Targeted integration of genes into the *Clostridium acetobutylicum* chromosome

Shane R. Wilkinson and Michael Young

Author for correspondence: Michael Young. Tel: +44 970 622348. Fax: +44 970 622350.
e-mail: miy@uk.ac.aber

Institute of Biological
Sciences, University of
Wales, Aberystwyth, Dyfed
SY23 3DA, UK

A number of non-replicative, integrational plasmids have been introduced into the chromosome of the NCIMB 8052 strain of *Clostridium acetobutylicum*. Integration occurred via homologous recombination, apparently by a Campbell-like mechanism. For plasmids containing internal fragments of *gutD* or *spo0A*, insertion was mutagenic leading, respectively, to an inability to use sorbitol as sole source of carbon for energy and growth or an inability to form endospores. One insertion was used to determine the map location of a randomly cloned DNA fragment and another underwent amplification in the bacterial chromosome. These results suggest that integrational plasmids will be very useful tools for genetic analysis in *Clostridium acetobutylicum*. To our knowledge, this is the first demonstration of gene transfer involving homologous recombination with the chromosome of any species of *Clostridium*.

Keywords: insertional mutagenesis, gene amplification, homologous recombination, integrational plasmid, *Clostridium acetobutylicum*

INTRODUCTION

Substantial advances have recently been made in the genetic analysis of mesophilic, saccharolytic clostridia (reviewed by Young *et al.*, 1989; Young & Cole, 1993). *Clostridium acetobutylicum*, well known for the acetone/butanol/ethanol fermentation that it undertakes (Jones & Woods, 1986), has become the favoured species for experimental genetics. A wide variety of plasmid vectors are now available (Minton *et al.*, 1993) and genes have been transferred to this organism by protoplast transformation (Azeddoug *et al.*, 1992), electro-transformation (Oultram *et al.*, 1988; Mermelstein *et al.*, 1992) and conjugation (Williams *et al.*, 1990). Streptococcal conjugative transposons have been used for isolating mutant strains (Woolley *et al.*, 1989; Bertram *et al.*, 1990) and they are also being employed for constructing a physical map of the bacterial chromosome (Wilkinson & Young, 1993). Gene transfer involving homologous recombination with the bacterial chromosome has not yet been documented. This remains a significant lacuna in the genetic analysis of all clostridia, including *C. acetobutylicum*.

Integrational plasmids may be employed to detect homologous recombination involving bacterial chromosomes. They contain a DNA segment from a host in which they cannot replicate, together with a genetic marker for which selection can be made. After transfer they become

established by inserting into the homologous region of the host replicon from which the DNA segment was derived. Integration is generally assumed to occur by a Campbell-like mechanism (Campbell, 1962). After insertion, plasmid sequences at the target site are flanked by two directly repeated copies of the DNA segment directing integration. These duplicated sequences are potential substrates for homologous recombination, resulting in loss of the integrated plasmid. Therefore, insertion mutations created by plasmid integration are not completely stable. Integrational plasmids have proved to be extremely powerful tools in the genetic analysis of Gram-positive bacteria (reviewed by Dowds *et al.*, 1988; Young & Hranueli, 1988; Perego, 1993). For example, insertion can be mutagenic if the host sequences providing homology for integration lie entirely within a gene or transcription unit (Piggot *et al.*, 1984). Another application is the controlled and relatively stable amplification of genes encoded by integrational plasmids (Young, 1984; Jannire *et al.*, 1985; Piggot & Curtis, 1987; Young & Ehrlich, 1989). This communication documents the establishment of integrational plasmids by homologous recombination in the chromosome of the NCIMB 8052 strain of *C. acetobutylicum*.

METHODS

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

Table 1. Bacterial strains and plasmids

Strain	Genotype	Source/reference
<i>E. coli</i>		
DH5 α	F ⁻ λ^- endA1 recA1 relA1 supE44 hsdR17(r _K ⁻ m _K ⁺) (argF-lacZYA)U169 thi-1 gyrA96(Nal ^R) [ϕ 80d/lacZ Δ M15]	Hanahan (1983)
HB101	F ⁻ λ^- hsdS20(r _B ⁻ m _B ⁻) supE44 aro-14 galK2 lacY1 proA2 rpsL20(Sm ^R) xyl-5 mtl-1 recA13	Boyer & Roulland-Dussoix (1969)
CA474	F ⁻ λ cI857 hsdS20(r _B ⁻ m _B ⁻) supE44 aro-14 galK2 lacY1 proA2 rpsL20(Sm ^R) xyl-5 mtl-1 recA13	λ cI857 lysogen of HB101 containing R702
<i>C. acetobutylicum</i>		
NCIMB 8052	Prototrophic	Laboratory stock
AA210	erm	Insertion of pSRW35 in NCIMB 8052
AA219	gutD::erm	Insertion of pJ1 in NCIMB 8052
AA225	erm	Insertion of pSRW38 in NCIMB 8052
AA243	spo0A::erm	Insertion of pSRW44 in NCIMB 8052
Plasmid	Relevant characteristics*/construction	Source/reference
R702	Km ^R ; Tra ⁺ Mob ⁺	Hedges & Jacob (1974)
pAT153	Ap ^R	Twigg & Sherratt (1980)
pUC19	Ap ^R	Norlander <i>et al.</i> (1983)
pMTL30	Ap ^R Em ^R ; Tra ⁻ Mob ⁺	Williams <i>et al.</i> (1990)
pCTC1	Ap ^R Em ^R ; Tra ⁻ Mob ⁺	Williams <i>et al.</i> (1990)
pBTM100†	Ap ^R ; pAT153 <i>Bam</i> HI containing 8.1 kbp partial <i>San</i> 3A fragment	N. P. Minton and J. D. Oultram, PHLS, Porton Down, UK
pCACB4†	Ap ^R ; pUC19 <i>Eco</i> RI/ <i>Bam</i> HI containing PCR- amplified 540 bp internal fragment of <i>spo0A</i>	D. Brown and P. Youngman, Univ. Georgia, Athens, USA
pJ1†	Ap ^R Em ^R ; pMTL30 <i>Xba</i> I containing 336 bp <i>Nhe</i> I- <i>Spe</i> I internal fragment of <i>gutD</i>	M. Mauchline and N. P. Minton, PHLS, Porton Down, UK
pSRW35†	Ap ^R Em ^R ; pMTL30 <i>Hind</i> III containing 0.8 kbp <i>Hind</i> III fragment	This study
pSRW38†	Ap ^R Em ^R ; pMTL30 <i>Eco</i> RI containing 3.8 kbp <i>Eco</i> RI fragment from the insert in pBTM100	This study
pSRW44†	Ap ^R Em ^R ; pMTL30 <i>Eco</i> RI/ <i>Bam</i> HI containing 540 bp internal fragment of <i>spo0A</i> from pCACB4	This study

* Km^R, Ap^R, Em^R, resistance to kanamycin, ampicillin and erythromycin, respectively; Tra, Mob, ability of plasmids to transfer by conjugation and to be mobilized by conjugation, respectively.

† Plasmids containing fragments of the *C. acetobutylicum* NCIMB 8052 chromosome.

Culture media and growth conditions. *Clostridium acetobutylicum* NCIMB 8052 was grown anaerobically at 37 °C in clostridial basal medium (CBM; O'Brien & Morris, 1971) solidified, as appropriate, with 1.5 % (w/v) agar. Derivatives harbouring integrational plasmids were selected on CBM supplemented with 10 μ g erythromycin ml⁻¹. All *Escherichia coli* strains were grown aerobically on Luria-Bertani medium (LB; Maniatis *et al.*, 1982) at 37 °C (for DH5 α derivatives) and 30 °C (for CA474 derivatives).

DNA isolation. Plasmids were isolated from *E. coli* using the method of Del Sal *et al.* (1988).

Chromosomal DNA was isolated from *C. acetobutylicum* using a method based on that of Stojanović *et al.* (1992). Bacteria from a 5 ml overnight culture were harvested, washed in

holding buffer (HB; 25 mM potassium phosphate, 10 mM MgSO₄, pH 7.0) and suspended in 250 μ l of a solution containing 50 mM Tris/HCl, 50 mM disodium EDTA, 25 % (w/v) sucrose, pH 8.0. Protoplasts were formed by adding 50 μ l of the same solution containing 100 mg lysozyme ml⁻¹ (freshly prepared) and incubating at 37 °C for 60–90 min. Protoplasts in the resulting suspension were lysed with 250 μ l of a solution containing 10 mM Tris/HCl, 5 mM disodium EDTA, 1 % (w/v) SDS pH 8.0. The above steps were carried out anaerobically to avoid the premature lysis and DNA degradation that ensues when *C. acetobutylicum* encounters oxygen. Thereafter, samples were removed from the anaerobic cabinet and deproteinized by extraction twice with an equal volume of phenol/chloroform pre-equilibrated with TE buffer (10 mM Tris/HCl, 1 mM disodium EDTA, pH 8.0). Residual phenol was removed by extraction with an equal volume of chloroform/isoamyl alcohol

(24:1, v/v) and nucleic acids were precipitated using 1.2 vols propan-2-ol. The fibrous peller of nucleic acids was rinsed with 70% (v/v) ethanol, vacuum-dried and dissolved by incubation for 15 min at 65 °C in 50 µl TE buffer.

The methods used for extraction of chromosomal DNA for pulsed-field gel electrophoresis were as described previously (Wilkinson & Young, 1993); electrophoresis conditions were as indicated in the Figure legend.

Construction of integrational plasmids. Integrational plasmids (see Table 1) were constructed by cloning fragments of *C. acetobutylicum* NCIMB 8052 DNA in the polylinker of plasmid pMTL30 (Williams *et al.*, 1990). This vector contains the *oriT* segment of the IncP plasmid RK2 and is efficiently mobilized by IncP plasmids from *E. coli* donors to *C. acetobutylicum*. Integrational plasmids were initially established in *E. coli* strain DH5α (Hanahan, 1983). They were then transformed into *E. coli* strain CA474 using the method of Mandel & Higa (1970), except that the heat-shock step was performed at 30 °C, to avoid induction of the λ prophage present in this strain.

Restriction endonucleases and T4 DNA ligase were used in accordance with the suppliers' instructions.

Filter mating procedure. The conjugative mobilization of integrational plasmids from *E. coli* CA474 derivatives to *C. acetobutylicum* NCIMB 8052 was carried out as previously described (Williams *et al.*, 1990), except that mating bacteria were incubated at 30 °C overnight to avoid induction of the λ prophage present in the donor strains. Transconjugants were selected anaerobically at 37 °C on CBM containing 10 µg erythromycin ml⁻¹. The donor:recipient ratio was approximately 100:1.

Southern blotting and hybridization. DNA fragments in agarose gels were depurinated (Wahl *et al.*, 1979) and transferred to nylon membranes (Hybond-N; Amersham) by capillary blotting (Southern, 1975; Maniatis *et al.*, 1982). Hybridization probes were prepared using a digoxigenin-based non-radioactive DNA labelling kit (Boehringer) and hybridizations were carried out as indicated by the manufacturer.

Measurement of recombination frequencies. Loss of integrational plasmids from the *C. acetobutylicum* chromosome was monitored, starting with overnight cultures grown in CBM containing 10 µg erythromycin ml⁻¹. The bacteria were diluted between 10³- and 10⁵-fold in pre-warmed CBM lacking erythromycin, to permit multiplication of recombinants lacking the integrated plasmid. Growth was monitored until the cell density reached a value corresponding to an OD₆₀₀ ~ 1.0. The culture was diluted again and growth monitored as previously. Subculturing steps were repeated until bacterial populations had undergone 20–30 generations of growth. At the beginning and end of each experiment, and at each subculturing step, samples were removed and the viable count was determined by plating serially diluted (in HB) samples on non-selective medium (CBM). The colonies that appeared after anaerobic growth for 24 h were replica-plated on selective medium (CBM with 1%, w/v, sorbitol instead of glucose for strains harbouring pJ1; CBM + 10 µg erythromycin ml⁻¹ for all other strains). The rate of plasmid loss per generation was approximated by dividing the proportion of antibiotic-sensitive (or sorbitol-utilizing) colonies by the number of generations of bacterial growth (Noirot *et al.*, 1987).

Assessment of spore formation. Spore formation was monitored by phase contrast microscopy of bacteria from colonies grown on CBM for 9 d.

RESULTS

Establishment of integrational plasmids in *C. acetobutylicum*

Filter matings were undertaken using *E. coli* CA474 derivatives harbouring integrational plasmids (see Table 1) as donors and *C. acetobutylicum* NCIMB 8052 as recipient. The results of a typical experiment are summarized in Table 2. In each experiment, *E. coli* CA474 donors harbouring pCTC1, a replication-proficient plasmid, and pMTL30, the vector used for constructing integrational plasmids, were employed as controls. The transfer frequencies observed with the pCTC1-containing donor strain were usually between 10⁻⁴ and 10⁻⁵ per recipient, whereas no transconjugants were detected with the pMTL30-containing donor. Donors harbouring integrational plasmids produced transconjugants at frequencies of between 10⁻⁶ and 10⁻⁷ per recipient. For donors harbouring integrational plasmids, the frequency of transconjugants was not strongly dependent on size of the chromosomal insert, over the range employed here.

Integrational plasmids undergo homologous recombination with the bacterial chromosome

Several lines of evidence indicate that the establishment of integrational plasmids in transconjugants results from homologous recombination between the chromosomal insert and the corresponding region of the bacterial chromosome. Southern hybridization experiments were undertaken with several transconjugants, using the chromosomal inserts from the relevant integrational plasmids as probes. In most cases, though not all (see below), the sizes of the fragments detected after cleavage with three different restriction endonucleases were as expected for integration of a single plasmid copy by a Campbell-like mechanism. The enzymes chosen had single sites in the pMTL30 vector backbone and no sites in the chromosomal insert. Accordingly, the single hybridizing band present in the parental strain was replaced in the transconjugants by two junction fragments, whose combined size corresponded to the sum of the sizes of the

Table 2. Frequencies of plasmid establishment in *C. acetobutylicum* after conjugative mobilization from *E. coli*

Plasmid	Insert size (kbp)*	Replication†	Frequency per recipient
pCTC1	–	Rep ⁺	1.2 × 10 ⁻⁴
pJ1	0.34	Rep ⁻	4.5 × 10 ⁻⁷
pSRW35	0.8	Rep ⁻	2.6 × 10 ⁻⁷
pSRW38	3.8	Rep ⁻	1.7 × 10 ⁻⁶
pSRW44	0.54	Rep ⁻	
pMTL30		Rep ⁻	< 4.8 × 10 ⁻⁹

* – indicates that the plasmid lacks an insert of chromosomal DNA.

† Ability to replicate in *C. acetobutylicum*.

Fig. 1. Insertion of plasmid pSRW35 into the bacterial chromosome. Lanes 1, 3 and 5, λ concatemers; lane 2, NCIMB 8052 *SmaI*; lanes 4 and 6, AA210 *SmaI*. Electrophoretograms are shown in lanes 1–4, Southern hybridization results in lanes 5 and 6, using a mixed probe of pSRW35 and λ DNA. Fragment SmC, which is visibly altered after integration of pSRW35 (to generate fragment C') is indicated in lanes 2 and 4. Samples in lanes 1 and 2 were subjected to electrophoresis (200 V for 24 h) through 1% (w/v) agarose in 0.5 $\times$ TBE (Maniatis *et al.*, 1982) maintained at 14 °C with a linear ramp from 1–60 s. Samples in lanes 3–6 were treated similarly except that electrophoresis was for 20 h and TAE buffer (Maniatis *et al.*, 1982) was employed.

integrated plasmid and the band present in the parental strain (Young & Hranueli, 1988).

DNA isolated from two transconjugants harbouring plasmid pSRW35 was digested with *SmaI* and the resulting macro-restriction fragments separated using pulsed-field gel electrophoresis. In both transconjugants plasmid pSRW35 had apparently inserted at the same site, located about 50 kbp from one end of SmC (see Wilkinson & Young, 1993). Since plasmid pSRW35 contains a *SmaI* site, the SmC fragment found in wild-type DNA was replaced by two *SmaI* fragments in transconjugant DNA (data for one transconjugant are shown in Fig. 1).

Two of the plasmids contain internal fragments of known genes. Plasmid pJ1 contains a 336 bp insert from within *gutD* (sorbitol dehydrogenase structural gene) and trans-

conjugants containing pJ1 were unable to grow using sorbitol as sole source of carbon. Plasmid pSRW44 contains a 540 bp insert from within *spo0A* and the single transconjugant obtained containing pSRW44 was unable to form endospores. A more detailed characterization of the phenotype of this Spo0A strain of *C. acetobutylicum* will be published elsewhere. As expected, Campbell-type integration of these plasmids by homologous recombination insertionally inactivated these genes.

Amplification of integrated plasmids

The results obtained with strain AA243 harbouring plasmid pSRW44 were not as expected for the integration of a single plasmid copy (see above). In experiments employing two different restriction endonucleases (*Bgl*II

Table 3. Amplification of an integrational plasmid in the bacterial chromosome

Strain	Size(s) of hybridizing fragments*	
	<i>Bgl</i> III	<i>Hind</i> III
NCIMB 8052	4.9	8.7
AA243	6.7†	7.3†
	5.1‡	6.5†
	3.5†	5.2‡

* The 540 bp *Eco*RI–*Bam*HI insert of *C. acetobutylicum* DNA was used as probe.

† Junction fragments.

‡ Amplified fragment corresponding to linearized pSRW44.

Table 4. Segregation of recombinants lacking integrated plasmids

Strain	Integrated plasmid	Insert size (kbp)	Recombination frequency*
AA210	pSRW35	0.8	$1.3 (\pm 0.5) \times 10^{-3}$
AA219	pJ1	0.336	$3.7 (\pm 1.9) \times 10^{-4}$
AA225	pSRW38	3.8	$5.6 (\pm 1.7) \times 10^{-4}$

* Values are means ($\pm$ SD) of three independent determinations.

and *Hind*III) with unique sites in the pMTL30 vector, two junction fragments would be expected if a single copy of the plasmid was integrated. However, in strain AA243 the expected junction fragments were accompanied by an additional intensely hybridizing band that co-migrated with the linearized integrational plasmid (Table 3). These data indicated that several tandemly repeated plasmid copies had become established by homologous recombination, a phenomenon that has been noted previously with integrational plasmids in Gram-positive bacteria (Young, 1984; Sargent & Bennett, 1985; Jannire *et al.*, 1985). The number of integrated copies of the amplified plasmid was estimated to be about five, based on the relative intensities of the amplified fragment and the junction fragments (note that the probe used in this experiment was the 540 bp internal fragment of *spo0A*).

Stability of strains harbouring integrated plasmids

Three strains of *C. acetobutylicum* harbouring different integrational plasmids were grown for 20–30 generations in the absence of selective pressure (i.e. erythromycin) and the frequency of segregation of erythromycin-sensitive recombinants was determined as indicated in Methods. The values obtained, which varied from 3.7×10^{-4} to 1.3×10^{-3} per generation, are summarized in Table 4. It appeared that segregation frequency was not directly

related to size of insert over the range employed here (0.34–3.8 kbp).

DISCUSSION

This paper establishes the occurrence of genetic exchange involving homologous recombination with the chromosome of the NCIMB 8052 strain of *C. acetobutylicum*. The frequency of integration of three replication-defective plasmids was two to three orders of magnitude less than that of the replication-proficient control (Table 2), suggesting that up to 1% of the incoming plasmid molecules integrated by homologous recombination. This is only a very rough estimate, since it is based on a comparison of the frequencies of transfer and establishment of different plasmids from different donors, but it does fall within the upper range of frequencies observed for integration of a plasmid showing thermosensitive replication in *Lactococcus lactis* at the non-permissive temperature (Maguin *et al.*, 1992; Biswas *et al.*, 1993). The frequency of conjugative mobilization from *E. coli* was sufficiently high to permit recovery of transconjugants containing non-replicative plasmids inserted into the bacterial chromosome. The reported frequencies of plasmid establishment following protoplast transformation of the NI-4081 strain (10^6 per μ g DNA; Azeddoug *et al.*, 1992) and electrotransformation of the NCIMB 8052 strain (10^3 per μ g DNA; Oultram *et al.*, 1988) suggest that these methods could also be employed for introducing integrational plasmids into these two very similar strains (Wilkinson & Young, 1993). Other commonly used laboratory strains of *C. acetobutylicum* (ATCC 824, DSM 1731 and NCP 262) are not, apparently, so closely related to the NCIMB 8052 strain (Wilkinson & Young, 1993) and it remains to be seen whether integrational plasmid technology will also be as readily applicable to these organisms.

In *Bacillus subtilis* and *L. lactis* the frequency of homologous recombination between DNA molecules is quite strongly dependent on length of the homologous DNA segment (Michel *et al.*, 1983; Michel & Ehrlich, 1984; Khasanov *et al.*, 1992; Biswas *et al.*, 1993). In the experiments reported here (Table 2) the plasmid bearing a 3.8 kbp segment of the bacterial chromosome became established at a frequency that was only four- to sevenfold higher than that observed for the two plasmids with shorter homologous DNA segments (336 bp and about 800 bp). Recombination frequency is affected by many factors in addition to length of the region of homology. These include chromosomal location, DNA base composition and sequence (Cox & Lehman, 1987; Vagner & Ehrlich, 1988; Gruss *et al.*, 1991; Biswas *et al.*, 1992). A carefully controlled study will be required before any firm conclusions can be drawn concerning the dependence of recombination frequency on extent of homology in *C. acetobutylicum*.

Integrational plasmids generate a duplication of host DNA sequences upon insertion. Excision (reversal of the integration event) generally occurs at a low frequency (less than 10^{-4} per generation) in *B. subtilis* and *L. lactis*

(Vagner & Ehrlich, 1988; Young & Ehrlich, 1989; Leenhouts *et al.*, 1989). The values obtained here are about an order of magnitude higher than those observed in *B. subtilis* and *L. lactis*. This may indicate the existence of a particularly active system of homologous recombination in the NCIMB 8052 strain of *C. acetobutylicum*. However, the explanation may be of a trivial nature. For example, recombinants lacking the integrated plasmids may have a more rapid growth rate than parental strains. Alternatively, the observed differences may simply reflect variations in the methods used to assess recombination frequency in the different organisms. For the experiments with *B. subtilis*, fluctuation tests (Vagner & Ehrlich, 1988) or establishment of bacterial cultures from single cell inocula (Young & Ehrlich, 1989) were employed. For the experiments with *C. acetobutylicum*, cultures were established with an initial inoculum density of between 5×10^2 and 5×10^4 c.f.u. ml⁻¹ because lower density inocula did not grow reproducibly. Further experiments will be required to distinguish between these possibilities.

Integrational plasmids have played a cardinal role in genetic analysis in *B. subtilis*. They have been employed for: (i) determining the map location and extent of cloned genes; (ii) undertaking insertional mutagenesis, mutational cloning and gene replacement; (iii) constructing gene fusions and merodiploid strains; (iv) controlled stable amplification of genes; (v) cloning replication origins (reviewed by Dowds *et al.*, 1988; Young & Hranueli, 1988; Perego, 1993). Integrational plasmids are also being used extensively in several other Gram-positive bacteria, including streptomycetes and lactic acid bacteria (Kieser & Hopwood, 1991; Chassy & Murphy, 1993). This paper establishes that they can be employed in *C. acetobutylicum* for at least three of the applications mentioned above (viz. determination of map location, insertional mutagenesis and gene amplification). It seems likely that integrational plasmids will prove to be powerful and versatile tools for genetic analysis in *C. acetobutylicum*.

ACKNOWLEDGEMENTS

We thank M. Mauchline, N. P. Minton and J. D. Oultram, PHLS, Porton Down, UK, and D. Brown and P. Youngman, University of Georgia, Athens, USA, for providing plasmids pBTM100, pJ1 and pCACB4. The financial support of the SERC Biotechnology Directorate is gratefully acknowledged.

REFERENCES

- Azeddoug, H., Hubert, J. & Reysset, G. (1992). Stable inheritance of shuttle vectors based on plasmid pIM13 in a mutant strain of *Clostridium acetobutylicum*. *J Gen Microbiol* **138**, 1371–1378.
- Bertram, J., Kuhn, A. & Dürre, P. (1990). Tn916-induced mutants of *Clostridium acetobutylicum* defective in regulation of solvent formation. *Arch Microbiol* **153**, 373–377.
- Biswas, I., Vagner, V. & Ehrlich, S. D. (1992). Efficiency of homologous intermolecular recombination at different locations on the *Bacillus subtilis* chromosome. *J Bacteriol* **174**, 5593–5596.
- Biswas, I., Gruss, A., Ehrlich, S. D. & Maguin, E. (1993). High-efficiency gene inactivation and replacement system for Gram-positive bacteria. *J Bacteriol* **175**, 3628–3635.
- Boyer, H. W. & Roulland-Dussoix, D. (1969). A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. *J Mol Biol* **41**, 459–472.
- Campbell, A. M. (1962). Episomes. *Adv Genet* **11**, 101–146.
- Chassy, B. M. & Murphy, C. M. (1993). *Lactococcus* and *Lactobacillus*. In *Bacillus subtilis and other Gram-positive Bacteria: Biochemistry, Physiology and Molecular Genetics*, pp. 65–82. Edited by A. L. Sonenshein, J. A. Hoch & R. Losick. Washington, DC: American Society for Microbiology.
- Cox, M. M. & Lehman, I. R. (1987). Enzymes of general recombination. *Annu Rev Biochem* **56**, 229–262.
- Del Sal, G., Manfioletti, G. & Schneider, C. (1988). A one tube plasmid DNA mini-preparation suitable for sequencing. *Nucleic Acids Res* **16**, 9878.
- Dowds, B. C. A., O'Kane, C., Gormley, E., McConnell, D. J. & Devine, K. M. (1988). Integrating plasmids in the genetic engineering of bacilli. In *Recombinant DNA and Bacterial Fermentation*, pp. 137–156. Edited by J. A. Thomson. Boca Raton, Florida: CRC Press.
- Gruss, A., Moretto, V., Ehrlich, S. D., Duwat, P. & Dabert, P. (1991). GC-rich DNA sequences block homologous recombination *in vitro*. *J Biol Chem* **266**, 6667–6669.
- Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557–579.
- Hedges, R. W. & Jacob, A. E. (1974). Transposition of ampicillin resistance from RP4 to other replicons. *Mol & Gen Genet* **132**, 31–34.
- Jannire, L., Niaudet, B., Pierre, E. & Ehrlich, S. D. (1985). Stable gene amplification in the chromosome of *Bacillus subtilis*. *Gene* **40**, 47–55.
- Jones, D. T. & Woods, D. R. (1986). Acetone–butanol fermentation revisited. *Microbiol Rev* **50**, 484–524.
- Khasanov, F. K., Zvingila, D. J., Zainullin, A. A. & Prozorov, A. A. (1992). Homologous recombination between plasmid and chromosomal DNA in *Bacillus subtilis* requires approximately 70 bp of homology. *Mol & Gen Genet* **234**, 494–497.
- Kieser, T. & Hopwood, D. A. (1991). Genetic manipulation of *Streptomyces*: integrating vectors and gene replacement. *Methods Enzymol* **204**, 430–458.
- Leenhouts, K. J., Kok, J. & Venema, G. (1989). Campbell-like integration of heterologous plasmid DNA into the chromosome of *Lactococcus lactis* subsp. *lactis*. *Appl Environ Microbiol* **55**, 394–400.
- Maguin, E., Duwat, P., Hege, T., Ehrlich, S. D. & Gruss, A. (1992). New thermosensitive plasmid for Gram-positive bacteria. *J Bacteriol* **174**, 5633–5638.
- Mandel, M. & Higa, A. (1970). Calcium-dependent bacteriophage DNA infection. *J Mol Biol* **53**, 159–162.
- Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Mermelstein, L. D., Welker, N. E., Bennett, G. N. & Papoutsakis, E. T. (1992). Expression of cloned homologous fermentative genes in *Clostridium acetobutylicum* ATCC 824. *Biotechnol* **10**, 190–195.
- Michel, B., Niaudet, B. & Ehrlich, S. D. (1983). Intermolecular recombination during transformation of *Bacillus subtilis* competent cells by monomeric and dimeric plasmids. *Plasmid* **10**, 1–10.
- Michel, B. & Ehrlich, S. D. (1984). Recombination efficiency is a quadratic function of the length of homology during plasmid transformation of *Bacillus subtilis* protoplasts and *Escherichia coli* competent cells. *EMBO J* **3**, 2879–2884.
- Minton, N. P., Swinfield, T.-J., Brehm, J. K., Whelan, S. M. & Oultram, J. D. (1993). Vectors for use in *Clostridium acetobutylicum*.

In *Genetics and Molecular Biology of Anaerobic Bacteria*, pp. 120–140. Edited by M. Sebold. New York: Springer.

Noirot, Ph., Petit, M.-A. & Ehrlich, S. D. (1987). Plasmid replication stimulates DNA recombination in *Bacillus subtilis*. *J Mol Biol* **196**, 39–48.

Norrander, J., Kempe, T. & Messing, J. (1983). Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. *Gene* **27**, 101–106.

O'Brien, R. W. & Morris, J. G. (1971). Oxygen and the growth and metabolism of *Clostridium acetobutylicum*. *J Gen Microbiol* **68**, 307–318.

Oultram, J. D., Loughlin, M., Swinfield, T.-J., Brehm, J. K., Thompson, D. E. & Minton, N. P. (1988). Introduction of plasmids into whole cells of *Clostridium acetobutylicum* by electroporation. *FEMS Microbiol Lett* **56**, 83–88.

Perego, M. (1993). Integrational vectors for genetic manipulation in *Bacillus subtilis*. In *Bacillus subtilis and other Gram-positive Bacteria: Biochemistry, Physiology and Molecular Genetics*, pp. 615–624. Edited by A. L. Sonenshein, J. A. Hoch & R. Losick. Washington, DC: American Society for Microbiology.

Piggot, P. J. & Curtis, C. A. M. (1987). Analysis of the regulation of gene expression during *Bacillus subtilis* sporulation by manipulation of the copy number of *spo-lacZ* fusions. *J Bacteriol* **169**, 1260–1266.

Piggot, P. J., Curtis, C. A. M. & de Lencastre, H. (1984). Use of integrational plasmid vectors to demonstrate the polycistronic nature of a transcriptional unit (*spoIIA*) required for sporulation of *Bacillus subtilis*. *J Gen Microbiol* **130**, 2123–2136.

Sargent, M. G. & Bennett, M. F. (1985). Amplification of a major membrane-bound DNA sequence of *Bacillus subtilis*. *J Bacteriol* **161**, 589–595.

Southern, E. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* **98**, 503–517.

Stojanović, S., Hranueli, D. & Young, M. (1992). Evidence that recombination between reiterated sequences in the *Bacillus subtilis* chromosome does not occur via unequal crossing over. *Biochimie* **74**, 713–721.

Twigg, A. J. & Sherratt, D. (1980). Trans-complementable mutants of plasmid ColE1. *Nature* **283**, 216–218.

Vagner, V. & Ehrlich, S. D. (1988). The efficiency of homologous recombination varies along the *Bacillus subtilis* chromosome. *J Bacteriol* **170**, 3978–3982.

Wahl, G. M., Stern, M. & Stark, G. R. (1979). Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. *Proc Natl Acad Sci USA* **76**, 3683–3687.

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

Williams, D. R., Young, D. I. & Young, M. (1990). Conjugative plasmid transfer from *Escherichia coli* to *Clostridium acetobutylicum*. *J Gen Microbiol* **136**, 819–826.

Woolley, R. C., Pennock, A., Ashton, R. J., Davies, A. & Young, M. (1989). Transfer of Tn1545 and Tn916 to *Clostridium acetobutylicum*. *Plasmid* **22**, 169–174.

Young, M. (1984). Gene amplification in *Bacillus subtilis*. *J Gen Microbiol* **130**, 1613–1621.

Young, M. & Cole, S. T. (1993). *Clostridium*. In *Bacillus subtilis and other Gram-positive Bacteria: Biochemistry, Physiology and Molecular Genetics*, pp. 35–52. Edited by A. L. Sonenshein, J. A. Hoch & R. Losick. Washington, DC: American Society for Microbiology.

Young, M. & Ehrlich, S. D. (1989). Stability of reiterated sequences in the *Bacillus subtilis* chromosome. *J Bacteriol* **171**, 2653–2656.

Young, M. & Hranueli, D. (1988). Chromosomal gene amplification in Gram-positive bacteria. In *Recombinant DNA and Bacterial Fermentation*, pp. 157–200. Edited by J. Thomson. Boca Raton, Florida: CRC Press.

Young, M., Staudenbauer, W. L. & Minton, N. P. (1989). Recent advances in the genetics of the clostridia. *FEMS Microbiol Rev* **63**, 301–325.

Received 11 June 1993; revised 6 August 1993; accepted 17 August 1993.