8			RsC (1,385)	1,380	SfA (2,750)	4
AA030/AA202[j]												
AA039[e]/**AA203**			SmD (362)	60	ApC (326)	9		325^R	RsC (1,385)	1,380	SfA (2,750)	4
p516(a)[g]		C										
p519[g]		C	SmN (178)	180	ApC (326)	10	180^R	325^R	RsC (1,385)	1,380	SfA (2,750)	4
pLDH[g]	*ldh*	B	SmH (300)	110	ApC (326)	11	300^R	325^R	RsC (1,385)	1,380	SfA (2,750)	4
p518[g]/p521[g]		C										
pKG1[k]	*dnaK*	43	SmH (300)	185	ApI (185)	12						4
p517[g]		C										
pGP16[g]		E	SmE (357)	170	ApJ″ (171)	13						5
pHZ208[h]	*glnA*	F										
AA193[e]			SmE (357)	50	ApP (50)	14						5
AA204[j]			SmE (357)	115	ApK (159)	15	250^S	160^S	RsA (2,065)	730	SfB (2,505)	5
p520[g]		C										
p252[g]		C	SmT (54)	55	ApK (159)	16						5
pRNA16[i]	*rrs*	D										
p256[g]/p260[g]		C										
pCPRP32[l]	*fed*	39	SmF (334)	230	ApF′ (231)	17	330^S	230^S				6
pRNA23[l]	*rrl*	D										
AA029			SmF (334)	110	ApH′ (189)	18	330^S	155^S	RsB (1,755)	730	SfA (2,750)	6
AA198												
pCPRD2(a)[l]	*rud*	37	SmK (250)	80	ApH′ (189)	19	200^S	35^S	RsB (1,755)	1,000	SfB (2,505)	6
p255[g]		C										

Continued on following page

TABLE 2—*Continued*

Strain[a] or plasmid[b]	Gene(s)	Reference no. or source[c]	Hybridizing fragment (size in kbp) with restriction enzyme:									Contig
			*Sma*I	*Sma*I + *Apa*I	*Apa*I	*Sma*I-*Apa*I profile	*Sma*I + *Rsr*II or *Sfi*I[d]	*Apa*I + *Rsr*II or *Sfi*I[d]	*Rsr*II	*Rsr*II + *Sfi*I	*Sfi*I	
AA200 p239[g]		C	SmK (250)	45	ApQ (45)	20						6
AA202[j]/AA204[j]			SmK (250)	130	ApJ (171)	21						6
p242[g]		C	SmG (327)	160	ApK′ (159)	22	190[R]	160[R]	RsB (1,755)	1,000	SfB (2,505)	7
pCACB4[g] p0A1[g] **AA199**/AA202[j]	*spo0A* *spo0A spoIVB*	7 58a	SmI (292)	290	ApC′ (326)	23	290[R]	300[R]	RsC (1,385)	1,380	SfA (2,750)	8
p240[g]		C	SmK′ (250)	160	ApK″ (159)	24	200[R]	160[R]	RsE (519)	520	SfB (2,505)	9
p516(b)[g]		C	SmK′ (250)	100	ApE (253)	25			RsF (251)	250	SfB (2,505)	9
pSORB[g] pKN11.21[m] pFN4[k] pCADH100[h] AA036	*gutABD* *leuBC* *groESL* *hdb*	B 23 42 61	SmL (231)	210	ApG (219)	26						10
pBTM100[g] pJC7[f] p241[g]/p250[g] p257[g] AA192	*ptb butK* *ptb butK*	B 14 C C	SmL (231)	30	ApM (99)	27	230[S]	85[S]	RsD (722)	640	SfA (2,750)	10
pALD1[g] pRNA16[i]	*ald* *rrs*	B D	SmU (47)	50	ApB′ (414)	28						11
AA256 AA032 **p515**[g]	*cysK*::Tn*1545*	C	SmM (221)	220	ApB′ (414)	29	120[R] 95[R]	215[R] 190[R]	RsD (722) RsC (1,385)	640 1,380	SfA (2,750) SfA (2,750)	11
pKN65.1[m] pCPRD2(b)[l] pSRW3[g]	*proAB* *rud*	23 37 E	SmO (166)	100	ApB′ (414)	30						11
pAT-1[h] pSRW3[g] pRNA23[i]	*spoIID* *rrl*	F E D	SmO (166)	55	ApO′ (58)	31						11
AA031[e]/AA196[e] AA204[j]			SmP (144)	75	ApN (85)	32			RsB (1,755)	730	SfA (2,750)	12
AA033/AA034 AA180 pRNA16[i]	 *rrs*	 D	SmP′ (144)	145	ApJ′ (171)	33			RsA (2,750)	1,345	SfC (1,430)	13
pGYRA[i]	*gyrA*	G	SmR (80)	13	Ap13	34	80[R]	13[R]	RsA (2,750)	1,345	SfC (1,430)	14

[a] The location of Tn*1545* was determined by using pAT187 (53) as a probe.
[b] Strains and plasmids in boldface were employed in hybridizations with *Rsr*II- and *Sfi*I-digested samples. p516 detected a primary signal denoted (a) and a secondary signal denoted (b). pCPRO2 gave two hybridization signals of similar intensity denoted (a) and (b), either or both of which may be associated with the structural gene for rubredoxin.
[c] A, subclone of pTeco-11 (48) containing the N terminus of a thiolase gene; B, N. P. Minton, PHLS, Porton, United Kingdom; C, random clone from a gene bank of partial *Sau*3A fragments of the NCIMB 8052 genome in pAT153; D, subclone of the *rrnA* operon of *C. perfringens* (25) obtained from B. Canard and S. T. Cole, Institut Pasteur, Paris, France; E, *Apa*I linking cone; F, D. R. Woods, University of Cape Town, Cape Town, South Africa; G, deletion derivative of pBC15 (25) containing part of *gyrA* obtained from T. Garnier and S. T. Cole, Institut Pasteur, Paris, France.
[d] Fragments detected after double digestion are indicated as follows: R, digestion with *Rsr*II plus *Apa*I or *Sma*I; S, digestion with *Sfi*I plus *Apa*I or *Sma*I.
[e] Contains two copies of Tn*1545*.
[f] Contains DNA segments from *C. acetobutylicum* ATCC 824.
[g] Contains DNA segments from *C. acetobutylicum* NCIMB 8052.
[h] Contains DNA segments from *C. acetobutylicum* NCP 262.
[i] Contains DNA segments from *C. perfringens* CPN50.
[j] Contains four copies of Tn*1545*.
[k] Contains DNA segments from *C. acetobutylicum* DSM 1731.
[l] Contains DNA segments from *Clostridium pasteurianum* ATCC 6013.
[m] Contains DNA segments from *C. acetobutylicum* ABkn8.

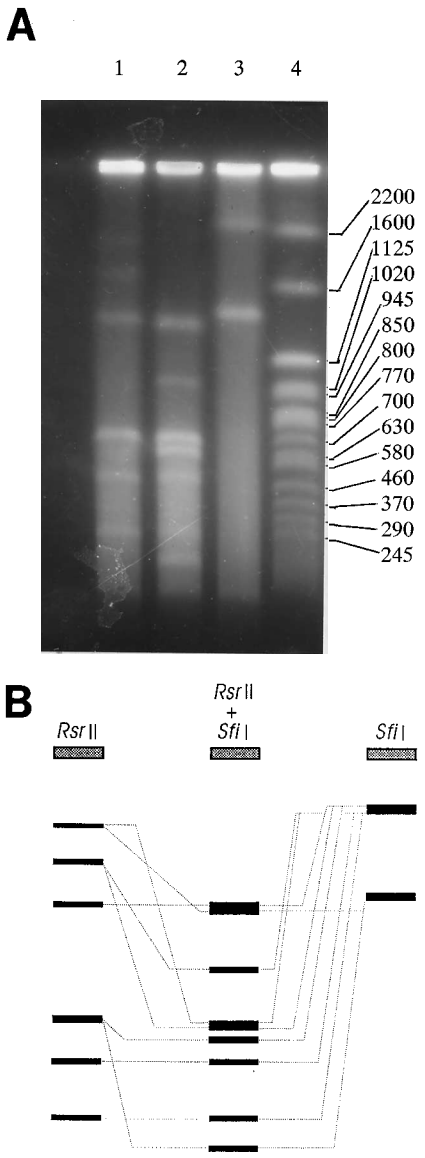


FIG. 1. Separation of DNA fragments produced after digestion with *Sfi*I and *Rsr*II. (A) Electrophoretogram. The sizes of the calibration markers are given in kilobase pairs. Electrophoresis using TAE buffer was for 20 h at 6 V cm^{-1} with a time ramp of 60 to 180 s. (B) Schematic representation showing relationships between the various fragments deduced from the data in Table 2.

effectively determine the location and orientation of contigs 1, 7, 8, 9, and 11, containing *Rsr*II sites, and contigs 5, 6, and 10, containing *Sfi*I sites, on the physical map (shown in black in Fig. 3). For fixed contigs 1, 6, and 11, rare restriction sites have been precisely positioned in relation to *Apa*I and *Sma*I sites on either side, whereas for fixed contigs 5, 7, 8, 9, and 10, rare restriction sites have been located in relation to *Apa*I and *Sma*I sites on one side only. RsE was located adjacent to RsA, and RsF was located adjacent to RsB in this series of experiments. The approximate locations of the remaining floating contigs, 2, 3, 4, 12, 13, and 14, which lack sites for either *Rsr*II or *Sfi*I, were also determined by hybridization (Table 2). Their orientations are considered later.

***rrn* operons.** In many eubacteria, the *rrs* and *rrl* genes encoding the 16S and 23S rRNAs occupy adjacent positions in operons (32), which are usually arranged such that their transcription is confluent with the direction of chromosome replication (5, 6). The *rrs* and *rrl* genes of *C. beijerinckii* NCIMB 8052 also appear to be organized in operons. This was deduced from analysis of three different *Apa*I linking clones. All three hybridized with *rrs*- and *rrl*-specific probes (data not shown), indicating that they contained rRNA genes (rDNA) corresponding to an *rrs* gene and part of an *rrl* gene. In one case, the presence of an *rrs* gene was confirmed by DNA sequencing. The published sequence of rDNA fragments generated by PCR amplification from *C. beijerinckii* (formerly *C. acetobutyl-*

TABLE 3. Sizes of *Rsr*II and *Sfi*I fragments of the *C. beijerinckii* NCIMB 8052 chromosome[a]

*Rsr*II		Fragment size (kbp) with *Rsr*II + *Sfi*I	*Sfi*I	
Fragment	Size (kbp)		Fragment	Size (kbp)
RsA	2,065 ± 72	1,380 ± 27[b]	SfA	2,750[c]
RsB	1,655 ± 37	1,345 ± 21[b]	SfB	2,505[c]
RsC	1,385 ± 22	1,006 ± 19	SfC	1,430 ± 33
RsD	722 ± 29	730 ± 29[d]		
RsE	519 ± 29	640 ± 23		
RsF	252 ± 17	518 ± 30		
		251 ± 17		
		90[e]		
Total	6,698	6,690		6,685

[a] Values are means ± standard deviations for five independent determinations, except where otherwise noted; chromosomes from *Saccharomyces cerevisiae* were used as size standards.
[b] The 1,380- and 1,345-kbp fragments comigrated on most electrophoretograms (see Fig. 1).
[c] The sizes of bands SfA and SfB were obtained by summing the sizes of fragments related to them in samples digested with both *Sfi*I and *Rsr*II (SfA = 1,380 + 730 + 640 = 2,750; SfB = 1,006 + 730 + 518 + 251 = 2,505).
[d] Fragment is a doublet.
[e] Fragment size was determined by using phage lambda concatemers as the size standard.

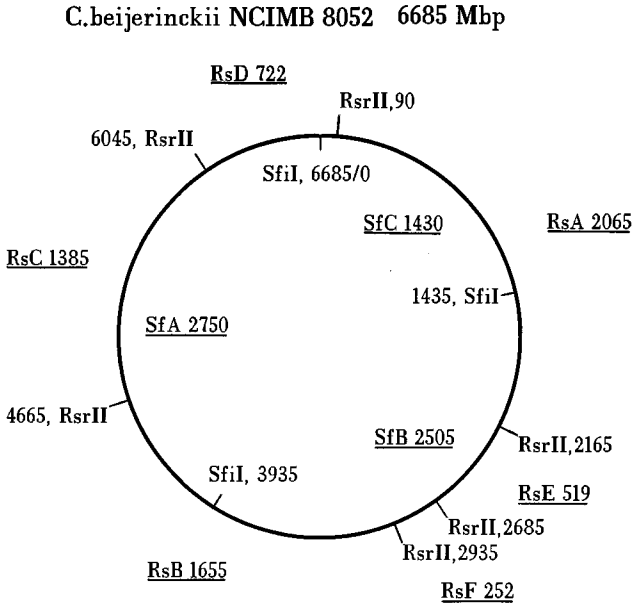
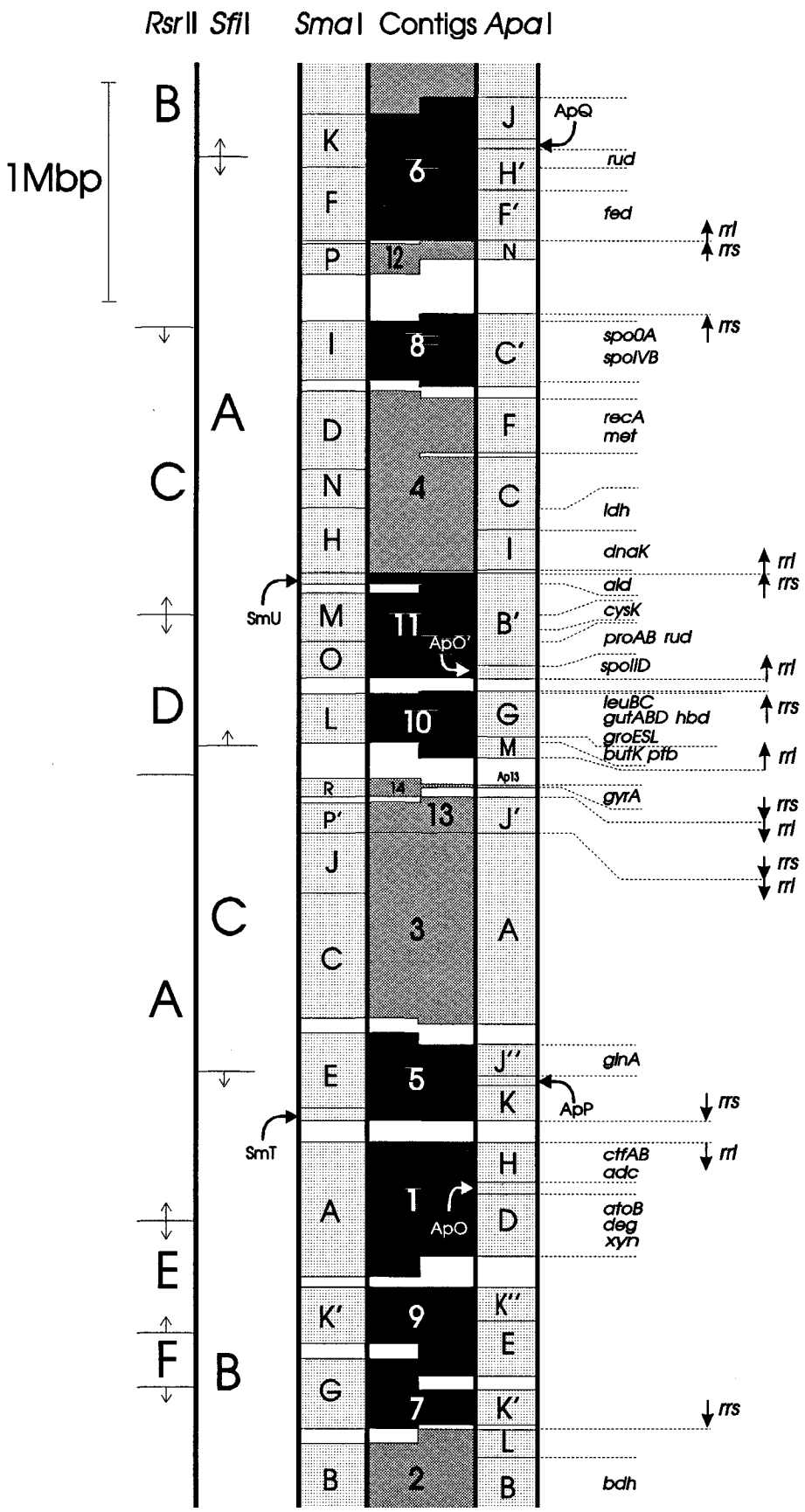


FIG. 2. Low-resolution physical map of the bacterial genome showing landmark *Sfi*I and *Rsr*II sites. The sites for *Sfi*I and *Rsr*II and their coordinates are indicated on the inside and the outside, respectively, of the circular map. The fragments generated by these two enzymes, together with their measured sizes (in kilobase pairs) are underlined.


Rsr II Sfi I
Sma I Contigs
Apa I
1Mbp
B
A
C
D
C
A
E
F
B
SmU
SmT
ApO'
ApO
ApQ
ApP
J
K
H'
F'
N
C'
F
C
I
B'
G
M
Ap13
J'
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K
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rud
fed
rrl
rrs
spo0A
spoIVB
recA
met
ldh
dnaK
ald
cysK
proAB rud
spoIID
leuBC
gutABD hbd
groESL
butK ptb
gyrA
glnA
ctfAB
adc
atoB
deg
xyn
bdh

FIG. 3. Map of the bacterial genome incorporating *Apa*I and *Sma*I sites. The circular map of landmark *Rsr*II and *Sfi*I sites is shown opened up in the region of the replication terminus, presumed to lie within fragments RsB and SfB. Fixed contigs are shown in black, and floating contigs are in grey. *Rsr*II and *Sfi*I sites that have been precisely located in relation to adjacent *Apa*I and *Sma*I sites on one or both sides are indicated with arrows. Approximate positions and orientations of the floating contigs have been determined as indicated in the text. The positions of the following genes are indicated: *adc*, acetoacetate decarboxylase; *ald*, aldolase; *atoB*, thiolase (acetyl-CoA acetyltransferase); *bdh*, butanol dehydrogenase; *butK*, butyrate kinase; *ctfAB*, acetoacetyl-CoA:acetate/butyrate:CoA transferase; *cysK*, *O*-acetylserine (thiol)-lyase; *deg*, degeneration (see text); *dnaK*, DNA biosynthesis; *fed*, [2Fe-2S] ferredoxin; *glnA*, glutamine synthetase; *groESL*, heat shock; *gutABD*, sorbitol utilization; *gyrA*, DNA gyrase; *hbd*, 3-hydroxybutyryl-CoA dehydrogenase; *ldh*, lactate dehydrogenase; *leuBC*, leucine biosynthesis (β-isopropylmalate dehydrogenase and α-isopropylmalate isomerase subunit); *met*, methionine biosynthesis; *proAB*, proline biosynthesis (γ-glutamyl phosphate reductase and γ-glutamyl kinase); *ptb*, phosphate butyryl transferase; *recA*, recombination; *rud*, rubredoxin; *spo0A*, *spoIID*, and *spoIVB*, sporulation; *xyn*, xylanase. The *adc*, *ctfAB*, *fed*, *groESL*, *gyrA*, *hbd*, *leuBC*, *proAB*, *rud*, *spoIID*, and *xyn* genes were detected by using heterologous probes containing flanking DNA sequences; the possibility that the observed hybridization signals are associated with the flanking sequences has not been ruled out.

icum) NCIMB 8052 DNA (18), and corroborated by our own sequence analysis of one *rrs* gene, indicates that the *rrs* genes contain one *Apa*I site and two *Sma*I sites towards their 3′ end. Figure 4 shows the hybridization profiles obtained when *Sma*I and *Apa*I macrorestriction fragments were analyzed with the *rrs*- and *rrl*-specific probes. The ranges of restriction fragments detected by the two probes were distinctly different. This is consistent with the presence of sites for both *Sma*I and *Apa*I between (or close to the extremities of) the sequences detected by the probes. The hybridization patterns in Fig. 4 included several profiles that had been encountered previously: the *rrs* probe detected profiles 16, 28, and 33, and the *rrl* probe detected profiles 3, 6, 17, and 31 (Table 2).

The *rrs*- and *rrl*-specific probes detected between 9 and 11 discrete *Sma*I, *Apa*I, or *Sma*I-plus-*Apa*I macrorestriction fragments (Fig. 4). Several additional smaller fragments were detected after conventional electrophoresis (data not shown). These results indicated that the NCIMB 8052 strain of *C. beijerinckii* contains at least 13 (possibly more) *rrn* operons.

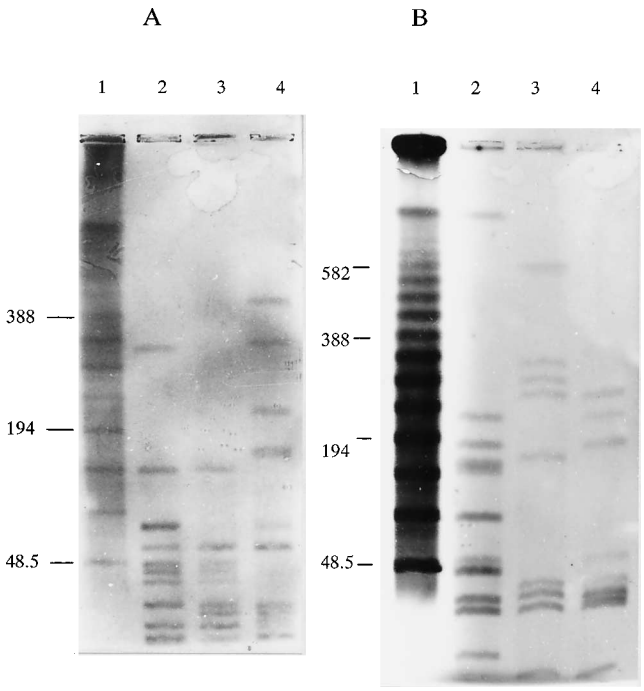

FIG. 4. *Apa*I and *Sma*I macrorestriction fragments detected with probes specific for *rrs* and *rrl* genes. (A) *rrs* probe (pRNA16; Table 2). Lane 1, λ concatemers; lane 2, *Sma*I fragments; lane 3, *Sma*I-plus-*Apa*I fragments, lane 4, *Apa*I fragments. (B) *rrl* probe (pRNA23; Table 2). Lane 1, λ concatemers; lane 2, *Apa*I fragments; lane 3, *Sma*I fragments; lane 4, *Sma*I-plus-*Apa*I fragments. Both probes are cloned segments of the *rrnA* operon of *C. perfringens*. The sizes of the calibration markers are given in kilobase pairs.

Seven of the eight contigs whose position and orientation are fixed (i.e., all except contig 9) are detected by one or other (or both) of the *rrs* and *rrl* probes (Table 2 and Fig. 3). These data effectively account for 9 of the 13 (or more) *rrn* operons. They are oriented on the fixed contigs such that they would be transcribed confluently with the direction of replication from a replication origin located somewhere within SfC.

In experiments with *Sfi*I- and *Rsr*II-digested DNA, all fragments except RsE and RsF gave hybridization signals with the *rrs* probe (data not shown). The fragments that gave the most intense hybridization signals were RsD, SfC, and the 90-kbp fragment in the *Sfi*I-plus-*Rsr*II-digested sample, corresponding to the overlap between them. This region therefore appears to contain multiple copies of the *rrn* operon, of which only one has so far been accounted for (Fig. 3).

Orientation of the floating contigs. Provisional orientations were assigned to contigs 3, 4, 12, 13, and 14 from information obtained with the *rrs* and *rrl* probes, by making the assumption that like the fixed contigs, they too are oriented such that the transcription of their *rrn* genes is coherent with the presumed direction of chromosome replication. Some of the *rrn* genes on floating contigs may form operons with *rrn* genes positioned on adjacent fixed contigs. This simplifying assumption has been made to allow the postulation of two putative operons containing the *rrs* and *rrl* genes on contigs 12 and 6 and contigs 11 and 4, respectively. Three of the floating contigs lie in the region of overlap between SfC and RsA. Contig 14 is detected by a *gyrA* probe and has provisionally been placed close to the *Rsr*II site separating RsA from RsD. The other two floating contigs in this region, 3 and 13, have been assigned provisional positions such that the *rrs* and *rrl* genes they contain make up two further *rrn* operons.

None of the *Apa*I and *Sma*I fragments making up contig 2 are detected by rDNA probes (Fig. 4). The orientation of contig 2 shown in Fig. 3 is probably correct as indicated, since the interval between the fixed extremities of contigs 6 and 7 is too small to accommodate it in the opposite orientation. However, this assignment will remain tentative until it has been confirmed independently.

There are 14 *Sma*I-*Apa*I contigs on the map (Fig. 3). In the absence of evidence to the contrary, 7 of the 14 gaps between them have provisionally been closed. Four of them disappear as a result of the simplifying assumption (see above) that the ends of contigs 13 and 3, 14 and 13, 11 and 4, and 12 and 6 represent the coding regions of *rrs* and *rrl* components of single *rrn* operons. Contigs 7 and 9 must overlap, since their positions are fixed in relation to landmark *Rsr*II sites. There is sufficient space for contig 2 between contigs 6 and 7 only if there is an overlap with the former and abutment with the latter. The number of lacunae between contigs could be further reduced from seven to five if contigs 3 and 5 and 4 and 8 also overlap, but there is currently no evidence apart from an approximate

correspondence between the sizes of their overhangs to support this.

DISCUSSION

A variety of methods, based on PFGE, have been employed to construct physical maps of bacterial genomes (see reference 17 for a review). Ideally, a map should be constructed by two different methods independent of each other. However, in many cases a single method used in isolation has not yielded all the information necessary to construct an unambiguous map. Most maps have therefore been constructed essentially by one method complemented, as necessary, by one or more others. In this investigation of the *C. beijerinckii* NCIMB 8052 genome, overlapping restriction fragments, generated by two pairs of endonucleases, were identified by hybridization with a variety of cloned DNA segments. This method, one of the so-called "top-down" approaches (17), yielded a circular, low-resolution map of the 6.7-Mbp chromosome of the NCIMB 8052 strain of *C. beijerinckii* for a pair of rarely cutting enzymes (*Sfi*I and *Rsr*II; 3 and 6 cleavage sites, respectively) and a collection of 14 contigs varying in size from 890 to 80 kbp for a second pair of enzymes that cleave the chromosome more frequently (*Apa*I and *Sma*I; more than 30 cleavage sites each). The 14 *Apa*I-*Sma*I contigs were then superimposed on the low-resolution map by using the four enzymes in various different combinations. Extensive use was made of a bank of strains harboring Tn*1545* insertions, which permitted unambiguous identification of comigrating DNA fragments. The resulting map for *Apa*I and *Sma*I is incomplete; each enzyme alone accounts for about 85% of the genome, and the gaps between contigs represent an estimated 8% of the genome. These gaps correspond to the positions of as-yet-unassigned *Apa*I and *Sma*I fragments, many of which are smaller than 50 kbp. There are 81 restriction sites on the map of the 6.7-Mbp chromosome, giving an average resolution of about 90 kbp between sites, though some map intervals are, of course, substantially larger than this. This level of resolution is adequate to determine the approximate locations of cloned genes (17). For higher-resolution mapping, it will be necessary to adopt a "bottom-up" approach based on ordered cosmid or phage lambda libraries.

Several additional methods were explored in order to confirm and extend these results, but in each case technical difficulties were encountered. Cross-hybridization, in which labelled fragments produced by one enzyme are hybridized with fragments produced by another, gave ambiguous results because fragments to be labelled could not be obtained in sufficient purity. Several bands contained comigrating fragments, and there was also a contaminating smear of partially replicated and degraded DNA in gel electrophoretograms. DNA degradation also interfered with two-dimensional electrophoretic separation of DNA fragments. *Apa*I and *Sma*I linking clones were of very limited utility in map construction, because there are sites for both enzymes in the reiterated *rrn* operons. Since the map obtained for *Rsr*II and *Sfi*I was effectively confirmed by assignment of all cleavage sites but one (the *Rsr*II site separating RsA from RsD) to the independently derived *Apa*I-*Sma*I contigs, a search for *Rsr*II and *Sfi*I linking clones was not undertaken.

Two pieces of evidence suggested that the replication origin of the bacterial chromosome probably lies close to the *Sfi*I site within RsD (Fig. 3). Firstly, *Sfi*I-*Rsr*II fragments from this region are overrepresented in electrophoretograms of DNA isolated from exponentially growing bacteria. Secondly, the nine *rrn* operons associated with fixed *Apa*I-*Sma*I contigs are oriented such that they would be transcribed divergently from an origin somewhere within SfC. Two additional observations are also consistent with the presumed location of the replication origin. Firstly, multiple copies of the *rrn* operons are often clustered close to the replication origin in other eubacteria (17). In conformity with this, the 90-kbp overlap between RsD and SfC apparently contains several *rrn* operons. Secondly, in several other bacteria, including the endospore formers *Clostridium perfringens* and *Bacillus subtilis*, the *gyrA* gene is located adjacent to *gyrB* in the vicinity of the replication origin (2, 10, 27). This is consistent with the assignment of contig 14, which contains *gyrA*, to SfC.

The *Rsr*II site separating fragments RsA and RsD is the only one of the nine landmark restriction sites that has not been assigned to one of the 14 *Apa*I-*Sma*I contigs, collectively covering 92% of the bacterial chromosome. It could perhaps lie very close to sites for *Sma*I and/or *Apa*I at one extremity of contig 3, 13, or 14 (whose order, relative to each other, has not been established unequivocally). Alternatively, it might reside in one of the small *Apa*I and *Sma*I fragments, not yet located on the map.

The 6.7-Mbp chromosome of the NCIMB 8052 strain of *C. beijerinckii* is in the upper range of sizes observed for bacterial genomes. Among the bacteria analyzed to date, only *Streptomyces coelicolor* (30), *Streptomyces lividans* (34, 35), *Myxococcus xanthus* (15), *Stigmatella aurantica* (44), and *Bradyrhizobium japonicum* (33) have larger genomes. Most endospore-forming bacteria have smaller genomes (2, 10, 11, 13, 27, 31), although a strain of *Bacillus cereus* with a 6.3-Mbp genome has been described (12).

The NCIMB 8052 strain of *C. beijerinckii* has a larger number of *rrn* operons than any other bacterium characterized to date (17, 32). Bacterial species capable of rapid growth tend to have more *rrn* operons than those that grow more slowly (32). In conformity with this, and in spite of the comparatively large size of its genome, the NCIMB 8052 strain of *C. beijerinckii* with its 13 (or more) *rrn* operons grows rapidly, with a mean generation time of about 30 min under optimum conditions. The positions of nine *rrn* operons, and the approximate locations of two more, associated with the floating contigs (14 and 13 and 13 and 3), are indicated on the physical map (Fig. 3). Additional *rrn* operons probably lie within the 90-kbp overlap between fragments RsD and SfC, close to the suspected location of the replication origin (Fig. 3).

Twenty-seven genetic loci corresponding to 32 protein-encoding genes have been located on the map. Their distribution is nonrandom, with at least 23 genes (possibly 25) lying on one side of the replication origin and at most 9 genes (possibly only 7, depending on the precise locations of *gyrA* and *bdh*) lying on the other. With so few genes currently assigned positions on the physical map, it is possible, though not very likely, that their nonrandom distribution is simply fortuitous. Several of the genes located on the right arm of the chromosome shown in Fig. 3 encode enzymes of fermentative metabolism (viz., thiolase, acetoacetate decarboxylase, coenzyme A [CoA] transferase, and, possibly, a butanol dehydrogenase), together with a locus, *deg*, identified by Tn*1545* insertion that has an unknown function in the process of strain degeneration (loss of solvent-forming ability) (29). Other genes of fermentative metabolism, whose products are phosphate transbutyrylase, butyrate kinase, and 3-hydroxybutyryl-CoA dehydrogenase, lie on the other arm of the chromosome.

The construction of a combined physical and genetic map of the *C. beijerinckii* NCIMB 8052 chromosome is a significant advance in the genetic analysis of this industrially important anaerobe. It will also facilitate comparative studies of problems of more general biological interest, such as the architecture

and organization of bacterial chromosomes and the regulation of genes concerned with solventogenesis and endospore formation.

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