

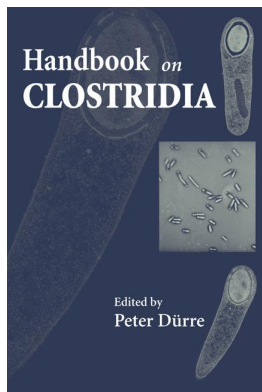
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## **Handbook on Clostridia**

Peter Dürre

### **Gene Cloning in Clostridia**

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# 3 Gene Cloning in Clostridia

Ian J. Davis, Glen Carter, Mike Young, and Nigel P. Minton

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## 3.1 INTRODUCTION

*Clostridium* is one of the largest prokaryotic genera, comprising a diverse assemblage of obligately anaerobic, Gram-positive, endospore-forming bacteria. As a whole, the genus exhibits extreme biocatalytic diversity. Some species such as *Clostridium acetobutylicum* are of considerable biotechnological interest as process organisms for the commercial production of chemical fuels and commodities from renewal biomass. However, the genus is best known as a consequence of the presence of organisms that cause human disease. Lethal intoxications are caused by *Clostridium botulinum* and *Clostridium tetani*, and to a lesser extent *Clostridium perfringens*, whereas debilitating infections are caused by *Clostridium difficile*, a major contributor to healthcare-associated illnesses within the U.K.

Following several years of intense activity, the genome sequences of representative strains of those species of greatest importance to the scientific community, namely *C. acetobutylicum*, *C. botulinum*, *C. tetani*, *C. perfringens*, and *C. difficile*, have now been determined; genome sequences for additional species, such as *Clostridium beijerinckii*, are in the pipeline. The availability of this information provides new opportunities for:

- Harnessing the biocatalytic potential of saccharolytic species in the commercial production of chemical fuels from renewable biomass
- Understanding the underlying mechanisms that contribute to the virulence of pathogenic species
- Assisting the development of improved methods for the detection and surveillance of the pathogenic organisms and of more effective countermeasures to prevent and treat infections

Effective means for genetically manipulating the organisms are required to maximize the potential utility of the wealth of available genome sequence information. In this chapter, we review the status of some of the genetic tools currently available, and we draw attention to the need for improved methods, where appropriate.

## 3.2 DNA ISOLATION

### 3.2.1 GENOMIC DNA

Protocols for the isolation of genomic DNA from different clostridia vary but tend to be based on the method first described by Marmur [1]. Examples include *C. acetobutylicum* [2], *C. thermocellum* [3], *C. botulinum* [4], and *C. cellulolyticum* [5]. This method involves an enzyme detergent lysis step, followed by extraction of cellular debris with phenol chloroform; the DNA is then precipitated out with ethanol. The method, described here in more detail, is taken from that used in *C. acetobutylicum* [2].

Cells are grown in clostridial basal media (CBM) [6] supplemented with glycine [0.4% (wt/vol)]. Pelleted cells (approx. 3 g wet weight) are then suspended in resuspension buffer [50 mM TRIS; 1 mM EDTA; 6.7% sucrose (wt/vol); pH 8.0] and incubated at 37°C for 15 min. Lysozyme solution (2.5 ml at 20 mg ml<sup>-1</sup>) is then added, and the mixture is incubated for 10 min at 37°C. Clostridial DNAases can be inhibited at this stage by the addition of 1.25 ml of 0.5 M EDTA solution (pH 8.0). Lysis is achieved by the addition of 0.75 ml lysis buffer [20% SDS (wt/vol); 50 mM TRIS; 20 mM EDTA; pH 8.0] and incubation for 10 min at 37°C. Proteinase K solution (0.1 ml at 2.5 mg ml<sup>-1</sup> in 10 mM TRIS; 1 mM EDTA) is added, and the mix incubated for 3 h at 37°C. Sodium perchlorate solution (5 M) is then added to a final concentration of 1 M and mixed. The emulsion is then extracted twice with chloroform/isoamylalcohol (24:1 vol/vol) by centrifugation for 5 min at 4000 to 10,000 g removing the aqueous phase. The DNA is then precipitated by the addition of 2 volumes of ethyl alcohol and can then be spooled out using a glass rod or pelleted via centrifugation. The DNA is then resuspended in TES (50 mM TRIS-HCl; 5 mM EDTA; 0.15 M NaCl). Treatment with Proteinase K and a further round of ethyl alcohol precipitation can be carried out to increase the purity of the DNA. Excess alcohol is removed by drying at room temperature and resuspension of the DNA in sterile water of TES.

It should be stated, however, that commercially available kits provide acceptable concentrations of purified genomic DNA for most applications and are less time-consuming to perform. They have been used successfully with many species, including *C. difficile* [7], *C. perfringens* [8], *C. acetobutylicum* [9] — and in our laboratory with *C. sporogenes* and *C. beijerinckii*. Anaerobic preincubation of the cell pellet in lysozyme (10 mg ml<sup>-1</sup>) for 15 to 60 min at 37°C may be required to aid lysis with some commercial kits.

### 3.2.2 PLASMID DNA ISOLATION

Published procedures for isolating plasmid DNA from *C. acetobutylicum* (now *C. beijerinckii*) [10], and *C. cellulolyticum* [5] and *C. beijerinckii*, are based on the standard alkaline lysis method described by Sambrook et al. [11] with the addition of a prelysis step. All centrifugation steps are at 12,000 *g* at 4°C. A 1.5 ml aliquot of cells, taken from an overnight culture, is centrifuged. The pellet is then resuspended in 100 µl prelysis buffer [25% (w/v) sucrose, 25 mM Tris/HCl pH 8.0, 10 mM EDTA, 10 mg ml<sup>-1</sup> lysozyme] and incubated anaerobically for 1 h at 37°C. A 200 µl aliquot of freshly prepared Solution II (0.2 N NaOH; 1% SDS) is added and mixed by inverting the tube followed by 150 µl of ice cold Solution III (5 M potassium acetate, 60 ml; glacial acetic acid, 11.5 ml; H<sub>2</sub>O, 28.5 ml). The mixture is vortexed and held on ice for 3 to 5 min before centrifuging for 5 min. The supernatant is transferred to a fresh tube. An optional step is to add an equal volume of phenol chloroform and mix by vortexing prior to centrifuging for 2 min; the supernatant is transferred to a fresh tube. The DNA is precipitated by adding 2 volumes of ethanol and mixing by vortexing. The mixture is left at room temperature for 2 min then centrifuged for 5 min. The supernatant is removed by aspiration. The DNA is rinsed with 1 ml 70% ethanol (chilled at 4°C), which is aspirated off. The DNA is air dried for 10 min then resuspended in 50 µl TE (10 mM TRIS-Cl; 1 mM EDTA; pH 8.0).

Again, commercially available kits provide acceptable concentrations of plasmid DNA. They have been utilized with many species including *C. difficile*, *C. beijerinckii* [12], *C. botulinum* [13], *C. cellulolyticum* [5], and *C. perfringens* [14]. The addition of the prelysis step (incubation with lysozyme) described above is required, and large-scale kits may be required for low-copy number plasmids.

## 3.3 TRANSFORMATION OF CLOSTRIDIA

There are no reported examples of natural competence in clostridia. In order to promote DNA uptake, transformation procedures thus rely on physical alteration of the cell envelope. The conversion of cells to protoplasts prior to transformation is one such method. However, the optimization of protocols for protoplast production is complex and time-consuming, and electrotransformation of intact cells is much more commonly used. The concentrations of antibiotics used for selection in clostridia vary between species; typical concentrations used are: chloramphenicol, 5 to 35 µg ml<sup>-1</sup>; thiamphenicol, 15 µg ml<sup>-1</sup>; spectinomycin, 250 µg ml<sup>-1</sup>; erythromycin, 10 to 50 µg ml<sup>-1</sup>; tetracycline, 5 to 10 µg ml<sup>-1</sup>.

### 3.3.1 PROTOPLAST PRODUCTION AND TRANSFORMATION

While this method has been largely superseded by electrotransformation, Reyssset et al. [15] have developed an efficient protoplast transformation protocol for use with *C. acetobutylicum* NI-4081. This strain gave high transformation frequencies (up to 10<sup>6</sup> transformants µg<sup>-1</sup> DNA) due to its reduced autolysin production (coupled with the use of autolysin inhibitors).

#### 3.3.1.1 Protoplast Production

Bacteria are grown to mid-exponential phase (approximately 10<sup>8</sup> cells ml<sup>-1</sup>) in T69 medium (per liter: 10 g glucose; 2 g ammonium acetate; 1 g yeast extract; 0.5 g casamino acids; 0.5 g bacto-tryptone; 0.5 g cysteine-HCl; 0.5 g KH<sub>2</sub>PO<sub>4</sub>; 0.3 g MgSO<sub>4</sub>·7H<sub>2</sub>O; 0.01 g FeSO<sub>4</sub>·7H<sub>2</sub>O; adjusted to pH 6.5 with NaOH). Solid sterile sucrose is added to a final concentration of 0.6 M. To remove the cell wall, lysozyme (100 µg ml<sup>-1</sup>) and penicillin G (20 µg ml<sup>-1</sup>) are added, and the cells incubated at 34°C for 1 h. The protoplasts are centrifuged at 3000 *g* and washed twice in protoplast wash buffer [T69 buffer supplemented with 0.6 M sucrose, 0.5% (w/v) bovine serum albumin (BSA) and 1 mM CaCl<sub>2</sub>]. Protoplasts are then resuspended in protoplast buffer [T69 supplemented with

0.5 M xylose, 0.5% (w/v) BSA, 25 mM MgCl<sub>2</sub>, and 25 mM CaCl<sub>2</sub>]. The number of potential L-form cells can be estimated by diluting a sample of cells in T69C regeneration medium [T69 supplemented with 0.3 M sucrose, 0.25 M xylose, 0.5% (w/v) BSA and 1 mM CaCl<sub>2</sub>] before plating on T69C solid medium [2.5% (w/v) agar]. Protoplast numbers can be estimated by phase-contrast light microscopy.

### 3.3.1.2 Protoplast Transformation

Plasmid DNA (50 to 800 ng), polyethylene glycol (PEG) 4000 [35% (w/v)], and 10<sup>9</sup> protoplasts are mixed and incubated at room temperature for 2 min. The mixture is then diluted 10-fold in T69 medium supplemented with 0.5 M xylose, 0.5% (w/v) BSA, 1 mM CaCl<sub>2</sub>, and 4 mg choline ml<sup>-1</sup>. The protoplasts are then centrifuged, washed, and resuspended in the same medium. Dilutions are then added to T69 top agar [T69 supplemented with 0.25 M xylose, 0.5% (w/v) BSA, 1 mM CaCl<sub>2</sub>, 4 mg choline ml<sup>-1</sup>, and 0.8% (w/v) agar]. This is then poured onto T69 agar [0.25 M xylose and 2.5% (w/v) agar] and incubated at 34°C for 20 h. To select for plasmids with erythromycin resistance markers, a further 3 ml of top agar [T69 supplemented with 0.25 M xylose, 1 mg erythromycin ml<sup>-1</sup>, and 0.8% (w/v) agar] is overlaid onto the plates and they are incubated for 4 to 6 days at 37°C.

### 3.3.2 TRANSFORMATION OF THERMOPHILIC CLOSTRIDIA

As previously reviewed by Mauchline et al. [16], procedures for the transformation of thermophilic clostridia remain poorly developed. *C. thermocellum* has not been reproducibly transformed. Reports of phenotypic conversion of transformed *C. thermocellum* protoplasts lack direct evidence of transformation [17]. The lack of reproducible transformation methods may be attributed to restriction systems such as that reported in *C. thermocellum* ATCC 27405 [18].

The biotechnologically important, ethanol-producing organism *C. thermohydrosulfuricum* has been successfully transformed [19]. Competence was induced by disruption of the surface S-layer and PEG-induced membrane permeabilization. The method was optimized for *C. thermohydrosulfuricum* DSM 568, which has low endogenous DNAase activity.

A fresh overnight culture is used to inoculate 50 ml of modified RCM broth (containing, per liter, 10 g tryptone; 4 g beef extract; 3 g yeast extract; 2.5 g glucose; 5 g NaCl; 0.5 g Na<sub>2</sub>S<sub>9</sub>H<sub>2</sub>O; 0.5 g cysteine HCl; 0.5 g resazurin; 2 g K<sub>2</sub>HPO<sub>4</sub>; pH 7.2). Cells are grown at 68°C to stationary phase (OD<sub>578</sub> = 0.45; approx. 1.5 × 10<sup>9</sup> cells ml<sup>-1</sup>). The bacteria are centrifuged at 6000 g for 8 min, washed in wash buffer [50 mM Tris-HCl, pH 8.3, 0.05% (w/v) Na<sub>2</sub>S<sub>9</sub>H<sub>2</sub>O, 0.05% (w/v) cysteine HCl], and then in 5 ml of electroporation buffer (wash buffer plus 0.35 M sucrose), and finally resuspended 0.2 ml of electroporation buffer. Plasmid DNA (2–5 µg) is added in TE buffer (25 mM Tris-HCl, pH 8.0, 10 mM EDTA) and incubated at 60°C for 5 min. A 1.5-ml sample of 40% PEG (mol. wt. 6000) is added, and the mixture is incubated for a further 60 min. A 2-ml sample of buffer [10 mM Tris-HCl, pH 8.3, 0.05% (w/v) Na<sub>2</sub>S<sub>9</sub>H<sub>2</sub>O, 0.15 M NaCl, 0.05% (w/v) cysteine HCl] is added and the cells centrifuged before resuspending them in 1 ml of RCM broth. Bacteria are finally plated on RCM agar (2%, w/v), supplemented with selective antibiotic, as appropriate, and incubated at 55°C for between 4 and 6 days.

### 3.3.3 ELECTROPORATION PROCEDURES

In this technique, cells are exposed to pulses of high-intensity electric fields that induce the transient formation of pores in the cell membrane, through which exogenous DNA can enter. The electroporation protocols are optimized for different species of clostridia by varying the method for cell preparation before electroporation; the electroporation parameters and the procedures for cell rescue post-electroporation. Numerous protocols have been documented. The most commonly used are

described here. All of these procedures benefit from placing the electroporation chamber within an anaerobic workstation.

### 3.3.3.1 Electrotransformation of *C. beijerinckii* NCIMB 8052

The protocol for electrotransformation of *C. beijerinckii* NCIMB 8052 was first described by Oultram et al. [20]. A 10-ml culture in 2 × YTG (per liter: 16 g tryptone; 10 g yeast extract; 5 g glucose and 5 g NaCl) is inoculated and then serially diluted in further 10-ml cultures for incubation for 16 h (overnight) at 37°C. A culture in exponential phase is used to inoculate (1:10) 100 ml of 2 × YTG. When the culture reaches an OD<sub>600</sub> = 0.6, it is cooled on ice and the cells pelleted by centrifugation at 2000 g for 10 min and washed in 10 ml precooled (4°C) electroporation buffer [270 mM sucrose, 1 mM MgCl<sub>2</sub> and 7 mM NaHPO<sub>4</sub> (pH7.4)]. The bacteria are then resuspended in 5 ml precooled electroporation buffer (4°C) and incubated on ice for 10 min before use.

Plasmid DNA (0.5 µg) is added to the prepared cells (0.8 ml), and the mixture is transferred to a precooled (4°C) electroporation cuvette (0.4 cm interelectrode distance). The mixture is incubated for 8 min on ice before electroporation. A 1.25 kV pulse (25 µF capacitance and 200 Ω resistance) is then applied using a Bio-Rad Gene Pulser. The cells are returned immediately to ice for 10 min. After dilution in 10 vol 2 × YTG and incubation at 37°C for 3 h, cells are concentrated by centrifugation at 2000 g for 10 min and resuspended in 100 µl 2 × YTG before plating on 2 × YTG plates containing the appropriate antibiotic. Plates are incubated at 37°C for 2 to 3 days.

### 3.3.3.2 Electrotransformation of *C. acetobutylicum* ATCC 824

*C. acetobutylicum* ATCC 824 contains a restriction system, Cac824I, that greatly reduces the electroporation efficiency of plasmid DNA lacking the appropriate methylation signature. To circumvent this, plasmid DNA may be methylated *in vivo* in *E. coli* containing the pAN1 plasmid, which encodes a bacteriophage methyltransferase [21]. The protocol described here (derived from Mermelstein et al. [22]) has been shown to produce 5 × 10<sup>5</sup> transformants µg<sup>-1</sup> DNA, with a pIM13-based vector (4.8 kb). The electroporation parameters have since been optimized to produce a 10-fold increase in transformation frequency [23]. However, the equipment required to obtain optimal results is not readily available.

A culture is grown in 60 ml RCM (pH 5.2) to late exponential phase. The cells are then centrifuged, washed, and resuspended in 2.1 ml precooled electroporation buffer [272 mM sucrose (pH7.4), 5 mM NaH<sub>2</sub>PO<sub>4</sub>]. A sample of the cell suspension (0.7 ml) is added to a cuvette (0.4 cm interelectrode distance) and held on ice for 5 min. Plasmid DNA (0.1–10 µg) is then added and the suspension held on ice for 2 min before electroporation with a 2.0 kV pulse and 25 µF capacitance. RCM (10 ml) is added to the bacterial suspension, and it is incubated at 37°C for 4 h before plating on RCM agar (pH 5.8) containing the appropriate selective agents.

### 3.3.3.3 Electroporation of *C. botulinum*

Electroporation protocols have been optimized in examples of proteolytic (ATCC 3502) and non-proteolytic (ATCC 25765) strains of *C. botulinum* [13,24]. The only major difference between the protocols is the field strength used. Both strains yield comparable transformation frequencies when electroporated with plasmid pGK12 (4.4 kb). The nonproteolytic group II strain ATCC 25765 encodes a restriction modification system. This barrier is circumvented *in vivo* by methylation in an *E. coli* host containing a gene encoding the *B. subtilis* M.BsuF1 methylase [13]. The protocol described below is for *C. botulinum* ATCC 25765, with alterations for ATCC 3502 given in square brackets.

A culture (20 ml) is grown for 16 h in TPGY medium (per liter: 20 g trypticase; 5 g yeast extract; 5 g peptone; 1 g glucose; 1 g cysteine-HCl) supplemented with glycine (1% w/v). A 300-ml

TPGY culture is then inoculated with this culture and grown to an  $OD_{660} = 0.8$ . The culture is equally divided, and the two aliquots are held on ice for 10 min before pelleting the cells by centrifugation at 6000 *g* for 10 min at 4°C. The supernatant is carefully removed and the bacterial pellet gently resuspended in 50 ml of precooled (4°C) electroporation buffer (10% PEG 6000 [PEG 8000 for ATCC 3502], 1 mM MgCl<sub>2</sub>, 7 mM Na phosphate, pH 7.5). The cells are centrifuged as before and then resuspended in 3 ml precooled electroporation buffer. DNA (0.1–2.0 mg ml<sup>-1</sup>) is added to a precooled electroporation cuvette (0.4 cm interelectrode distance) and mixed with 0.8 ml of cell suspension by gentle inversion 2 to 3 times. The bacteria are electroporated using a 2.0 kV pulse [2.5 kV for ATCC 3502] (25 μF capacitance and 400 Ω resistance). The cuvette is then immediately placed back on ice for 5 min. The bacterial suspension is added to 10 ml prewarmed (37°C) TPGY broth (supplemented with 25 mM MgCl<sub>2</sub>), and the cells are left to recover for 5 h at 37°C. The bacterial suspension is then distributed into 6 eppendorf tubes, centrifuged for 3 min, the cells in each tube gently resuspended in 150 μl of TPGY broth and spread on individual TPGY agar (2.5%, w/v) plates containing the appropriate selective agent. Plates are incubated for between 24 and 48 h.

### 3.3.3.4 Electroporation of *C. cellulolyticum*

Electroporation of *C. cellulolyticum* ATCC 35319 was optimized using ATP leakage assays [25]. This strain encodes a restriction system, which is circumvented by *in vitro* methylation of vector DNA with *MspI* methylase (1 U per μg of DNA incubated at 37°C for 3 h). Bacteria are grown on a defined medium containing (per liter): 6.7 g cellulose; 1.4 g KH<sub>2</sub>PO<sub>4</sub>; 2.9 g K<sub>2</sub>HPO<sub>4</sub>·3H<sub>2</sub>O; 1 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 0.1 g MgCl<sub>2</sub>·6H<sub>2</sub>O; 0.02 g CaCl<sub>2</sub>; 25 μl 5% (w/v) FeSO<sub>4</sub> dissolved in 50 mM H<sub>2</sub>SO<sub>4</sub>; 1 ml trace element solution (described below); 10 ml vitamin solution (described below); 0.5 g Na<sub>2</sub>S; and 0.5 ml 0.2% (w/v) resazurin [26].

The trace element solution contains (per liter): 5 g FeSO<sub>4</sub>·7H<sub>2</sub>O; 1.44 g ZnSO<sub>4</sub>·7H<sub>2</sub>O; 1.12 g MnSO<sub>4</sub>·7H<sub>2</sub>O; 0.25 g CuSO<sub>4</sub>·5H<sub>2</sub>O; 0.2 g Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>; 1 g (NH<sub>4</sub>)<sub>6</sub>(Mo)<sub>7</sub>O<sub>24</sub>·4H<sub>2</sub>O; 0.04 g NiCl<sub>2</sub>; 0.02 g CoCl<sub>2</sub>; 0.03 g H<sub>3</sub>BO<sub>3</sub>; 0.02 g Na<sub>2</sub>SeO<sub>3</sub>; 50 ml HCl (10M).

The composition of the vitamin solution is (per 100 ml distilled water): 10 mg d-biotin; 25 mg p-aminobenzoic acid; 15 mg nicotinic acid; 25 mg ribofavin; 25 mg pantothenic acid; 25 mg thiamin; 10 mg cyanocobalamin. The vitamin solution is sterilized by passage through a 0.2-μm filter.

All incubations are at 34°C. Cells are grown to mid-log phase in 40 ml synthetic medium then washed with ice-cold electrotransformation buffer [270 mM sucrose, 5 mM K<sub>2</sub>HPO<sub>4</sub> (pH 6.5)] and resuspended in 1.5 ml of the same buffer. A 0.5 ml aliquot of the cell suspension is added to 5 μl (0.5–1.0 μg) of methylated DNA in an electroporation cuvette (0.4 cm gap). The bacterial suspension is electroporated with a 2.0 kV pulse (25 μF capacitance and 1000 Ω resistance) and then immediately added to 5 ml prewarmed (34°C) synthetic medium for 6 h. Cells are then plated on selective medium (as above, but solidified with agar) and incubated for 3 to 5 days.

### 3.3.3.5 Electroporation of *C. perfringens*

Various methods have been devised for electroporation of *C. perfringens* [27,28]. The method most commonly used is based on that of Allen and Blaschek [28] and is given here.

Cells are incubated in TYG broth (3% biotrypticase, 2% yeast extract, 0.1% sodium thioglycolate, 0.5% glucose, pH 7.4) for 16 h at 37°C. A 5-ml sample of cell suspension is centrifuged for 10 min at 5000 *g*. The cells are washed with 5 ml of electroporation buffer (0.272 M sucrose, 7 mM K<sub>2</sub>HPO<sub>4</sub>, and 1 mM MgCl<sub>2</sub>) and resuspended in 1 ml of electroporation buffer. DNA (1 μg ml<sup>-1</sup>) and 350 μl of the cell suspension are mixed in a cuvette (0.4 cm gap) and electroporated at 2.5 kV (25 μF capacitance and 200 resistance). The cuvette is incubated on ice for 5 min, after which 1 ml of YTG broth is added and incubation is at 37°C for 3 h. Cells are then spread on YTG agar (2%, w/v) containing the appropriate selective agent.

It should be noted that transformation frequency appears to be very strain-specific. Between 100- and 1000-fold differences have been reported between the transformation frequencies of different strains of this organism [28,29].

### 3.4 CONJUGATIVE TRANSFER

Conjugation is an important route for genetic exchange between bacteria — and indeed between bacteria and other organisms. It is thought to have played an important role in the spread of antibiotic resistance genes between organisms, particularly through the agency of conjugative transposons. The process requires close cell-to-cell contact and involves both a *cis*-acting nick site (*oriT*), as well as a number of *trans*-acting functions (most often plasmid encoded) that are necessary for mating pair formation as well as DNA processing and transfer of the conjugative plasmid to the recipient cell.

#### 3.4.1 CONJUGATIVE PLASMIDS

Conjugative plasmids are widespread throughout the bacterial kingdom. However, in clostridia, naturally occurring conjugative plasmids appear to be relatively rare and, indeed, they have only been described in *C. perfringens*. In this species, all such plasmids are closely related, suggesting that they may have arisen from a common progenitor [30]. They encode two highly conserved antibiotic resistance genes. One of these, *tetP*, confers resistance to tetracycline via an efflux mechanism [31] and the other, *catP*, confers resistance to chloramphenicol [32] and resides on a transposable element [33].

These plasmids are readily transferred between different strains of *C. perfringens* [34], but despite this, they have so far proved to be of little use in the development of gene transfer technologies. To date, there is only a single report of the transfer of pIP401 to *C. difficile*, in which it was not stably inherited [35].

The conjugative plasmids from a number of other bacteria have very broad host ranges, and several examples from enterococci and streptococci, including pAM $\beta$ 1 and pIP501, have been conjugated into *C. acetobutylicum* [36,37]. Plasmid pAM $\beta$ 1 has also been conjugated into *C. butyricum* and *C. pasteurianum* [38]. The transfer efficiency of these plasmids is highly dependent on the donor organism and is typically most efficient when a Gram-positive species such as *Lactococcus lactis* is employed [36]. Transfer of nonconjugative plasmids from *B. subtilis* into *C. beijerinckii*, has also been achieved using the conjugative functions of pAM $\beta$ 1. It relies on the formation of a co-integrate molecule, which is then transferred into the recipient cell [39]. This technique is inefficient and prone to the occurrence of large deletions affecting the conjugative functions of pAM $\beta$ 1 in *B. subtilis* [40], and it has not been used widely.

#### 3.4.2 CONJUGATIVE TRANSPOSONS

In addition to conjugative plasmids, a number of Gram-positive organisms, including various clostridia, harbor conjugative transposons, such as Tn916 of *E. faecalis* [41], Tn1545 of *S. pneumoniae* [42], and Tn4451 of *C. perfringens* [33]. They are large mobile genetic elements that generally reside within the bacterial chromosome and encode all of the functions necessary for their own transfer. Transposition of conjugative transposons relies on the formation of a covalently closed supercoiled circular DNA intermediate, which can then either reintegrate into the chromosome of the same cell or, following transfer by conjugation, insert into a recipient's genome. Insertion takes place without duplication of the target site, and they are replicated as part of the host chromosome [43]. They often possess an extremely wide host range [44] and have been transferred to a number of different clostridia, including *C. tetani* [45], *C. beijerinckii* [46],



*C. acetobutylicum* [2], *C. perfringens* [30], *C. botulinum* [4], and *C. difficile* [47,48]. These elements have been utilized as tools for the delivery of cloned gene fragments [49,50] and for transposon mutagenesis strategies (see Chapter 4).

### 3.4.3 CONJUGATIVE MOBILISATION FROM *E. COLI*

The conjugation strategy with perhaps the widest potential utility, however, is the transfer of plasmids from *E. coli* into clostridia. Indeed, the transfer of genetic material from *E. coli* into several species of clostridia has now been documented. These include *C. beijerinckii* (formerly known as *C. acetobutylicum*) [10], *C. perfringens* [51], *C. cellulolyticum* [5], *C. botulinum* [52], and *C. difficile* [12,53]. In all cases, transfer was reliant on components of the broad host range IncP family of plasmids.

A number of mobilizable *Clostridium/E. coli* shuttle vectors are available carrying the transfer origin (*oriT*) of an IncP plasmid, such as RP4 or RK2 (see Table 3.1). This region, in conjunction with several additional trans-acting functions (Tra functions), is absolutely required for the conjugative transfer of plasmid DNA to recipient cells. The Tra functions are provided by the *E. coli* (Tra<sup>+</sup>) donor and may either be plasmid-encoded (e.g., carried by a IncP-type helper plasmid such as R702) or integrated into the chromosome, as is the case with *E. coli* strain SM10. In many instances, the transfer of IncP-containing vectors from *E. coli* (Tra<sup>+</sup>) donors is remarkably efficient, particularly if a high donor-to-recipient ratio is used during conjugation. A typical protocol for conjugative plasmid transfer from *E. coli* to *Clostridium* is described below.

Inoculate the donor strain (e.g., *E. coli* HB101 containing R702, as well as the plasmid to be mobilized) into 5 ml of Brain Heart Infusion Broth (BHIB, Oxoid) containing the appropriate antibiotic selections, and incubate aerobically overnight at 37°C with shaking (200 rpm). Grow serial dilutions of the clostridial recipient in 5 ml aliquots of BHIB anaerobically overnight at 37°C. The following morning, dilute back one of the serial dilutions of the clostridial recipient that has not entered stationary phase to give an OD<sub>600</sub> = 0.05 – 0.1 and grow anaerobically. Concurrently, dilute the *E. coli* donor culture to give an OD<sub>600</sub> = 0.01 and grow aerobically. When both cultures have reached an OD<sub>600</sub> = 0.45 – 0.6, place the donor culture into the anaerobic chamber, harvest the bacteria from 1 ml of culture, and wash them with 1 ml anaerobic phosphate buffered saline (PBS). Gently resuspend the bacterial pellet in 100 µl of the recipient clostridial culture (giving a ratio of 10:1, donor:recipient) and gently spread the mixture onto the surface of a 0.2 µm filter (Whatman) that has been placed on an anaerobic reinforced clostridial medium (RCM) agar plate. Incubate anaerobically overnight. Following this period of cocultivation, resuspend the bacterial growth from the filter in 1 ml anaerobic BHIB or PBS by vortex-mixing and spread 0.1 ml samples onto RCM agar containing the appropriate antibiotics to select for transconjugants (and counter-select against the donor and the recipient). To determine the transfer frequency, make serial dilutions of the donor-recipient mixture and spread onto appropriate agar plates to permit the separate growth of donor and recipient strains. Transconjugants are usually visible after anaerobic incubation at 37°C for 24 to 48 h. Most *E. coli* and *Bacillus subtilis* donors can be counter-selected using 10 µg trimethoprim ml<sup>-1</sup>, and this is particularly efficient if the donor is a phage lambda *cI857* lysogen, since plating at 42°C provokes donor lysis, as a result of prophage induction. In the case of *C. difficile* recipients selection with D-cycloserine (250 µg ml<sup>-1</sup>) and cefoxitin (8 µg ml<sup>-1</sup>) will select against nonclostridial donors.

Gene transfer procedures based on conjugation are perhaps more labor-intensive than electrotransformation techniques. Despite this, they can offer distinct advantages. Most notably, conjugation may result in substantially higher plasmid transfer frequencies than can routinely be obtained with electrotransformation. Moreover, in some cases conjugation represents the only available option. Furthermore, it is not affected by extracellular nucleases because of the need for close cell-to-cell contact [69], and it does not require specialized and expensive equipment.

**TABLE 3.1**  
**Clostridial Plasmid Cloning Vectors**

Plasmid	Size (kb)	Replicon <sup>a</sup>	Comments	Markers <sup>b</sup>	Ref.
pCAK1	11.6	CAK1 ( <i>C. acetobutylicum</i> )	Phagemid ssDNA intermediate	Em <sup>R</sup> [Ap <sup>R</sup> ]	54
pTYD101	4.0	pSC86 ( <i>C. acetobutylicum</i> )	Deletion variant	Cm <sup>R</sup>	55
pTYD104	7.6	pSC86 ( <i>C. acetobutylicum</i> )	Deletion variant	Cm <sup>R</sup> [Ap <sup>R</sup> ]	55
pCB3	7.03	pCB101 ( <i>C. butyricum</i> )	ssDNA replication	Em <sup>R</sup> [Ap <sup>R</sup> ]	56
pCTC511	7.85	pCB101 ( <i>C. butyricum</i> )	IncP mobilizable	Em <sup>R</sup> [Ap <sup>R</sup> ]	10
pMTL540E	5.23	pCB102 ( <i>C. butyricum</i> )	Segregationally stable	Em <sup>R</sup> [Ap <sup>R</sup> ]	57
pMTL540F	5.5	pCB102 ( <i>C. butyricum</i> )	Expression vector	Em <sup>R</sup> [Ap <sup>R</sup> ]	57
pMTL9401	4.6	pCB102 ( <i>C. butyricum</i> )	IncP mobilizable	Em <sup>R</sup>	12
pCB5	9.5	pCB103 ( <i>C. butyricum</i> )	Uncharacterized replicon	Em <sup>R</sup> [Ap <sup>R</sup> ]	56
pSYL2	8.7	pCBU2 ( <i>C. butyricum</i> )		Em <sup>R</sup> Tc <sup>R</sup>	58
pMTL9301	7.1	pCD6 ( <i>C. difficile</i> )	IncP mobilizable, transferred to <i>C. difficile</i> genome strain	Em <sup>R</sup>	12
pAK201	8.0	pHB101 ( <i>C. perfringens</i> )	Stable in <i>C. perfringens</i>	Cm <sup>R</sup>	59
pJIR1456	6.8	pIP404 ( <i>C. perfringens</i> )	IncP mobilizable	Cm <sup>R</sup>	51
pJIR1457	6.2	pIP404 ( <i>C. perfringens</i> )	IncP mobilizable, transferred to <i>C. botulinum</i> and <i>C. difficile</i>	Em <sup>R</sup>	51
pJIR418	7.4	pIP404 ( <i>C. perfringens</i> )	General purpose cloning vector	Em <sup>R</sup> Cm <sup>R</sup>	60
pMTL9611	5.5	pIP404 ( <i>C. perfringens</i> )	IncP mobilizable	Em <sup>R</sup>	12
pJU12	11.6	pJU121 ( <i>C. perfringens</i> )		Tc <sup>R</sup>	61
pHR106	7.9	pJU122 ( <i>C. perfringens</i> )	Replicates in <i>C. beijerinckii</i>	Tc <sup>R</sup> [Ap <sup>R</sup> ]	62
pRZE4	10.0	pJU122 ( <i>C. perfringens</i> )	Replicates in <i>C. beijerinckii</i>	Em <sup>R</sup> [Ap <sup>R</sup> ] Cm <sup>R</sup>	62
pRZL3	10.8	pJU122 ( <i>C. perfringens</i> )	Replicates in <i>C. beijerinckii</i>	Tc <sup>R</sup> [Ap <sup>R</sup> ]	62
pSYL7	9.2	pJU122 ( <i>C. perfringens</i> )		Em <sup>R</sup> Tc <sup>R</sup>	63
pBC16Δ1	2.8	pBC161 ( <i>B. cereus</i> )	Unstable in <i>C. acetobutylicum</i>	Tc <sup>R</sup>	63
pECII	4.5	pIM13 ( <i>B. subtilis</i> )	Transferred to <i>C. cellulolyticum</i>	Em <sup>R</sup>	5
pFNK1	2.4	pIM13 ( <i>B. subtilis</i> )		Em <sup>R</sup>	64
pIA		pIM13 ( <i>B. subtilis</i> )	PACYC-based vector	Em <sup>R</sup>	65
pIM13	2.3	pIM13 ( <i>B. subtilis</i> )	ssDNA replication	Em <sup>R</sup>	63
pKNT11	6.5	pIM13 ( <i>B. subtilis</i> )	General purpose cloning vector	Em <sup>R</sup> [Ap <sup>R</sup> ]	63
pKNT14	4.3	pIM13 ( <i>B. subtilis</i> )	No <i>E. coli</i> replicon	Em <sup>R</sup>	63
pKNT19	4.9	pIM13 ( <i>B. subtilis</i> )	pUC19 polylinker	Em <sup>R</sup> [Ap <sup>R</sup> ]	66
pSYL14	4.4	pIM13 ( <i>B. subtilis</i> )		Em <sup>R</sup> [Ap <sup>R</sup> ]	63
pXYLgusA		pIM13 ( <i>B. subtilis</i> )	Xylose inducible expression vector	Em <sup>R</sup> [Ap <sup>R</sup> ]	67
pAMβ1	25.5	pAMβ1 ( <i>E. faecalis</i> )	Conjugative theta replicating	Em <sup>R</sup>	36
pMTL500E	6.43	pAMβ1 ( <i>E. faecalis</i> )	Broad host range cloning vector	Em <sup>R</sup> [Ap <sup>R</sup> ]	20
pMTL500F	6.69	pAMβ1 ( <i>E. faecalis</i> )	Expression vector	Em <sup>R</sup> [Ap <sup>R</sup> ]	56
pMTL502E	7.52	pAMβ1 ( <i>E. faecalis</i> )	Low copy version of pMTL500E	Em <sup>R</sup> [Ap <sup>R</sup> ]	56
pMTL513	7.29	pAMβ1 ( <i>E. faecalis</i> )	Stability cloning vector	Em <sup>R</sup> [Ap <sup>R</sup> ]	56
pMU1328	7.5	pAMβ1 ( <i>E. faecalis</i> )	Deletion variant in <i>C. acetobutylicum</i>	Em <sup>R</sup>	56
pSYL9	8.9	pAMβ1 ( <i>E. faecalis</i> )	Broad host range cloning vector	Em <sup>R</sup> [Ap <sup>R</sup> ] Tc <sup>R</sup>	63
pVA1	11.0	pAMβ1 ( <i>E. faecalis</i> )	Broad host range cloning vector	Em <sup>R</sup>	15
pIP501	35.0	pIP501 ( <i>E. faecalis</i> )	Conjugative	Em <sup>R</sup> Cm <sup>R</sup>	37
pGK12	4.4	pWV01 ( <i>L. lactis</i> )	Very broad host range vector	Em <sup>R</sup> Cm <sup>R</sup>	68
pT127	4.4	pT127 ( <i>S. aureus</i> )	Unstable in <i>C. acetobutylicum</i>	Tc <sup>R</sup>	63
pMK419	5.6	pUB110 ( <i>S. aureus</i> )	Transferred to <i>C. thermocellum</i>	Cm <sup>R</sup> [Ap <sup>R</sup> ]	17
pUB110	4.5	pUB110 ( <i>S. aureus</i> )	ssDNA replication, thermostable	Km <sup>R</sup>	19
pMTL30/31	4.36	None	IncP mobilizable integration vector	Em <sup>R</sup> [Ap <sup>R</sup> ]	10

<sup>a</sup> Progenitor plasmid and host from which it was originally isolated.

<sup>b</sup> Em<sup>R</sup>, erythromycin resistance; Tc<sup>R</sup>, tetracycline resistance; Cm<sup>R</sup>, chloramphenicol/thiamphenicol resistance; Km<sup>R</sup>, kanamycin resistance; [Ap<sup>R</sup>], ampicillin resistance in Gram-negative intermediate.

### 3.5 CIRCUMVENTION OF RESTRICTION BARRIERS

As indicated in Section 3.3, in many instances, the successful transfer of extrachromosomal elements, either by transformation or conjugation, has required the circumvention of the activity of endogenous restriction-modification (RM) systems. This is achieved through appropriate methylation of the vector DNA to be introduced. Organisms for which such a strategy has proven necessary include *C. acetobutylicum* ATCC 824 [21], *C. cellulolyticum* ATCC 35319 [5], *C. botulinum* ATCC 25765 [13], and *C. difficile* CD3 and CD6 [12]. In all cases, restriction activity was initially detected in bacterial lysates, after which the restriction and the methylation specificity of the RM system was determined. As more and more genome sequences become available, gene transfer strategies may be based on the characterization of cloned methylase genes identified by *in silico* approaches [70].

To isolate restriction enzyme activities, cell wall and associated nonspecific nucleases are enzymatically (lysozyme) removed in isotonic buffer before releasing the contents of the protoplasts formed by osmotic shock. A typical procedure, exemplified by the methodology employed in *C. difficile* [12], is as follows: Organisms are grown in 50 ml BHIB to an  $OD_{600} = 0.4$  to  $0.6$ , whereupon cells are harvested by centrifugation at  $6000\text{ g}$  for  $5\text{ min}$  and resuspended in  $2\text{ ml}$  of  $0.5\text{ M}$  sucrose/ $0.05\text{ M}$  Na phosphate buffer,  $\text{pH}7.0$  containing lysozyme ( $10\text{ mg ml}^{-1}$ ). This cell suspension is held anaerobically at  $37^\circ\text{C}$  for  $1$  to  $4\text{ h}$ , during which protoplast formation occurs. Cells may be monitored under a light microscope following staining with safranin red and harvested by centrifugation once  $>90\%$  of the cells are deemed to have formed protoplasts. The resulting pellets are resuspended in  $500\text{ }\mu\text{l}$  of  $0.05\text{ M}$  Na phosphate buffer,  $\text{pH}7.0$ , and lysis allowed to proceed for  $5$  to  $10\text{ min}$  at room temperature. The cell debris may then be removed by centrifugation, and the resulting lysate stored at  $4^\circ\text{C}$ . Aliquots of a lysate ( $2$  to  $8\text{ }\mu\text{l}$ ) are incubated with  $0.5\text{ }\mu\text{g}$  of DNA of a plasmid of known nucleotide sequence in a range of restriction enzyme buffers for  $4\text{ h}$  at  $37^\circ\text{C}$ , in a final volume of  $20\text{ }\mu\text{l}$ , and the reaction products analyzed by agarose gel electrophoresis.

Using this type of approach, it has invariably proven possible to identify the point at which the marker plasmid is cleaved, most simply by comparison with the fragmentation pattern observed using commercially available restriction enzymes. Novel specificities require a more detailed analysis, in which fragmented DNA may be directly sequenced, or cloned and sequenced thereafter [12]. Having determined the specificity of the restriction enzyme, it is necessary to determine the methylation specificity of the RM system involved. This involves testing the ability of available methylase enzymes known to act on the identified recognition sequence. However, as the majority of clostridial methylases appear cytosine-specific, the methylation point may be determined directly, using a modification of the method of Feil et al. [71].

Approximately  $2\text{ }\mu\text{g}$  of linearized plasmid DNA isolated from the strain under investigation is suspended in  $100\text{ }\mu\text{l}$  of deionized water in a siliconized,  $1.5\text{-ml}$  eppendorf tube and then denatured by adding  $11\text{ }\mu\text{l}$  of  $3\text{ M}$  NaOH and incubating at  $37^\circ\text{C}$  for  $20\text{ min}$ . The tube is placed on ice, and  $1.1\text{ ml}$  of  $3.5\text{ M}$   $\text{NaHSO}_3$ / $1\text{ mM}$  hydroquinone,  $\text{pH} 5.0$ , is added. The solution is then overlaid with  $150\text{ }\mu\text{l}$  of mineral oil and incubated in the dark for  $24\text{ h}$  at  $0^\circ\text{C}$ . The sample is removed from beneath the mineral oil and transferred to a  $1.5\text{-ml}$  siliconized eppendorf tube where the DNA is extracted using a GeneClean II kit (Strattech Scientific Ltd.) for  $30\text{ min}$  at  $4^\circ\text{C}$ . The precipitated DNA is resuspended in  $100\text{ }\mu\text{l}$  of deionized water and desulfonated by adding  $11\text{ }\mu\text{l}$  of  $2\text{ M}$  NaOH and incubating for  $10\text{ min}$  at  $20^\circ\text{C}$ . This treatment converts all unmethylated cytosine bases to uracil. The modified DNA is precipitated by adding  $5\text{ M}$  ammonium acetate,  $\text{pH} 7.0$ , to a final concentration of  $3\text{ M}$ , and  $3$  volumes of ethanol. Following resuspension in  $100\text{ }\mu\text{l}$  of deionized water, a  $4\text{ }\mu\text{l}$  sample is used as a template in a PCR reaction using "modified" primers, which can only anneal if all of the targeted cytosine residues have been converted to uracil. The amplified region is then cloned and sequenced. Any cytosines present in the sequence obtained were methylated in the original DNA.

The application of this approach is reliant on being able to PCR-amplify a known sequence from the organism under investigation, which is likely (i.e., GC-rich DNA in an AT-rich organism),

or experimentally known, to be cleaved by the endogenous enzyme. In the study of [13], this was made possible by the fact that a relatively GC-rich, foreign plasmid element (pGK12) could be transformed into *C. botulinum* at low efficiencies. In other cases, where transformation cannot be demonstrated, known genomic sequences may be targeted. Alternatively, it may prove possible to introduce foreign DNA using either a conjugative plasmid or a conjugative transposon. In this instance, the delivered element may be engineered to contain GC-rich DNA, either through the simple insertion of appropriate DNA fragments or by cointegration of a suitable plasmid vector with the conjugative element [47,72].

Prevention of DNA transfer by host RM systems is highly strain-specific. Indeed, there are many instances where restriction has not been a problem e.g., *C. beijerinckii* NCIMB 8052 [21], *C. perfringens* strain 13 [73], *C. difficile* strains CD37 [12] and CD630 [70], and *C. botulinum* ATCC 3502 [16]. Genome sequencing has shown that many of these organisms carry at least one type II methylase gene (most often more than one), but they lack genes encoding the cognate restriction enzymes. Thus, the recently completed genomes of *C. botulinum* ATCC 3502 and *C. perfringens* contain orphan copies of methylase genes (three and one, respectively), and are both readily transformable in the absence of any measures to circumvent restriction barriers [16,73]. In the case of *C. acetobutylicum*, a total of six methylase genes are present, two of which have an adjacent gene encoding a restriction enzyme; *C. thermocellum* has seven methylase genes, but only three restriction enzyme genes; while *C. tetani* has three methylase genes and a single restriction enzyme gene. The latter two organisms have yet to be transformed. On the other hand, *C. acetobutylicum* requires that the incoming DNA be protected from the activity of just one restriction endonuclease (*Cac824I*) for successful DNA transfer [21]. The circumvention of a single restriction activity is similarly required for transformation of *C. botulinum* ATCC 25765 [13], *C. cellulolyticum* [5], and *C. difficile* CD3 [12]. It now seems likely that the majority of type II methylase genes in clostridia plays no role in restriction/modification. To date, the potential effects of type I restriction systems on gene transfer in *Clostridium* spp. have not been analyzed.

### 3.6 AVAILABLE PLASMID CLONING VECTORS

Over the past two decades, genetic manipulation of several clostridia has been achieved, and a number of vectors are now available for use in these organisms. Despite this, the genus as a whole remains relatively recalcitrant to “laboratory based” genetic transfer, and the use of more genetically amenable organisms such as *E. coli* and *B. subtilis* as intermediates is necessary for undertaking plasmid constructions. While the available vectors are almost exclusively based on replicons derived from native clostridial plasmids, or from plasmids found in other Gram-positive hosts, they must also carry a second replication region, such as that of ColE1, which facilitates their maintenance in the intermediate host (normally *E. coli*).

#### 3.6.1 NATIVE PLASMIDS

Plasmids are widespread among the clostridia. Most remain cryptic, but in some cases functions have been ascribed. For example, pSOL1 of *C. acetobutylicum* ATCC 824 is a megaplasmid that encodes the genes needed for the production of acetone and butanol by this organism [74]. In addition, some important virulence factors of several pathogenic clostridia are plasmid-encoded, including the tetanus toxin of *C. tetani* [75], type G neurotoxin of certain strains of *C. botulinum* [76], and several of the toxins produced by some strains of *C. perfringens* [77].

#### 3.6.2 SHUTTLE VECTORS

The naturally occurring plasmids represent the most obvious candidates for use in the development of clostridial shuttle vectors, and it is not therefore surprising to find that several have been

extensively characterized, including pIP404 from *C. perfringens* [78], pCB101 and pCB102 from *C. butyricum* [79], and, more recently, pCD6 from *C. difficile* [12]. As a result, a number of clostridial replicons have been identified and analyzed. The replication regions of pCB101 and pIP404 were identified by their ability to promote the stable maintenance of an otherwise nonreplicative vector in *B. subtilis* [80,81], while *C. beijerinckii* NCIMB 8052 was used to identify the replication regions of pCB102 [56] and pCD6 [12]. In addition, the use of sequence databases proved a valuable tool in predicting the location of the pCD6 replicon.

The replicon of pCB101 exhibits many of the characteristic features of plasmids that replicate via a rolling circle mechanism. One ORF is present, which shares homology with the replication proteins of other plasmids from Gram-positive bacteria that use this mode of replication [82]. The replication region of pCB102, on the other hand, shares no similarity at either the DNA or the protein level with any other characterized plasmid, and as such, the mechanism by which this plasmid replicates remains unknown. Likewise, it is not clear how pCD6 and pIP404 replicate. They do, however, possess several shared features, such as two large, distantly related, putative replication proteins and an extensive region of DNA repeats, which is often associated with plasmids that replicate by a theta mechanism [12]. In spite of our rudimentary understanding of the replication mechanisms of several of these plasmids, they have all been used successfully to develop clostridial cloning vectors (see Table 3.1).

An alternative strategy for vector construction uses replicons derived from other Gram-positive organisms. Plasmids such as pWV01 from *Lactococcus lactis* [83], pIM13 from *B. subtilis* [63], and pUB110 from *Staphylococcus aureus* [19] are good examples. The lack of specificity of the replication and partitioning systems of these particular plasmids, as well as the functionality of some of their associated antibiotic-resistance genes in clostridia, has permitted the development of many of the cloning vectors currently in use (see Table 3.1). Although many of these nonclostridial plasmids appear able to replicate in *Clostridium* spp. [5,10], none of them are segregationally stable (i.e., they tend to be lost in the absence of positive selection for bacteria that maintain them). With the notable exception of pAM $\beta$ 1, which replicates via a unidirectional theta mechanism, the majority of these plasmids replicate using a rolling circle mechanism, involving a single stranded DNA (ssDNA) intermediate [84]. The highly recombinogenic ssDNA molecules may account for the structural instability of several of the recombinant vectors that have been constructed [56].

In addition to the creation of basic shuttle vectors, a number of specialized plasmids have been constructed, including expression vectors. These have been used to bring about heterologous gene expression, and of antisense RNA expression, in a number of species, including *C. acetobutylicum* [85–87] and *C. tetani* [88]. pMTL500F and pMTL540F are two examples of versatile expression vectors for use in clostridia. The former is based on the pAM $\beta$ 1 replicon, while the latter utilizes the pCB102 replication region. Both carry an expression cassette that contains the promoter region of the *C. pasteurianum* ferredoxin (Fd) gene. They have been used in a number of situations, most prominently to bring about the production of prodrug converting enzymes useful in cancer therapy [56,57,89]. More recently, an inducible expression vector, pXYLgusA, which utilizes *xylR* and the *xylA* promoter-operator regions from the xylose operon of *Staphylococcus xylosus*, has been tested in *C. acetobutylicum* ATCC 824. This expression vector carries the *B. subtilis* pIM13 replication region and reportedly leads to a 17-fold increase in reporter gene activity when xylose, which functions as the inducer, is added to the system [67]. A more detailed description of such vectors, and other specialized vectors, is presented in other chapters in this volume.

### 3.7 CONCLUDING REMARKS

The basic gene transfer technologies described in this chapter have underpinned the recent development of strategies for targeted gene disruption via allelic replacement, generation of unmarked deletions, and antisense regulation of genes in several species of *Clostridium*. These approaches are documented elsewhere in this volume. They will permit exploration of the biological roles of

the multitude of genes, whose presence has been revealed by genome sequencing projects, as well as the exploration of problems of fundamental biological interest, such as the contributions made by individual genes to metabolic regulation in response to environmental change. Nevertheless, considerable ingenuity will be required to perfect and further refine the tools already available for the small number of clostridia that we can now manipulate.

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