Stable inheritance of shuttle vectors based on plasmid pIM13 in a mutant strain of *Clostridium acetobutylicum*

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New shuttle vectors for *Clostridium acetobutylicum* were constructed, using as replicons the Gram-positive plasmid pIM13, and derivatives of the Gram-negative plasmid pBR322, including pUC19. These vectors transformed *C. acetobutylicum* at a high frequency (up to 10^6 transformants per μg DNA) by PEG-mediated protoplast transformation. A mutant host strain, NI-4082, was isolated on the basis of its ability to maintain plasmid pIM13 stably in the absence of selection pressure. The shuttle vectors showed no segregational or structural instability in this mutant strain. Moreover, the results suggested a relationship between segregational instability and the multimerization of pIM13 in *C. acetobutylicum*. The host/vector system described possessed all the properties required for efficient gene cloning in this species.

Introduction

Considerable work has been carried out in recent years towards the development of genetic tools for the butanol-acetone clostridia. The first, recently achieved, step was the development of efficient gene transfer procedures. These were performed either by conjugation (Oultram *et al.*, 1987, 1988*a*), or by electrotransformation (Oultram *et al.*, 1988*b*) of vegetative cells and, in the case of *Clostridium acetobutylicum* strain NI-4081, by PEG-mediated transformation of protoplasts (Reysset *et al.*, 1988). Cloning and shuttle vectors have been constructed using either indigenous clostridial plasmids or heterologous replicons, originating largely from streptococci or bacilli. Unfortunately, irrespective of the plasmid/host systems used, the constructed replicons tended to show segregational instability (for details see the review of Young *et al.*, 1989). The instability of plasmids bearing the replication functions of pIM13, originally from *Bacillus subtilis* (Mahler & Halvorson, 1980), has now been resolved by selecting a mutant strain of *C. acetobutylicum* denoted NI-4082. In this strain, plasmid pIM13 was stably maintained in the absence of selection pressure. This allowed the construction of shuttle vectors capable of replicating in this organism, in *Escherichia coli* and in *B. subtilis*.

Methods

Bacterial strains. The *E. coli* strains used in this study were HB101 [F^- hsdS20 (r_B^- m_B^-) *recA13* *ara-14* *lacY1* *proA2* *galK2* *rpsL20* (Sm^r) *xyl-5* *mtl-1* *supE44* (λ^-)] (Boyer & Roulland-Dussoix, 1969) and GT869 [*thrB1004* *pro* *thi* *strA* *hsdS* *lacZ* Δ *M15* (F' *lacZ* Δ *M15* *lacI^s* *traD36* *proA^+* *proB^+*)] (Parsot, 1986). *C. acetobutylicum* strain NI-4081 (Rif^r Pro⁻ Lys⁻) (Reysset *et al.*, 1987) was a derivative of strain NI-4 (Hongo, 1960). Strain NI-4082 was selected from NI-4081 for its ability to maintain pIM13 stably (this report). *B. subtilis* strain BD224 (*recE4* *thr-5* *trpC2*) was used as recipient for plasmid transformation (Dubnau *et al.*, 1973).

Plasmids. The *E. coli* plasmid pBR322 was constructed by Bolivar *et al.* (1977) and pUC19 by Yanisch-Perron *et al.* (1985). The *B. subtilis* plasmid pIM13 (Mahler & Halvorson, 1980), and *Staphylococcus aureus* plasmids pT127 (Novick & Brodsky, 1972) and pBC16 Δ 1 (Polak & Novick, 1982), were also used. The *C. acetobutylicum* cloning vector pKNT14 has been described previously (Truffaut *et al.*, 1989). The shuttle vector pCTC511 (Williams *et al.*, 1990) was also used as a control. pKNT15 and pKNT19 are new *E. coli*/*B. subtilis*/*C. acetobutylicum* shuttle vectors (this report).

Media and growth conditions. *E. coli* strains were grown aerobically at 37 °C in LB medium (containing, per litre: tryptone, 10 g; yeast extract, 5 g; NaCl, 10 g) or in LB medium solidified with 1.5% (w/v) agar (Difco). Ampicillin (Ap) or erythromycin (Em), were added to the media when required to a final concentration of 100 and 300 $\mu\text{g ml}^{-1}$, respectively. For β -galactosidase assay on plates, the chromogenic substrate 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-Gal) and the *lacZ* inducer isopropyl β -D-thiogalactopyranoside (IPTG) (Boehringer Mannheim) were added to LB medium at final concentrations of 50 and 150 $\mu\text{g ml}^{-1}$, respectively.

B. subtilis strains were grown at 37 °C in Difco nutrient broth

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(Schaeffer *et al.*, 1965); erythromycin was added to the medium when required to a final concentration of $5 \mu\text{g ml}^{-1}$.

For *C. acetobutylicum*, TYA medium (Ogata & Hongo, 1973) was used for routine growth and plasmid segregation studies. T70 medium, derived from the previously described T69 medium (Reyssset *et al.*, 1987), was also used; it contained, per litre: KH_2PO_4 , 0.5 g; $\text{CH}_3\text{COONH}_4$, 3 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 g; MOPS, 10 g; NaHCO_3 , 0.5 g; bioglytone (bio-Mérieux), 1 g; Casamino acids (Difco), 1 g. Both media were adjusted to pH 6.5 with NaOH. Glucose and CaCl_2 were added aseptically to a final concentration of 1% (w/v) and 1 mM, respectively. For plating, media were solidified with agar (Difco) at 1.8% (w/v). Erythromycin or tetracycline (Tc) were added to media when required to a final concentration of 50 and $8 \mu\text{g ml}^{-1}$, respectively.

Media derived from T70 were used for protoplast formation and regeneration (see below).

Media and supplements were pre-reduced and anaerobically sterilized, except for lysozyme, BSA and choline solutions, which were sterilized by filtration (0.45 μm pore size; Millipore).

All manipulations with *C. acetobutylicum* were carried out in an anaerobic chamber (Celser La Calhene) with an atmosphere of $\text{N}_2/\text{CO}_2/\text{H}_2$ (90:5:5, by vol.). Cultures were incubated in this chamber at 35 or 25 °C when specified.

Protoplast formation and regeneration. Cells were grown in T70 medium to mid-exponential phase (about 1×10^8 cells ml^{-1}). Cultures were adjusted with pre-weighed solid sterilized sucrose to 0.6 M. Protoplasts were obtained by adding lysozyme ($100 \mu\text{g ml}^{-1}$) plus benzylpenicillin ($20 \mu\text{g ml}^{-1}$), and incubating at 35 °C for 60 min. After addition of BSA, 0.5% final concentration, the protoplasts were recovered by centrifugation (4 min at 13000 r.p.m., MSE-micro Centaur) at room temperature, washed twice with T70 medium containing 0.5 M-xylose, 0.5% BSA (T70-2X), and resuspended in T70-2X. The protoplasts were then diluted and incorporated into soft agar regeneration medium (T70-X), i.e. T70 basal medium containing 0.25 M-xylose (pre-weighed solid sterilized), 0.5% BSA and 0.4% (w/v) choline, plus 0.8% (w/v) agar (Difco), and spread onto the same medium solidified with 2.5% (w/v) agar (Difco).

Plasmid stability. Exponential-phase cultures ($\text{OD}_{550} = 0.3$) of *C. acetobutylicum* were diluted and grown in TYA medium under non-selective conditions at 25 °C or 35 °C so that the original OD_{550} was recovered after about 20 generations. The dilutions were repeated to obtain about 40, 60 and 80 generations. The percentage of clones maintaining resistance to erythromycin was determined by the replica-plating method. At every dilution step (about 20–40–60–80 generations) 24 erythromycin-resistant clones were checked for the presence of plasmid.

Plasmid transformation procedure. *E. coli* HB101 and GT869 were transformed by electroporation (Dower *et al.*, 1988). Competent cells of *B. subtilis* were prepared according to the method of Anagnostopoulos & Spizizen (1961). Protoplast transformation of *C. acetobutylicum* strains NI-4081 and NI-4082 was performed as follows. Plasmid DNA (0.1 to 1 μg) was added to about 2×10^8 to 1×10^9 protoplasts prepared as described above. A solution of 40% (w/v) PEG 4000 prepared in T70 containing 0.5 M-xylose and 20 mM- MgCl_2 was filter-sterilized, and then added at a final concentration of 35% to the protoplast suspension. This was gently mixed and incubated for 2 min at room temperature, then diluted with about 5 vols T70-2X medium, centrifuged (6 min at 13000 r.p.m.), washed with the same medium and resuspended after centrifugation (4 min at 13000 r.p.m.) in T70-X medium. Appropriate dilutions of *C. acetobutylicum* protoplasts were incorporated into soft agar and poured onto regeneration plates (see above). After 20 h, 3 ml of T70-X soft agar containing erythromycin (1 mg) or tetracycline (160 μg) was poured onto the plates. Em^r or Tc^r transformants appeared after 4 to 6 d incubation.

Plasmid isolation. The procedure of Birnboim & Doly (1979) was used for small- and large-scale isolation of plasmid DNA from *E. coli* cells. Similar methods were developed for *C. acetobutylicum* (Reyssset *et al.*, 1988) and were also used for *B. subtilis*.

DNA manipulation. Restriction endonuclease and T_4 DNA ligase were obtained from Boehringer Mannheim, Appligene, Gibco-BRL or Pharmacia. Digestion and ligation were carried out using the buffers and the concentrations suggested by the manufacturers.

Agarose gel electrophoresis was performed with horizontal 9×11 cm slab gels in either TBE (90 mM-Tris/borate, 2 mM-EDTA) or TAE (40 mM-Tris, 5 mM-sodium acetate, 1 mM-EDTA adjusted to pH 7.8 with glacial acetic acid) buffers. Agarose concentrations of 0.7 and 1% (w/v) were used, depending on the fragment sizes to be resolved. After electrophoresis, the gels were stained in ethidium bromide ($1 \mu\text{g ml}^{-1}$) for 10 min and the DNA bands were visualized under a UV-light transilluminator (302 nm; UVP, Inc.). Fragment sizes were determined by the method of Southern (1979) using phage λ DNA digested with *Hind*III or *Hind*III and *Eco*RI as size standards (Daniels *et al.*, 1980).

The isolation and detection of single-stranded (ss) plasmid DNA in whole-cell lysates were as described by te Riele *et al.* (1986).

Plasmid curing. The elimination of plasmids in regenerating protoplasts was first observed by Novick *et al.* (1980) for *Staphylococcus aureus*. Protoplasts of *C. acetobutylicum* strain NI-4082(pIM13) were prepared as previously described, except that the liquid and solid regeneration medium was devoid of choline and that 0.25 M-xylose was substituted by 0.3 M-sucrose. After regeneration, the colonies were replicated on plates with or without erythromycin ($50 \mu\text{g ml}^{-1}$). About 3% of the regenerated protoplasts were found to be Em^s . These clones were checked for the absence of plasmid using the miniscale preparation.

Results

Construction and properties of shuttle vectors

The shuttle vector pKNT15 is a chimaeric plasmid consisting of pKNT14 and a derivative of pBR322, pBR322 Δ , lacking a 1.9 kb *Eco*RV-*Pvu*II fragment. pBR322 Δ was digested with *Hind*III and ligated to pKNT14 partially digested with the same restriction enzyme. The mixture was used to transform *E. coli* HB101 and recombinants were selected for both ampicillin and erythromycin resistance. Among the four types of recombinant plasmids of the expected size (6.8 kb), one, pKNT15, was retained for further studies. The construction and restriction map of this plasmid are shown in Fig. 1.

To construct pKNT19, plasmids pIM13 and pUC19 were linearized with *Cl*aI and *N*arI, respectively. The fragments were ligated and used to transform *E. coli* GT869. Transformants were selected for erythromycin resistance and screened for β -galactosidase activity. The construction of one hybrid plasmid recovered, named pKNT19 (4.9 kb), is shown in Fig. 1.

The plasmids pKNT15 and pKNT19 prepared in *dam*⁺ strains of *E. coli* (Azeddoug *et al.*, 1989) transformed *C. acetobutylicum* strain NI-4081 with high

Fig. 1. Construction of hybrid plasmids pKNT15 and pKNT19. Plasmid pKNT15 was obtained by ligation of a partial *HindIII* digest of pKNT14 and a total *HindIII* digest of pBR322Δ (pBR322 deleted of the 1.9 kb *EcoRV*–*PvuII* fragment). Plasmid pIM13, digested by *ClaI*, was ligated to *NarI*-digested pUC19 to generate pKNT19. The main unique restriction sites available for cloning are shown in bold type. Nucleotide numbering starts for all plasmids at the first A of the *ClaI* site of pIM13 (Monod *et al.*, 1986). The arrows represent the junction sites used in the construction of the hybrid plasmids. The *AccI*, *HincII*, *HindIII* and *SacI* sites of the multiple cloning sequence (MCS) of pUC19 were also present in pIM13.

efficiency, ranging from 2×10^6 to 6×10^6 transformants per μg of DNA, using the PEG-mediated protoplast procedure. The phenotypic properties of the two vectors either in *E. coli* or *C. acetobutylicum* are summarized in Table 1, and insertional cloning sites are given in Fig. 1.

Isolation of strain NI-4082

A high degree of structural and segregational stability is essential for efficient host/vector cloning systems. Although the recombinant plasmids showed good structural stability in *C. acetobutylicum* strain NI-4081, they

Table 1. General properties of plasmid pIM13 and derivatives

Plasmid	Size (kb)	Replicon		Phenotypic traits expressed in	
		Gram + ve	Gram - ve	Gram + ve	Gram - ve
pIM13	2.2	pIM13	—	Em ^r	—
pKNT14	4.3	pIM13	—	Em ^r Tc ^r	—
pKNT15	6.8	pIM13	pBR322Δ*	Em ^r Tc ^r	Em ^r † Ap ^r
pKNT19	4.9	pIM13	pUC19	Em ^r	Em ^r † Ap ^r Lac ⁺

* The deletion (Δ) of 1.9 kb was generated between the *EcoRV* and *PvuII* sites of pBR322.

† Selection for erythromycin resistance in *E. coli* was feasible, but the efficiency of transformation was half that for ampicillin resistance and clones appeared 8 h later.

exhibited poor segregational stability. As the Gram-positive replicon of a range of shuttle vectors, the segregational stability of pIM13 was evaluated in strain NI-4081. The results given in Fig. 2 clearly demonstrate the high segregational instability of pIM13. Moreover the instability was higher at 25 °C than at 35 °C.

As shown in Fig. 2, after 43 generations only 0.1% of the NI-4081 cells remained Em^r. However, when transfers without antibiotic were continued, this percentage was maintained, even after 90 generations. At this step the cells were grown in TYA medium containing 50 µg erythromycin ml⁻¹ and then transferred at mid-exponential phase into TYA lacking the antibiotic. After three successive subcultures into TYA plus erythromycin followed by TYA without erythromycin, all 2500 clones tested by replica plating exhibited erythromycin resistance. All 24 clones chosen at random and checked for

Fig. 2. Segregational stability of plasmid pIM13 in *C. acetobutylicum* strain NI-4081 at 25 and 35 °C. *C. acetobutylicum* strain NI-4081 harbouring plasmid pIM13 was grown in antibiotic-free TYA medium for approximately 90 generations at 25 °C (Δ) or 35 °C (□). The proportion of Em^r cells was determined by replica-plating from non-selective media onto erythromycin-containing plates. At least 200 colonies were tested for each sample.

the presence of plasmid DNA possessed the entire pIM13 replicon. One clone, NI-4082, was cured of the resident plasmid by the regeneration procedure (Novick *et al.*, 1980).

Fig. 3. Segregational stability of cloning vectors in *C. acetobutylicum* strain NI-4081 and NI-4082 at 25 and 35 °C. *C. acetobutylicum* strains NI-4081 and NI-4082 harbouring plasmid pKNT15 or pKNT19 were tested for segregational stability at (a) 25 °C and (b) 35 °C as described in the legend to Fig. 2. (□), NI-4081(pKNT15); (Δ), NI-4081(pKNT19); (■), NI-4082(pKNT15); (▲), NI-4082(pKNT19).

Fig. 4. Segregational stability of different replicons in *C. acetobutylicum* strains NI-4081 and NI-4082 at 35 °C. *C. acetobutylicum* strains NI-4081 (a) and NI-4082 (b) harbouring plasmid pBC16Δ1 (□), pT127 (■), pIM13 (△) or pCTC511 (▲) were tested for segregational stability at 35 °C as described in the legend to Fig. 2. The proportion of *Emr* or *Tc^r* cells was determined by replica-plating from non-selective media onto antibiotic-containing plates.

Studies on the segregational stability of the two strains of *C. acetobutylicum*

The segregational stability of pIM13 derivatives was evaluated in strains NI-4081 and NI-4082 at 25 °C (Fig. 3a) and 35 °C (Fig. 3b). In strain NI-4081, the cloning vectors had the same instability as pIM13 at 25 °C but were slightly more stable at 35 °C. In contrast, no significant segregation was observed in strain NI-4082 cultivated at either temperature for 100 generations or more, whatever the vector tested. This suggested that the stability of the pIM13 replicon in strain NI-4082 was a property of the host strain rather than the plasmid during the selection procedure. It was also notable that stability of the pIM13 replicon in strain NI-4082 was independent of the temperature. Both strains harbouring pKNT19

Fig. 5. Detection of single-stranded pKNT19 and pCTC511 DNA in strain NI-4081 of *C. acetobutylicum*. Cell lysates were prepared from *C. acetobutylicum* NI-4081 harbouring pKNT19 (lanes 1 and 2) or pCTC511 (lanes 3 and 4). Lysates were treated (lanes 2 and 4) or not treated (lanes 1 and 3) with nuclease S1, then electrophoresed through a 0.8% agarose gel. After transfer of DNA onto a nylon membrane, the filter was probed with ³⁵S-labelled pKNT19. The single-stranded plasmid DNAs are indicated by stars.

were cultured in complex and semi-synthetic minimal media; no noticeable growth rate differences were observed between the two strains on either medium. But here also, the vector instability was observed in both media for strain NI-4081 only (data not shown).

Three other plasmids (pT127, pBC16Δ1 and pCTC511) replicating like pIM13 in *B. subtilis* via rolling circle replication were introduced by transformation into both strains and tested for segregational stability at 35 °C. Results obtained in strains NI-4081 and NI-4082 are shown in Fig. 4(a) and 4(b) respectively. For plasmids pT127 and pBC16Δ1 only about 0.01% of the clones remained *Tc^r* after 20 generations in the absence of selection pressure for either strain NI-4081 or NI-4082. On the other hand, plasmid pCTC511 was rather stably maintained in both strains.

Fig. 6. Analysis of plasmid DNA preparations of pIM13, pKNT19 and pCTC511. Agarose gel (0.8%) electrophoresis of small-scale preparations of pIM13, pKNT19 and pCTC511 plasmid DNA isolated from strains NI-4082 and NI-4081 of *C. acetobutylicum* and BD224 of *B. subtilis*. A ccc DNA ladder (bands of about 2, 3, 4, 5, 6, 7, 8, 10, 12, 14 and 16 kb; Bethesda Research Laboratories) is shown in lane 4. Plasmid preparations are: pIM13 in strains NI-4082 (lane 1), NI-4081 (lane 2) and BD224 (lane 3); pKNT19 in strains NI-4082 (lane 5), NI-4081 (lane 6) and BD224 (lane 7); pCTC511 in strains NI-4082 (lane 8), NI-4081 (lane 9) and BD224 (lane 10). The monomeric forms of plasmids pIM13 (2.2 kb), pKNT19 (4.9 kb) and pCTC511 (7.8 kb) are indicated by arrows. The upper band present on the gel corresponds to the chromosomal DNA (chr). Note that the band in front of the monomeric form of plasmid pKNT19 in strain NI-4081 was probably the relaxed form of the plasmid.

Vector pCTC511 was constructed using the *Clostridium butyricum* plasmid pCB101 *rep* region, consisting of two ORFs (B and C) essential for replication. As the ORF B polypeptide exhibited significant homology to the Rep proteins of staphylococcal plasmids pC194 and pUB110 and phage ϕ X174 (Minton *et al.*, 1990a, b), it was suggested that pCB101 replicates via rolling circle replication, and belongs to the ssDNA family of Gram-positive plasmids. It has been classified by Gruss & Ehrlich (1989) in the pC194-type sub-group. Nevertheless, transformation of *B. subtilis* by pCTC511 strain BD224 occurred with poor efficiency as already observed by Collins *et al.* (1985) and Luczak *et al.* (1985), and plasmid DNA was barely detectable in isolates (see Fig. 6). In DNA preparations of plasmids pKNT19 and pCTC511 from strain NI-4081, ssDNA material sensitive to S1 nuclease activity was observed, demonstrating

that pIM13 and pCB101 replicated via rolling circle replication in *C. acetobutylicum* (Fig. 5). Plasmid DNAs of pIM13, pKNT19 and pCTC511, prepared from an identical number of exponentially growing cells of strains NI-4081 and NI-4082, were electrophoresed on a 0.8% agarose gel, and compared with identical plasmids extracted from *B. subtilis* strain BD224 (Fig. 6). The same intensity of material stained with ethidium bromide was observed in the DNA preparations of the two *C. acetobutylicum* strains. This suggested that the stability of the pIM13 replicon in strain NI-4082 was not related to a variation in plasmid copy number. Nevertheless, the banding patterns of the preparations were very different. Almost all DNA of plasmids pIM13 and pKNT19 from strain NI-4082 was present in the monomeric form, whereas in strain NI-4081 a significant amount of the plasmid DNA was present in multimeric forms (largely dimeric), as in *B. subtilis* (Monod *et al.*, 1986). It was also observed that the same amount of plasmid pCTC511 was found, mostly in the monomeric form, in the two strains of *C. acetobutylicum*. For *B. subtilis*, only the monomeric form was detectable in highly concentrated preparations of pCTC511 (Fig. 6, lane 10).

Discussion

The small multicopy plasmids isolated from Gram-positive aerobic bacteria (mainly from staphylococci and bacilli) have been divided into four groups (Novick, 1989) on the basis of similarity at the nucleotide sequence and functional organization levels. They replicate by an asymmetric rolling circle replication generating monomeric single-stranded circular DNA (ssDNA plasmids) (Gruss & Ehrlich, 1989). These plasmids have been used extensively during the last decade to develop cloning vectors for Gram-positive eubacteria (Novick, 1989; Gruss & Ehrlich, 1989), because they were shown to replicate in a wide variety of species. It was previously observed (Truffaut *et al.*, 1989, and unpublished results) that amongst representative plasmids from the four groups (pT127; pC194 and pBC16 Δ 1; pIM13; pE194), only pIM13 replicated efficiently in strain NI-4081, but it was lost rapidly in the absence of a selective antibiotic. The *E. coli*-*B. subtilis*-*C. acetobutylicum* shuttle vectors pKNT15 and pKNT19 constructed with this replicon were similarly unstable in strain NI-4081. This difficulty has finally been overcome by the isolation of a mutant host strain, NI-4082, in which the vectors exhibited no detectable segregational instability. In contrast to experimental results reported for *B. subtilis* by Gruss & Ehrlich (1988) and Bron *et al.* (1988, 1991), for cloning vectors based on ssDNA replicons, it was not possible, from gel electrophoretic analysis, to detect any structural instability.

The mutation(s) selected during the enrichment procedure of strain NI-4082 appeared to be specific to pIM13 replication as two other ssDNA plasmids, pT127 and pBC16Δ1, were still segregationally unstable in this new strain. In contrast, plasmid pCTC511, based on the clostridial pCB101 replicon, was easily established in *C. acetobutylicum* but not in *B. subtilis* (Collins *et al.*, 1985; Luczak *et al.*, 1985), and showed no segregational instability even in the parent strain NI-4081. This plasmid was shown to belong to the ssDNA family, but its replication strategy seems to be quite different from the Gram-positive plasmids of aerobic bacteria (Minton *et al.*, 1990a, b).

We have clearly shown that the vectors based on pIM13 or pCB101 replicate in *C. acetobutylicum* strain NI-4081 and NI-4082 via rolling circle replication. A relationship between multimeric DNA formation and instability is suggested by the electrophoretic pattern of plasmid DNA prepared in both strains. In *C. acetobutylicum*, segregational stability should occur when most of the DNA is in the monomeric form. For vectors based on pIM13 replicon, this property was only shown in strain NI-4082; for plasmid pCTC511 this pattern was observed in both strains. A possible explanation for this observation is that resolution of multimeric forms of the pIM13-based plasmids is more efficient in strain NI-4082.

A similar association between multimerization and segregational instability for pIM13 and the closely related plasmid pE5 was also observed by Projan *et al.* (1987) in *S. aureus* but not in *B. subtilis*. These authors suggested that the multimerization was a property of the plasmid rather than of the strain. In contrast, the results presented here suggest that in *C. acetobutylicum* at least, the host functions may play an important role in plasmid stability.

The host genes involved in the stabilization of the pIM13 replicon have not yet been characterized, but they are of obvious interest. It will be necessary to understand why the genetic background of strain NI-4082 was inefficient for the replication of other ssDNA plasmids of aerobic Gram-positive bacteria. It should be noted here that plasmids of the pIM13 family are the only ssDNA plasmids in which the direction of replication is opposite to that of the direction of transcription of the *rep* gene (Novick, 1989). But even if valuable for one group only of ssDNA plasmids, a genetic modification of host strains may be of great value for improving plasmid stability.

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