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# Development of a Transformation and Gene Reporter System for Group II, Non-Proteolytic *Clostridium botulinum* Type B Strains

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**Abstract**

**Non-proteolytic, Group II strains of *Clostridium botulinum* are of particular concern to the food industry because of their ability to survive and grow in REPFEDs (refrigerated processed foods of extended durability). Their analysis would benefit from the availability of a gene transfer system. In the present study we have been able, for the first time, to demonstrate transformation in a representative Group II strain, ATCC 25765. Initial attempts to transform ATCC 25765 with existing clostridial cloning vectors (pMTL540E and pMTL500E) were, however, prevented by a restriction barrier. Through a combination of classical and molecular approaches we were able to show that strain ATCC 25765 possesses a restriction endonuclease (*Cbo*I) and a methylase activity (M.*Cbo*I) which have the same specificity as *Msp*I and M.*MspI*, respectively. *Cbo*I cleaves the palindrome 5'-CCGG-3' to generate a 3'-GC sticky end, whilst M.*Cbo*I specifically methylates the external C residue. An *E. coli* host was generated which expressed a *Bacillus subtilis* methylase enzyme (M.*Bsu*F1) with equivalent specificity to M.*Cbo*I. Plasmids (pMTL540E and pMTL500E) prepared in this strain were subsequently shown to be capable of transforming ATCC 25765. The highest frequencies (0.8 X $10^4$ transformants per μg of DNA) were obtained when cells were cultivated in media supplemented with 1% (w/v) glycine, and when the electroporation was undertaken at 10 kV/cm, 25 μF and at 400 Ω. Having developed an effective transformation procedure, we went on to construct reporter cassettes based on the *Therman-aerobacterium sulfurigenes lacZ* and the *Vibrio fischeri luxAB* genes. Using the former, and promoter regions isolated from the botulinum toxin genes, we have obtained preliminary evidence that reporter genes may be used to evaluate the physiological factors that affect toxin production in the food environment.**

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## Introduction

Clostridia capable of producing botulinum neurotoxin (BoNT) are widely distributed in nature (Hatheway *et al*., 1992). Their presence in the food environment is, therefore, not uncommon. The consequent risk of botulism is countered either by heat killing of any organisms present (cooking), or by preventing bacterial growth. Current preventative measures rely on a lowering of aω, a reduction in pH or maintaining food at a temperature below that at which clostridia can grow. Equally important is an appreciation of the physiological factors which promote/diminish toxin production, and their relationship to growth. In all cases, BoNT is released from bacterial cells in association with other non-toxic proteins, the resultant toxin complex ranging in size from 300 to 900 kDa (Hatheway *et al*., 1992). These associated proteins afford BoNT protection from the extreme environments encountered in food, and in the gut when ingested, and are therefore crucial to BoNT potency. Toxin complex size and constituents, and therefore potency, appear to be influenced by the same physiological conditions which regulate production (Schantz and Johnson, 1992). These factors are little understood, but undoubtedly act at the level of transcription through complex regulatory cascades composed of alternate sigma factors and so-called two-component systems (Hoch and Silhavy, 1995).

Previous studies using cultured cells have shown that important factors affecting both toxin complex production and constituents include metal ion levels and the nature and concentration of exogenous carbon and nitrogen (Patterson-Curtis and Johnson, 1989). Determination of these factors is, however, hindered by reliance on the mouse lethality assay for estimating levels of toxin. This could be overcome through the exploitation of reporter gene technology. The application of reporter gene technology, however, requires the availability of gene transfer systems. When considering the development of such systems it is important to realise that *C. botulinum* is not a homogenous assemblage (Hatheway *et al*., 1992; Hutson *et al*., 1994). Cases of human botulism arising through food contamination are limited to intoxification by types A, B, E and F BoNT. Types A, B and F are elaborated by proteolytic Gp I *C. botulinum* strains, which, aside from their ability to produce BoNT, are indistinguishable from *C. sporogenes*. Gp II, non-proteolytic *C. botulinum* strains are physiologically and phylogenetically distinct from Gp I strains and elaborate type B, E and F BoNT. The successful transformation of a representative of the Gp I family has been reported (Zhou and Johnson, 1993). However, of the two groupings, the Gp II strains are of particular concern to the food industry because of their ability to survive and grow in REPFEDs (refrigerated processed foods of extended durability) (ACMSF report, 1992).

It was the purpose of this present study to derive a reporter system for Gp II *C. botulinum* strains which could be used to better understand the physiological factors that affect toxin production. This has necessitated the derivation of a gene transfer system for these organisms based on electroporation.

## Results

### Initial Transformation Experiments

Initial transformation experiments were undertaken utilising the method previously developed for the electroporation of *Clostridium beijerinckii* NCIMB 8052 (Oultram *et al.*, 1988a), the clostridial shuttle vectors pMTL500E (Swinfield *et al.*, 1990) and pMTL540E (Fox *et al.*, 1996), the broad host range plasmid pGK12 (Kok *et al.*, 1984), and the *C. botulinum* hosts ATCC 25765, BL229 and BL151. Electroporation was undertaken with 0.8 ml cell volumes in 0.4 cm cuvettes, at 2.5 kV (field strength 6.25 kV/cm) 25 µF and a resistance of 100 Ω. However, in no instance were any cells obtained which were able to form colonies on agar media supplemented with erythromycin (Em). Thereafter, the procedure was modified such that relatively high cell densities ($10^9$ to $10^{10}$ per cuvette) were employed and the electroporator was sited within the anaerobic cabinet. Under these conditions a small number (1-10) of Em resistant ($^R$) colonies were invariably obtained from the strains ATCC 25765 and BL229 with plasmid pGK12, but no such colonies arose when either pMTL500E or pMTL540E was employed (Table 1). Because it is difficult to isolate plasmid DNA from clostridial cells, to confirm that the colonies obtained were true transformants, crude cell lysates were prepared and used to transform *E. coli* to $Em^R$ . Thereafter, *E. coli* transformants were subjected to a small scale plasmid purification procedure, and the plasmid isolated shown to possess the same restriction fragment profile as pGK12 when cleaved with *Mbo*I and *Xmn*I and electrophoresed on an agarose gel.

Having obtained transformation, an attempt was made to try and optimise the efficiency of transformation, through the introduction of a number of variations to the protocol. The changes evaluated included: (i) the addition of the autolysin inhibitor polyanetholsulfonic acid (Sigma, at a concentration of 1 mg/ml) to the electroporation buffer; (ii) the inclusion of the buffering agent calcium carbonate in the recovery media to compensate for acetic and butyric acid production, and; (iii) the growth of the organisms in media containing 2.3% glycine prior to the preparation of "competent" cells. No significant increase in transformation frequency was noted following the introduction of any of these changes. As the production of non-specific, extracellular nucleases can affect transformation frequencies (Blaschek and Klacik, 1984), the relative production of such an activity by the three *C. botulinum* strains was compared to two other clostridial strains which are relatively efficiently transformed, *C. beijerinckii* NCIMB 8052, and the *C. botulinum* strain, ATCC 3502. This was accomplished by growth on DNAse agar and subsequent precipitation of the remaining unhydrolysed DNA with 1M HCl. Both of the strains ATCC 25765 and BL229 appeared to produce equivalent quantities of extracellular nuclease as the reference strains, *C. botulinum* ATCC 3502 (Type A) and *C. beijerinckii* NCIMB 8052. Interestingly, the *C. botulinum* strain which could not be transformed by pGK12, strain BL151, clearly produced higher levels of extracellular nuclease.

In addition, as *Clostridium* are known to possess endonuclease restriction-modification systems (Young *et al.*, 1989), plasmid DNA was isolated from a number of sources, including an ATCC 25765 transformant, two group I *C. botulinum* strains (NCTC 2916 and ATCC 3502) which had been transformed with pGK12 by the method of Zhou and Johnson (1993), and *E. coli* strains (Top10, GM2163 and HB101) with a range of DNA-methylation specificities. Transformation efficiency remained poor and inconsistent. Plasmid pGK12 DNA isolated from the *E. coli* strain GM2163 and *C. botulinum* ATCC 25765 proved to give the 'highest' and most reliable transformation frequencies, with between 10 and 20 transformants per µg of DNA being repeatedly obtained (Table 1).

Table 1. Electroporation Frequencies of *C. botulinum* ATCC 25765

| Plasmid | Protocol[a] | Unmethylated | Methylated[b] *in vitro* | Methylated[b] *in vivo* |
|---|---|---|---|---|
| pGK12 | I | 0.5 X10$^1$ | 1.0 X 10$^2$ | 0.9 x 10$^3$ |
| pMTL500E | I | 0 | 0.6 X 10$^1$ | 1.1 X 10$^2$ |
| pMTL540E | I | 0 | 0.9 X 10$^2$ | 0.8 X 10$^3$ |
| pMTL540E | II | ND | ND | 2.5 X 10$^3$ |
| pMTL540E | III | ND | ND | 8.1 X 10$^4$ |

[a] the original conditions (I) were 0.8 ml cell volumes in 0.4 cm cuvettes, at 2.5 kV (field strength 6.25 kV/cm) 25 µF and a resistance of 100 Ω. In protocol II the same electroporation conditions were employed, but cells were grown in media containing 1% (w/v) glycine prior to electroporation. In protocol III, cells were grown in the presence of glycine, but the electroporation conditions were altered to 2.0 kV (field strength 10 kV/cm) 25 µF and a resistance of 400 Ω.
[b] for *in vitro* methylation, plasmid DNA was treated with M.MspI methylase prior to transformation. *In vivo* methylation of DNA was achieved by preparing the plasmid DNA in an *E. coli* host carrying a gene encoding the *B. subtilis* M.*Bsu*F1 methylase.
ND = not determined.

### Determination of the Restriction Profiles

Having derived some indication that restriction of DNA may be important in determining the transformation frequency we elected to characterise any endogenous activity in more detail. Accordingly, attempts were made to prepare lysates of a number of *C. botulinum* Group II strains (ATCC 25765, BL229, BL151 and BL194) and to assay them for specific restriction activities using purified pGK12 and pMTL540E plasmid DNA as a substrate. Pilot experiments to determine the conditions required to form protoplasts established that lysozyme-induced (Hen egg white) protoplasts could be formed using only strain ATCC 25765. A crude extract (1-2 µl) from this strain was incubated at both 37°C and 50°C with substrate plasmid DNA in a range of commercial restriction endonuclease buffers; NBL reaction buffers 1-11 and BCL reaction buffers A, B, L, M and H. Samples were incubated for 2 and 16 hr before analysing the results by agarose gel electrophoresis against uncut plasmid DNA standards. After 2 hr of incubation at either temperature, a high degree of fragmentation was evident in pMTL540E DNA, consistent with the presence of a large number of restriction recognition sites in this plasmid. These profiles were clear cut after a 16 hr incubation (Figure 1).

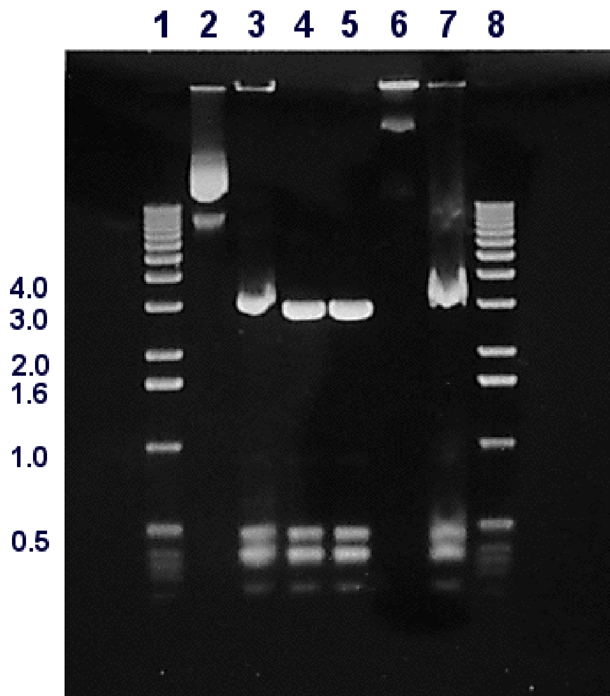


Figure 1. Restriction/Methylase Specificity of ATCC 25765
Plasmid pMTL540E DNA was used as the substrate in all digestions. DNA kilobase markers (lane 1 and 8), untreated pMTL540E DNA (lane 2), pMTL540E DNA treated with ATCC 25765 lysate (lane 3), pMTL540E cut with *Msp*I (lane 4), pMTL540E cut with *Hpa*II (lane 5), pMTL540E treated with M.*Msp*I and then ATCC 25765 lysate (lane 6) and pMTL540E treated with M.*Hpa*II and then ATCC 25765 lysate (lane 7).

The approximate sizes of the fragments produced from pMTL540E DNA were compared with those predicted by computer analysis (DNASTAR Inc.) for all known restriction endonuclease recognition sequences. These data suggested that if a single restriction endonuclease activity was present, it was likely to recognise a four bp site consisting of either 5'-CCGG-3', 5'-GGCC-3', 5'-GCGC-3' or 5'-CGCG-3'. Digestion of pGK12 plasmid DNA with the crude extract from strain ATCC 25765 resulted in a simple linearisation of the plasmid, consistent with the presence of a single recognition sequence. This suggested that the targeted restriction site was 5'-CCGG-3', and was therefore of an equivalent sequence to that recognised by the commercially available enzymes *Hpa*II or *Msp*I (Roberts and Macelis, 1997). This assumption was supported by the observation that cleavage of pGK12 and pMTL540E with ATCC 25765 lysate gave identical restriction profiles to that obtained when either *Hpa*II or *Msp*I were employed (Figure 1).

**Endonuclease Cleavage Specificity**

The cleavage of the recognition sequence 5'-CCGG-3' by a type II restriction endonuclease can produce a number of different types of 5'- or 3'- overhangs. These overhangs may be compatible with the sticky ends generated by cleavage of the various unique restriction enzyme cloning sites present on the vector pMTL20 (Chambers *et al.*, 1988). To establish if this was the case, plasmid pJIR418 DNA (Sloan *et al.*, 1992) was incubated with the ATCC 25765 crude lysate and the DNA fragments generated isolated. These were then individually ligated with pMTL20 DNA which had been cleaved with a number of different restriction enzymes. The resulting ligation mixes were transformed into an appropriate *E. coli* host, and the number of recombinants obtained assessed through the appearance of white transformant colonies on agar supplemented with XGal. Clones were only obtained when pMTL20 had been cleaved with *Cla*I.

The enzyme *Cla*I cleaves the recognition sequence 5'-ATCGAT-3' to produce a 3'-GC sticky end. An identical overhang is produced following cleavage of the recognition sequence 5'-CCGG-3' by *Hpa*II and *Msp*I. Therefore, it was concluded that restriction endonuclease produced by ATCC 25765 has the same specificity as *Hpa*II and *Msp*I, namely it cleaves 5'-CCGG-3' to produce a 3'-GC overhang. Confirmation of the identity of the sticky end produced was obtained by sequencing the pMTL20::pJIR418 clones.

**Characterisation of the Methylation Specificity of ATCC 25765**

Both *Hpa*II and *Msp*I endonucleases have an equivalent methylase which covalently modifies the 5'-CCGG-3' palindrome by attaching a methyl residue at the internal and external cytosine residues, respectively, at the $C^5$ position (Roberts and Macelis, 1997). These two methylases (M.*Hpa*II and M.*Msp*I) were purchased from New England Biolabs and used to *in vitro* methylate both pGK12 and pMTL540E plasmid DNA. Treated samples were purified and subjected to a 16 hr incubation with the ATCC 25765 crude extract, *Hpa*II and *Msp*I in combination with unmethylated control plasmids. Subsequent analysis of the DNA revealed that *in vitro* methylation with M.*Msp*I had completely protected pGK12 and pMTL540E from digestion with the ATCC 25765 crude lysate (Figure 1). In contrast, *in vitro* methylation with M.*Hpa*II had only afforded partial protection. The commercial restriction endonucleases *Hpa*I and *Msp*I had failed to digest their respective methylated DNA controls, demonstrating that the methylation process was complete (data not shown).

These results suggest that the endonuclease present in ATCC 25765 possesses the same specificity as the *Msp*I-M.*Msp*I system. Thus, methylation probably occurs at the external cytosine residue 5'-<u>C</u>CGG-3'. Confirmation that the restriction-modification system of ATCC 25765 is analogous to the *Msp*I-M.*Msp*I system, and not the *Hpa*II-M.*Hpa*II system, was obtained by showing that genomic DNA isolated from ATCC 25765 is fragmented by *Hpa*II, but remains unaffected by digestion with *Msp*I (data not shown).

Definitive data to support this conclusion was obtained using a procedure previously developed to map methylation sites in eukaryotic DNA (Feil *et al.*, 1994). In this procedure DNA is treated with sodium bisulphite, whereupon every cytosine residue is converted to uracil unless it is otherwise protected by methylation. Thus, pGK12 plasmid DNA was isolated from a transformed cell of ATCC 25765 and subjected to sodium bisulphite treatment. DNA primers were then designed capable of amplifying a 900 bp region of the plasmid known to encompass two *Hpa*II sites. These primers were designed to take into account the predicted mutagenic changes. Indeed, they were shown to be incapable of amplifying the desired fragment from untreated pGK12 DNA, with a 900 bp fragment only being obtained

following sodium bisulphite treatment. The fragment obtained in this latter PCR was cloned and its entire nucleotide sequence determined. The sequence obtained was found to be absolutely devoid of cytosine residues, with the exception of the external “C” of the two 5'-CCGG-3' palindromes.

**Effect of *Msp* I Methylation on Transformation**

To test the effect of appropriate methylation on transformation frequencies, plasmid DNA was subjected to *in vitro* methylation with M.*Msp*I, purified and then introduced into *C. botulinum* ATCC 25765 by electroporation. In the initial experiments, the plasmid DNA employed was that of pGK12. The number of transformants routinely obtained using the treated DNA was increased by greater than 10-fold, with frequencies of $10^1$-$10^2$ transformants per µg plasmid DNA being consistently obtained (Table 1). Furthermore, following their *in vitro* methylation, it now proved possible to transform this strain with both pMTL540E and pMTL500E plasmid DNA (Table 1). However, whereas plasmid pMTL540E transformed at equivalent frequencies to pGK12, the number of transformants obtained with plasmid pMTL500E was significantly lower.

The necessity of pre-treating plasmid DNA with purified methylase enzyme would be circumvented if the plasmid could be prepared in an *E. coli* host which possessed the requisite methylase activity. The gene encoding M.*Bsu*F1, an enzyme with equivalent specificity to M.*Msp*I, has previously been cloned from *Bacillus subtilis* (Walter *et al*., 1990). Therefore, a 3kb *Eco*RI fragment carrying this gene was isolated from pBW20 (Walters *et al*., 1990), cloned into pMTL23 (Chambers *et al*., 1988), re-isolated as an equivalent sized *Hin*dIII-*Stu*I fragment, inserted between the *Hin*dIII and *Eco*RV sites of plasmid pACYC184 (Rose, 1988) and introduced into the methylase deficient *E. coli* host Top10. The plasmid generated was designated pBSU1. As plasmid pBSU1 is based on the p15A replicon (Rose, 1988), it is compatible with both ColE1 based vectors, such as pMTL540E and pMTL500E, and with pGK12. Accordingly, these plasmids were transformed into Top10 harbouring pBSU1 where they were shown to be completely protected from subsequent digestion with *Msp*I. A purified large-scale pMTL540E plasmid preparation (1.4 mg/ml) was then made from this strain and used in transformation experiments with ATCC 25765. Transformants were now obtained in the order of $10^3$ transformants per µg of DNA (Table 1).

**Optimisation of Transformation Frequencies**

Having prepared appropriately *in vivo* methylated pMTL540E DNA, electroporation parameters were re-examined to establish the optimum conditions for transformation. In the initial experiments the effect of culturing the organisms prior to electroporation in media containing glycine was re-assessed. These experiments demonstrated that under standard electroporation conditions (2.5 kV, 25 µF and at 100 Ω) prior growth in the presence of 1% (w/v) glycine resulted in an approximate 3-fold increase in electroporation frequencies. Thereafter, the effect of field strength on electroporation was examined. These experiments indicated that field strengths of between 6.25 kV and 10 kV were required for efficient transformation. Finally, the effect of resistance was evaluated. The data obtained revealed that the highest frequencies, at field strengths of both 5.0 kV and 10 kV, occurred at a resistance of 400 Ω, with 1.2 X$10^3$ and 8.1 X $10^3$ transformants per µg DNA, respectively. The final electroporation procedure was therefore adapted to reflect these results. Thus, cells were always cultured in TPGY broth supplemented with 1% glycine, and electroporation was undertaken in 0.2 cm cuvettes at 2.0 kV (10.00 kV/cm field strength) at a resistance of 400 Ω. Under these conditions we routinely obtain frequencies of between $10^3$ and $10^4$ transformants per µg of DNA.

**Construction of a β-Galactosidase Gene Reporter Cassette**

In other bacterial species, genes encoding β-galactosidase have proven to be of great utility as reporters of gene expression, as the enzyme has a simple, inexpensive assay and colourimetric reagents are widely available for phenotypic detection. Moreover, a *lacZ* gene isolated from *Thermanaerobacterium sulfurigenes* (formerly known as *Clostridium thermosulfurogenes*) by Burchhardt and Bahl (1991) has been shown to be a useful reporter of gene expression in *Clostridium acetobutylicum* ATCC 824 (Tumala *et al*., 1999). We therefore elected to base our initial studies on this gene.

Before initiating the construction of the β-galactosidase cassette, confirmatory experiments were undertaken to establish that *C. botulinum* ATCC 25765 did not produce an endogenous activity. Accordingly, ATCC 25765 was cultivated in TPGY media and assays for of β-galactosidase activity undertaken on cells harvested from the mid and late exponential phase of growth, and from cultures which had entered stationary phase. No such activity was detected. Thereafter, reporter plasmid, pMTL542E, was constructed as described in the Experimental Procedures section (see Figure 2). This plasmid essentially consists of a reporter cassette inserted into the shuttle vector pMTL540E (Fox *et al*., 1996). The reporter cassette is composed of a promoter-less copy of the *T. sulfurigenes lacZ* gene flanked at its 3'-end by the transcription termination signals of the *Clostridium pasteurianum* ferredoxin gene (Graves and Rabinowitz, 1986), and at its 5'-end by the transcriptional terminator of the *Clostridium thermocellum celA* gene (Béguin *et al*., 1985). The cassette was designed such that a unique *Xho*I site was positioned between the *celA* terminator and the ribosome binding site (RBS) of the *lacZ* gene into which the botulinum toxin-derived promoter signals could be inserted.

The BoNT gene of all strains examined to date is transcribed as part of a bicistronic message encoding both the nontoxic nonhaemagglutinating protein (NTNH) and the BoNT, where the promoter responsible immediately precedes the NTNH gene (Henderson *et al*., 1997). In addition, in strains such as the ATCC 25765, a second operon is present encoding the haemagglutinating proteins. These are counter-transcribed relative to the NTNH/BoNT operon from a second promoter. The two promoters share a high degree of sequence homology (Henderson *et al*., 1997). In certain strains, for example *C. botulinum* type C strains, these two promoter elements lie immediately adjacent to one another (see Henderson *et al*., 1997). In ATCC 25765, however, the two promoters are separated by an open reading frame encoding a transcriptional factor (*orfX*/P22, see Henderson *et al*., 1997) now known to be a

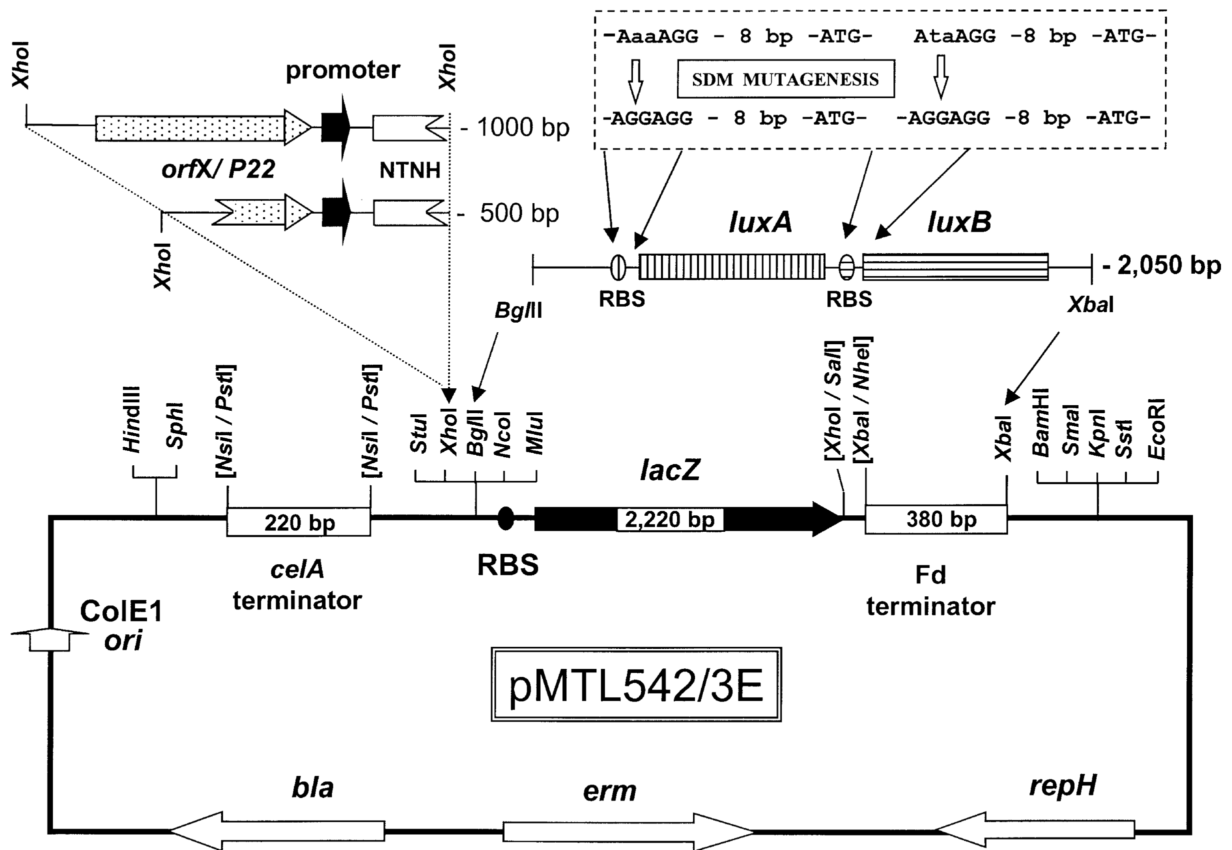


Figure 2. Construction of Reporter Plasmids pMTL542E and pMTL543E
The derivation and source of all fragments is described in the Experimental Procedures section. Plasmid pMTL542E is essentially pMTL540E into which has been inserted the *celA* and Fd terminator and the *T. sulfurigenes lacZ* gene. Plasmid pMTL543E was derived from pMTL542E by substitution of *lacZ* and the Fd terminator with a modified, promoter-less *luxAB* operon. Plasmid pMTL540E carries the ColE1 replication origin, the pBR322 ampicillin resistance gene (*bla*), the pAMβ1 $Em^R$ gene (*erm*) and the replicon (*repH*) of the *C. butyricum* plasmid pCB102 (see Fox *et al.,* 1996). Insertion of the 500 bp fragment carrying just the NHHT/BoNT/B promoter in pMTL542E and pMTL543E generated plasmids pTOM42 and pTOM43. Insertion of the 1000 bp fragment additionally carrying the transcriptional regulator encoded by *orfX*/P22 yielded pTOM42R and pTOM43R, respectively.

positive regulator of the two operons (Marvaud *et al.,* 1998). Bearing this in mind, two separate *Xho*I restriction fragments were isolated from ATCC 25765. The one fragment encompassed only the promoter region upstream of the NTNH gene, the other additionally carried *orfX*/P22. Both fragments were then inserted into the unique *Xho*I fragment orientated to transcribe the *lacZ* gene.

**Construction of a Luciferase Gene Reporter Cassette**
In addition to *lacZ*, we also chose to examine the possibility of using the *Vibrio fischeri* luciferase genes, *luxAB* (Foran and Brown, 1988) as the reporter system. Such a system would have some advantage over β-galactosidase as assays for luciferase do not require cell breakage and are extremely unlikely to be compromised either by endogenous activity or the existence of contaminating activities (Meighen, 1991). Furthermore, a modified *luxAB* cassette has previously been employed to monitor transcriptional activity in *Clostridium perfringens* (Phillips-Jones, 1993).

Accordingly, the *luxAB* genes (Foran and Brown, 1988) were PCR amplified from the *V. fischeri* ATCC 7744 genome and cloned. The primers utilised, in addition to incorporating unique flanking restriction sites for subsequent cloning steps, were also designed to alter the RBS preceding the *luxA* gene such that it more closely matched that of clostridial genes (Young *et al*., 1989). Subsequently, the *luxB* RBS was also altered via SDM using a complementary oligonucleotide approach (Stratagene Quick Change Mutagenesis kit) (Figure 2). Positive clones were selected on the basis of the ability of *E. coli* extracts to give bioluminescence activity, measured using an LKB 1250 luminometer by the method of Park *et al.* (1992). The *lacZ* gene of plasmid pMTL542E was then replaced with the created *luxAB* cassette using complementary *Bgl*II and *Xba*I sites (Figure 2) such that transcription of luxAB was under the control of the NTNH/ BoNT promoter. This new plasmid was designated pMTL543E.

**Assessment of a Reporter Gene Expression**
Methylated plasmid constructs were introduced into *C. botulinum* ATCC 25765 via electrotransformation and transformed cells grown to an $OD_{600}$ of 1.0 and then assayed for the appropriate enzyme activity. High levels of both luciferase and β-galactosidase were detected in

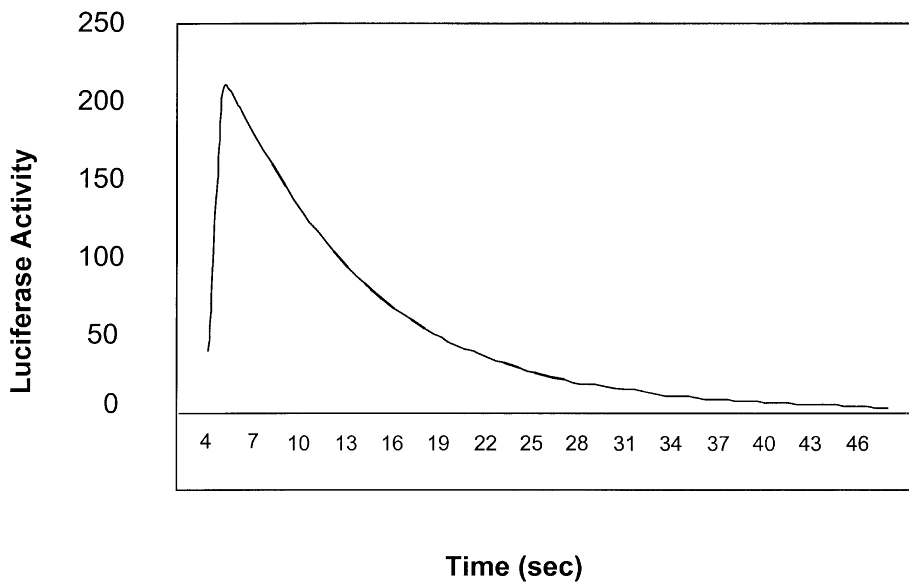


Figure 3. Luciferase Activity in ATCC 25765
A typical bioluminescence activity curve obtained when ATCC 25765 cells carrying pTOM43R were incubated with decanal.

the appropriate cell line. In the case of luciferase activity, however, the decay of bioluminescence observed with the ATCC 25765 (pTOM43R) cells (Figure 3), did not resemble results obtained in aerobes where bioluminescence decay is relatively slow, but did resemble the results obtained with another anaerobe *C. perfringens* (Phillips-Jones, 1993). However, maximal bioluminescence was only obtained after cell suspensions were exposed to air for 3 hr (Figure 4). This was in stark contrast to previous reports where the exposure time to air was not considered to be a limiting factor (Phillips-Jones, 1993). This requirement for oxygen saturation of samples introduced an unacceptable degree of variability into the assay and called into question the reproducibility and degree of quantitation that could be achieved using this enzyme. We therefore focussed our attention on the use of the *lacZ* gene.

For these experiments, single colonies of ATCC 25765 cells carrying pTOM42 and pTOM42R were inoculated into 20 ml seed cultures, incubated for 16 hr, and then used to inoculate 300 ml of fresh TPGY media. Culture aliquots (1 ml) were extracted at hourly intervals from which turbidity and β-galactosidase levels were determined. The data obtained indicated that significant levels of β-galactosidase could only be detected in cells carrying pTOM42R (Figure 5). This activity did not become measurable until late exponential phase, and then showed a steady increase in

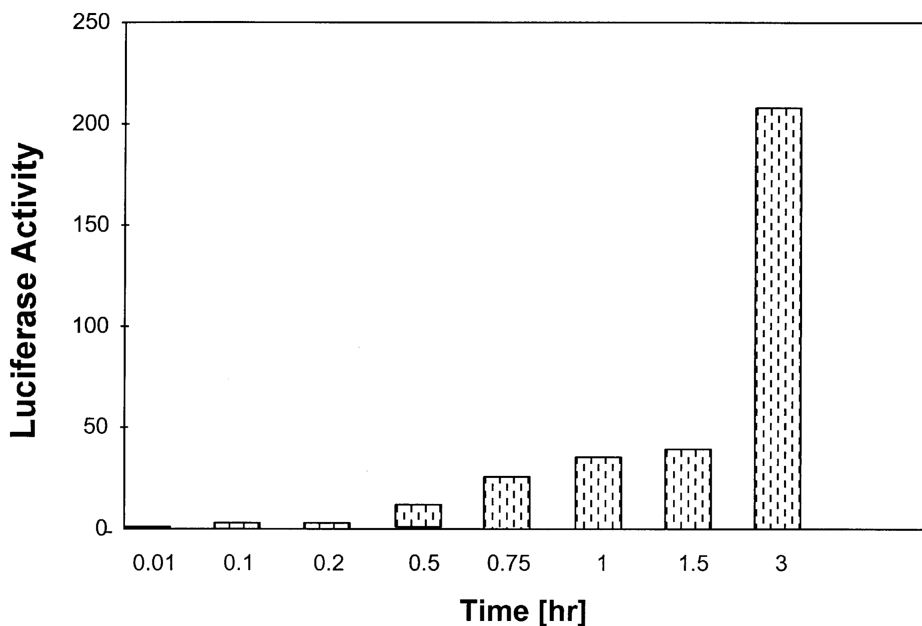


Figure 4. Effect of Aeration on Bioluminescence
Culture aliquots were removed from an anaerobic environment and then left to stand for a range of times in an aerobic atmosphere prior to luciferase assays.

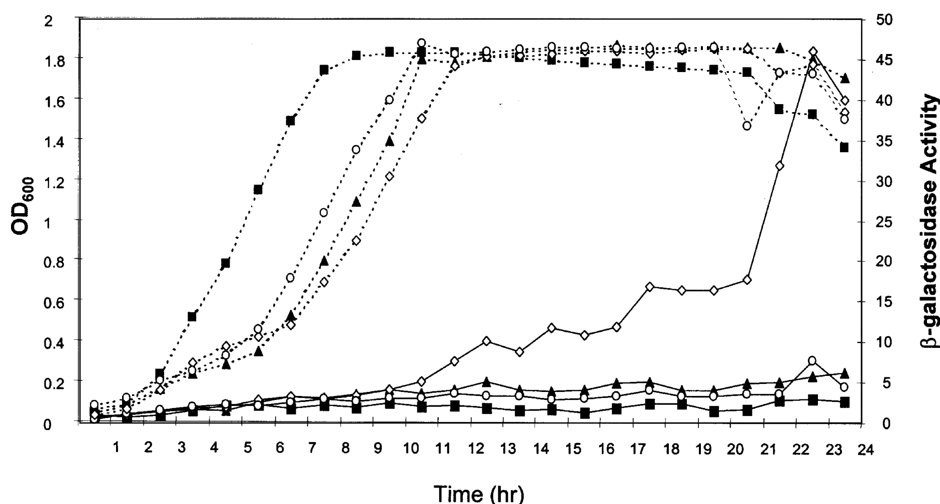


Figure 5. Toxin Promoter Controlled Production of β-Galactosidase in ATCC 25765 Cells of ATCC 25765 containing either no plasmid (■), plasmid pMTL542E (O), plasmid pTOM42 (▲), and plasmid pTOM42R (◊). Cell growth (---), β-galactosidase activity ( — ).

levels through early to late stationary phase, achieving a maximum value some 23 hours after inoculation of the culture. This time of appearance of enzyme activity in late log/early stationary phase correlates with the known properties of toxin production in *C. botulinum* (Schantz and Johnson ,1992).

Having established that the production of β-galactosidase correlated with the known growth phase dependent production of toxin, a preliminary evaluation of reporter enzyme production in media of different nutritional status with regard to nitrogen source was undertaken. These experiments utilised a modification of the minimal media described by Whitmer and Johnson (1988) in which the arginine supplementation was reduced 10-fold. Four different media were prepared, containing : (i) 50 mM glucose; (ii) 50mM glucose and 50 mM $NH_4Cl$; (iii) 50 mM arginine, and; 50 mM casein hydrolysate. *C. botulinum* ATCC 25765 carrying plasmid pTOM42R was grown in all four media supplemented with Em for 48 hr, at which point cells were harvested and lysates assayed for β-galactosidase activity (Figure 6). The results obtained indicated ammonia repressed enzyme production when cells were grown on glucose by a factor of 2.5-fold, whereas growth on a complex nitrogen source (casein hydrolysate) resulted in 4.5-fold higher levels of β-galactosidase compared to when grown on a single amino acid, arginine. These results are in agreement with previous studies which investigated the effect of nitrogen source on toxin production (Patterson-Curtis and Johnson, 1988).

**Attempted Genomic Integration of Reporter Cassettes**

Whilst useful information may be obtained using a reporter system based on an autonomous plasmid vector, the expression data obtained would better reflect the natural situation if the reporter gene was present as a single copy in the host genome. A number of methods were therefore

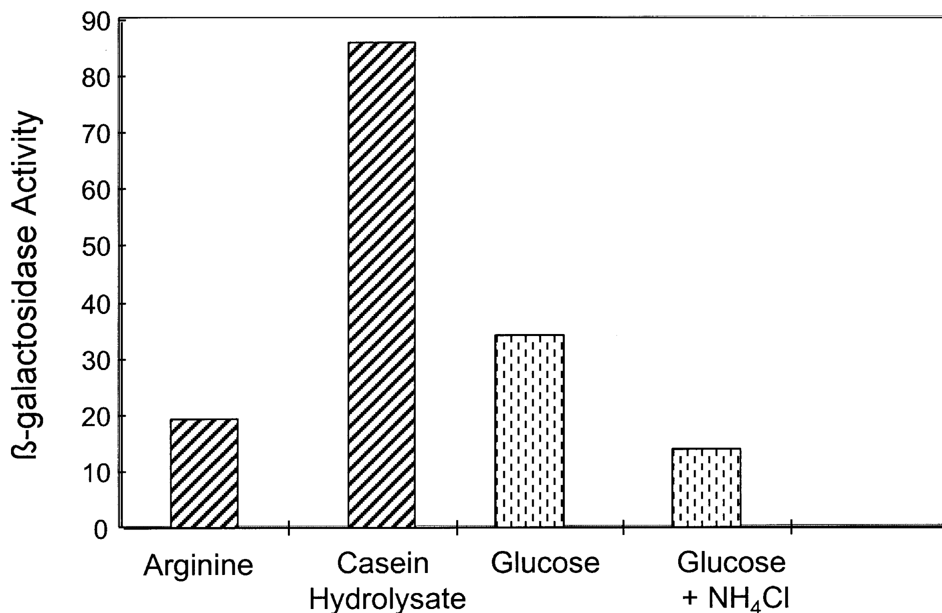


Figure 6. Effects of Nitrogen Source on β-Galactosidase Production
*C. botulinum* ATCC 25765 harbouring pTOM42R was cultivated in an arginine deficient minimal media (Whitmer and Jonson, 1988) supplemented with Em (50 µg/ml), containing; 50 mM glucose, 50 mM glucose and 50 mM $NH_4Cl$, 50mM arginine, and 50mM casein hydrolysate. Cells were harvested after 48 hr growth and levels of β-galactosidase determined.

investigated aimed at replacing the chromosomal copy of the toxin genes with the promoter::*lacZ* gene cassette. In the initial experiments a copy of the BoNT/B gene was insertionally inactivated through the insertion of the *erm* gene of pAMβ1 (Swinfield *et al.,* 1990) and inserted into a plasmid, pMTL20, unable to replicate in clostridia. However, despite the relative high transformation frequencies obtained using a control plasmid ($10^4$ per µg) , no $Em^R$ transformants were obtained.

As an alternative strategy the use of specialised plasmids which are temperature sensitive (ts) for replication in other Gram-positive bacteria was investigated. However, a ts derivative of pE194, plasmid pAUL-A (Chakraborty *et al.*, 1992), proved unable to transform either ATCC 25765 or ATCC 25765. In contrast, plasmid pGH4 (Quandt and Hynes, 1993) (which contains a ts pWVO1 replicon) and pGH5 (Maguin *et al.*, 1992) (which contains a ts pWVO1 replicon and an *E. coli* replicon) were both found to transform ATCC 25765 at the permissive temperature (28 °C). However, when the resultant transformants were subsequently grown at 40 °C, plasmids were found to be maintained in virtually all colonies examined, indicating a low degree of segregational instability. Finally, a plasmid (pECII) based on the replicon of pIM13 (Monod and Dubnau, 1986) has been found to exhibit extreme segregational instability in *Clostridium acetobutylicum* ATCC 824, where it has been successfully employed to generate integrants (Phillippe Soucaille, personal communication). However, in *C. botulinum* ATCC 25765 this plasmid proved remarkably stable, with the plasmid being retained by >99% of cells following growth for 20 generations in non-selective media.

## Discussion

An efficient transformation system has been developed for *C. botulinum* ATCC 25765 based on electroporation. This is the first report of the successful transformation of a Gp II *C. botulinum* strain. The development of our protocol was reliant on circumvention of an endogenous restriction enzyme activity. We have shown that this enzyme possesses the same specificity (both in terms of recognition sequence and cleavage point) as the restriction enzymes *Hpa*II and *Msp*I, and that a cognate methylase activity displays the same specificity as M.*Msp*I. We have designated this new enzyme *Cbo*BI and its corresponding methylase, M.*Cbo*BI. In total, 24 restriction enzymes have been characterised from clostridial species (http://rebase.neb.com/rebase/rebase.enz.html). This is the first enzyme, however, to be shown to recognise the palindrome 5'-CCGG-3'.

The method employed to determine the methylation specificity of the *C. botulinum* ATCC 25765 restriction/modification system proved particularly effective, and clearly would be of great benefit to other workers characterising equivalent systems that cleave GC-rich DNA. In such instances, the application of this approach is reliant on being able to PCR amplify a known sequence from the organism under investigation, which is likely (*ie.*, GC-rich DNA in an AT-rich organisms), or experimentally known, to be cleaved by the endogenous enzyme. In our study this was made possible by our ability to introduce, by transformation, a relatively GC-rich, foreign plasmid element (pGK12) into our host at low efficiencies. In other cases, where transformation cannot be demonstrated, then known genomic sequences may be targeted. Alternatively, it may prove possible to introduce foreign DNA by a conjugative procedure using either a conjugative plasmid system 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 conjugal element (Oultram *et al*., 1988b; Mullany *et al.,* 1994).

Plasmid cloning vectors, such as pMTL540E and pMTL500E, which are not protected against digestion by *Cbo*BI are unable to transform *C. botulinum* ATCC 25765. This is not unsurprising, as these plasmids contain 15 and 12 *Cbo*BI restriction sites, respectively. Plasmid pGK12 contains a single *Cbo*B1. This dearth of sites may alone explain the ability of pGK12 plasmid to transform *C. botulinum* ATCC 25765 in the absence of methylase protection. However, it is interesting to note that, in contrast to pMTL500E and pMTL540E, the replicon of pGK12 belongs to the ssDNA family (Gruss and Erhlich, 1989). This could offer an alternative explanation as to why pGK12 was able to transform *C. botulinum* ATCC 25765, despite the presence of a *Cbo*B1 site. This is because plasmid DNA preparations based on pGK12 would contain single stranded DNA which would be immune to restriction but would display an intrinsic low efficiency in transforming cells.

During this investigation we have examined the utility of two separate reporters viz., *lacZ* and *luxAB*. Using the former it proved possible to demonstrate that production of β-galactosidase mirrored that of botulinum toxin, both in terms of growth phase and affect of nitrogen source. However, effective detection of reporter enzyme production was reliant on the presence on the plasmid of the transcriptional regulator encoded by *orfX*/P22. As this transcriptional activator is presumed to act *in trans* (Marvaud *et al*., 1998), this suggest that there is insufficient production of this transcriptional factor from the chromosomally located copy of the gene to effect sufficient production of β-galactosidase in cells carrying pTOM42, *ie.*, the plasmid carrying only the NHNT/BoNT/B promoter. The result may also be consistent with the suggestion by Marvaud *et al.* (1998) that the activity of the NHNT/BoNT/B promoter may be greater when *orfX*/P22 is in the *cis* position. Further work will be required to test whether the production of β-galactosidase is sensitive enough to provide an effective marker of "toxin" production under dif ferent physiological conditions. The question mark over this issue of sensitivity may justify a re-evaluation of the merits of luciferase, and the development of an assay protocol that standardises the oxygenation of cells prior to measurement of bioluminescence.

### Experimental Procedures

#### Bacterial Strains, Plasmids and Culture Media

The strains and plasmids employed in this study are listed in Table 2. Clostridial strains were cultured in TPGY media (2% Trypticase, 0.5% peptone, 0.1 % Glucose, 0.5% Yeast extract and 0.1% Cysteine HCl) at 37°C in an atmosphere of 87% $N_2$, 9% $CO_2$ and 4% $H_2$ using a Don Whitely Mk III anaerobic cabinet. Broth and agar versions of this media were placed in the anaerobic environment for 24-36 hr to become free of oxygen. *E. coli* strains were cultured in L-broth and on L-agar at 37 °C, supplemented with antibiotics (Erythromycin; 500 µg/ml, ampicillin; 100 µg/ml, tetracycline; 10 µg/ml).

Table 2. Bacterial Strains and Plasmids

| Organism/ plasmid | Feature | Source/reference |
|---|---|---|
| **Strains:** | | |
| *Clostridium botulinum* ATCC 25765 (17B) | non-proteolytic, BoNT/B | TA Roberts |
| *Clostridium botulinum* BL229 | non-proteolytic, BoNT/B | M Eklund |
| *Clostridium botulinum* BL151 | non-proteolytic, BoNT/B | Unilever |
| *Clostridium botulinum* ATCC 3502 (Hall) | proteolytic, BoNT/A | ATCC |
| *Clostridium botulinum* NCTC 2916 | proteolytic, BoNT/A | NCTC |
| *Clostridium beijerinckii* NCIMB 8052 | formerly *C. acetobutylicum* | JG Morris, UCW Aberystwyth |
| *Clostridium pasteurianum* ATCC 6013 | source of the Fd gene | JG Morirs, UCW Aberystwyth |
| *Vibrio fischeri ATCC 7744* | source of *luxAB* genes | ATCC |
| *Eschericihia coli* Top10 | F- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ*ΔM15 Δ*lacX74 recA1 deoR araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (str$^R$) *endA1 nupG* | Invitrogen |
| *Eschericihia coli* GM2163 | F- *ara-14 leuB6 thi-1 fhuA31 lacY tsx-78 galK2 galT22 supE44 hisG4 rpsL136 (Str$^R$) xyl-5 mtl-1 dam13::Tn9 (Cam$^R$) dcm-6 mcrB1 hsdR2 ($r_K$-$m_K$+)* mcrA | Novagen |
| *Eschericihia coli* HB101 | *thi-1 hsdS20* ($r^-_B$, $m^-_B$) *supE44 recAB ara-14 leuB5 proA2 lacY1 galK rpsL20* (str$^R$) *xyl-5 mtl-1* | Novagen |
| **Plasmids:** | | |
| pMTL20, pMTL21, pMTL23 | *E.coli* cloning vector, ColE1, Ap$^R$ | Chambers *et al.* (1988) |
| pCR2.1-TOPO | *E.coli*, PCR cloning vector, ColE1, Km$^R$ | Invitrogen |
| pMTL500E | *E.coli/Clostridium* shuttle, pAMβ1, Em$^R$ | Swinfield *et al.* (1990) |
| pMTL540E | *E.coli/Clostridium* shuttle, pCB102, Em$^R$ | Fox *et al.* (1996) |
| pJIR418 | *E.coli/Clostridium* shuttle, pIP404, Em$^R$ | Sloan *et al.* (1992) |
| pGK12 | broad host range, pWVO1, Em$^R$ , Cm$^R$ | Kok *et al.* (1984) |
| pHG4 | ts derivative of pWV01, Em$^R$ | Quandt and Hynes (1993) |
| pHG5 | ts derivative of pWV01, Em$^R$ | Maguin *et al.* (1992) |
| pAUL-A | ts derivative of pE194, Em$^R$ | Chakraborty *et al.* (1992) |
| pECII | segregationally unstable pIM13 derivative, Em$^R$ | Phillippe Soucaille |
| pCT101 | source of the *T. sulfurigenes lacZ* gene | Burchhardt and Bahl (1991) |
| pBW20 | source of B. subtilis M.*Bsu*F1 gene | Walter *et al.* (1990) |
| pBSU1 | pACYC184 carrying the M.*Bsu*F1 gene | this study |
| pMTL542E | pMTL540E + the *T. sulfurigenes lacZ* gene | this study |
| pMTL543E | pMTL540E + the *V. fischeri luxAB* genes | this study |
| pTOM42 | pMTL542E + the NTNH/BoNT/B promoter | this study |
| pTOM42R | pMTL542E + the NTNH/BoNT/B promoter + *orfX/*P22 | this study |
| pTOM43 | pMTL543E + the NTNH/BoNT/B promoter | this study |
| pTOM43R | pMTL543E + the NTNH/BoNT/B promoter + *orfX/*P22 | this study |

**Electroporation**

An overnight 20 ml culture, cultivated in TPGY media (g/l: trypticase, 20 g; peptone, 5 g; glucose, 1 g; yeast extract, 5 g; cysteine-HCl, 1 g) supplemented with 1% (w/v) glycine was used to inoculate a 300 ml volume of TPGY and allowed to grow to an $A_{660}$ of 0.8. The culture was divided in two, cooled on ice for 10 min and then centrifuged at 6000 g outside of the anaerobic cabinet for 10 min, at 4°C. The centrifuge pots were returned to the cabinet and the supernatant carefully removed. Cells were gently resuspended in 50 ml of chilled electroporation buffer (10 % PEG mol. wt. 6000 or 270 mM sucrose, 7 mM sodium phosphate, 1 mM $MgCl_2$) and the centrifugation procedure repeated. The cell pellet was eventually resuspended in 3 ml of fresh, chilled electroporation buffer. In the initial experiments, DNA (between 0.1 to 2.0 mg/ml) was added to a chilled electroporation cuvettes of 0.2 cm inter-electrode distance, followed by 0.3 ml of cells and the two mixed by 2 to 3 gentle inversions of the cuvette. The cuvette was wiped dry and subjected to a pulse of magnitude 2.0 kV, 25 µF (filed strength 10 kV/cm) and 400 Ω. The cuvette was immediately replaced on ice and left for 5 min. The contents of the cuvette were resuspended in 10 ml of warm TPGY broth supplemented with 25 mM MgCl and the cells left to recover for 5 hr. Thereafter, the 10 ml of broth was separated into 6 eppendorfs, centrifuged for 3 min, the supernatant decanted and the cells gently resuspended in 150 µl of TPGY broth and spread on individual TPGY 2.5% (w/v) agar plates containing 5 µg/ml erythromycin. Plates are incubated for 24-48 hr.

**Isolation of Plasmid DNA**

Plasmid DNA was isolated from standard laboratory *E. coli* K12 strains DH5α, ER1648 and HB101 using standard methods. Plasmid DNA was isolated from clostridia using a column purification system supplied by Qiagen Ltd. This essentially uses the alkaline lysis method (Birnboim & Doly, 1979), however, a slightly modified resuspension buffer containing lysozyme (10 mg/l) and preincubation for 15 min at 37°C was employed to enhance cell wall degradation in preference to the buffer P1 supplied by Qiagen.

**Cloning DNA Restriction Fragments**

DNA restriction fragments created from digestion with clostridial endonucleases were separated by agarose gel electrophoresis using a 1x TAE buffer. Fragments were then excised and purified using the 'Sephaglas' system (Pharmacia). Individual fragments were then ligated into a dephosphorylated *E. coli* cloning vector, pMTL20 (Chambers *et al.*, 1988) and introduced into an *E. coli* host.

**Nucleotide Sequence Analysis**

DNA samples were prepared using an *E. coli* strain with an *end*A genotype. 1-2 µg of template DNA were mixed with 4.5 pmol of oligonucleotide primer and the sequencing reaction completed using *Amplitaq* FS dye terminator chemistry in a robotic work station. Samples were separated and recorded using an ABI377 DNA Sequencer. Primary data was manipulated using software in the *Lasergene* program, DNA STAR Inc.

**Isolation of *C. botulinum* Extracts for Restriction Assays**

Cell-wall free extracts of clostridia were prepared by an adaptation to the method described by Mermelstein *et al.* (1992). An 'overnight' batch culture was used to inoculate 100 ml of TPGY broth supplemented with glycine (0.4% w/v) at a 3% (v/v) ratio. The culture was grown to an $O.D_{600}$=0.4-0.5. Cells were harvested in air tight centrifuge pots and resuspended in 4 ml of clostridial basal media supplemented with lysozyme 5 mg/ml (w/v) and reinforced with 10% sucrose (w/v), 25 mM $MgCl_2$ and 25 mM $CaCl_2$. The cells were incubated for 1 hr at 37°C before then the extent to which the cell wall had degenerated and protoplasts formed was determined by light spectroscopy. The protoplasts were pelleted and lysed in hypotonic buffer

(4 mM Tris 7-9 pH 8.0, 10 mM EDTA, 25 mM KCl, 6.6 mM 2-Mercaptoethanol). A cleared lysate was obtained by centrifugation at 15,000 x *g* for 30 min. The supernatant was removed and stored on ice until use.

**Restriction Endonuclease Assays**
Restriction endonuclease activity was determined using Clostridial-*E. coli* plasmid shuttle vectors as substrate, which had been prepared from an *E. coli* host. 2 µg of plasmid DNA was incubated with an appropriate volume of commercial restriction endonuclease buffer (10x) in a 20-30 µl volume. Sterile $H_2O$ was used to equate final volumes. Reaction samples were typically incubated at 37°C for 2-16 hr. Samples were then analysed by agarose gel electrophoresis using kilobase ladder (Gibco BRL) as a molecular weight standard.

***In vitro* Methylation**
Methylation of specific DNA sequences was achieved *in vitro*. Methylases; M.*HpaII*, M.*Msp*I and M.*Ss*I were purchased from New England Biolabs Inc. with their respective buffers and S-adenosylmethionine cofactor. Methylation of double stranded plasmid DNA was performed at 37°C using 15 units of methylase. 1-60 µg of DNA was incubated for 3-6 hr.(Unit definition = 1 unit protects 1 µg of λ DNA per hr). Modified DNA was then purified from the contaminating reaction constituents using the Sephaglas kit (Pharmacia).

**Methylation Site Analysis**
The methylation status of cytosine residues present on plasmid DNA maintained in a clostridial host was determined using a modification of the method of Feil *et al.* (1994). Approximately 2 µg of linearised plasmid DNA was suspended in 100 µl of deionised water in a siliconised 1.5 ml eppendorf and then denatured by adding 11 µl of 3N NaOH and incubating at 37 °C for 20 min. The tube was placed on ice and 1.1 ml of 3.5 M $NaHSO_3$/1 mM hydroquinone, pH 5.0, was added. The solution was then overlayed with 150 µl of mineral oil and incubated in the dark for 24 hr at 0°C. The sample was removed from beneath the mineral oil and transfer to a 1.5 ml siliconised eppendorf tube where the DNA was extracted using a Gene clean II kit ( Stratech Scientific Ltd) for 30 min at 4°C. The precipitated DNA was resuspended in 100 µl of deionised water and desulfonated by adding 11 µl of 2 N NaOH and incubating for 10 min at 20°C. The DNA was precipitated by adding 5 M ammonium acetate, pH 7.0, to a final concentration of 3 M, and three volumes of ethanol. Following resuspension in 100 µl of deionised water, a 4 µl aliquot was used as a template in a PCR reaction using the "mutagenic" primers P1 (5'-GGGGAAA TGTGtGtGGAAtttt-3') and P2 (5'-aCCTaACTCCCCaTCaTaTAa-3'), where the anticipated changes required to complement the mutagenised DNA are indicated in lower case. These two primers are complementary to the target sequence of one strand of plasmid pGK12 (positions 5220 to 5199, and position 4308 to 4328 of pGK12 for P1 and P2, respectively) assuming that all cytosine residues have been changed to uracil. The approximately 900 bp product of the PCR reaction was cloned into the vector pCR2.1, and sequenced.

**Reporter Vector Construction Based on *lacZ***
The DNA sequence encoding a promoter-less version of the *T. sulfurigenes* β-galactosidase gene was isolated by PCR amplification as a 2.2 kb fragment from plasmid pCT101 template DNA (Burchhardt & Bahl) using the mutagenic primers lac-1 [5'-CAGATACGCGTGAGAAAG AATATTCCTATTAATAATAATTGG-3'] and lac-2 [5'-ATTCTTTGCCTCGA GGATGTGGAT-3']. The amplified fragment was cloned into pCR2.1-TOPO and its nucleotide sequence verified. Thereafter, it was re-excised using the *Mlu*I and *Xho*I restriction sites created by lac-1 and lac-2, respectively, and inserted between the *Mlu*I and *Sal*I sites of pMTL21 vector (Chambers et al), yielding pMTL21*lacZ*. A region of DNA flanking the transcriptional terminator of the *C. pasteurianum* ferredoxin gene (Fd) was amplified as a 220 bp fragment from the *C. pasteurianum* genome using the primers Fd-1 [5'-GTTAATGCTAGCAGTCAAGGA–3'] and Fd-2 [5'-ATTTTCTTCAATC TAGAGTATAACATA-3'] and cloned into pCR2.1-TOPO and its complete nucleotide sequence verified. The fragment was then re-isolated using unique *Xba*I and *Nhe*I sites created by Fd-1 and Fd-2, respectively, and inserted into the *Xba*I site of pMTL21*lacZ* located immediately 3' to *lacZ*. The plasmid obtained was designated pMTL21*lacZ*Fdt. The *lacZ* gene together with the Fd terminator where then isolate from pMTL21*lacZ*Fdt as a 2.4 kb *Xho*I/*Xba*I fragment and cloned into the polylinker of similarly cleaved pMTL540E, yielding pMTL540E *lacZ*Fdt. The unique *Pst*I site upstream of the β-galactosidase/Fd transcriptional terminator was used to insert another transcriptional terminator, that of the *Clostridium thermocellum celA* gene (Beguin *et al.,* 1985). This element was PCR amplified from *C. thermocellum* chromosome DNA using the primers cel-1 [5'-CTG AAGAGTGTTACCAATATAAACAG-3'] and cel-2 [5'-AAATGCATCCCT TTACAAAACAATTTC-3'], and the 246 bp fragment obtained cloned into pCR2.1-TOPO and its sequence verified. The fragment was then re-excised as a 220 bp *Nsi*I fragment, using the restriction sites created by cel-1 and cel-2, and inserted into the unique *Pst*I site of pMTL540E*lacZ*Fdt which precedes the promoter-less *lacZ* gene. The plasmid obtained was designated pMTL542E.

**Reporter Vector Construction Based on *luxAB***
The luciferase-based reporter plasmids were essentially made by replacing the lacZ gene of pMTL542E with the *luxAB* operon (Foran and Brown, 1988). A 2.0 kb DNA fragment encompassing *luxAB* was PCR amplified from the *Vibrio fischeri* genome using the primers lux-1 309 [5'-AGATCTGAATAAATAGGAGGAATAGAGTATGAAGTTTGGAAATATTTG-3'] and lux-2 [5'-TCTAGATATTCAGTATGGACAGTCATACCC-3']. The former primer, in addition to creating a flanking 5' *Bgl*II site, was also designed to alter the ribosome binding site (RBS) preceding *luxA* from 5'-AAAAGG-3' to 5'-AGGAGG-3', such that it more closely matched RBS sequences found in clostridial genes (Young *et al.,* 1989). Following the cloning of this fragment into pCR2.1-TOPO and verification of its sequence, the RBS sequence preceding the *luxB* gene was mutated from 5'-ATAAGG-3' to 5'-AGGAGG-3', using a "Quick change TM" kit (Stratagene) and the primers lux-3 [5'-TTGATACTAGAGATAATAAGGAACAAGTTATG-3'] and lux-4 [5'-CATAACTTGTTCCTCCTTATCTCTAGTATCAA-3']. Thereafter, the *lacZ* gene of pMTL543E was excised by cleavage of the plasmid with *Bgl*II and *Xba*I, and the altered promoter-less *luxAB* operon was inserted in its place following its isolation from pCR2.1-TOPO as a 2.0 kb *Bgl*II/*Xba*I fragment. The plasmid obtained was designated pMTL543E.

**Insertion of Botulinum Promoters into the Reporter Plasmids**
Two fragments were isolated. A 500-bp fragment encompassing only the NTNH/BoNT/B promoter element was PCR amplified from *C. botulinum* ATCC 25765 chromosome DNA using the primers bot-1 [5-GCCATAATATCTCTCTCGAGCCACCCAAATATT-3'] and bot-2 [5'-TAGTTATTCAAGTTCTGCAGAAGTTGAATTTAATGA-3'] and cloned into pCR2.1-TOPO for sequence verification. A second, larger 1.0 kb fragment was also amplified, using bot-1 and bot-3 [5'-AGTATCGGCCTTA CTCGAGATGGTAACAATTTCGTC-3'], which encompassed a transcription factor (OrfX/P22) residing immediately 5' to the NTNH/BoNT/B promoter. Following authentication of the sequence of both fragments, they were re-excised from pCR2.1-TOPO as *Xho*I fragments and inserted into the unique *Xho*I sites of plasmids pMTL542E and pMTL543E which immediately precedes the promoter-less *lacZ* and *luxAB* genes, respectively. The pMTL542E-based plasmids carrying the NTNH/BoNT/B promoter with and without OrfX/P22 were designated pTOM42 and pTOM42R, respectively. The equivalent plasmids based on pMTL543E were respectively designated pTOM43 and pTOM43R.

**Enzyme Assays**
Assays for β-galactosidase activity were essentially undertaken as described by Miller (1972). Culture aliquots (100, 200 or 500 µl) were made up to 1.0 ml with Z-buffer (0.06 M $Na_2HPO_4.7H_2O$, 0.04 M $NaH_2PO_4$. $H_2O$, 0.1 M KCl, 0.001 M $MgSO_4.7H_20$, 0.05M β-mercaptoethanol, pH 7.0) and then 2 drops of chloroform added and 1 drop of 0.1% (w/v) SDS. The cells were mixed and incubated for 5 min at 28°C and then 200 µl of *o*-nitrophenol-β-D-galactosidase (ONPG, 4 mg/ml in 0.1M phosphate buffer, pH 7.0). Whilst this particular enzyme has a temperature optimum of around 65°C, for convenience, the assays were undertaken at 28°C. The activities obtained at this lower temperature are perfectly adequate, being around 80% of those observed at 65°C. The reaction was stopped after 60 min through the addition of 500 µl of 1M $NaCO_3$, and the absorbance was measured at 420 nm and 550 nm. In addition, to counter the effects of media constituents on absorbance at 420 nm, a control reaction was undertaken in which no ONPG was added and which was then used as the blank in measurement of the absorbance at 420 nm of samples to which ONPG had been added. Units were calculated according to the equation 1000 X ($OD_{420}$ – (1.75 X $OD_{550}$ ) / reaction time in min X culture $OD_{600}$ X reaction culture volume in ml.).

Assays for luciferase activity was as described by Park *et al.* (1992). A 1.0 ml aliquot of culture was added to the luminometer cuvette (51 mm X 12 mm, Starstedt, No. 68.750) followed by 20 µl of decanal solution (1% (w/v) in ethanol) mixed, and the resultant luminescence measured using a luminometer (LKB. Model 1251). Readings were blanked against a sample to which no decanal had been added.

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