THE DEVELOPMENT OF MOLECULAR TOOLS FOR THE EXPRESSION OF PRODRUG CONVERTING ENZYMES IN *CLOSTRIDIUM SPOROGENES*

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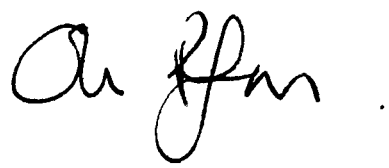
DECLARATION

Unless otherwise acknowledged, the work presented in this thesis is my own. No part has been submitted for another degree in the University of Nottingham or any other institute of learning.



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ABSTRACT

Despite intensive research, cancer remains one of the major causes of worldwide morbidity. It is widely believed, however, that if currently available anti-cancer drugs could be delivered specifically to tumours then the disease would have been mastered. The delivery of prodrug converting enzymes by clostridial spores specifically to the anoxic centres of tumours is one potential delivery mechanism. This is due to the extreme selectivity of spores to germinate solely in the hypoxic regions of tumours. Once germinated, the expression of a prodrug converting enzyme converts a systemically administered prodrug to a highly toxic drug only in the tumour. Previous studies using *Clostridium acetobutylicum* and *Clostridium beijerinckii* as the delivery vehicle highlighted that prodrug converting enzyme expression is only found in tumours. However, no significant anti-tumour affect was observed. Two possible reasons were evolved. Firstly, expression of the prodrug converting enzyme may be low, and/or, secondly, the tumours may not be colonised sufficiently to promote an anti-tumour effect.

Preliminary studies identified that *Clostridium sporogenes* NCIMB 10696 may represent a more suitable host. Higher spore titres could be prepared and, once administered, higher cell counts are found in the colonised tumours. Prodrug converting enzymes with improved kinetics over pre-existing enzymes have also been identified. Once effective gene transfer systems and expression systems had been developed, suitably high levels of several different prodrug converting enzymes, in particular nitroreductases, were obtained. Initial *in vivo* studies on one of the early recombinant strains identified a definite anti-tumour effect. Since those initial studies, further improvements to expression have been made. It is hoped that a more significant anti-tumour affect would result from using these improved strains.

It is the ultimate aim of CDEPT to have the prodrug converting enzymes integrated into the host genome so as to negate the use of antibiotics. Towards this, studies on the use of both classical and novel integrative technologies have been investigated.

List of publications relating to these studies:

Pennington, O.J., van Mallaert, L., Theys, J., Barbé, S., Lambin, P., Anné, J., and Minton, N.P. (2004) Recombinant Clostridial Spores in Tumour Therapy. In *Bacterial Spore Formers: Probiotics and Emerging Applications*. Ricca, E., Henriques, A.O., and Cutting, S.M. (ed). Wymondham, Norfolk: Horizon Bioscience, pp. 207-215.

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Theys, J., **Pennington, O.J.,** Dubois, L., Landuyt, W., Anné, J., Burke, P.J., Anlezark, G., Dürre, P., Wouters, B.G., Minton, N.P., and Lambin, P. (2006) Repeated systemic treatment cycles of *Clostridium*-directed enzyme prodrug therapy results in sustained anti-tumour effects *in vivo*. *Br J Cancer* (submitted).

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ABBREVIATIONS

%	Percentage
°C	degrees Celsius
μF	micro Faraday
μg	microgram
μl	microlitre
μM	micromolar
A	absorbance
amp	ampicillin
bp	base pairs
CD	cytosine deaminase
cfu	colony forming units
cm	centimetre
Cm	chloramphenicol
dsDNA	double stranded DNA
Erm	erythromycin
g	grams
g	gravity
h	hour(s)
IR	inverted repeat
IS	insertion sequence

Kan	kanamycin
kb	kilobase pairs
kV	kilovolt
l	litre(s)
M	molar
min	minute(s)
ml	millilitre
mM	millimolar
mm	millimetre
nM	nanomolar
NTR	nitroreductase
OD	optical density
Ω	ohms
psi	pounds per square inch
sec	second(s)
ssDNA	single stranded DNA
Ta	annealing temperature
tet	tetracycline
Tm	melting temperature
Tn	transposon
TNFα	tumour necrosis factor alpha

U	units
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight

CHAPTER 1

INTRODUCTION

1.1 THE GENUS *CLOSTRIDIUM*

The genus *Clostridium* comprises one of the largest prokaryotic genera (Minton and Clarke, 1989). Characterised as anaerobic Gram-positive rods they are unified by their ability to form spores. As a grouping, they have achieved greatest prominence as a consequence of their more notorious representatives, *Clostridium botulinum, Clostridium tetani, Clostridium perfringens* and *Clostridium difficile*. In this context, spore production represents one of the, if not the most important, virulence factor. Thus, the capacity of *C. botulinum* spores to survive extreme adversity has meant that this organism has been the principal target in food processing for almost a century. Similarly, within the healthcare setting, the ability of *C. difficile* spores to survive in the hospital and nursing home environment makes this particular clostridia one of the major causes of nosocomial infection. It is also one of the most difficult examples of microbial contamination to eradicate.

Paradoxically, it is the very ability to form spores that presents the genus with perhaps its greatest potential to benefit mankind, through the exploitation of clostridial spores as a delivery system for treating cancer. This is because intravenously administered spores localise to, and then exclusively germinate in, the hypoxic centres of solid tumours. The net result is the establishment of an actively growing population of vegetative cells, specifically restricted to the anaerobic environment within the tumour mass. This unique feature provides the opportunity to selectively deliver therapeutic agents to solid tumours, by endowing the clostridial species used with appropriate genes capable of directing the production of the desired therapeutic.

1.2 CLOSTRIDIAL TUMOUR ONCOLYSIS

The notion of using bacteria to treat tumours has been around for nearly 140 years. The first documented case of a cancer patient being purposefully injected with live bacteria as a form

of cancer treatment was undertaken by the German physician W. Busch in 1868 (Hall, 1988). The patient, who had an inoperable sarcoma, was infected with *Streptococcus pyogenes* by placing her into a bed previously occupied by a patient with a *S. pyogenes* infection. Within a week the primary tumour had decreased by 50%, and the lymph nodes in the neck had also shrunk in size. This provided the first indication that tumours can be reduced by bacterial infection. Treatment with *S. pyogenes* was also performed by William B. Coley at New York Hospital, and Friedrich Fehleisen in Germany around 1880 – 1890 who achieved some degree of tumour regression and patient survival (Pawelek *et al.*, 2003). Subsequent animal studies indicated that the results were in part attributed to the infection and in part to the stimulation of the host immune response.

The concept of using bacteria as tumour vectors has, however, been most vigorously pursued using *Clostridium* species. As early as 1813, Vautier reported that cancer patients who suffered gas gangrene (*C. perfringens*) were apparently cured of their cancer following the clostridial infection (*cited in* Hall, 1988). The potential of deliberately using clostridia was explored in 1935, when Connell (1935) used sterile filtrates of *Clostridium histolyticum* for the treatment of advanced cancers. The clinical improvements observed were attributed to the production of proteolytic enzymes that preferentially degraded cancerous tissue without affecting normal tissue. These observations subsequently led to several experiments in which proteolytic enzymes were produced *in situ* by deliberately infecting tumours with clostridial spores. Thus, Parker and colleagues (Parker *et al.*, 1947) demonstrated that intratumoural injection of spores of *C. histolyticum* into the transplanted sarcomas of mice resulted in germination of the clostridia and a noticeable lysis of the tumour tissue. These studies gave the first suggestion of the potential to use clostridial spores to induce the lysis of tumours.

Following on from Parker's work, Malmgren and Flanigan (1955) further demonstrated the extreme selectivity of clostridia for the hypoxic areas of tumours. Systemic administration of *C. tetani* into mice bearing a variety of tumours resulted in the mice succumbing to

tetanus within 48 hours. The healthy control mice, which were not transplanted with a tumour, remained unaffected, demonstrating no signs of disease over the entire time of the study. This observation demonstrated that spore germination and production of the tetanus toxin occurred exclusively in the hypoxic cores of tumours.

Möse and Möse (1959) were the first to consider using non-pathogenic clostridial spores as a means of treating cancer via clostridial-induced lysis. For this purpose they isolated *Clostridium butyricum* M55, a strain they later termed *Clostridium oncolyticum* M55 (now reclassified as *Clostridium sporogenes* ATCC 13732). The intravenous injection of *C. butyricum* M55 spores into mice transplanted with solid Ehrlich tumours resulted in the softening of tumours followed by the spontaneous discharge of a "brownish liquid necrotic mass". Of the few animals that survived, most had recurrence of the tumour at the same site, indicating the presence of an outer viable rim of cells from which tumour re-growth could occur. The results were confirmed with studies that used other rodent tumour models and non-pathogenic *Clostridium* species (Gericke and Engelbart, 1964; Möse and Möse, 1964; Thiele *et al.*, 1964).

The data obtained in these early studies was sufficient for the initiation of clinical studies. These were undertaken by Möse and Möse using *C. oncolyticum* M55. In the study, they demonstrated that this organism lacked human pathogenicity by administering a spore suspension to themselves (*cited in* Carey et al., 1967). Initial trials were performed with 36 patients, who received a dose of 10^6 to 10^9 spores either intratumourally or intravenously. The injections were well tolerated, but were followed by a high-grade fever that then decreased to a low-grade fever for 1–3 days. Oncolysis occurred in the largest of the tumours 5–8 days later, but was not evident in the smaller tumours, metastases, and the surrounding tissues. Trials were also performed in the USA (Carey *et al.*, 1967) with five patients with advanced neoplastic disease. Oncolysis occurred in three cases only in the largest of tumours, and in one case a transient clinical benefit was attributed to clostridial-induced oncolysis. Several clinical trials have assessed the use of clostridia for the treatment

of inoperable malignant brain tumours (Heppner and Möse, 1978; Heppner *et al.*, 1983; Kretschmer, 1972). Oncolysis of the glioblastomas did occur, but complications arose before the completion of oncolysis and the tumours had to be removed via conventional surgery. These clinical trials showed that most patients could tolerate *Clostridium* well, although the presence of an outer viable rim of cells hampered progress.

Experiments with wild type clostridial species clearly demonstrated that spore treatment is remarkably well tolerated and that vegetative growth frequently leads to the destruction of large parts of the tumour. Invariably, however, an outer viable rim remains from which tumour regrowth occurs. This led to the concept of combining drug treatment with spore administration to enhance the already observed therapeutic effect. In early studies, alkylating agents of the ethyleneimino type (such as tetramin, E-39, trenimon and mitomycin C) were shown to produce positive results when the dose and timing of drug/spore administration was adjusted to optimum levels (Thiele *et al.*, 1964). The concept of enhancing the therapeutic properties of the clostridial cells used through genetic manipulation was first suggested by Schlechte and Elbe (1988). These workers attempted to make a recombinant strain of *C. butyricum* M55 that produced Colicin E3, an *E. coli* bacteriocin suggested to possess canceriostatic properties. At the time the methodology required to generate such a recombinant strain was not available, and the evidence presented to support the creation of such a strain was not convincing. It has only been since the advances made in clostridial gene transfer systems in the late 1980s and early 1990s (Mauchline *et al.*, 1999; Minton *et al.*, 1993), that the desired genetic changes could be reproducibly made. As these gene systems were developed for non-pathogenic saccharolytic strains, the initial experiments were undertaken with *Clostridium acetobutylicum* and *Clostridium beijerinckii*.

1.3 USE OF SACCHAROLYTIC STRAINS

As a prelude to the enhancement of the therapeutic properties of clostridial strains through recombinant approaches, the ability of the various saccharolytic clostridial species to colonise tumours was assessed. Lemmon *et al* (1997) systemically applied 10^8 spores of *C. beijerinckii* NCIMB 8052 to mice bearing mammary EMT6 tumours and showed that vegetative Gram-positive clostridial rods were only present in the hypoxic and necrotic regions of the tumour. Histological samples of brain, heart, kidney, lung, and spleen tissues indicated the complete absence of spores. In another study (Lambin *et al.*, 1998), the colonisation of four different saccharolytic clostridia: *C. beijerinckii* ATCC 17778, *C. limosum* DSM 1400, *C. acetobutylicum* ATCC 824 and NI-4082 (now reclassified as *C. saccharoperbutylacetonicum*), were compared to *C. oncolyticum*. It was found that a spore titre of at least 10^7 was required for colonisation of the WAG/Rij rats, and that *C. acetobutylicum* colonised better than the other three saccharolytic strains obtaining a similar population level to that obtained by *C. oncolyticum*. Heat treatment of the tumours indicated that the numbers of colony forming units (cfu) obtained (up to 10^9 cfu per gram of tissue after 4 – 5 days) was due to vegetative cells. 10^4 to 10^6 cfu were found in normal tissues. These colonies were proven to be entirely due to spores, as heat treatment had no effect on colony counts. This result was confirmed with Gram-staining and histochemical staining of the tumour and normal tissues. No clostridial spores were present in urine samples taken at 4 and 8 days after spore injection.

Having established that saccharolytic clostridial strains are able to colonise tumours, efforts have focussed on the introduction of genes encoding anti-cancer agents. To date, the introduction of two classes of recombinant therapeutic protein have been explored: (i) proteins that are antitumourogenic in their own right such as toxins and cytokines, and; (ii) enzymes which turnover a prodrug to a toxic drug, so-called prodrug converting enzymes.

1.3.1 Tumour necrosis factor (TNFα)

TNFα acts as a therapeutic agent in several ways. These include selective destruction of the neovasculature leading to thrombosis and necrosis of tumours, stimulation of T-cell immunity, and direct cytotoxicity to tumour cells, mainly via apoptosis (Fiers, 1991; Larrick and Wright, 1990; Laster *et al.*, 1988; Zheng *et al.*, 1995). Furthermore, enhancement of the anti-tumour effect of TNFα has been demonstrated when combined with irradiation (Hallahan *et al.*, 1995; Sersa *et al.*, 1988). The downside to its use, however, is the systemic toxicity that ensues from direct administration. Delivery via recombinant clostridia therefore provides an opportunity for the localised production, thereby minimising any toxic side effects.

In the investigations of Theys *et al* (1999), mouse TNFα (mTNFα) was placed under the control of the *eglA* promoter and *eglA* signal sequence for secretion of mTNFα into the tumour mass. Biologically active mTNFα was detected by spectrophotometric assay and the presence of mTNFα in both the culture supernatants and cell lysates was demonstrated by Western blot. The cleaved processed form of mTNFα was detectable in culture supernatants, with both the precursor and processed forms of mTNFα detectable in cell lysates. Lysates and supernatants were taken at various times throughout bacterial growth and added to the highly TNFα sensitive WEHI164 clone 13 cells. Up to $10^{5.5}$ U TNFα per ml of lysate and approximately 10^{4} U TNFα per ml of supernatant was detectable. However, it was noted that the TNFα activity in the supernatant decreased after 20 hours. This is unlike the TNFα activity in the cell lysates, which was maximal at mid-log growth phase and maintained at that level for at least 20 hours. Acidification of the media during growth was attributed to be influencing TNFα stability, as this phenomenon was not seen in pH buffered media.

1.3.2 Prodrug converting enzymes

Greatest attention has focussed on the use of prodrug converting enzymes. These represent the crucial component of Directed Enzyme Prodrug Therapy (DEPT). In DEPT strategies the anti-cancer drug is introduced into the bloodstream as a harmless "prodrug". This is subsequently converted into the active drug by an "enzyme" that is specifically targeted ("directed") to tumour cells, prior to injection of the prodrug. Specific targeting of the enzyme ensures that high therapeutic doses of the drug are exclusively achieved within the vicinity of the tumour, and not elsewhere in the body. As each enzyme molecule is able to catalyse the generation of large quantities of therapeutic drug, not every tumour cell needs to be specially targeted, *i.e.* the 'bystander effect'.

In order for enzyme/prodrug therapy to be effective, both the enzyme and prodrug should meet certain requirements. The enzyme should be either of a non-human origin or a human protein that is absent or expressed only at a low concentration in normal tissues (Rainov *et al.*, 1998; Rigg and Sikora, 1997). The protein must achieve a sufficient level of expression in the tumours and have high catalytic activity (Niculescu-Duvaz *et al.*, 1998). The prodrug should be a good substrate for the expressed enzyme and not be activated by exogenous enzymes present in normal tissue. Additionally, the prodrug must be able to pass through the tumour cell membrane in order for it to take its effect on the tumour cell. It is also preferential to have a high as possible cytotoxicity differential between the prodrug and toxic drug. Finally, it is beneficial if the drug is highly diffusible or is actively taken up by non-expressing cancer cells in order to elicit a 'bystander effect' (Niculescu-Duvaz *et al.*, 1998).

The first DEPT strategy was proposed by Bagshawe (1987). Here, enzyme delivery is mediated by the fusion of the prodrug converting enzyme to a monoclonal antibody raised against a tumour-specific antigen, *i.e.* Antibody-Directed Enzyme Prodrug Therapy (ADEPT). Whilst such an approach remains under investigation (Francis *et al.*, 2002; Spooner *et al.*, 2003), ADEPT suffers from a number of fundamental drawbacks. Tumours

exhibit great heterogeneity with regard to the type of antigen present, necessitating the generation of a multitude of different antibodies for different forms of cancer. Moreover, in some instances tumours do not possess an enriched antigen, and so may not be targeted by this approach. This has led, in part, to a greater emphasis in recent years on strategies which seek to deliver the gene encoding the prodrug converting enzyme, most often through the use of a viral vector (Green *et al.*, 2003; Okabe *et al.*, 2003); in so-called Gene-Directed or Viral-Directed Enzyme Prodrug Therapy (G/VDEPT). However, GDEPT/VDEPT approaches also exhibit fundamental deficiencies, most notably a lack of tumour specificity, poor levels of transgene expression and inefficient distribution of the vector throughout the tumour mass (Xu and McLeod, 2001).

The suggested (Minton *et al.*, 1995) delivery of prodrug converting enzymes through the use of clostridial spores (Clostridial-Directed Enzyme Prodrug Therapy, CDEPT) would overcome many of these problems. The strategy does not rely on the presence or absence of a particular antigen. Rather, it achieves its specificity through the existence of the hypoxic environment present in the centre core of the majority of solid tumours. Two prodrug converting enzymes have been explored to date, based on the bacterial enzymes nitroreductase (NTR) and cytosine deaminase (CD). Both enzymes essentially have no human equivalent.

1.3.2.1 Nitroreductase

The first prodrug-converting enzyme to be cloned and expressed in clostridia was the *E. coli* B nitroreductase NfnB (Michael *et al.*, 1994), which was introduced into *C. beijerinckii* NCIMB 8052 (Minton *et al.*, 1995). Nitroreductase reduces the prodrug CB1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide) into either a 2-hydroxylamine, or 4-hydroxylamine product (Knox *et al.*, 1988b) (see Figure 1.1). The 4-hydroxylamine product (5-(aziridin-1-yl)-4-hydroxylamine-2-nitrobenzamide) is known to be particularly toxic (10^4- to 10^5-fold more cytotoxic than the CB 1954 progenitor). A further non-enzymatic conversion of the

4-hydroxylamine derivative by Acetyl CoA to a 4-acetoxy derivative results in the formation of DNA cross-links. The DNA cross-links lead to apoptosis of the cancer cell (Knox *et al.*, 1988a; Knox *et al.*, 1991).

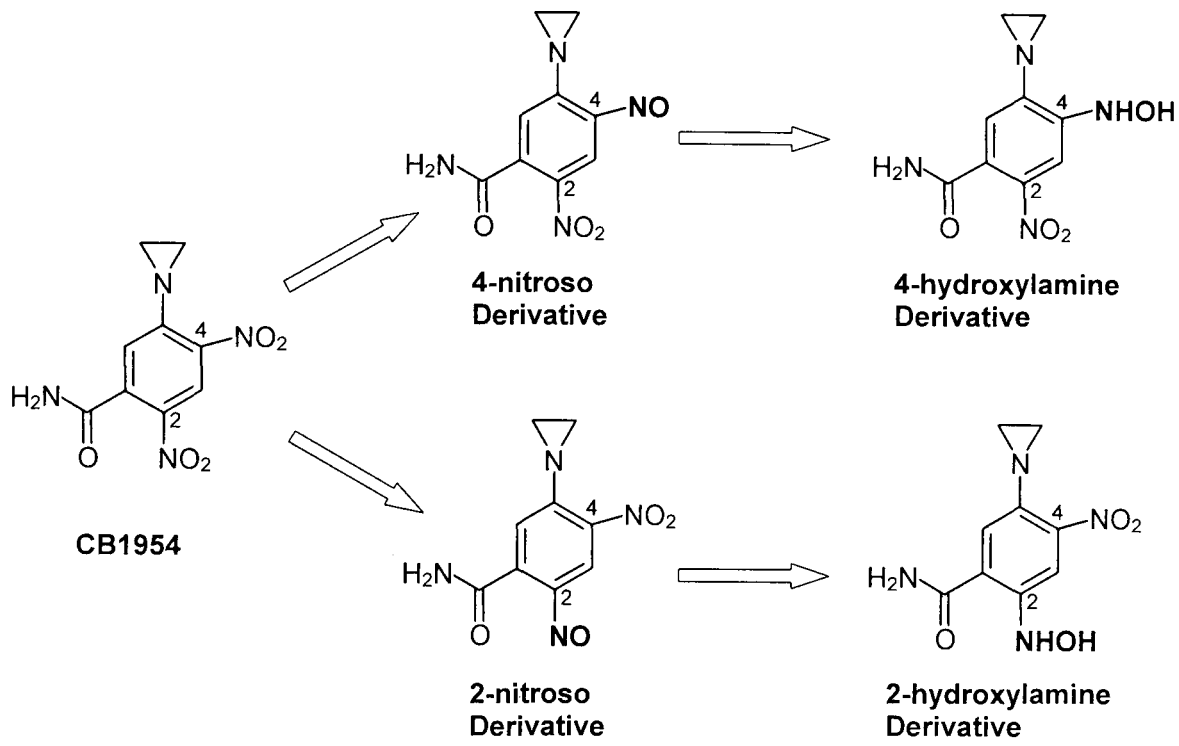


Figure 1.1: Nitroreductase reduction of CB1954 to the 4-hydroxylamino (4-HX) toxic drug. The arrows represent the nitroreductase enzyme catalysed two electron reduction. NfnB converts CB1954 to the 4-HX plus a less toxic 2-HX derivative. Most other nitroreductases studied, except NfnB, convert CB1954 to the toxic 4-HX only. The numbered positions in the benzene ring are indicated. Adapted from AbuKhader *et al* (AbuKhader *et al.*, 2005).

The *nfnB* gene was efficiently expressed in *C. beijerinckii* NCIMB 8052, where recombinant NfnB was estimated to represent 8% of the cells soluble protein. Following the intravenous injection of the recombinant spores into mice bearing EMT6 tumours, tumour lysates were shown, by Western blots, to contain the *E. coli*-derived enzyme (Minton *et al.*, 1995). Subsequently, Lemmon *et al* (1997) confirmed the result obtained with *C. beijerinckii* and measured a 22-fold increase in killing in EMT6 tumours treated with CB1954. However,

despite the fact that recombinant nitroreductase could be detected in tumours, enzyme activity could not be detected in tumour tissue.

1.3.2.2 Cytosine deaminase

The other type of prodrug converting enzyme to be expressed in clostridia is the *E. coli* cytosine deaminase CodA. This enzyme converts the non-toxic prodrug 5-fluorocytosine (5-FC) into the cytotoxic drug 5-fluorouracil (5-FU). 5-FU acts as an anticancer agent as it is further metabolised into 5-fluorouridine-5′-triphosphate and 5-fluoro-2′-deoxyuridine 5′-monophosphate, which inhibit DNA and RNA synthesis (Polak *et al.*, 1976). This enzyme is of particular interest in DEPT strategies as the difference in toxicity between prodrug (5-FC) and drug (5-FU) is large (10^4). Moreover, both 5-FU and 5-FC are currently approved for clinical applications in the treatment of breast and gastrointestinal cancers. Fox *et al* (1996) were the first to report expression of *codA* in *C. beijerinckii*. Supernatants taken from the genetically modified *C. beijerinckii* culture increased the sensitivity of murine EMT6 carcinoma cells to 5-FC 500-fold, a level comparable to other studies where the *codA* gene is transfected into mammalian cells.

More recently, *C. acetobutylicum* was engineered to secrete CodA specifically at the site of the tumour by fusion of the clostripain (*closI*) signal sequence to the 5′ end of *codA* (Theys *et al.*, 2001a). Western blot analysis revealed the 52-kDa CodA enzyme in both culture filtrates and cell lysates from early-logarithmic growth phase samples. *In vitro* and *in vivo* tests ascertained a maximal enzyme activity of 700 pmol 5-FC converted to 5-FU per ml in recombinant bacterial supernatants during early-log phase. This level would equate to a 3% conversion efficiency of 5-FC to 5-FU *in vivo*, an effectiveness considered to be therapeutically viable. The level of CodA activity decreased after early-log phase possibly due to denaturation of the enzyme from acidification of the media or by proteolytic breakdown from extracellular proteases produced by the bacterial host.

1.4 USE OF PROTEOLYTIC STRAINS

The lack of anti-tumour effects seen following administration of prodrug to animals colonised with engineered saccharolytic clostridia may have been due to a number of factors, including segregational instability of the expression vector used and insufficient production of the recombinant prodrug converting enzyme gene. The latter could most simply be accounted for by the attainment of relatively low cell densities of saccharolytic clostridia in the colonised tumour, compared to, for instance *C. sporogenes.* Indeed, it was apparent that the bacterial cell numbers in tumours colonised by *C. beijerinckii* NCIMB 8052 was two orders of magnitude lower than comparable tumours colonised by *C. sporogenes* M55 (Liu *et al.*, 2002).

These experiments suggest that a strain such as *C. sporogenes* M55 may represent a more effective delivery vehicle for anti-cancer agents. As this strain initially proved recalcitrant to gene transfer, another *C. sporogenes* (NCIMB 10696) was identified into which plasmid DNA could be transformed. Accordingly, an expression plasmid (pMTL540CD) carrying *codA* was successfully introduced into strain NCIMB 10696 and its *in vivo* effectiveness assessed as follows (Liu *et al.*, 2002): cell extracts taken from CodA recombinant *C. sporogenes* increased the cytotoxicity of 5-FC to SCCVII cells by a factor of 10^4. Western blot analysis of CodA and detection of enzyme activity *in vitro* were performed both 7 and 14 days post-intravenous injection of 10^8 spores of recombinant *C. sporogenes* into SCCVII tumour-bearing mice. CodA was detected by Western blot analysis in both the 7 and 14 day tumour samples. Also, extracts from tumours from mice injected with recombinant spores increased the cytotoxicity of 5-FC by a factor of 10^3 with no increase observed for mice injected with wild-type spores. The exquisite selectivity of the process was highlighted by Western blot of several different tissue homogenates. CodA was found to be confined to the tumour and could not be found in any of the other tissues screened (brain, heart, lung, liver, kidney and spleen).

To further confirm that *C. sporogenes* was producing sufficient CodA to convert 5-FC to 5-FU at a clinically relevant rate, colonised mice were injected with either 5-FC or 5-FU at maximally tolerated doses. Control groups of animals received either recombinant spores alone, or saline as a negative control. Injection of spores alone caused a small amount of tumour lysis as seen in other experiments, whereas a combination of 5-FC and recombinant spores produced a greater growth delay than that given by the maximally tolerated dose of 5-FU. However, in both cases, the tumours became mildly resistant to 5-FU after the first week of injections.

The benefits of a more aggressive coloniser have additionally been shown in a study that utilised a non-toxinogenic, proteolytic strain of *C. novyi*, *C. novyi*-NT (Dang *et al.*, 2001). These authors assessed a variety of anaerobic bacteria for their capacity to grow extensively and uniquely in the anoxic zones of transplanted tumours. Of the 26 bacterial species tested, 15 species were of the non-pathogenic anaerobe *Bifidobacterium*, 2 species were *Lactobacillus* and 9 were members of the genus *Clostridium*. Intravenous injection of *Bifidobacterium* species resulted in tight clusters of bifidobacteria uniquely in the tumour, rather than dispersed throughout the necrotic regions as preferred. Intratumoural injection of clostridia, in particular *C. novyi* and *C sordellii*, resulted in an extensive spread of vegetative cells throughout the poorly vascularised area of the tumour mass. Toxicity of *C. novyii* and *C. sordellii* is a barrier when injected intravenously. Injection of up to 10^8 spores led to the death of all tumour-bearing mice within 16-18 hours, due to the release of potent lethal bacterial toxins. To overcome this toxicity, the lethal toxin gene was eliminated from *C. novyi* by heat shock, the toxin gene being located within a phage episome. The result was confirmed by PCR and led to a new strain, *C. novyi*-NT. *C. sordellii* was not chosen due to the presence of two homologous toxin genes. Intravenous injection of spores of *C. novyi*-NT led to germination of the spores in the tumour, and resulted in greatly expanded areas of necrosis, without the toxicity side effects observed earlier. However, as in earlier studies, a viable rim of tumour cells still existed at the periphery of the tumour.

1.5 COMBINATION THERAPIES

At an early stage in the evolution of clostridial-based strategies, it was recognised that procedures that enhance tumour hypoxia might increase oncolysis. Thus, Dietzel *et al* (1978) looked at increasing hypoxia in tumours by raising the temperature within the tumour using microwaves (High-Frequency Hyperthermia, H-FH). When this was combined with irradiation in repeated cycles, a larger reduction in tumour weight in animal tumour models was noticed (Gericke *et al.*, 1979). Increasing the degree of hypoxia through restriction of the oxygen level in the respiratory air supplied to animals has also been investigated. A reduction of between 11 and 12% in the air supplied to animals carrying both Ehrlich and Hardy Passey melanomas dramatically improved the extent of tumour lysis, resulting in complete tumour eradication in 30% of cases (Möse, 1979). More recently, an effective route to increasing tumour hypoxia has been demonstrated using vascular targeting agents.

1.5.1 Vascular targeting agents

It had been observed that the efficiency with which tumours are colonised following spore inoculation was directly proportional to tumour volume (Theys *et al.*, 2001b). A tumour size of approximately 3 cm^3 is necessary to guarantee colonisation. This size constraint is directly linked to the degree of hypoxia and necrosis present, *i.e.* the larger the tumour the greater the extent of hypoxia and necrosis within the centre. This led to the hypothesis that the use of angiogenesis inhibitors and/or vascular targeting agents would increase the level of hypoxia and thereby lead to more effective colonisation by clostridia. Moreover, it would allow smaller, more aerobic tumours to be colonised.

This hypothesis was tested in WAG/Rij rats bearing rhabdomyosarcomas using Combretastatin A4-phosphate (CombreAp, OXIGENE, Lund, Sweden), an example of a new class of tumour vasculature targeting agent. CombreAp selectively attacks and destroys tumour-specific blood vessels formed by angiogenesis (Dark *et al.*, 1997) leaving normal

vasculature unharmed, due to the morphologically and functionally abnormal tumour blood vessels (Denekamp, 1993). Systemic administration of CombreAp was shown to result in severe tumour vascular shutdown some 3 to 6 hours after administration, leading to obvious necrosis within 1 to 3 days (Landuyt *et al.*, 2000). Moreover, if administration of CombreAp was preceded 4 hours earlier by an intravenous dose of clostridial spores then small tumours (< 1 cm^3) were colonised. Equivalent sized tumours in the control animals that did not receive any CombreAp were not colonised (Theys *et al.*, 2001b), indicating a strong relationship between the level of hypoxia/necrosis and the likelihood of tumour colonisation.

The benefits of vascular targeting agents were also shown in the studies of Dang *et al* (2001) using *C. novyi*-NT. In this case the vascular targeting agent employed was dolastatin-10 (D10), which was also used in combination with the DNA damaging agent mitomycin C (MMC). Treatment with spores, D10, and MMC resulted in dramatic effects on the large subcutaneous tumours of the colorectal cancer cell line HCT116 in nude mice. 24 hours post-administration of the spores the tumour mass swelled. D10 was then given intravenously and followed by MMC 24 hours later. 6 hours after D10 treatment a zone of necrosis could be seen. This zone increased in size over 24 hours and often completely enveloped the tumour. The necrotic masses then shrunk over the following 2 to 4 weeks. In the control mice, which received only D10 and MMC, no reduction in tumour mass was noted. The downside to this combination bacteriolytic therapy (termed 'COBALT') is the association of significant toxicity: 15–45% of animals died depending on tumour size, when *C. novyi*-NT spores were combined with chemotherapy. A possible explanation could be tumour lysis syndrome, a phenomenon seen in other cases where large tumours are rapidly destroyed by antineoplastic agents.

1.5.2 Temporal regulation of gene expression

A further avenue that is under investigation is to position the gene encoding the therapeutic agent under the control of a radiation-inducible promoter. This would place the production

of the desired therapeutic protein, in combination with the appropriate dose of ionising irradiation, under both temporal and spatial control. In bacteria, DNA damage is restored by a number of systems, the most important of which is the SOS repair mechanism (Miller and Kokjohn, 1990). The *recA* gene is a central component of this system. Using both Northern blot analysis and promoter systems, the expression of the *recA* gene of *C. acetobutylicum* has been shown to be induced by some 30% following irradiation at clinically relevant doses of 2 Gy (Nuyts *et al.*, 2001a; Nuyts *et al.*, 2001b). The feasibility of using the *recA* promoter to regulate mTNFα production was tested by Nuyts and co-workers (Nuyts *et al.*, 2001c). To enable secretion of the protein, the *eglA* signal sequence was fused to mTNFα. Secretion of mTNFα was shown to increase by 44% 3.5 hours after a single irradiation dose of 2 Gy. As radiotherapy is usually given in smaller fractionated doses, a second dose of 2 Gy was given 3 hours after the first. This lead to a 1.33- to 1.36-fold increase in TNFα production. The level of induction achieved correlates well with the increase seen after a single dose indicating that the *recA* promoter can be reactivated by a second dose of radiotherapy (Nuyts *et al.*, 2001c).

Radioinducibility is mediated by binding of DinR (equivalent to LexA in *E. coli*) to operator sequences in the *recA* promoter, termed 'Cheo boxes'. In the original experiments, expression from the *recA* promoter was not completely repressed, as TNFα was produced even in the absence of irradiation (Nuyts *et al.*, 2001c). To exert greater regulatory control, a second Cheo box was subsequently incorporated into the *recA* promoter (Nuyts *et al.*, 2001d). This resulted in an increase in mTNFα secretion from 44% for the wild-type promoter to 412% for the promoter with an extra Cheo box after a single irradiation dose of 2 Gy. Moreover, these authors were able to show that the constitutive endo-β-1,4-glucanase (*eglA*) promoter could be converted to an ionising irradiation responsive promoter through the introduction of a Cheo box. Thus, the presence of a Cheo box resulted in a 242% increase in mTNFα secretion. The increase in mTNFα secretion, from irradiated and non-irradiated control cultures, was confirmed by RT-PCR to be caused by enhanced

transcription.

Radiation-inducible promoters show promise for use in CDEPT strategies as the therapeutic protein is only induced in response to irradiation. Therefore, radiation may be employed as a 'molecular switch', activating transcription/expression of the therapeutic gene only in tumour tissues rather than in other non-tumoural hypoxic tissues, such as abscesses or infarcted tissues.

1.6 OTHER BACTERIAL DELIVERY SYSTEMS

Whilst clostridia have been under the greatest scrutiny, other bacterial-based tumour delivery systems are under investigation, including the facultative anaerobe *Salmonella typhimurium* and the obligate anaerobe *Bifidobacterium*. Growth of *S. typhimurium* within tumours is mediated by severe disablements that both reduce toxicity (by alteration of the lipopolysaccharide) and impose a requirement for adenine. The latter is presumed not to be readily available in normal healthy tissues, but is enriched in the tumour environment as a consequence of cellular breakdown. This attenuation allows the organism to accumulate and persist in tumours and exert an inherent anti-tumour efficacy in their own right. However, whilst this efficacy has been observed in animal models (Low *et al.*, 1999; Pawelek *et al.*, 1997), it was not seen in human trials (Toso *et al.*, 2002). As with clostridia, the use of *S. typhimurium* has been combined with DEPT approaches, most notably with *codA*, where appropriately manipulated strains have been shown, in combination with 5-FC, to cause significant tumour regression in rodent models (Zheng *et al.*, 2000). This particular system has been taken into a Phase I clinical trial (Cunningham and Nemunaitis, 2001), but the final outcome has yet to be published.

The obligate anaerobe *Bifidobacterium longum* has also been shown to selectively colonise solid tumours (Yazawa *et al.*, 2000). Compared to clostridia, the population densities

achieved in tumours are significantly less, *i.e.* less then 10^3 cfu g^{-1} tumour tissue compared to 10^9 cfu g^{-1} in the case of *C. sporogenes*. Given the result obtained with saccharolytic clostridial species, these low numbers might be predicted to be insufficient for the production of effective levels of a recombinant therapeutic protein. Surprisingly, however, recent studies have shown that a strain engineered to produce CodA caused significant regression of DMBA-induced mammary tumours in rats following administration of 5-FC (Fujimori *et al.*, 2002). Similarly, *Bifidobacterium adolescentis* has been used to deliver an endostatin gene to solid tumours in mice, where a strong inhibition of angiogenesis stemmed tumour growth (Li *et al.*, 2003).

1.7 GENETIC MANIPULATION OF CLOSTRIDIA

Anti-tumour effects have been demonstrated using recombinant clostridia to target expression of prodrug converting enzymes and cytotoxic agents to the hypoxic areas of tumours. Nevertheless, further improvements to the current rudimentary genetic manipulation systems available for the clostridia would enable even greater enzyme expression and therefore superior anti-tumour effects.

1.7.1 Introduction

Natural bacterial competence has been reported in several bacteria, most notably in *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Bacillus subtilis*. However, there has never been reported natural competence in the clostridia. Therefore, to introduce foreign DNA into clostridia, transformation procedures that rely on induced competence via the alteration of the cell envelope need to be devised. It has been some 20 years since the development of the first plasmid transformation procedure for clostridia. Cryptic *C. perfringens* plasmids were introduced into a plasmid cured derivative of *C. perfringens* 11268, strain 11268 CDR (Heefner *et al.*, 1984; Squires *et al.*, 1984). In this early work, L-

phase variants (protoplasts unable to regenerate to the bacillary form) of *C. perfringens* 11268 CDR were generated by growth in the presence of penicillin G and 0.4 M sucrose. Being osmotically sensitive, it was thought that L-phase variants would be susceptible to polyethylene glycol (PEG) mediated transformation. Protoplasts were transformed with the tetracycline resistance encoding cryptic plasmid pJU124 from *C. perfringens* 12502 and tetracycline resistant transformants arose at a frequency of approximately 10^2 transformants per μg of plasmid DNA. The plasmid was also introduced by a filter mating technique using *C. perfringens* 12502 as the donor and *C. perfringens* 11268 as the recipient at a frequency of 10^{-5} cells per donor.

Included in the publication on the work on transformation procedures was the development of a variety of *E. coli*/*C. perfringens* shuttle vectors (Squires *et al.*, 1984). A series of plasmid vectors were constructed based on small (< 4 kb) cryptic plasmids from *C. perfringens* fused to pBR322 into which had been inserted a tetracycline resistant determinant to allow for antibiotic selection in both *E. coli* and *C. perfringens*. The plasmids contained restriction sites to allow the insertion of additional heterologous/homologous DNA. Transformation of *C. perfringens* occurred at a frequency of 10^2 transformants per μg of the chimeric plasmids. Following this initial work there have been numerous reports on the improvement of clostridial transformation.

1.7.2 Transformation procedures

PEG mediated transformation has had limited success mainly due to the difficulty in improving the regeneration step such that the L-form cells can regenerate to bacillary form. This difficulty was finally overcome using the partially autolysin deficient *C. acetobutylicum* NI-4081 (Azeddoug *et al.*, 1989; Truffaut *et al.*, 1989). Transformation frequencies of around 1×10^6 transformants per μg plasmid DNA were obtained in this strain. However, the inherent difficulty in transforming and regenerating protoplasts led to the development of transformation of vegetative cells by electric field permeabilisation of the cell membrane, a

process now termed electrotransformation or alternatively electroporation.

1.7.2.1 Electroporation

There have been several reports of the successful introduction of plasmid DNA at a high frequency to a variety of clostridial species including *C. beijerinckii* (Oultram *et al.*, 1988), *C. acetobutylicum* (Mermelstein *et al.*, 1992), proteolytic (Zhou and Johnson, 1993) and non-proteolytic (Davis *et al.*, 2000) *C. botulinum*, *C. perfringens* (Allen and Blaschek, 1988), *C. cellulolyticum* (Jennert *et al.*, 2000) *C. sporogenes* (Liu *et al.*, 2002) and *C. thermocellum* (Tardif *et al.*, 2001). In summary, a pulse of high-intensity electrical field is applied to the cell that induces the temporary formation of pores in the cell membrane through which exogenous DNA can enter the cell. The protocols have been optimised for each species with variations at the stage of cell preparation and in the buffers utilised, the electroporation parameters, and at the stage of cell rescue post-electroporation.

1.7.2.2 Conjugation

As an alternative to electroporation, the conjugal transfer of plasmids from *E. coli* to a clostridial host has been documented for several species including *C. beijerinckii* (Williams *et al.*, 1990), *C. botulinum* (Bradshaw *et al.*, 1998), *C. celluloyticum* (Jennert *et al.*, 2000), *C. difficile* (Purdy *et al.*, 2002), and *C. perfringens* (Lyras and Rood, 1998). In all the reported cases, plasmid transfer from the donor to the recipient was dependent on the transfer mechanism of the broad host range IncP family of plasmids. A transfer origin (*oriT*) provided on the shuttle vector, and several *trans*-acting functions (Tra functions) supplied by the *E. coli* donor are required for conjugal transfer. The Tra functions may be either plasmid-encoded, as is the case for the IncP-type helper plasmid R702, or integrated into the chromosome, as is the case with the donor *E. coli* SM10.

1.7.2.3 Restriction modification

During the early development of genetic systems for *C. acetobutylicum* it was found that *C. acetobutylicum* ATCC 824 contains a restriction system designated *Cac*824I, that greatly reduces the electroporation efficiency of plasmid DNA that lacks the appropriate methylation signature (Mermelstein and Papoutsakis, 1993). The cognate methylase M.*Cac*824I protects host DNA from restriction digestion by the *Cac*824I endonuclease. The *Cac*824I restriction system endonuclease recognises the sequence 5′-GCNGC-3′, which is prevalent in *E. coli* plasmids but occurs infrequently in the *C. acetobutylicum* genome. Protecting plasmid DNA by methylation with the ϕ3TI methyltransferase encoded by *Bacillus subtilis* phage ϕ3T, was shown to protect plasmid DNA from restriction by *Cac*824I and the isoschizomer *Fnu*4HI.

Since these findings, restriction modification systems have been found in the non-proteolytic group II strain of *C. botulinum*, ATCC 25765 (Davis *et al.*, 2000), where the restriction endonuclease *Cbo*I cleaves the palindrome 5′-CCGG-3′. Restriction is overcome by methylation of the shuttle vector in an *E. coli* host containing the *B. subtilis* M.*Bsu*FI methylase which is equivalent to M.*Cbo*I. The *Cce*I (5′-CCGG-3′) restriction activity has been found to exist in *C. cellulolyticum* ATCC 35319 (Jennert *et al.*, 2000), with methylation of the external C by M.*Msp*I (*Msp*I is an isoschizomer of *Cce*I) affording protection of transformed plasmid DNA. Finally, *C. difficile* strains CD3 and CD6 were also found to possess restriction modification systems (Purdy *et al.*, 2002). In the case of CD3 no methylase exists to protect against the enzyme present (*Cdi*I cleaves 5′-CATCG-3′) so all restriction sites need to be removed from shuttle vectors prior to transformation (Purdy *et al.*, 2002). In strain CD6 the two restriction systems, *Cdi*CD6I (cleaves 5′-GGNMCC-3′, equivalent to *Sau*96I) and *Cdi*CD6II (cleaves 5′-GATC-3′ equivalent to *Mbo*I) exist (Purdy *et al.*, 2002). It was found that methylation by M.*Sau*96I protected against the *Cdi*CD6I restriction activity, and the resident *dam* methylation system of *E. coli* protected against *Cdi*CD6II.

Additional to the restriction modification system, some clostridial species contain extracellular endonucleases, in particular, DNases. These have been reported to be present at low levels in *C. thermohydrosulfuricum* DSM 568 (Soutschek-Bauer *et al.*, 1985) and putatively in *C. sporogenes* NCIMB 10696 (Liu *et al.*, 2002). PEG-mediated transformation in the case of *C. thermohydrosulfuricum*, and the addition of DNase inhibitors to the electroporation buffer in the case of *C. sporogenes* NCIMB 10696, negated the activity of extracellular DNases on the introduced plasmid DNA allowing successful transformation.

1.7.3 Plasmid replicons and antibiotic selection

Two decades have elapsed since the development of gene transfer technology for clostridia. During this time an arsenal of vectors has been developed to allow manipulation of the clostridia at the genetic and protein level. Despite this, the clostridial genus as a whole remains relatively intractable to *in vitro* derived genetic transfer such that the use of more genetically amenable organisms like *E. coli* and *B. subtilis* as intermediates is required for plasmid construction. This has led to the exclusive use of 'shuttle' vectors, which can replicate in the intermediate, normally *E. coli*, by the provision of a second replication region. Shuttle vectors therefore require a Gram-negative replication signal and antibiotic resistance marker for selection and maintenance in *E. coli*. Equivalent signals also need to be present for the Gram-positive host.

1.7.4 Shuttle vectors

Native plasmids have been identified in several clostridia, for example in *C. acetobutylicum* ATCC 824 the pSOL1 megaplasmid encodes the genes necessary for acetone and butanol production by this organism (Cornillot *et al.*, 1997). In addition to house keeping genes, some clostridia have plasmid-encoded virulence factors. These include the tetanus toxin of *C. tetani* (Finn *et al.*, 1984) and several toxins produced by *C. perfringens* (Katayama *et al.*, 1996). Native plasmids are one source of replication functions that have been utilised in the

construction of shuttle vectors since they are known to be functional in the host species and should be relatively segregationally stable. Several native plasmids have been extensively characterised, including pCB101 and pCB102 from *C. butyricum* (Minton and Morris, 1981), pCD6 from *C. difficile* (Purdy *et al.*, 2002) and pIP404 from *C. perfringens* (Garnier and Cole, 1986). As well as these well defined plasmids, several other clostridial plasmids have been utilised in the construction of shuttle vectors, namely pCS86 from *C. acetobutylicum* strain No. 86 (Yoshino *et al.*, 1990), and the *C. perfringens* plasmids pHB101, pJU121, and pJU122 (Blaschek and Solberg, 1981; Squires *et al.*, 1984).

The replication regions of the well-defined clostridial plasmids were identified by cloning putative minimal replication regions into otherwise non-replicative vectors. The vectors constructed were then transformed into either *B. subtilis* in the case of pCB101 and pIP404 (Collins *et al.*, 1985; Garnier and Cole, 1988), or *C. beijerinckii* in the case of pCB102 and pCD6 (Collins *et al.*, 1985; Purdy *et al.*, 2002). Analysis of the replication regions from these plasmids identified several different modes of replication. Firstly, pCB101 displays many of the characteristics of other Gram-positive plasmids which replicate via a rolling circle mechanism (Brehm *et al.*, 1992). Sequence analysis of the replication protein of pCB102 on the other hand revealed no homology to any currently known plasmid, thus it is unclear as to the mechanism by which this plasmid replicates. The replication mechanisms of pCD6 and pIP404 are also not definitely known, although they do both share features found in plasmids that replicate via the theta replication method. These are a large putative replication protein and an extensive region of AT rich DNA repeats (Purdy *et al.*, 2002).

An alternative to the use of clostridial plasmids to construct shuttle vectors is the use of replicons derived from other Gram-positive bacteria. The *B. subtilis* plasmid pIM13 (Monod *et al.*, 1986) has been widely used. It replicates via a single stranded DNA (ssDNA) intermediate and putatively based on that, a rolling circle mechanism. The *Enterococcus faecalis* plasmid pAMβ1 (Clewell *et al.*, 1974) has also been extensively employed. It replicates via a unidirectional theta replicating mechanism. Other plasmids that have been

utilised to a lesser extent are pIP501 from *E. faecalis* (Saunders and Guild, 1980), pWV01 from *Lactococcus lactis* (Otto *et al.*, 1982), and the *Staphylococcus aureus* plasmids pT127 and pUB110 (Lacey and Chopra, 1974; Novick and Brodsky, 1972). Generally, plasmids from other Gram-positive bacteria are not as segregationally stable as their clostridial counterparts. This is in part due to these plasmids replicating via a rolling circle mechanism, which generates highly recombinogenic ssDNA intermediates (Gruss and Ehrlich, 1989), and as such they are not as good a choice for the construction of stable shuttle vectors. The one exception to this rule is pAMβ1 which is generally stable in the variety of clostridial hosts which have been tested (Allen and Blaschek, 1988; Jennert *et al.*, 2000; Oultram *et al.*, 1988; Reysset and Sebald, 1985).

In the absence of a 100% segregationally stable plasmid, antibiotic selection is required for positive selection of transformants. Ampicillin is routinely used in *E. coli* for antibiotic selection of plasmids, the resistance being encoded by β-lactamase (*bla*). However, Bla is not functional in Gram-positive bacteria. The erythromycin resistance genes *ermB* from pAMβ1 (Clewell *et al.*, 1974) or *ermC* from pIM13 (Monod *et al.*, 1986) are the most widely used selective marker to screen for clostridial transformants. Other antibiotic resistance markers that are frequently used include the tetracycline resistance gene *tetM* from Tn*916* (Franke and Clewell, 1981) and the *C. perfringens* Tn*4451* chloramphenicol resistance gene, chloramphenicol acetyl transferase (*catP*) (Abraham and Rood, 1987). In the case of CatP, thiamphenicol is used instead of chloramphenicol for the selection of clostridial transformants.

1.7.5 Expression vectors

Expression vectors have been used in clostridia to obtain both expression of a particular protein and the expression of antisense RNA as a means of down-regulating protein expression.

For the over expression of prodrug converting enzymes it is desirable to have strong constitutive expression such that the maximal level of protein is produced all throughout the growth cycle of the bacterium. Also, as the prodrug converting enzyme genes are not native to clostridia it is unlikely that the native prodrug converting enzyme promoter signals will function efficiently in clostridia. Therefore, the gene cannot be cloned with its own promoter, a technique often used for the expression of clostridial genes.

Clostridial species produce a variety of sigma factors throughout growth and in response to different growth and environmental conditions. These alter the promoter recognition by RNA polymerase allowing coordinate transcription of different gene sets for a variety of bacterial responses. Furthermore, numerous transcriptional regulators exist leading to activation or repression of transcription. For this reason, most of the promoters that have been characterised are unsuitable for CDEPT due to their temporal expression. For example, in *C. acetobutylicum* these are promoters related to solventogenesis or acidogenesis, or to sporulation (Boynton *et al.*, 1996a; Boynton *et al.*, 1996b; Harris *et al.*, 2002; Mermelstein *et al.*, 1992; Nair *et al.*, 1999; Papoutsakis and Bennett, 1993). As a consequence, only a few promoters have been isolated and utilised to promote prodrug converting enzyme production. These are the promoters preceding the β-1,4-endoglucanase (*eglA*) region of *C. acetobutylicum* P262 (Zappe *et al.*, 1988), the clostripain gene (*closI*) gene of *C. histolyticum* DSM 1126 (Dargatz *et al.*, 1993), and the *C. pasteurianum* ferredoxin (*fdx*) gene (Graves and Rabinowitz, 1986). These promoters all generally conform to the promoter consensus sequence recognised by the major form of *Clostridium* RNA polymerase, which shows homology to other bacteria (see Figure 1.2).

	-35		-10	
eglA	AAAAA	TTATTA ATGTAAAAATATACTAAG	TATAGA	ATATTTA
closI	AATAA	TGTAAA ACTTTAAATAATAACTCT	TATAAT	GGTTTTT
fdx	ACACT	TTTAAA AAGTTTAAAAACATGA	TACAAT	AAGTTAT
Consensus		**TTGACA** (17 bp)	**TATAAT**	

Figure 1.2: Putative promoter sequences of the β-1,4-endoglucanase (*eglA*), clostripain (*closI*) and ferredoxin (*fdx*) genes used within clostridial prodrug converting enzyme expression vectors. The consensus sequence for the -35 and -10 is based on that recognized by the vegetative RNA polymerase holoenzyme. The -35 and -10 regions are underlined; bold characters within the consensus sequence indicate the highly conserved nucleotides, modified from Van Mellaert *et al* (Van Mellaert *et al.*, 2005).

As well as expressing the prodrug converting enzyme, it is desirable in some circumstances that the clostridial cells secrete the enzyme into the tumour mass. This can be obtained by fusing a signal sequence in frame to the 5′ end of the gene of interest. The signal sequence encodes a signal peptide that consists of an NH_2-terminal extension of the secretory peptide, which is required for successful translocation of the cell membrane. The signal peptide is cleaved by a signal peptidase once the protein is in the extracellular environment. The native signal peptide that is encoded by a secreted prodrug converting enzyme is usually not recognised by clostridial cells such that secretion is not very effective, if it occurs at all. Therefore, to enable efficient secretion of the prodrug converting enzyme from the clostridial cell two such signal peptides have been successfully developed, based on either the *C. histolyticum* DSM 1126 *closI* gene, or on the *eglA* gene of *C. acetobutylicum* P262 (Dargatz *et al.*, 1993; Zappe *et al.*, 1988). Both signal peptides proved functional and secreted protein was detectable in the culture medium when the signal peptides were fused to either cytosine deaminase (*codA*) (Theys *et al.*, 2001a) or tumour necrosis factor (m-TNFα) (Theys *et al.*, 1999), respectively.

1.7.6 Gene integration in clostridia

The recent and dramatic increase in antibiotic resistance and the rise in the occurrence of multidrug resistant bacteria in both community and hospital settings has led to the reduction in the use of antibiotic markers for clinical applications such as CDEPT. It is therefore necessary to have the prodrug converting enzyme integrated into the clostridial chromosome. This negates the use of plasmid-borne expression systems that are reliant on antibiotic selective markers.

1.7.6.1 Homologous recombination

The most common way of introducing genes into the bacterial chromosome is via homologous recombination, a process that results in genetic exchange between homologous DNA sequences from different sources. If the homologous DNA that is being inserted is interrupted in the middle with an antibiotic resistance marker (or other gene of interest) then following recombination, the target gene will be inactivated and the presence of the antibiotic resistance marker will allow selection of appropriate clones. Usually this technique is used to inactivate a gene of interest in a process called allelic exchange mutagenesis. This process involves the exchange of the wild type chromosome encoded copy of the gene with a non-functional truncated copy of the gene. This is usually achieved by introducing the non-functional copy of the gene onto a replication deficient suicide vector such that once introduced the only antibiotic resistant clones will be ones where the gene of interest has been mutated. The preferable way of introducing the non-functional copy of the gene is on a conditionally replicative vector, for example a vector containing a temperature sensitive replicon. This would result in a plasmid that replicates only at the permissive condition. Changing to the non-permissive condition would inhibit plasmid replication thereby forcing segregational loss of the plasmid from the bacterial cell. The only way antibiotic resistant bacteria can be recovered is if the antibiotic resistance marker has integrated into the chromosome. The advantage to this approach is that a suitably high

number of cells will contain the plasmid prior to selecting for the rare integration event.

Gene inactivation by allelic exchange can occur in two ways:

1) Single crossover integration

This process involves genetic exchange between a chromosomal copy of a gene and a plasmid-borne copy of the same gene that is truncated at the 5′ and 3′ ends (see Figure 1.3). Homologous recombination between the two DNA sequences results in the integration of the entire plasmid into the chromosome. The final outcome being the presence of two dysfunctional truncated copies of the target gene, one truncated at the 5′ end the other truncated at the 3′ end. However, mutations achieved in this way are intrinsically unstable due to the presence of large repeated flanking regions that may recombine to revert to the parent genotype.

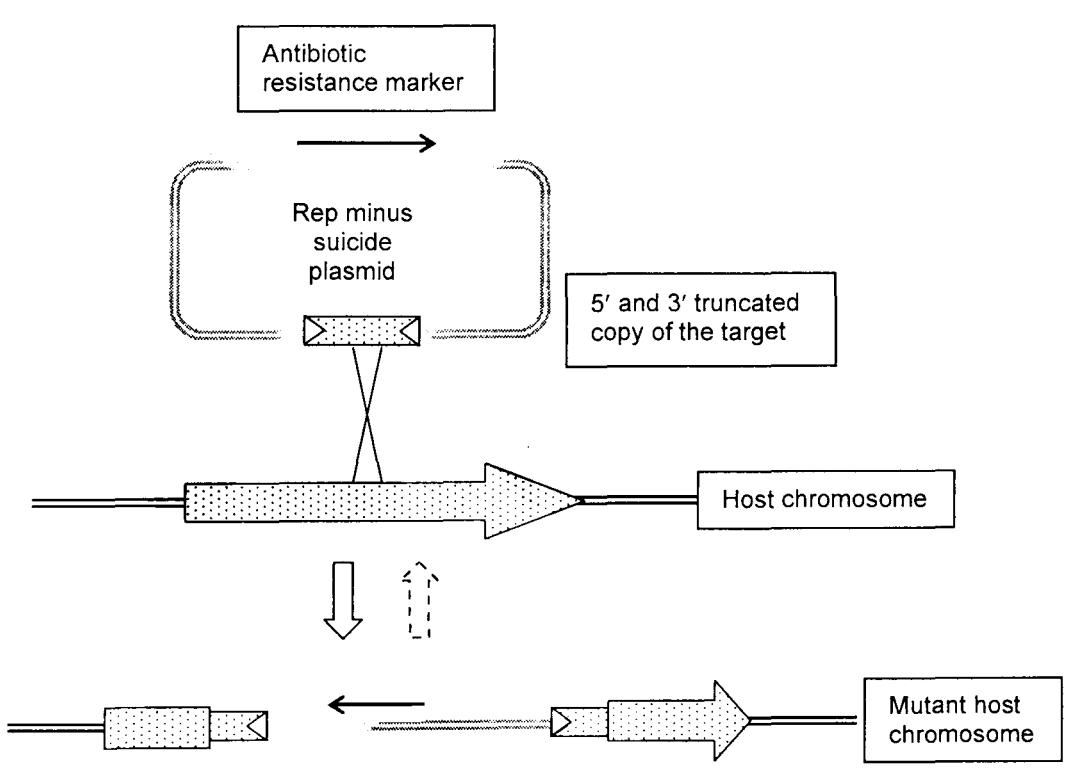


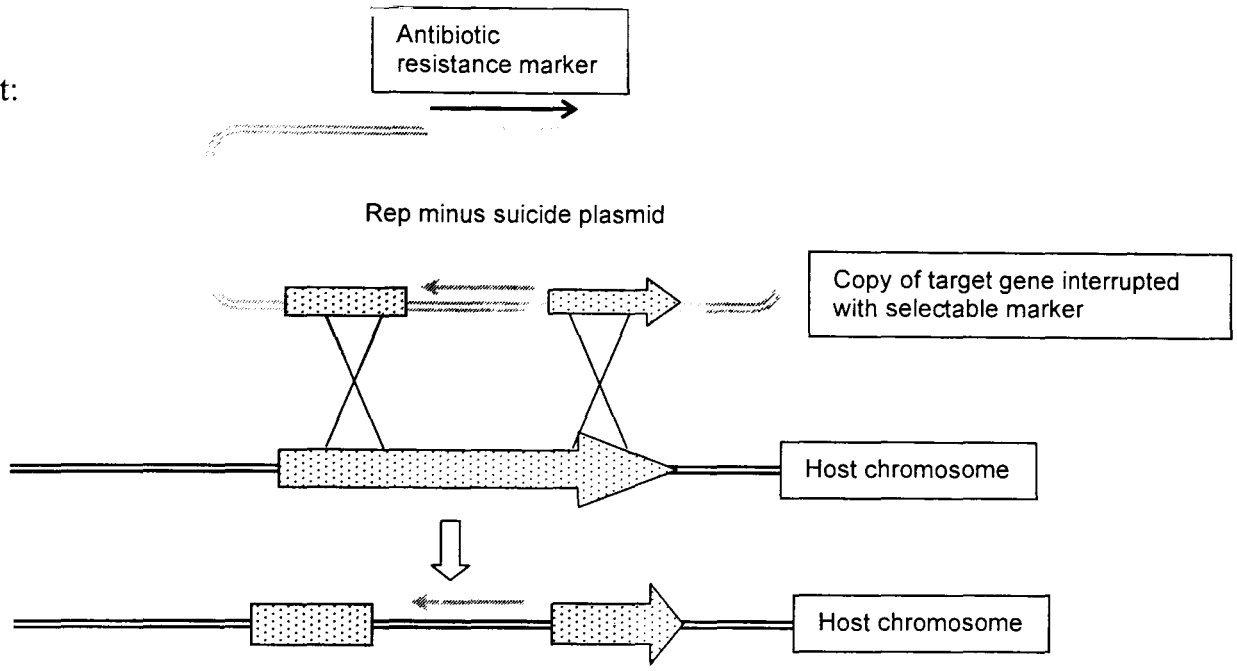
Figure 1.3: A schematic representation of single crossover recombination.

2) Double crossover integration

The more desired situation is double crossover integration as there is less chance of reversion to the wild type genotype and only the antibiotic resistance marker persists in the host. Allelic exchange by double crossover relies on the exchange between the target gene and a plasmid-borne copy of the target gene disrupted with a selectable marker, which is usually an antibiotic resistance marker (see Figure 1.4). The process of double crossover can occur either directly or indirectly. Direct double crossovers occur in one recombination event such that a correct mutant can be directly selected. Alternatively, the process of double crossover integration can be split into a two-stage event. Initially the process is identical to single crossover integration, in that the entire plasmid integrates into the host chromosome. However, a second recombination event occurs between two homologous stretches of DNA resulting in two possibilities. In 50% of cases there is resolution of the entire plasmid and disrupted target gene and reversion to the parent genotype. The alternative and desired event is re-excision of the plasmid leaving an interrupted dysfunctional target gene.

Allelic exchange has been used successfully for the knock out of several genes in both *C. acetobutylicum* and *C. perfringens*. The first reported clostridial mutant was generated by Wilkinson and Young (1994), where both *gutD* and *spoOA* single crossover mutants were made in *C. acetobutylicum* NCIMB 8052 (now renamed *C. beijerinckii* NCIMB 8052) by conjugal transfer of a suicide plasmid from an *E. coli* donor. Since then, a variety of mutants have been made in *C. acetobutylicum* ATCC 824: a single crossover mutant in aldehyde/alcohol dehydrogenase (*aad*) (Green and Bennett, 1996), an uncharacterised mutant in the sporulation specific sigma E-processing enzyme (*orfA*) (Wong and Bennett, 1996), and single crossovers in phosphotransacetylase (*pta*) and butyrate kinase (*buk*) (Green *et al.*, 1996). In *C. perfringens* double crossover mutants were obtained in theta-toxin (*pfo*A) and alpha toxin (*plc*) (Awad *et al.*, 1995), in the enterotoxin (*cpe*) (Sarker *et al.*, 1999), in collagenase (*colA*) (Awad *et al.*, 2000), and in the toxin regulator (*virX*) (Ohtani *et al.*, 2002).

Direct:



Indirect:

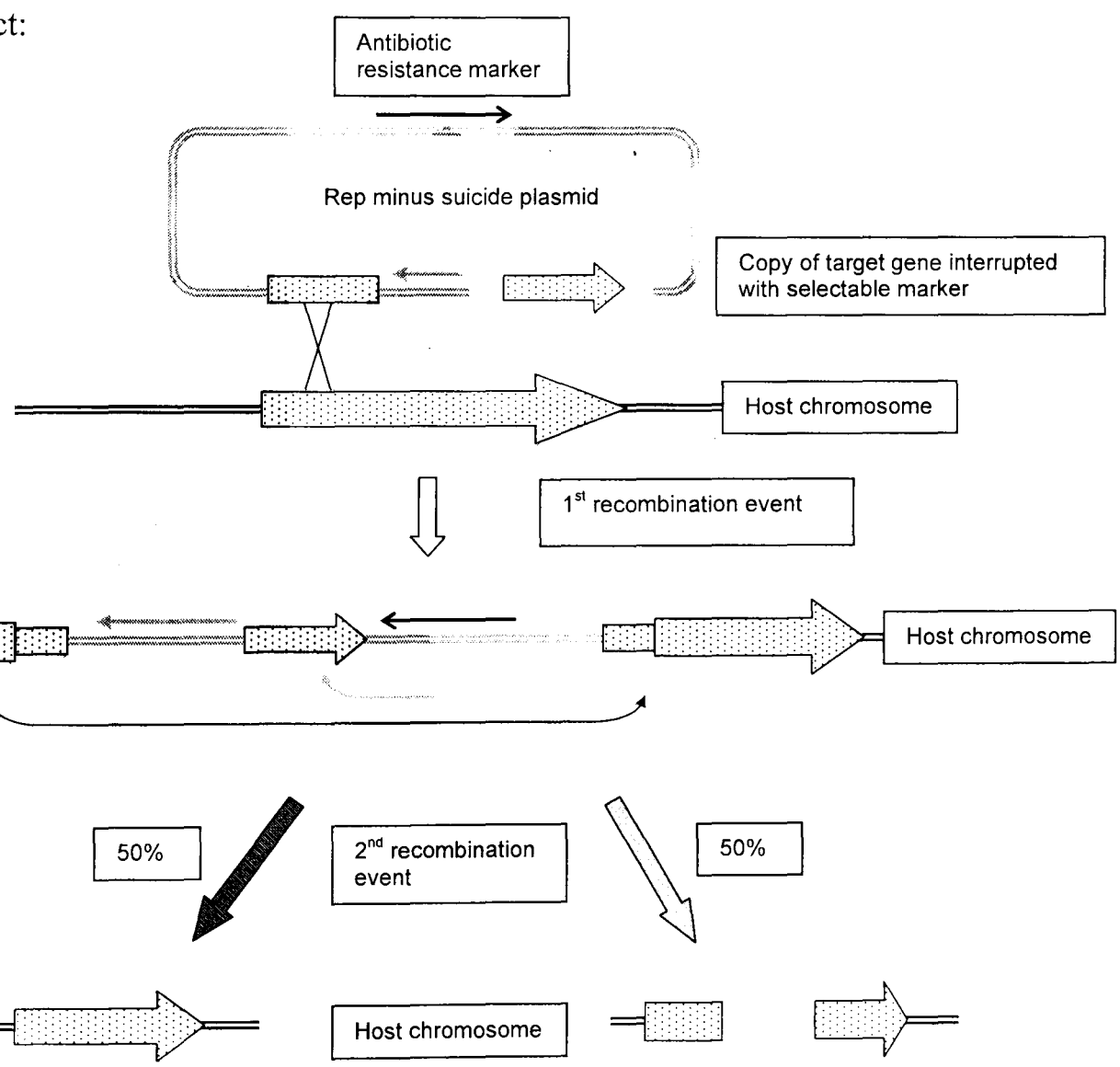


Figure 1.4: Schematic representation of direct and indirect double crossover recombination.

Delivery of the single crossover or double crossover fragments to clostridia is usually by a replication deficient suicide plasmid. However, this method is somewhat unreliable and burdensome with sometimes several thousand colonies needing to be screened in order to obtain a correct knockout. Also, the experiment may need several attempts to obtain a knockout, and sometimes a knockout will not occur at all.

Due to the problems experienced by Harris and colleagues in obtaining a *spoOA* knockout mutant in *C. acetobutylicum* ATCC 824 using the suicide method, they developed a replicative plasmid based approach (Harris *et al.*, 2002). In order to disrupt *spoOA*, a 580 bp internal *spoOA* fragment was PCR amplified and cloned into a plasmid based on the Gram-positive replicon from the *B. subtilis* plasmid pIM13. Serial passage of the recombinant *C. acetobutylicum* ATCC 824 harbouring the *spoOA* knockout plasmid on non-selective media was performed every 24 hours for 5 consecutive days with replica plating. Cells that grew on erythromycin (selects for integration event) but not on thiamphenicol (selects for plasmid) were isolated by comparing cultures that were replica plated onto medium containing either antibiotic. Isolates of *C. acetobutylicum* ATCC 824 that met the thiamphenicol sensitive and erythromycin resistant phenotype were then analysed by PCR amplification and sequencing.

One isolate, SKO1, which was obtained in the first experiment with this method was analysed further by sequencing the interrupted region as the expected PCR product size for the interrupted gene was not obtained. From the sequencing data they determined that the following sequence of events occurred: first, the entire plasmid was apparently integrated into the chromosome at the *spo0A* gene, due to the presence of the duplicated internal *spoOA* fragment. It was expected that a second recombination event would occur somewhere in the duplicated region, resulting in either reversion to the parent phenotype or deletion of the plasmid DNA except for the marker resulting in an ideal double crossover. However, a second recombination event occurred between two 10 bp homologous sequences (5′-ACGACCAAAA-3′) that were present in the 3′ end of the Gram-positive replicon *repL* structural gene and upstream of the erythromycin resistance marker. The result was

inactivation of *spoOA* by insertion of a slightly larger fragment than predicted, an abnormal double crossover mutant. Potential reasons why a double crossover could occur on a replicative vector are due to the method of replication of the vector. pIM13 is known to replicate via a rolling circle method (Projan *et al.*, 1987) that generates highly recombinogenic ssDNA intermediates. Secondly, by not implying selection, the resistance markers would only be maintained by the plasmid integrating due to the segregational instability of non-native rolling circle plasmids.

1.7.6.2 Non-homologous recombination

Recombination between DNA molecules can occur in a variety of other ways that are not dependent on regions of homologous DNA cloned onto a plasmid, or on the hosts recombination machinery. Mobile genetic elements, namely insertion sequences (IS) and transposons, are one such example of plasmid or chromosomally encoded genetic elements that perform non-homologous (illegitimate) recombination.

Insertion sequences are the simplest type of mobile genetic element. Usually they consist of one or more transposase enzymes that are necessary for the movement of the element from one site to another. At the ends of most insertion sequences is an almost perfect inverted repeat (IR) sequence. The complementary nature of the IR sequences is in part responsible for the mobile nature of IS elements. Short duplicated target sequences are usually found flanking IS elements. This is not part of the IS element *per se*, but arises from the DNA at the site of insertion as a consequence of the molecular events involved.

Transposons are similar to IS elements in that they consist of a transposase flanked with IR sequences and short duplicated target sequences. However, transposons also contain a resolution site, a resolvase and an identifiable genetic marker (usually an antibiotic resistance gene). More complex transposons, composite transposons, exist that consist of two copies of an IS element in inverted orientation or as direct repeats (one usually contains a non-

functional defective transposase) surrounding a set of resistance genes. Finally, a transposon can exist as an integron that is built up by insertion of additional antibiotic resistance genes within an existing transposon.

Insertion sequences have been found in a variety of bacteria, although only a few have been reported in the clostridia. Several have been reported in *C. perfringens* (Brynestad *et al.*, 1997; Daube *et al.*, 1993), and one, IS*CbI*, has been found in *C. beijerinckii* NCIMB 8052 (Liyanage *et al.*, 2000a). To date, IS elements have not been utilised as a genetic tool for the manipulation of the clostridia.

Transposons have also been found in a few clostridia, namely a Tn*916*-like tetracycline resistant determinant in *C. difficile* (Hachler *et al.*, 1987), and two Tn*3*-like transposons, Tn*4451* and Tn*4452*, in *C. perfringens* (Abraham and Rood, 1987). Although potentially useful, these transposons have not been shown to be capable of transposition. Among the several well characterised transposons, the 16.4 kb tetracycline resistance encoding conjugative transposon Tn*916* from *E. faecalis* DS16 (Franke and Clewell, 1981) has been shown to be successfully transferred by conjugation to *C. acetobutylicum* (Woolley *et al.*, 1989), *C. botulinum* (Lin and Johnson, 1991), *C. tetani* (Volk *et al.*, 1988), *C. difficile* (Mullany *et al.*, 1991), and *C. perfringens* (Lyristis *et al.*, 1994).

Utilising Tn*916*, several different mutants were obtained in a variety of clostridia. In *C. perfringens*, virulence gene sensor histidine kinase regulator (*virS*) (Lyristis *et al.*, 1994) and alpha, theta and kappa toxin mutants (Awad and Rood, 1997) were achieved. Granulose mutants (Mattsson and Rogers, 1994) and a tRNA mutant (Sauer and Durre, 1992) were obtained in *C. acetobutylicum*. Finally, several different uncharacterised auxotrophic mutants have been obtained in *C. botulinum* (Lin and Johnson, 1991).

Tn*1545*, originally isolated from *Streptococcus pneumoniae*, has also been used but to a lesser extent than Tn*916*, in the generation of mutants in *C. beijerinckii* (Evans *et al.*, 1998;

Liyanage *et al.*, 2000b).

One major disadvantage to the use of Tn*916* and transposons as a whole as a genetic tool is that integration is not targeted. Tn*916* is reported to integrate into a hot spot in *C. difficile* CD37 (Mullany *et al.*, 1991; Wang *et al.*, 2000) and *C. beijerinckii* (Woolley *et al.*, 1989). In other clostridial hosts, Tn*916* was found to integrate at multiple host sites, namely in *C. difficile* 79-685 (Roberts *et al.*, 2003), *C. tetani* (Volk *et al.*, 1988), *C. acetobutylicum* (Babb *et al.*, 1993) and *C. botulinum* (Lin and Johnson, 1991). Tn*1545* is reported to integrate at multiple sites in *C. beijerinckii* (Woolley *et al.*, 1989). From the perspective of CDEPT, non-targeted integration is not desired as integration in unknown genes may change the characteristics of the host such that the colonisation of the tumour may be affected, expression of the prodrug may be changed or the formation of spores reduced.

1.8 CONCLUSIONS

It is now over 10 years since the CDEPT concept was first proposed (Minton *et al.*, 1995) as a means of treating solid tumours. In the intervening years a number of key steps have been taken towards proof of principle and the strategy now shows considerable promise as a novel therapy for treating solid tumours. Whilst concerns over the concept of deliberately 'infecting' patients with a live clostridial species may surface, it is to be anticipated that clinical evaluations should dispel any such fears, allowing the full potential of CDEPT to be realised. However, there are still a number of additional refinements that have to be put in place before this approach can move into a clinical setting.

Whilst proteolytic strains such as *C. sporogenes* and *C. novyi* are more effective in tumour colonisation and achieve higher population densities, these more aggressive tumour colonisers may cause toxicity (Dang *et al.*, 2001). The data obtained with *B. longum* has shown that high bacterial cell numbers are not necessarily required (Fujimori *et al.*, 2002).

The higher levels of therapeutic protein necessary could be achieved through the use of more effective transcription and translation signals. Therefore, to obtain the maximal benefit from CDEPT a suitable expression system needs to be employed. Regardless of the host used, for clinical evaluation, strains need to be generated which do not carry bacterial antibiotic resistance markers. This may be achieved through the integration of the therapeutic genes concerned into the genome.

In addition to optimising the production of recombinant protein, the strategy may also benefit from maximising the catalytic activity of the enzyme utilised, through the use of the most effective enzyme and prodrug. The benefit of a more soluble nitroreductase prodrug has already been demonstrated (Liu *et al.*, 2002). The use of more effective enzymes is also likely to prove fruitful (Anlezark *et al.*, 2002). The deliberate secretion of the therapeutic protein may extend the area of drug generation away from the immediate vicinity of the foci of colonisation within the tumour. However, secreted enzymes are prone to proteolysis. This may present a particular problem with proteolytic clostridial strains. Moreover, a wider distribution of enzyme may give rise to a specific immune response directed against the protein. Immune responses in ADEPT prevent repeat therapies. On the other hand, recent studies have shown that the immunogenicity of specific enzymes can be attenuated (Spencer *et al.*, 2002).

1.9 PROJECT AIMS

This study forms part of a European Union 5th Framework consortium consisting of six partners. The overall goal was to further develop the CDEPT strategy, ultimately to phase I clinical trial.

The specific aims of this study are:

- To obtain over-expression of either of two prodrug converting enzymes. Both carboxypeptidase G2 and novel nitroreductase enzymes will be cloned and over-expressed in an optimised expression vector and transformed into an optimally colonising clostridial host. Once obtained, engineered strains will be assessed for the production of the prodrug converting enzyme both *in vitro* and in an *in vivo* mouse model.
- To assess the tools currently available for gene integration in clostridia and modify them for integration of the promoter/prodrug converting enzyme fusion into suitable genome targets, such that the colonisation capabilities of the recombinant strain remain unaltered.

CHAPTER 2

MATERIALS AND METHODS

2.1 CHEMICALS AND KITS

Invitrogen Ltd., Paisley, U.K.

TOPO TA Cloning® Kit (with pCR2.1-TOPO or pCRII-Blunt vector), NuPAGE® gels and buffers, Zymogram gels and buffers

New England Biolabs (U.K.) Ltd., Herts, U.K.

Restriction endonucleases and buffers, Klenow DNA polymerase I fragment, T4 DNA polymerase, Antarctic phosphatase, T4 DNA ligase,

Novagen, Merk Biosciences, Nottingham, U.K.

Bugbuster protein extraction reagent, rLysozyme™ , Benzonase™

QIAGEN Ltd., West Sussex, U.K.

QIAprep spin miniprep kit, QIAquick PCR purification kit, QIAquick gel extraction kit, RNeasy miniprep kit, One-Step RT-PCR kit, Hot Star Taq, DNeasy tissue kit.

Sigma Aldrich Company Ltd., Dorset, UK

All chemicals were supplied by Sigma Aldrich unless otherwise stated.

Nucleic acids

Oligonucleotides were synthesised by Sigma-Genosys Ltd., Haverhill, U.K.. Synthetic genes were supplied by Entelechon GmbH, Regensburg, Germany.

Cambio

Failsafe PCR system

2.2 BACTERIAL STRAINS AND PLASMIDS

Strain	Genotype	Reference/source
E. coli TOP10	F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lac*X74 *deoR recA1 araD139* Δ(*ara-leu*)7697 *galU galK rspL* (Str^R) *endA1 nupG*	Invitrogen Ltd.®
E. coli TOP10 F′	F′{*lacI*^{q} Tn*10* (Tet^R)} *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lacZ*ΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L(*Str*^{R}) *end*A1 *nup*G	Invitrogen Ltd.®
E. coli HMS174 (DE3)	F⁻, *recA,1 hsdR*(r_{K12} -m_{K12}), Rif^R lambda (DE3)	Invitrogen Ltd.®
E. coli HB101	*thi*-1 hsdS20 (r_B-, m_B-) *supE44 recAB ara-14 leuB5proA2 lacY1 galK rpsL20* (str^R) *xyl-5 mtl-1*	N. P. Minton
Bacillus subtilis BS34A	Tn*916* conjugative donor strain	A. Roberts, UCL, London
Clostridium sporogenes NCIMB 10696		N. P. Minton
Variovorax paradoxus ATCC 25301		N. P. Minton

Table 2.1 - Bacterial strains used in this study

Vector	Replicon(s)	Antibiotic selection	Features	Reference/ source
pMTL20	ColEI	Amp	*E. coli* cloning vector	(Chambers *et al.*, 1988a)
pMTL20E	ColEI	Amp, Erm	*E. coli* cloning vector	M. Young, University of Wales Aberystwyth
pCR2.1-TOPO	ColEI	Amp, Kan	*E. coli* PCR T/A cloning vector	Invitrogen
pIMP1	ColEI, pIM13	Amp, Erm	*Clostridium* shuttle vector	(Mermelstein and Papoutsakis, 1993)

pMTL9341aLS2	ColEI, pCD6	Erm	*Clostridium* shuttle vector, antisense expression vector	(Carter *et al.*, 2005)
pCR4-TOPO::*fac*2	ColEI	Amp, Kan	*E. coli* PCR T/A cloning vector, *fac2* promoter	Entelechon GmbH
pMTL30	ColEI	Amp, Erm	Mobilisable suicide plasmid	(Williams *et al.*, 1990)
pCR-Blunt II-TOPO	ColEI	Kan, Zeocin	*E. coli* PCR blunt cloning vector	Invitrogen
R702	IncPβ conjugative	Tet, Kan	Tra^+, Mob^+, conjugative plasmid	(Hedges and Jacob, 1974)
pMTL9301	ColEI, pCD6	Erm	*Clostridium* shuttle vector	(Purdy *et al.*, 2002)
pHZ117	ColEI	Amp	*eglA* promoter and coding sequence	(Abratt *et al.*, 1993)
pMTL21	ColEI	Amp	*E. coli* cloning vector	(Chambers *et al.*, 1988a)
pMTL1010	ColEI	Tet	*E. coli* expression vector	R. Melton, Protherics, Salisbury
pCR4-TOPO::CPG2-synth	ColEI	Amp, Kan	*E. coli* PCR T/A cloning vector, synthetic CPG2	Entelechon GmbH
pCR4-TOPO::*Hin*NTR-synth	ColEI	Amp, Kan	*E. coli* PCR T/A cloning vector, synthetic *Hin*NTR	Entelechon GmbH
pF25	ColEI	Amp	*nfnB* cloned into pTrc99A	R. Naylor, Protherics, Salisbury
pMTL9361*gusA*	ColEI, pCD6	Erm	GusA reporter vector	J. Scott, University of Nottingham
pKNT19-c*closI*$_p$*codA*	ColEI, pIM13	Erm	Source of *closI* promoter	(Theys *et al.*, 2001a)

pMTL9361*gusA*::$fac2_p$	ColEI, pCD6	Erm	*fac2* promoter *gusA* reporter fusion	G. Carter, Nottingham
pMTL9361*gusA*::$glnA_p$	ColEI, pCD6	Erm	*glnA* promoter *gusA* reporter fusion	G. Carter, Nottingham
pMTL9361*gusA*::$hydA_p$	ColEI, pCD6	Erm	*hydA* promoter *gusA* reporter fusion	G. Carter, Nottingham
pMTL9361*gusA*::$eglA_p$	ColEI, pCD6	Erm	*eglA* promoter *gusA* reporter fusion	G. Carter, Nottingham
pMTL9361*catP*	ColEI, pCD6	Erm	CatP reporter vector	J. Scott, University of Nottingham
pMTL5200	ColEI	Amp, Erm	*Hind*III/*Xba*I pBP1 replicon	N. Minton, University of Nottingham
pMTL9401	ColEI, pCB102	Erm	*Clostridium* shuttle vector	(Purdy *et al.*, 2002)
pMTL9511	ColEI, pAMβ1	Erm	*Clostridium* shuttle vector	M. Herbert, HPA Porton Down, Salisbury
pMTL9611	ColEI, pIP404	Erm	*Clostridium* shuttle vector	(Purdy *et al.*, 2002)
pJIR418	ColEI, pIP404	Erm, Cm	*Clostridium* shuttle vector	(Sloan *et al.*, 1992)
pMTL1015::NTR-N	ColEI	Tet	*E. coli* overexpression plasmid, source of NTR-N	J. Heap, University of Nottingham
pCRScript::*Hin*NTR-synthv2	ColEI	Amp	Synthetic gene – *Hin*NTR version 2	Entelechon GmbH
pCRScript::NTR-N-synth	ColEI	Amp	Synthetic gene – NTR-N	Entelechon GmbH
pMTL31	ColEI	Amp, Erm	Mobilsable suicide vector	(Williams *et al.*, 1990)

pMTL900	ColEI	Amp, Cm	Tn*916* integrative suicide plasmid	(Roberts *et al.*, 2003)
pWG3	ColEI	Erm, Cm	Suicide vector contains *C. sporogenes spo0A* interrupted with CatP	C. Schwarz, University of Ulm, Germany
pMTL4	ColEI	Amp	*E. coli* cloning vector	This study
pMTL4-Em	ColEI	Erm	*E. coli* cloning vector	This study
pMTL5100	ColEI, pIM13	Erm	*Clostridium* shuttle vector	This study
pMTL20::*fdx*T:*fac*aLS2:*celA*T	ColEI	Amp	*fac* expression cartridge	This study
pMTL20::*celA*T: *fac*2:*fdx*T	ColEI	Amp	*fac*2 expression cartridge	This study
pMTL5102	ColEI. pIM13	Erm	*Clostridium* shuttle *fac*2 expression vector	This study
PEOriT	ColEI	Amp, Kan	pCR2.1-TOPO carrying RK2 OriT region PCR amplified from pMTL30	This study
pMTL5112	ColEI, pIM13	Erm	Mobilisable *Clostridium* shuttle *fac*2 expression vector	This study
pCR2.1::LacZ alpha	ColEI	Amp, Kan	PCR amplified lacZ alpha	This study
pMTL5122	ColEI, pIM13	Erm	Mobilisable *Clostridium* shuttle *fac*2 expression vector blue/white selection	This study
pCR2.1::CPG2	ColEI	Amp, Kan	PCR amplified wild type CPG2	This study

pCR2.1::*eglA*SSCPG2SOE	ColEI	Amp, Kan	*eglA* signal sequence fused to 5′ end of CPG2	This study
pMTL21::*eglA*SSCPG2	ColEI	Amp	eglA signal sequence fused to 5′ end of CPG2	This study
pMTL1010::CPG2-synthv1	ColEI	Tet	*E. coli* overexpression plasmid harbouring synthetic sequence CPG2 version 1	This study
pMTL1010::CPG2-synthv2	ColEI	Tet	*E. coli* overexpression plasmid harbouring synthetic sequence CPG2 version 2	This study
pCR2.1::*Hin*NTR	ColEI	Amp, Kan	PCR amplified wild type *Hin*NTR	This study
pMTL5112::CPG2	ColEI, pIM13	Erm	*Clostridium fac*2 based expression vector expressing wild type sequence CPG2	This study
pMTL5112::CPG2-synth	ColEI, pIM13	Erm	*Clostridium fac*2 based expression vector expressing synthetic sequence CPG2	This study
pMTL5122::*Hin*NTR	ColEI, pIM13	Erm	*Clostridium fac*2 based expression vector expressing wild type sequence *Hin*NTR	This study
pMTL5122::*Hin*NTR-synth	ColEI, pIM13	Erm	*Clostridium fac*2 based expression vector expressing synthetic sequence *Hin*NTR	This study

pMTL5122::*nfnB*	ColEI, pIM13	Erm	*Clostridium fac*2 based expression vector expressing *nfnB*	This study
pMTL9361*gusA*:*closI*p	ColEI, pCD6	Erm	*closI* promoter *gusA* reporter fusion	This study
pCR2.1::botHallA*fdx*p	ColEI	Amp, Kan	PCR amplified ferredoxin promoter from *C. botulinum* Hall A ATCC 3502	This study
pCR2.1::bot2916*fdx*p	ColEI	Amp, Kan	PCR amplified ferredoxin promoter from *C. botulinum* ATCC 2916	This study
pCR2.1::perst13*fdx*p	ColEI	Amp, Kan	PCR amplified ferredoxin promoter from *C. perfringens* strain 13	This study
pCR2.1::per8327*fdx*p	ColEI	Amp, Kan	PCR amplified ferredoxin promoter from *C. perfringens* NCTC 8327	This study
pCR2.1::spo*fdx*p	ColEI	Amp, Kan	PCR amplified ferredoxin promoter from *C. sporogenes* NCIMB 10696	This study
pMTL9361*catP*:spo*fdx*p	ColEI, pCD6	Erm	*C. sporogenes* ferredoxin promoter *catP* reporter fusion	This study
pMTL9361*catP*:perst13*fdx*p	ColEI, pCD6	Erm	*C. sporogenes* ferredoxin promoter *catP* reporter fusion	This study
pMTL5210	ColEI, pBP1	Amp, Erm	Mobilisable *Clostridium* shuttle vector	This study

pIMP1::OriT	ColEI, pIM13	Amp, Erm	Mobilisable *Clostridium* shuttle vector	This study
pMTL5612	ColEI, pIP404	Erm	*fac*2 based *Clostridium* expression vector	This study
pMTL5622::*nfnB*	ColEI, pIP404	Erm	Stable *Clostridium fac*2 based expression vector expressing *nfnB*	This study
pMTL5123::*nfnB*	ColEI, pIM13	Erm	*C. sporogenes fdx* promoter based expression vector expressing *nfnB*	This study
pMTL5623::*nfnB*	ColEI, pIP404	Erm	Stable *C. sporogenes fdx* promoter based expression vector expressing *nfnB*	This study
pMTL5622::*Hin*NTR-synth	ColEI, pIP404	Erm	Stable *Clostridium fac*2 based expression vector expressing synthetic sequence *Hin*NTR	This study
pMTL5122::NTR-N	ColEI, pIM13	Erm	*Clostridium fac*2 based expression vector expressing NTR-N	This study
pMTL5123::NTR-N	ColEI, pIM13	Erm	*C. sporogenes fdx* promoter based expression vector expressing NTR-N	This study
pMTL5623::NTR-N	ColEI, pIP404	Erm	Stable *C. sporogenes fdx* promoter based expression vector expressing NTR-N	This study

pMTL5122::*Hin*NTR-synthv.2	ColEI, pIM13	Erm	*Clostridium fac*2 based expression vector expressing synthetic sequence *Hin*NTR version 2	This study
pMTL5122::NTR-Nsynth	ColEI, pIM13	Erm	*Clostridium fac*2 based expression vector expressing synthetic sequence NTR-N	This study
pCR2.1::Cspo*pyrF*	ColEI	Amp, Kan	PCR amplified *C. sporogenes pyrF* crossover fragment	This study
pMTL31::*pyrF*(Csp)	ColEI	Erm	*pyrF* single crossover suicide plasmid	This study
pMTL900::*pyrF*(Csp)	ColEI	Cm	*pyrF* single crossover *Tn*916 integrative plasmid	This study
pMTL5122::*spoOA*:*catP* ori1	ColEI, pIM13	Erm	*spoOA* double crossover fragment cloned into rolling circle plasmid orientation 1	This study
pMTL5122:: *spoOA*:*catP* ori2	ColEI, pIM13	Erm	*spoOA* double crossover fragment cloned into rolling circle plasmid orientation 2	This study
pIMP1OriT::*spoOA*:*catP* ori1	ColEI, pIM13	Amp, Erm	*spoOA* double crossover fragment cloned into rolling circle plasmid orientation 1	This study

pIMP1OriT::*spoOA*:*catP* ori2	ColEI, pIM13	Amp, Erm	*spoOA* double crossover fragment cloned into rolling circle plasmid orientation 2	This study

Table 2.2 - Plasmids used in this study

2.3 GROWTH MEDIA

2.3.1 Luria-Bertani medium

Luria-Bertani (LB) broth was prepared as described by Sambrook *et al.* (1989) and consisted of 10 g tryptone (Oxoid), 5 g yeast extract powder (Oxoid) and 5 g sodium chloride (NaCl) made up to 1 l with distilled water. Sterilisation was achieved by autoclaving at 120 °C and 15 psi for 20 min.

LB agar was prepared by addition of 1% (w/v) Number 1 Bacteriological Agar (Oxoid).

2.3.2 2 x YT medium

2 x YT broth was prepared as described by Sambrook *et al.* (1989) and consisted of 16 g tryptone (Oxoid), 10 g yeast extract powder (Oxoid) and 5 g sodium chloride (NaCl) made up to 1 l with distilled water.

2 x YT agar was prepared by addition of 1% (w/v) Number 1 Bacteriological Agar (Oxoid).

2.3.3 TYG medium

TYG medium consisted of 30 g tryptone (Oxoid), 20 g yeast extract powder (Oxoid) and 1 g sodium thioglycolate (thioglycolic acid, $HSCH_2COOH$) made up to 1 l with distilled water.

If required, glucose supplementation was added to a final concentration of 0.5% (w/v) after sterilisation.

TYG agar was prepared by addition of 1% (w/v) Number 1 Bacteriological Agar (Oxoid).

2.3.4 Spizizen's minimal salts medium (SMM) [pH7.0]

SMM consisted of 14 g dibasic potassium phosphate (K_2HPO_4), 6 g monobasic potassium phosphate (KH_2PO_4), 1 g sodium citrate and 0.2 g magnesium sulphate ($MgSO_4.7H_2O$) made up to 1 l with distilled water. The medium was filter sterilised prior to use.

2.3.5 *B. subtilis* competence medium

B. subtilis competence medium consisted of 10 ml SMM (as described in **Section 2.3.4**), 0.5 ml glucose (10% [w/v]), 5 µl calcium chloride ($CaCl_2$, 0.1 M stock solution), 0.1 ml magnesium sulphate ($MgCl_2$, 250 mM stock solution), 0.1 ml manganese chloride ($MnCl_2$, 50 nM stock solution), 0.2 ml casamino acids 10% (w/v) and 0.25 ml tryptophan (10 mM stock solution). The individual components were filter sterilised prior to use.

2.3.6 *B. subtilis* transformation medium

B. subtilis transformation medium consisted of 1 ml SMM (as described in **Section 2.3.4**), 50 µl glucose, 10% (w/v), 20 µl magnesium sulphate ($MgCl_2$, 250 mM stock solution), 1 µl casamino acids, 10% (w/v) and 25 µl tryptophan (10 mM stock solution). The individual components were filter sterilised prior to use.

2.4 SUPPLEMENTS

Growth medium was supplemented at the following final concentrations as required: ampicillin (Amp), 100 µg ml^{-1}; chloramphenicol (Cm), 25 µg ml^{-1} (for *B. subtilis* 5 µg ml^{-1});

folate (Fol) 0.1% v/v; thiamphenicol (Thi), 20 μg ml^{-1}; tetracycline (Tet), 10 μg ml^{-1} (for clostridia 20 μg ml^{-1}), erythromycin (Erm), 300 μg ml^{-1} in liquid broth, 500 μg ml^{-1} on solid media (for clostridia 10 μg ml^{-1}); kanamycin (Kan), 100 μg ml^{-1}; trimethoprim (Tri), 10 μg ml^{-1}; cycloserine, 250 μg ml^{-1}; isopropyl-1-thio-β-D-galactopyranoside (IPTG), 64 μg ml^{-1}; 5-bromo-4-chloro-3 indolyl-β-D-galactoside (X-Gal), 40 μg ml^{-1}. Stock solutions were prepared as described by Sambrook *et al.* (1989) and stored according to the manufacturer's instructions.

2.5 GROWTH CONDITIONS

E. coli was grown aerobically at 37 °C unless otherwise stated. Liquid cultures were grown in Luria Bertani broth, or 2 x YT broth, with gentle agitation at 200 rpm. Strains of *E. coli* were stored at -80 °C using the Microbank™ system (Prolab Diagnostics). Stocks were revived by plating onto LB media supplemented with the appropriate antibiotics and incubated at 37 °C. Cells were maintained at 4 °C on LB agar plates for up to 2 weeks.

C. sporogenes was cultured and manipulated in a Mk 3 Anaerobic Work Station (Don Whitely Scientific Ltd, West Yorks, U.K.). The atmosphere of nitrogen (N_2), carbon dioxide (CO_2), and hydrogen (H_2) was maintained at a ratio of 80% (v/v):10% (v/v): 10% (v/v) respectively and at a temperature of 37 °C. Clostridial strains were stored in 10 ml cooked meat medium (Oxoid) and incubated anaerobically for 3 weeks at 37 °C to enable sporulation to occur. After this time the cultures were removed from the anaerobic cabinet and stored in the laboratory at room temperature. TYG medium supplemented with 0.5% (w/v) glucose was used for the propagation of *C. sporogenes*.

Variovorax paradoxus was grown aerobically at 30 °C. Liquid cultures were grown in 2 x YT broth with gentle agitation at 200 rpm. Plate cultures were grown on 2 x YT agar

supplemented with folate (0.1% v/v) when appropriate.

Bacillus subtilis was grown aerobically at 37 °C. Liquid cultures were grown in LB broth with gentle agitation at 200 rpm.

Growth of all bacterial cultures in liquid medium was monitored by measuring optical density at 600 nm (OD_{600}) using a Pharmacia Novaspec II.

2.6 DNA MANIPULATIONS AND ANALYSIS

2.6.1 Chromosomal DNA preparation

Chromosomal DNA was prepared using the QIAGEN DNeasy® Tissue kit (Qiagen Ltd., UK). For extraction of clostridial DNA, lysis was aided by the addition of rLysozyme (Novagen) (10 KU ml^{-1} final concentration).

2.6.2 Plasmid preparation

Plasmid preparation was carried out by the alkaline lysis method described by Sambrook *et al.* (1989) using the QIAprep Spin Miniprep Kit (Qiagen Ltd., UK) as described in the manufacturer's instructions.

2.6.3 Restriction digests

Restriction enzymes were obtained from New England Biolabs, or Promega. Digests were performed as described in the manufacturer's instructions. Briefly, digests contained 1 x buffer supplied by manufacturer, bovine serum albumin (1 μg ml^{-1} final concentration) was added if required, 0.5 – 2 μg DNA, 5 – 20 U restriction endonuclease, unless the endonuclease exhibited star activity in which case 1 U was added. The final volume was

made up with water and maintained < 5% glycerol concentration to assist in minimising star activity.

2.6.4 Blunt-ending of DNA fragments using Klenow polymerase

DNA digested with restriction enzymes which resulted in 5′ overhanging ends was blunt-ended using DNA Polymerase I, Large (Klenow) Fragment, obtained from New England Biolabs or Promega as described in the manufacturer's instructions. Briefly, to a restriction digest (described above), 1 x DNA polymerase buffer (supplied by manufacturer), 2.5 U Klenow polymerase and 33 μM each dNTP was added, followed by incubation at 25 °C for 15 min.

2.6.5 Blunt-ending of DNA fragments using T4 DNA polymerase

DNA with 3′ overhanging ends was blunt-ended using T4 DNA Polymerase, obtained from New England Biolabs, as described in the manufacturer's instructions. Briefly, to a restriction digest (described above), 1 x T4 DNA polymerase buffer (supplied by manufacturer), 5 U T4 DNA polymerase and 100 μM each dNTP was added, followed by incubation at 12 °C for 15 min.

2.6.6 Dephosphorylation of linearised DNA fragments

Linearised DNA fragments were dephosphorylated using Antarctic Phosphatase (AP) obtained from New England Biolabs, as described in the manufacturer's instructions. Briefly, to a restriction digest (described above), 1 x AP buffer (supplied by manufacturer) and 5 U AP were added, followed by incubation at 37°C for 1 h.

2.6.7 Ligation of DNA fragments

Ligation of DNA fragments was carried out using T4 DNA ligase, obtained from NEB

according to manufacturer's instructions. Briefly, purified DNA fragment inserts were added to purified vector fragments at ratios of 1:1, 3:1 and 5:1. Ligations were carried out in 10µl volumes also containing 1 x T4 DNA ligase buffer (supplied by manufacturer) and 3 U T4 DNA ligase, followed by incubation at 16 °C overnight.

2.6.8 Annealing of oligonucleotides prior to ligation

Each oligonucleotide was diluted in sterile water to a concentration of 1 nM. 1 µl of each was then added to 5 µl REact buffer 3 (Invitrogen) and 5 µl 100 mM $MgCl_2$. The solution was then made up to a final volume of 50 µl with sterile water, and heated at 90 °C for 5 min before being allowed to slowly cool to room temperature over 45 min to 1 h.

2.6.9 Ligation of annealed oligonucleotides and vector DNA

The molarity of the annealed oligonucleotides and vector DNA was calculated and ratios of 3:1, 5:1 and 0:1 (oligonucleotides: vector) DNA were mixed. Ligations were carried out in 10 µl volumes also containing 1 x T4 DNA ligase buffer (supplied by manufacturer) and 3 U T4 DNA ligase. The reactions were incubated at 16 °C overnight.

2.6.10 PCR and restriction digest clean up

DNA fragments from PCR reactions, restriction digests and blunt-ending reactions were routinely purified away from contaminating oligonucleotides and enzymes using Qiagen's QIAquick PCR Purification Kit (Qiagen Ltd., UK) as described in the manufacturer's instructions.

2.6.11 Extraction of DNA fragments from agarose

DNA fragments were visualised using ethidium bromide and the desired band excised from the agarose gel using a clean scalpel. The DNA was then extracted from the excised agarose

gel using Qiagen's QIAquick Gel Extraction Kit (Qiagen Ltd., UK) as described in the manufacturer's instructions.

2.7 AGAROSE GEL ELECTROPHORESIS

Agarose gels were prepared and run as described by Sambrook *et al.* (1989). Gels were prepared in 1 x TAE (40 mM Tris Acetate, 2 mM EDTA, pH 8.5) at a concentration of 1-2% (w/v) agarose. Ethidium bromide was added to a final concentration of 10 $\mu g\ ml^{-1}$. Gels were run in 1 x TAE buffer at 50 – 120 V. DNA was visualised using a UV transilluminator.

2.8 POLYMERASE CHAIN REACTION

2.8.1 Primers

Primer Name	Nucleotide Sequence (5′ – 3′)	Primer Tm (°C)
ERMBF	ATGACTGATATCACTGATGCTAGCGAAATGATAC ACCAATCAG	71
ERMBR	CTTAGTGTTAACACAGCTGTAGGCGCTAGGGACC TC	69
ORITF	CCTGCTTCGGGGTCATTATGC	56
ORITR	CCTGCTTCGGGGTCATTATAGC	57
LACZALPHAF	CTATGGCGTGCTGCTAGCG	55
LACZALPHAR	ACAGGAAACACATATGAC	43
CPG2F	ACCCGAACGAACAATGCGTAGA	58
CPG2R	GTGGCTTCCTGGTCTGCTGTC	56
EGLASOEF	GGGGGTATTCATATGTTTTC	56
EGLASOER	CGCGCTTCTGAGCTTCAGCTTTATAAGT	62

CPG2SOEF	CAGAAGCGCGACACAACG	58
CPG2SOER	GCCGCCCTTGTCGTCG	56
CPG2correction1	CTAGGAGATTATATATGGCTGCAAGATTGATAAT GGATCTAGGTGCTGGTAAATAACTGCA	76
CPG2correction2	GTTATTTACCAGCACCTAGATCCATTATCAATCTT GCAGCCATATATAATCTC	72
HINNTRF	GAGGAAATCATATGACTCAAC	51
HINNTRR	CTGCAGGCCTTTTTTAAT	50
HINWTRTF	ATGACTCAACTTACTCGTGAACAAG	54
HINWTRTR	TTACCCCACCCATTTCACC	51
HINSYNTHRTF	ATGACTCAATTAACAAGGGAACAA	51
HINSYNTHRTR	TTAACCAACCCATTTTACTACTTCATC	54
NFNBF	CATATGGATATCATTTCTGTCGCC	54
NFNBR	AGGCCTTCTAGATTACACTTCGGTTAAGGTGAT	63
BOTFDXF	GCGGCCGCGTGTAGTAGCCTGTGAAATAAGTAA	70
BOTFDXR	GTATGCCATATGTAACACACC	53
PERFDXF	GCGGCCGCTTAAATTTTTAATACGGTATAGGGGT	69
PERFDXR	CATATGAAAACACCTCCTAAAATG	57
NMERTF	ATGACAGTATTAAGCAAAGAGCAGG	54
NMERTR	TTATGCCCAAATAACGGTTTC	49
PYRFF	GCATGCCTTGATACGGATATAAGTTATCTACC	61
PYRFR	CAGCTGCTTGCACATTCTTCAAAGTTTTTTCCTTC	62
TETMF	GGGGACGCGTGATAGCGGGAACAAATAATTG	62
TETMR	GGGGACGCGTCAACATAAAATACACTAAG	44
CATPR	GCGGAGACGGAGAAAATC	50
PYRCF	GTAGATTGTATAGGGACGGATCATGCACC	62
PYRKR	GCTCTTATCATATAAAACTGTCCTGGCTTCC	60

Table 2.3 Nucleotide sequence and melting temperatures of oligonucleotides used in this study.

2.8.2 General PCR parameters

PCR reactions were carried out using the Failsafe PCR system (Cambio) as described in the manufacturer's instructions. Briefly, on ice, 100 ng DNA template, 0.5 µl of each primer (from 100 µM stock), and 1.25 U of Failsafe PCR enzyme mix were mixed and the reaction was made up to a final volume of 25 µl with sterile, nuclease free water. Finally, 25 µl of 2 x Failsafe PCR premix buffer was added bringing the final reaction volume to 50 µl. The mixture was then subjected to PCR in a thermal cycler block (Applied Biosystems Gene Amp PCR system 2400).

Failsafe PCR premix buffer E was routinely used for all PCR reactions. If however, premix buffer E failed to yield a product, then the reaction was repeated with the remaining 11 premix buffers (A-D & F-L); the one that gave the highest yield was used in subsequent repeat PCR's with identical template and primer pairs.

Annealing temperatures (Ta) varied and were dependent upon the melting temperatures (Tm) of the primers used. Tm's for oligonucleotides shorter than 20 bases was calculated using the Wallace Rule: $T_m = 2(A + T) + 4(G + C)$. For oligonucleotides longer than 20 bases, Tm was calculated using the Nearest Neighbour formula (Breslauer *et al.*, 1986). The Ta used in the individual PCR's was 5 °C below the lowest calculated Tm of the primer pair.

Extension times varied and were dependent on the size of the product being amplified. A 1 min extension time was allowed for every kilobase of DNA amplified.

2.8.3 Colony PCR

A single colony was taken from an agar plate and resuspended in 20 µl of sterile distilled water. The cell suspension was then lysed by heating to 98 °C for 10 min in a thermal cycler block. The lysate was cleared by centrifugation at 16,000 x *g* in a bench top centrifuge. 4 µl was carefully removed (avoiding any cellular debris) and added to the PCR reaction.

2.8.4 Gene splicing by overlap extension ("gene SOEing")

Genes were spliced according to the method of Horton *et al* (1990). Briefly, a first round of PCR was performed with two primers, one of the primers containing a region of DNA sequence homologous to the region to which it is to be fused. This was followed by a second round of PCR using both fragments required to be fused together as templates, and the outer primers. This results in the common sequence between the two fragments allowing the strands from the two different fragments to hybridise to one another, forming an overlap. Extension of the overlap by DNA polymerase yields the required dsDNA recombinant molecule. Further amplification was achieved using the outer primers to obtain the full length PCR fusion product.

2.9 TRANSFER OF PLASMID DNA INTO BACTERIAL CELLS

2.9.1 Dialysis of DNA ligation reactions

Ligation reactions were dialysed against water using 0.025 μm nitrocellulose dialysis filters (Millipore Corporation). The filter was allowed to float, reflective side up, in a petri dish containing sterile deionised water. The ligation reaction was then added to the uppermost surface by pipette and left to dialyse for 30 min.

2.9.2 Preparation of electrocompetent *E. coli*

Electrocompetent *E. coli* were prepared as follows: a 1 ml aliquot from a 10 ml overnight culture of *E. coli* (grown with selection if necessary) was used to inoculate 100 ml LB broth. The cells were grown at 37 °C with shaking at 200 rpm to an $OD_{600\,nm}$ of 0.5 – 1.0 (an optical density indicative of exponential growth). Cells were harvested by chilling on ice for 15 – 30 min followed by centrifugation at 4,000 x *g* for 15 min at 4 °C. The supernatant was

aspirated and the pellet resuspended in 100 ml ice-cold sterile deionised water. The cell suspension was centrifuged as before. The pellet was then resuspended in 2 ml of 10% glycerol, and centrifuged as before. The pellet was then resuspended to a final volume of 0.5 ml in 10% (v/v) glycerol, resulting in a cell concentration of approximately 1 x 10^{10} cells ml^{-1}. The cells were stored at – 80 °C in 40 µl aliquots.

2.9.3 Electroporation of plasmid DNA into *E. coli*

Plasmid DNA or dialysed ligation reactions were added to 40 µl of electrocompetent *E. coli* (as prepared in **Section 2.9.2**) that had been allowed to thaw on ice for 10 min. This mixture was then transferred to a pre-chilled 2 mm gap elecroporation cuvette (BioRad). A pulse of 2.5 kV (25 µF, 200 Ω) was delivered to the cuvette using a BioRad Gene Pulsar according to the manufacturer's instructions. 400 µl of SOC or 2 x YT recovery medium was immediately added, and the cell suspension was incubated at 37 °C for 1 h with shaking (200 rpm). Appropriate serial dilutions were then made from the cell suspension prior to spreading onto selective 2 x YT agar.

2.9.4 Preparation of chemically competent *E. coli*

A 1 ml aliquot from a 10 ml overnight culture of *E. coli* (grown with selection if necessary) was used to inoculate 100 ml LB broth. The cells were grown at 37 °C with shaking at 200 rpm to an $OD_{600\ nm}$ of 0.5 – 1.0 (an optical density indicative of exponential growth). To harvest the cells, the cells was chilled on ice for 15 to 30 minutes and centrifuged at 4,000 x *g* for 15 min at 4 °C. The cell pellet was then resuspended in 25 ml of ice cold 0.1 M $MgCl_2$. The cells were harvested as before and the pellet gently resuspended in 2 ml of ice cold 0.1 M $CaCl_2$. The cell suspension was then left on ice for a minimum of 2 h, to achieve chemical competence. 100 µl aliquots could then be used for chemical transformations for up to 48 h provided the cell suspension was kept chilled.

2.9.5 Transformation of plasmid DNA into chemically competent *E. coli*

Plasmid DNA or ligation reactions were added to 100 µl of competent *E. coli* cells that had been kept chilled on ice and gently mixed. The transformation reactions were then incubated for 30 min on ice, followed by heat-shock at 42 °C for 30 sec. The mixture was then incubated on ice for a further minute, before the addition of 200 µl SOC or 2 x YT recovery medium. The reaction was then incubated at 37 °C for one hour with shaking at 200 rpm before plating onto 2 x YT agar containing the appropriate antibiotic selection.

2.9.6 Transformation of plasmid DNA into *B. subtilis*

Plasmid DNA was introduced into *B. subtilis* by transformation as follows: a heavy inoculum of fresh overnight growth was taken and added to 20 ml of competence medium (detailed in **Section 2.3.5**) with appropriate selection if necessary, followed by incubation at 37 °C with shaking (200 rpm), until an OD_{600} equivalent of 3.0 or more was achieved. The cells were then diluted ten-fold in transformation medium (detailed in **Section 2.3.6**). 1 ml aliquots were removed and added to 1 µg of plasmid DNA, followed by incubation at 37 °C with agitation, for a further 90 min. Aliquots were then spread onto LB agar plates containing the appropriate selection.

2.9.7 Conjugation of plasmid DNA into *C. sporogenes*

Plasmids were introduced into *C. sporogenes* by conjugation from *E. coli* CA434 (*E. coli* HB101 carrying the helper plasmid R702) essentially as described by Purdy *et al* (2002). A 1 ml aliquot from a 5 ml overnight culture of the *E. coli* donor strain, grown in LB broth with appropriate selection, was taken and the cells harvested by centrifugation in a bench top microfuge at 5,000 x *g* for 1 min. The pellet was gently resuspended with a pipette tip in 1 ml sterile PBS and the cells harvested as before. The pellet was then transferred to the anaerobic cabinet and resuspended in 200 µl of *C. sporogenes* that had been grown

anaerobically, overnight at 37 °C. The cell suspension was then spotted in 10 µl volumes onto non-selective agar plates, followed by incubation at 37 °C for 7 h under anaerobic conditions. The cells were then harvested by flooding the agar plates with 0.5 – 1.0 ml of sterile PBS. The resulting cell slurry was removed, serially diluted and spread on selective agar plates, followed by incubation at 37 °C for 16 - 24 h in an anaerobic workstation.

2.10 ESTIMATION OF PLASMID SEGREGATIONAL STABILITY

The method utilised is a modification of experiments by Bron and Luxen (1985). Recombinant *C. sporogenes* was grown overnight in TYG medium supplemented with the appropriate selective antibiotic. A 30 µl aliquot of the overnight culture was then subcultured into 3 ml non-selective TYG medium. Cultures were then incubated for 12 h before being subcultured as before into fresh non-selective medium and incubated for a further 12 h overnight. This subculturing regime was repeated four times. At the end of each 12 h growth period, cells were serially diluted in PBS up to 10^{-7} and plated to single colonies on TYG agar either with or without antibiotic selection. The colonies that arose on non-selective plates were picked onto fresh TYG agar without and then with antibiotic supplementation.

Segregational stability was expressed as percentage cells having lost the plasmid after n generations. It can also be expressed as plasmid loss per generation and was calculated according to the formula:

$$R = (1-x)^n$$

Where R = fraction of plasmid containing cells, x = the rate of plasmid loss/generation, ^{n} = number of generations (Swinfield *et al.*, 1991).

Therefore, to calculate plasmid loss per generation (x):

$$x = 1 - R^{1/n}$$

2.11 T/A CLONING

PCR products were cloned into pCR2.1-TOPO (Invitrogen) as described in the manufacturer's instructions. PCR products were ligated directly into the vector due to the presence of precleaved ends in the vector, which have been treated with terminal deoxynucleotidyl transferase to create ddT overhangs on both 3′ ends. The addition of TOPO isomerase to the TOPO reaction negates the need for DNA ligase and PCR products can be ligated directly into the vector due to the addition of 3′ deoxyadenosine overhangs by the action of *Taq* polymerase during PCR.

2.12 BLUE/WHITE SELECTION

Blue/white selection was possible when DNA inserts were cloned into the *E. coli lacZ* alpha (*lacZ*α) region present on many standard cloning vectors. This leads to disruption of the protein's function, giving rise to an easily identifiable phenotypic marker. Colonies were screened on agar plates containing 40 μg ml^{-1} X-Gal and 1 mM IPTG. Colonies harbouring vector containing the insert grew white, whilst those without grew blue due to a functional β-galactosidase activity and hence uninterrupted gene. This screening process was only possible in *E. coli* strains that carry a mutation in the native *lacZα* region, namely TOP10 and TOP10F′. Complementation of the mutant LacZα with the vector-borne copy of LacZα enables a functional enzymatic activity.

2.13 RNA ANALYSIS

To minimise RNase contamination, all RNA work was carried out in a designated clean area, which had been thoroughly cleaned with RNaseZap (Ambion). Certified RNase clean tips and microfuge tubes (Eppendorf) were used and where possible all solutions were treated with 1% (v/v) diethyl pyrocarbonate (DEPC). In addition, all glassware and apparatus were wiped over with RNaseZap and rinsed with DEPC-treated water before use.

2.13.1 Preparation of total RNA from *C. sporogenes*

Prior to harvesting cells, 2 volumes (1 ml) of RNAprotect Bacteria Reagent (QIAGEN) was added to a bijou. 0.5 ml of an overnight culture of *C. sporogenes* cells grown in TYG medium (~1 x 10^7 cells) was then added to the RNAprotect Bacteria Reagent and vortexed for 5 sec followed by incubation at room temperature for 5 min. The crude RNA prep was harvested by centrifugation at 5,000 x *g* for 10 min in a benchtop microfuge. The supernatant was thoroughly aspirated and the pellet loosened by briefly vortexing. The bacteria were then lysed by resuspended in 100 µl of TE containing 3 mg ml^{-1} lysozyme and incubating at room temperature for 10 min.

Total RNA was then extracted from the *C. sporogenes* lysate using Qiagen's Rneasy Kit, as described in the manufacturer's instructions. Briefly, 350 µl of buffer RLT (supplied with kit) to which β-mercaptoethanol had been added, was mixed with the lysate, followed by the addition of 250 µl of cold absolute ethanol. This solution was then applied to an RNeasy column followed by the addition of 5U of Turbo DNase (Ambion) as a preliminary on-column DNase digestion. The column was then incubated at room temperature for 5 minutes and centrifuged in a bench top microfuge at 8,000 x *g* for 15 sec. The flow-through was discarded and 700 µl of buffer RW1 (supplied with kit) was applied to the column for 5 min. The column was again centrifuged at 8,000 x *g* for 15 sec and the flow-through again discarded. The column was then washed by addition of 500 µl of buffer RPE (supplied with

kit) and centrifuged as before. The column was then washed with 500 µl of buffer RPE for a second time and centrifuged at 8,000 x *g* for 2 min. The flow-through was again discarded and the RNA eluted by the addition of 50 µl of DEPC treated water and centrifugation at 8,000 x *g* for 1 min.

2.13.2 Removal of contaminating genomic DNA from total RNA

Contaminating DNA was removed from RNA samples using Ambion's Turbo DNase as described in the manufacturer's instructions. The appropriate volume of 10 x Turbo DNase buffer was added to the RNA sample, 5 U of Turbo DNase per µg of RNA was then added and the final volume adjusted with DEPC-treated water. The reaction was incubated at 37 °C for 30 min.

2.13.3 DNase I treated RNA sample clean up

Total RNA that had been treated with DNase I was cleaned up prior to use in RT-PCR using Qiagen's RNeasy columns as described in the manufacturer's instructions. Briefly, the RNA sample was adjusted to 100 µl with DEPC-treated water and 350 µl of buffer RLT (supplied with kit) was added to the sample, followed by the addition of 250 µl of ice cold absolute ethanol. This solution was then applied to an RNeasy column and centrifuged in a bench top microfuge at 8,000 x *g* for 15 sec. The column was then washed by addition of 500 µl of buffer RPE (supplied with kit) and centrifuged as before. The column was washed for a second time by the addition of 500 µl of buffer RPE and was then centrifuged at 8,000 x *g* for 2 min. The flow-through was again discarded and the RNA eluted by the addition of 30 µl of DEPC-treated water and centrifugation at 8,000 x *g* for 1 min.

2.13.4 One-tube RT-PCR

Before use in RT-PCR, RNA was treated with Turbo DNase (as described in **Section 2.13.2**)

and subsequently cleaned up as described in **Section 2.13.3**. RT-PCR was carried out using Qiagen's One-tube RT-PCR kit and HotStar Taq (QIAGEN), as described in the manufacturer's instructions. Briefly, RNA samples, buffers and reagents were allowed to thaw on ice. On ice, 10 µl 5 x QIAGEN OneStep RT-PCR buffer, 400 µM of each dNTP, 0.6 µM forward and reverse primer and 2 µl QIAGEN OneStep RT-PCR enzyme mix were added to 10 ng of RNA (quantified using a NanoDrop ND-1000 spectrophotometer), and the final volume was adjusted to 50 µl with DEPC-treated water. Reverse transcription was then allowed to proceed at 50 °C for 30 min. After which time, the reaction was then transferred to a nuclease free PCR tube and PCR amplification was carried out using an Applied Biosystems Gene Amp PCR system 2400 thermocycler (see Table 2.4).

Step	Time	Temperature (°C)	Details
1	15 min	95	Inactivation of Omnicsript and Sensiscript ReverseTranscriptases, denaturation of the cDNA template, and activation of the HotStarTaq DNA Polymerase
2	1 min	94	Denaturation.
3	1 min	x	Primer annealing. The temperature was calculated according to the primer pair used in the reaction, i.e. 5°C below the lowest Tm.
4	x sec	72	Extension. (The time was dependant on the size of the product, with 60 sec extension allowed for every kilobase of DNA amplified).
5	10 min	72	Final extension
6	hold	4	

Table 2.4: PCR programme utilised for RT-PCR amplification of mRNA.

Control reactions were performed where the RNA template was exchanged for DEPC treated water to check for RNA contamination of RT-PCR buffers, dNTPs e.t.c. Also several HotStarTaq control PCR reactions were performed with a variety of templates: DNA template as a positive control, RNA template to check for DNA contamination of the RNA, and water template to check for DNA contamination of HotStar Taq components.

If the RT-PCR failed, the procedure was repeated with the inclusion of either Q-solution (changes the melting behaviour of DNA) and/or additional $MgCl_2$.

RT-PCR products were visualised with ethidium bromide by agarose electrophoresis as described in **Section 2.7.**

2.14 PROTEIN EXPRESSION AND PURIFICATION

2.14.1 Over-expression of proteins in *E. coli*

5 ml cultures of *E. coli* harbouring the expression vectors were grown in LB broth under selective conditions at 37 °C overnight with shaking (200 rpm). The culture was then diluted 100-fold in sterile selective LB broth and grown at 37 °C with shaking (200 rpm). 1.5 ml aliquots of cells were taken at the appropriate time (usually 4 h, 8 h, and 24 h) and harvested by centrifugation at 4,000 x *g* for 20 min, the supernatant discarded and the pellet stored at -20 °C overnight.

2.14.2 Preparation of cell lysates and protein extraction from *E. coli*

E. coli cell lysates were prepared using BugBuster protein extraction reagent (Novagen) as described in the manufacturer's instructions. Briefly, cell pellets that had been stored at -20 °C overnight were allowed to thaw on ice for approximately 15 min. The pellet was resuspended in BugBuster reagent, which had been previously diluted to 1 x by addition of

the appropriate buffer, by gentle vortexing. 300 µl of reagent per 1.5 ml culture was used and 25 U of benzonase per ml of BugBuster was added in order to reduce the viscosity of the final lysate. The cell suspension was then incubated at room temperature with gentle shaking for 30 min. The insoluble cell debris was removed by centrifugation at 16,000 x *g* for 20 min at 4 °C and the supernatant, which contains the soluble protein, was retained.

To isolate insoluble proteins packaged in inclusion bodies, the pellet obtained after the BugBuster lysis was subjected to the following further modifications: the pellet was resuspended in the same volume of BugBuster reagent that was used to resuspend the original cell pellet, by pipetting up and down followed by vortexing to obtain an even suspension. Complete resuspension of the pellet is essential to ensure solubilisation and removal of any contaminating soluble proteins. 1 KU ml^{-1} rLysozyme was then added and the solution mixed by gentle vortexing followed by a 5 min room temperature incubation. 6 volumes of a 1:10 dilution BugBuster reagent (in deionized water) was then added to the suspension and mixed by vortexing for 1 min. The suspension was then centrifuged at 5,000 × *g* for 15 min at 4°C to collect the inclusion bodies. The supernatant is removed with a pipette. The inclusion bodies were then resuspened in half the original culture volume (0.5 volume) of 1:10 diluted BugBuster reagent, mixed by vortexing, and centrifuged at 5,000 × *g* for 15 min at 4°C. This step is repeated twice. A final resuspension is followed by a centrifugation at 16,000 × g for 15 min at 4°C, and the supernatant removed. The final pellet of purified inclusion bodies is resuspended in the Tris buffer appropriate for the protein being studied.

2.14.3 Over-expression of proteins in *C. sporogenes*

5 ml cultures of *C. sporogenes* harbouring the expression vectors were grown in TYG broth under selective conditions anaerobically at 37 °C overnight. The culture was then diluted 100 fold in sterile selective TYG broth and grown at 37 °C anaerobically. 1 ml aliquots of cells were taken at the appropriate time intervals (usually 4 h, 8 h, and 24 h), the OD_{600}

recorded, and harvested by centrifugation at 5,000 x *g* for 5 min. The supernatant was discarded and the pellet stored at - 20 °C overnight.

2.14.4 Preparation of cell lysates and protein extraction from *C. sporogenes*

C. sporogenes cell lysates were prepared using BugBuster protein extraction reagent (Novagen) as described in the manufacturer's instructions. Briefly, cell pellets that had been stored at –20 °C overnight were allowed to thaw on ice for approximately 15 min. The pellet was resuspended by gentle vortexing in 1/5th culture volume of BugBuster reagent that had been previously diluted from 10 x stock to 1 x by addition of the desired enzyme assay buffer. If a larger volume of culture had been harvested then 1/20th culture volume of 1 x BugBuster was used. 25 U of benzonase per ml of BugBuster was added in order to reduce the viscosity of the final lysate. 1 KU rLysozyme per ml of 1 x BugBuster was added to the resuspended pellet. The cell suspension was then incubated at room temperature with gentle shaking for 30 min. The insoluble cell debris was removed by centrifugation at 16,000 x *g* for 20 min at 4 °C and the supernatant, which contains the soluble protein, was retained.

2.15 PROTEIN VISUALISATION

2.15.1 NuPAGE gel electrophoresis

NuPAGE 4 – 12% precast gels (Invitrogen) in MES running buffer (50mM 2-(*N*-morpholino)ethane sulphonic acid, 50 mM Tris, 0.1% (w/v) SDS, 1 mM EDTA, [pH 7.3]) were used for protein analysis by gel electrophoresis. Cell lysates were diluted in 4 x NuPAGE LDS sample buffer (106 mM Tris-HCl, 141 mM Tris-base, 2% (w/v) LDS, 10% (w/v) glycerol, 0.51 mM EDTA, 0.22 mM Serva blue G250, 0.175 mM Phenol red [pH 8.5]) and heated at 95 °C for 5 min. Protein samples were then loaded into the wells of the

gel and run at 150 V for 90 min using the Novex XcellII minicell as described in the manufacturer's instructions. Pre-stained broad size range protein markers (BioRad) were used to estimate sizes of purified proteins.

2.15.2 Novex Zymogram gel electrophoresis

For the detection and characterisation of proteases, more specifically collagenases, Novex 10% Zymogram gelatin gels (Invitrogen) were used. Proteases are visualised as clear bands against a dark blue background where the protease has digested the substrate, in this case gelatin. 5 ml overnight cultures of *C. sporogenes* NCIMB 10696 were centrifuged at 5,000 x *g*. A 5 µl aliquot of a 10^{-1} dilution of supernatant was diluted in 2 x Novex Tris-Glycine SDS sample buffer. Culture supernatant samples were loaded into the wells of the gel and run in 1 x Tris-Glycine running buffer at 125 V for 1 h using the Novex XcellII minicell as described in the manufacturer's instructions. Pre-stained broad range protein markers (BioRad) were used to estimate the size of proteases. After electrophoresis, the gel was incubated in 1 x Zymogram renaturing buffer for 30 min at room temperature with gentle agitation. The buffer was decanted off and the gel was equilibrated prior to being developed by the addition of 1 x Zymogram developing buffer. The gel was equilibrated for 30 min at room temperature with gentle agitation. The buffer was decanted and replaced with fresh 1 x Zymogram developing buffer followed by overnight incubation at 37 °C.

2.15.3 Simply blue staining

After electrophoresis, NuPAGE gels were rinsed with 100 ml of deionised water for 5 min with gentle agitation three times, before being stained with 20 ml SimplyBlue for 1 – 3 h, again with gentle agitation. The stain was discarded and the gel destained with water for 1 – 3 h. Zymogram gels were stained after being developed by the addition of 20 ml of SimplyBlue. The gel was stained with gentle agitation until the zones of clearance were visible, usually after 1 – 2 hours.

2.15.4 Densitometry

Overexpression of proteins was quantified by densitometry using a GS-800 Calibrated Densitometer with Quantity One software (BioRad).

2.16 QUANTIFICATION OF PROTEIN SAMPLES

Protein concentration of cell lysates was determined using the Bradford assay (Bradford, 1976). Briefly, bovine serum albumin (BSA) standards were prepared in 200 µl volumes ranging from 1 to 20 µg protein/200 µl. The standards along with 200 µl of lysates and serial dilutions up to 10^{-3} were added to 1 ml light path plastic cuvettes containing 800 µl Bradford Reagent (Sigma-Aldrich Company Ltd. Dorset, U.K.) which contains Coomassie Brilliant Blue G-250, and incubated for 5 min. The absorbance at 595 nm (A_{595}) was measured relative to a water blank. The data created from the BSA standards was used to create a standard curve, from which the concentration of the lysates could be calculated based on the A_{595} obtained.

2.17 ENZYME ASSAYS

2.17.1 Chloramphenicol acetyl transferase assay

C. sporogenes lysates were prepared as described previously (see **Section 2.14.3** and **2.14.4**). A 100 µl aliquot of lysate was added to 1 ml reaction buffer (10 mM 5,5'-dithio-bis(2-nitrobenzoic acid) in 1 M Tris-HCl [pH 7.8], and 0.1 mM Acetyl-CoA) which had previously been equilibrated to 37 °C for at least 30 min, in a clean plastic cuvette. The cuvette was then placed in a spectrophotometer, blanked, and 20 µl of 5 mM chloramphenicol added and

quickly mixed by inverting. The reaction was left to run for 1 min in the spectrophotometer, after which time the increase in absorbance at 412 nm was recorded. This process was repeated for each sample lysate, with appropriate dilutions made of the lysates where necessary.

Units per ml of CAT were calculated using the following equation,

$$\frac{(\Delta A_{412} / \text{min}) / V}{13.6}$$

ΔA_{412}/ min = the increase in A_{412} per min

V = the volume of lysate added (ml)

In order to obtain Units mg^{-1} protein the Units per ml were divided by the protein concentration (mg ml^{-1}) of the sample.

2.17.2 β-Glucuronidase assay

β-Glucuronidase levels were measured by modification of the method described by Melville *et al* (1994). *C. sporogenes* was serially diluted into 3 ml of TYG medium and grown anaerobically at 37 °C overnight. The lowest dilution showing growth was used to inoculate fresh sterile selective media 1 in 100 and grown anaerobically at 37 °C. A 1 ml sample was removed every hour or at the appropriate time interval (usually 4 h, 8 h, and 24 h) and the OD_{600} recorded. Each sample was then centrifuged at 5,000 x *g* for 5 min, the supernatant aspirated and the pellet frozen at – 70 °C overnight. The pellets were thawed on ice for 5 min and resuspended in 0.8 ml Z buffer (60 mM $Na_2HPO_4.7H_2O$ [pH 7.0], 40 mM $NaH_2PO_4.H_2O$, 10 mM KCl, 1 mM $MgSO_4.7H_2O$ and 50 mM 2-mercaptoethanol). Cells were lysed by addition of 8 µl of toluene followed by vigorous vortexing for 1 min. Each sample was incubated on ice for 10 min, and then with caps open for 30 min at 37 °C in a fume hood. 150 µl from each sample was then transferred in duplicate to the wells of a flat

bottom 96 well plate and the enzyme reaction started by addition of a 6 mM solution of p-nitrophenyl-β-$_D$- glucuronide. The plate was then immediately placed in a 37 °C preheated ELISA plate reader (Wallac Victor^2 1420 Multilabel counter). Absorbance at 405 nm was recorded every 30 sec until the reaction stopped.

The specific activity was calculated based on the change in A_{405} while the reading was increasing linearly, using the following equation:

$$\frac{A_{405} \times 1{,}000}{OD_{600} \times t\ (\text{min}) \times 1.25 \times \text{sample vol (ml)}}$$

In order to obtain specific activity per mg protein, the value for the specific activity was divided by the total protein concentration (mg ml^{-1}).

2.17.3 Carboxypeptidase assay

E. coli and *C. sporogenes* lysates were prepared as described previously (see **Section 2.14.1, 2.14.2, 2.14.3** and **2.14.4**). A quartz cuvette was prepared containing 0.1 ml of methotrexate solution (0.6 mM methotrexate [0.27 mg ml^{-1} methotrexate] in Tris buffer [0.1 M Tris-HCl pH 7.3, containing 0.2 mM $ZnSO_4$]) and 0.9 ml Tris buffer (0.1 M Tris-HCl pH 7.3, containing 0.2 mM $ZnSO_4$). The initial A_{320} was recorded (a value of at least 0.8 is required after zeroing with a Tris buffer blank). The cuvette was pre-warmed to 37 °C, and the reaction initiated by the addition of 10 µl of cell lysate. The decrease in absorbance at 320 nm was recorded.

To calculate Units ml^{-1} in the lysate:

$$\frac{\Delta A_{320} \times 100 \times \text{dilution factor}}{8.3}$$

ε_{max} for methotrexate is 8300

2.17.4 Nitroreducatse menadione assay

E. coli and *C. sporogenes* lysates were prepared as described previously (see **Section 2.14.1, 2.14.2, 2.14.3** and **2.14.4**). A 1 ml quartz cuvette was prepared containing 780 µl of Tris buffer (10 mM Tris-HCl pH7.5 at 37 °C) preheated to 37 °C. Immediately prior to adding the lysate the following were added to the cuvette and mixed: 10 µl 1mM menadione, 100 µl 10 mM NADH, and 100 µl 700 µM cytochrome C. The cuvette was returned to the spectrophotometer and the absorbance zeroed. 10 µl of lysate, or serial dilutions of the lysate, was then added to the cuvette and the reaction mixture immediately mixed. The increase in A_{550} being recorded.

Units per ml of nitroreductase activity were calculated using the following equation,

$$\frac{(\Delta A_{550} / \text{min}) / \text{ml}}{14.79}$$

where ΔA_{550}/min = the increase in A_{550} per min.

In order to get Units mg^{-1} protein, the Units per ml were divided by the protein concentration of the sample in mg ml^{-1}.

2.18 PHENOTYPIC ASSAY

2.18.1 Spore assay

Sporulation efficiencies were calculated using a modification of the method described by Kamiya *et al* (1992). In essence, 5 ml overnight cultures of *C. sporogenes* were used to inoculate (1:100) 35 ml of selective TYG broth. Broths were incubated anaerobically at 37°C for 72 h. Following incubation, 500 µl of cell culture was heat inactivated at 80 °C for

20 min. Dilutions of heat inactivated cells, and a non heat inactivated sample as a total cell count control, were plated out on selective TYG agar. Sporulation efficiency was recorded as % of spores relative to total cell count.

CHAPTER 3

DEVELOPMENT OF A CLOSTRIDIAL EXPRESSION VECTOR AND GENE TRANSFER STUDIES IN *C. SPOROGENES*

3.1 INTRODUCTION

It is the ultimate aim of CDEPT to have the gene encoding the prodrug converting enzyme stably integrated into the genome, so as to negate the use of antibiotics in the clinic. In the interim, an effective segregationally stable plasmid-based expression system is required in order to assess the effectiveness of new enzyme/prodrug systems in the *in vivo* animal model.

A variety of vectors are available for the manipulation of clostridia, however they could not be employed without becoming party to a restrictive materials transfer agreement. This would impose restrictions on the consortium's freedom to develop any process generated. Therefore, the required vectors needed to be reconstructed. In this chapter the construction of an *E. coli/Clostridium* shuttle expression vector will be described.

For the initial expression studies, the ferredoxin promoter (fdx_p) from *C. pasteurianum* was chosen for the shuttle expression vector. Ferredoxin (Fdx) is a low molecular weight electron carrier protein that functions as an electron acceptor in redox reactions. Clostridia grown in iron-rich media are capable of expressing Fdx at a level up to 2% total cellular protein (Marczak *et al.*, 1985; Rabinowitz, 1972). Also, considering that Fdx is a low-molecular weight protein that is encoded by a single-copy gene (*fdx*) (Graves *et al.*, 1985; Graves and Rabinowitz, 1986), clostridial *fdx* appears to be transcribed at high levels. Previous work (Minton *et al.*, 1995) utilised the *fdx* promoter from *C. pasteurianum* to express the nitroreductase gene *nfnB* from *E. coli* to a level equivalent of 8% total cellular protein in a *C. beijerinckii* NCIMB 8052 host. If necessary, studies to identify an improved promoter will be performed at a later stage.

An IPTG inducible ferredoxin promoter, *fac*, has been previously generated (CAMR, Porton Down, Salisbury). However, due to ownership issues, *fac* was resynthesised taking the opportunity to incorporate some improvements. These were enhancement to the spacing of

the ribosome binding site from the start codon and the alteration of the orientation of the *lac* operator. The resulting promoter was then utilised to generate an expression cartridge.

3.2 RESULTS

3.2.1 Construction of an *E. coli/Clostridium* shuttle vector

Plasmid nomenclature was derived as follows. All plasmids constructed belong to the pMTL family of plasmids. For plasmids generated for this work the prefix 5 was used therefore plasmids were called pMTL5xxx (i.e. 5 followed by three digits). A description of the nomenclature is shown in Appendix I.

3.2.1.1 Vector backbone construction

The first stage in the generation of an *E. coli/Clostridium* shuttle vector was to construct the basic plasmid backbone on which all future shuttle vectors could be based, that of pMTL4 (Chambers *et al.*, 1988a). pMTL20 (Chambers *et al.*, 1988a; Oultram *et al.*, 1988), a derivative of pMTL4, was digested with the restriction endonuclease *Hae*II in order to excise *lacZα*. This was performed so that *lacZα* could be recloned back into the shuttle vector in a more useful location at a later stage. Religation of the *lacZα* deleted plasmid yielded pMTL4 (see Figure 3.1). For antibiotic selection, erythromycin can be utilised as the sole antibiotic due to its selectivity in both *E. coli* and a variety of *Clostridium* species (Minton, N.P. personal communication).

The next stage was to amplify up the erythromycin resistance marker, *ermB*, from pMTL20E (Oultram *et al.*, 1988) using ERMF and ERMR primers. PCR amplified *ermB* was digested with *Eco*RV and *Hpa*I and cloned into pMTL4 digested with *Eco*RV to generated pMTL4-Em (see Figure 3.1). To reduce the overall plasmid size, useful in generating as

segregationally stable a plasmid as possible, and due to the fact that erythromycin can be used as the sole resistance marker, the ampicillin resistance marker was excised on a *Bsp*HI (blunt-ended) fragment. In place of *bla* a Gram-positive replicon was cloned.

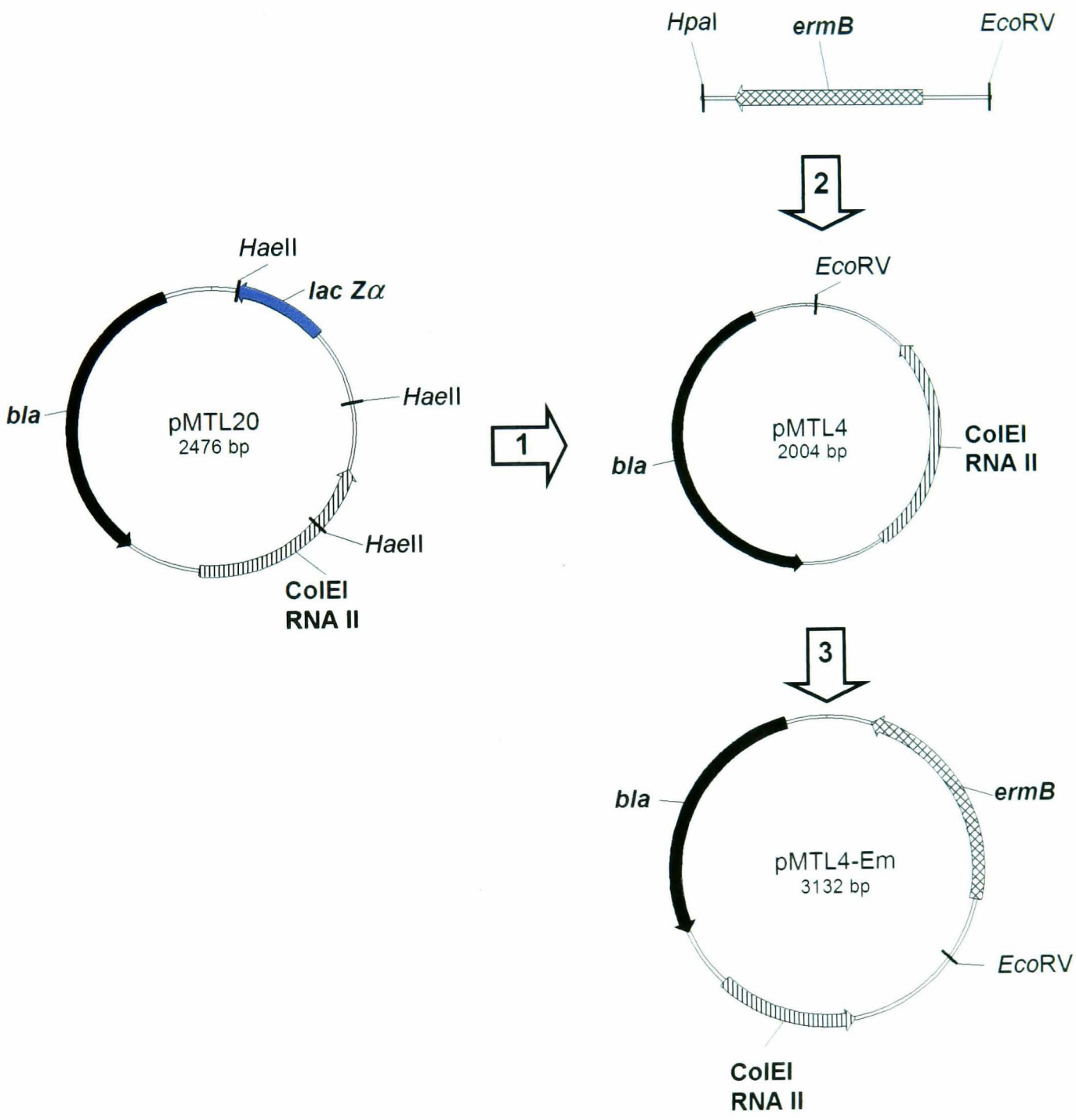


Figure 3.1: Schematic representation of the cloning strategy for the construction of pMTL4-Em. A partial *Hae*II digest was performed on pMTL20 in order to delete out *lacZα* (such that *lacZα* could be cloned into a more suitable location) to generate pMTL4 (**1**). *ermB* was then PCR amplified from pMTL20E and cloned into the *Eco*RV site of pMTL4 (**2**) to generate pMTL4-Em (**3**).

3.2.1.2 Choice of replicon and minimal region of replicon

Initial studies on the genetic manipulation of clostridia for use in CDEPT involved *C. acetobutylicum* (Theys *et al.*, 1999) and the closely related *C. beijerinckii* (Minton *et al.*, 1995). Expression vectors utilised in these preliminary CDEPT studies were based on the replicon from the *B. subtilis* plasmid pIM13 (Monod *et al.*, 1986), and on the *E. faecalis* plasmid pAMβ1 (Clewell *et al.*, 1974; LeBlanc and Lee, 1984), respectively. *C. acetobutylicum* NI-4082 was the initial CDEPT host of choice. Therefore, vectors based on the pIM13 replicon were generated due to their high segregational stability in this strain (Azeddoug *et al.*, 1992).

pIM13 is a 2.2 kb multicopy plasmid from *B. subtilis* that contains two ORFs over 100 amino acids in length. The first ORF (*ermC*) confers resistance to the macrolide-lincosomide-streptogramin B (MLS) antibiotics (Mahler and Halvorson, 1980). The second ORF constitutes a 16 kDa replication protein that has high homology to the replication protein of the cryptic *Staphylococcus aureus* plasmid pSN2. It is thought that the pSN2 family of plasmids, including pIM13, replicate via asymmetric rolling-circle replication (Dempsey *et al.*, 1995).

The approximate minimal region of pIM13 required for replication was determined by performing a variety of deletions on pIM13 (Monod *et al.*, 1986). The *ermC* determinant was not essential as deletion of the minor segment from the *Mbo*I site at position 1369 to the *Cfo*I site at position 17 still yielded a stable high copy number plasmid. The smaller *Hin*dIII fragment from positions 882 to 1098 could also be deleted. The resultant plasmid was fully capable of stable replication with a high copy number. A *Hin*fI digest followed by Klenow DNA polymerase blunt-ending failed to give a functional plasmid. Finally a *Bal*31 digest from the *Cfo*I site at position 17 showed that removal of approximately 100 bp in the clockwise direction still yielded a viable plasmid. Therefore, the minimal region of pIM13 required for replication encompasses the region from the *Cfo*I site at position 17 to the

*Hin*dIII site at position 882 (Monod *et al.*, 1986) (see Figure 3.2).

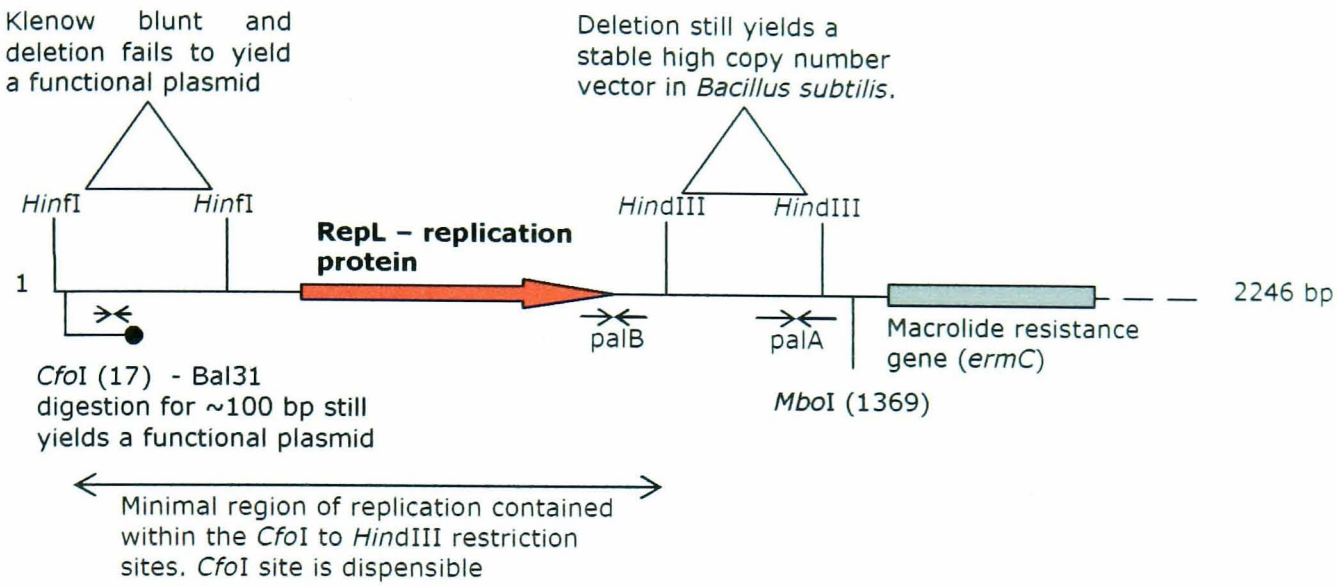


Figure 3.2: Derivation of minimal region required for replication in pIM13. Converging arrows represent repeat regions. To generate the clostridial shuttle plasmid pIMP1, pUC9 digested with *Hin*dIII was cloned in place of the small *Hin*dIII fragment of pIM13 (Mermelstein *et al.*, 1992).

Practically, this *Cfo*I/*Hin*dIII region has been fused to the chloramphenicol resistance determinant (*cat*) of pC194 to yield a fully functional replicating plasmid, pBD347 (Projan *et al.*, 1987). Additionally, the small *Hin*dIII region (bases 882 to 1098) has been deleted and replaced with pUC9 to yield pIMP1 (Mermelstein *et al.*, 1992), a vector widely used in *C. acetobutylicum*. It has also been found that pIM13-based vectors expressing the cytokine mouse tumour necrosis factor alpha (mTNFα) and the prodrug converting enzyme cytosine deaminase (*codA*) are structurally and segregationally stable in *C. acetobutylicum* DSM 792 (Theys *et al.*, 1999; Theys *et al.*, 2001b).

To generate the clostridial shuttle vector, the Gram-positive replicon from pIMP1 (Mermelstein *et al.*, 1992) containing the minimal region of replication was excised by digestion with *Sma*I and *Fsp*I and cloned into *Bsp*HI (blunt-ended) digested pMTL4-Em giving plasmid pMTL5100 (see Figure 3.3).

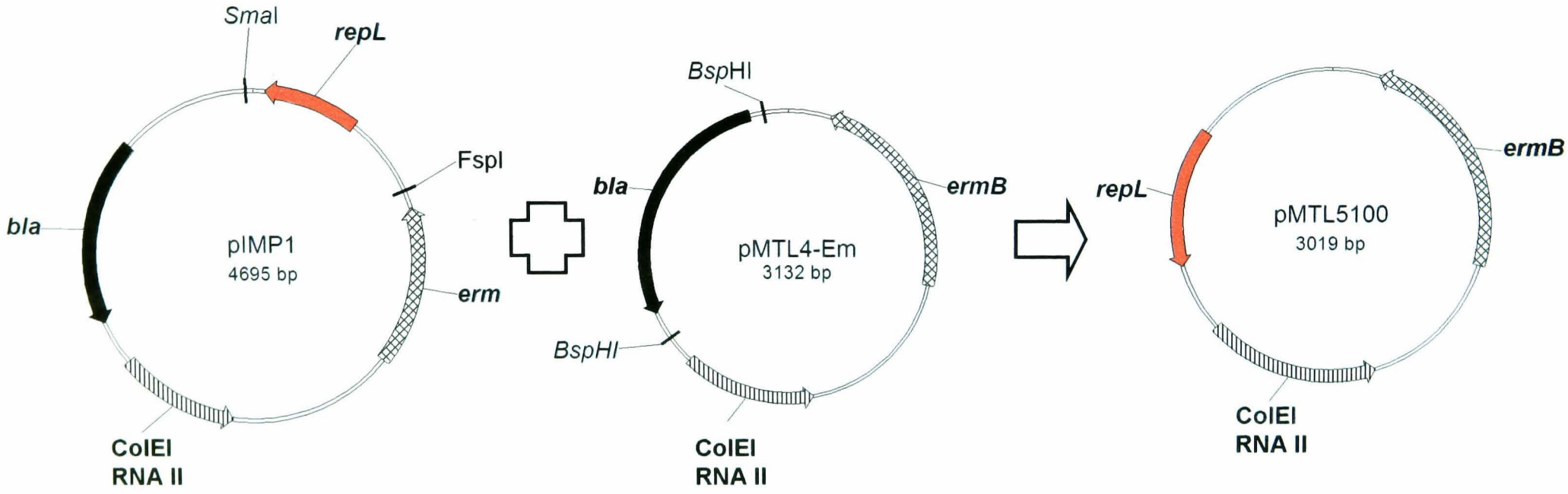


Figure 3.3: Schematic representation of the cloning strategy for the construction of pMTL5100. The Gram-positive pIM13 replicon (*repL*) from pIMP1was cloned on a *Sma*I and *Fsp*I fragment and cloned into pMTL4-Em in place of *bla* which was previously deleted on a *Bsp*HI fragment, the result being pMTL5100.

3.2.1.3 Shuttle expression vector construction.

An IPTG inducible ferredoxin promoter expression cartridge has been previously constructed (Carter *et al.*, 2005). The cloning of a *lac* operator into the ferredoxin promoter (Graves and Rabinowitz, 1986) generated the *fac* promoter. The *fac* promoter is flanked by the ferredoxin and cellulase (*celA*) transcriptional terminators.

The ferredoxin expression cartridge was obtained from pMTL9341aLS2 (Carter *et al.*, 2005) and cloned as an *Nhe*I/*Sph*I into the *Xba*I site in the multiple cloning site of pMTL20 (Chambers *et al.*, 1988a) to yield pMTL20::*fdx*T:*fac*aLS2:*celA*T (see Figure 3.4). The ferredoxin promoter (*fac*) and antisense LuxS fragment (aLS2) was replaced with a synthetic optimised ferredoxin promoter (*fac*2) (synthesised and supplied by Entelechon GmbH on plasmid pCR4::*fac*2). *Xba*I and *Not*I digests on both plasmids yielded pMTL20::*celA*T:*fac*2:*fdx*T (see Figure 3.4). The sequence of *fac*2 is identical to that published for *fac* (Minton *et al.*, 1990) apart from a two nucleotide reduction in the spacing between the ribosome binding site (RBS) and the ATG start codon, such that the sequence changed from AGGAGG**TGTATTT**CATATG to AGGAGG**TTAGT**CATATG. An *Nde*I restriction site was included 'over' the ATG start to enable optimal positioning of the gene encoding the prodrug converting enzymes to the promoter. Additionally, a *lac* operator sequence was included in *fac*2 to enable IPTG induction. The orientation of the *lac* operator sequence was reversed in *fac*2 in relation to *fac*.

The entire expression cartridge was then cloned as an *Aat*II/*Bam*HI (both sites blunt-ended) fragment into pMTL5100 linearised with *Eco*RV to give pMTL5102 (see Figure 3.4).

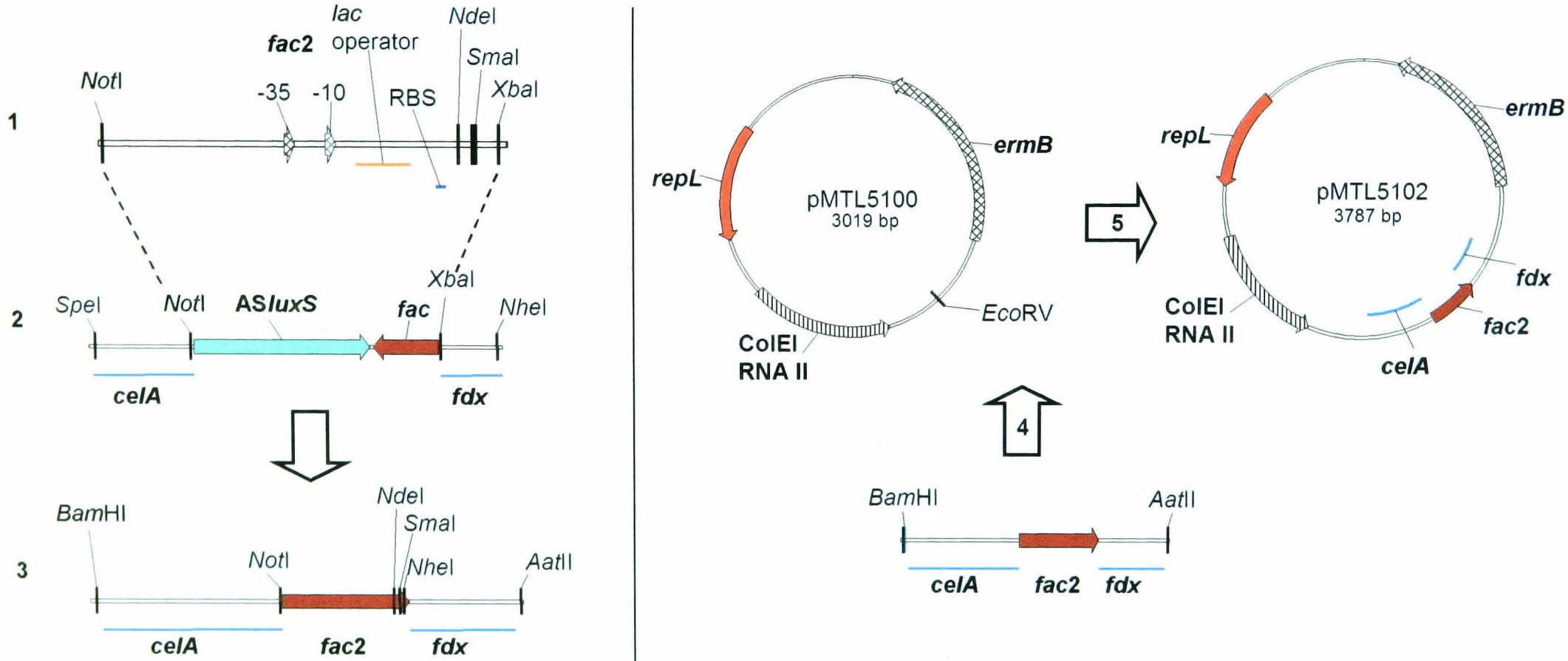


Figure 3.4: Schematic representation of the cloning strategy for the construction of the *fac*2 expression cartridge (**1**) and the consequent cloning of the *fac*2 expression cartridge into pMTL5100. For ease of cloning, the *fdx*T:*facASluxS*:*celA*T expression cartridge (**2**) was digested with *Nhe*I and *Spe*I and cloned into pMTL20 digested with *Xba*I. *fac*2 was digested with *Not*I and *Xba*I and cloned into pMTL20::*fdx*T:*facASluxS*:*celA*T digested with *Not*I and *Xba*I which excises the *fac*AS*luxS* fragment, the result being pMTL20::*celA*T:*fac*2:*fdx*T (**3**). The *fac*2 expression cartridge was then digested with *Bam*HI and *Aat*II and cloned into pMTL5100 linearised with *Eco*RV (**4**) generating pMTL5102 (**5**). Blue bars represent transcriptional terminators.

For conjugative mobilisation of the plasmid, *oriT* was first PCR amplified from pMTL30 (Williams *et al.*, 1990) using ORITF and ORITR primers, and cloned into pCR-Blunt II-TOPO (Invitrogen Ltd, Paisley, U.K.) to yield pEOriT. *oriT* was then excised on a *Sma*I/*Eco*RV fragment and cloned into the *Pvu*II site of pMTL5102 yielding pMTL5112 (see Figure 3.5). Finally to enable blue/white selection for cloning purposes *lacZα* was PCR amplified from pMTL20 using LACZALPHAF and LACZALPHAR primers to yield pCR2.1::lacZalpha. *lacZα* was then digested with *Nde*I/*Nhe*I (blunt-ended) and cloned into pMTL5112 digested with *Nde*I/*Sma*I to give pMTL5122 (see Figure 3.6).

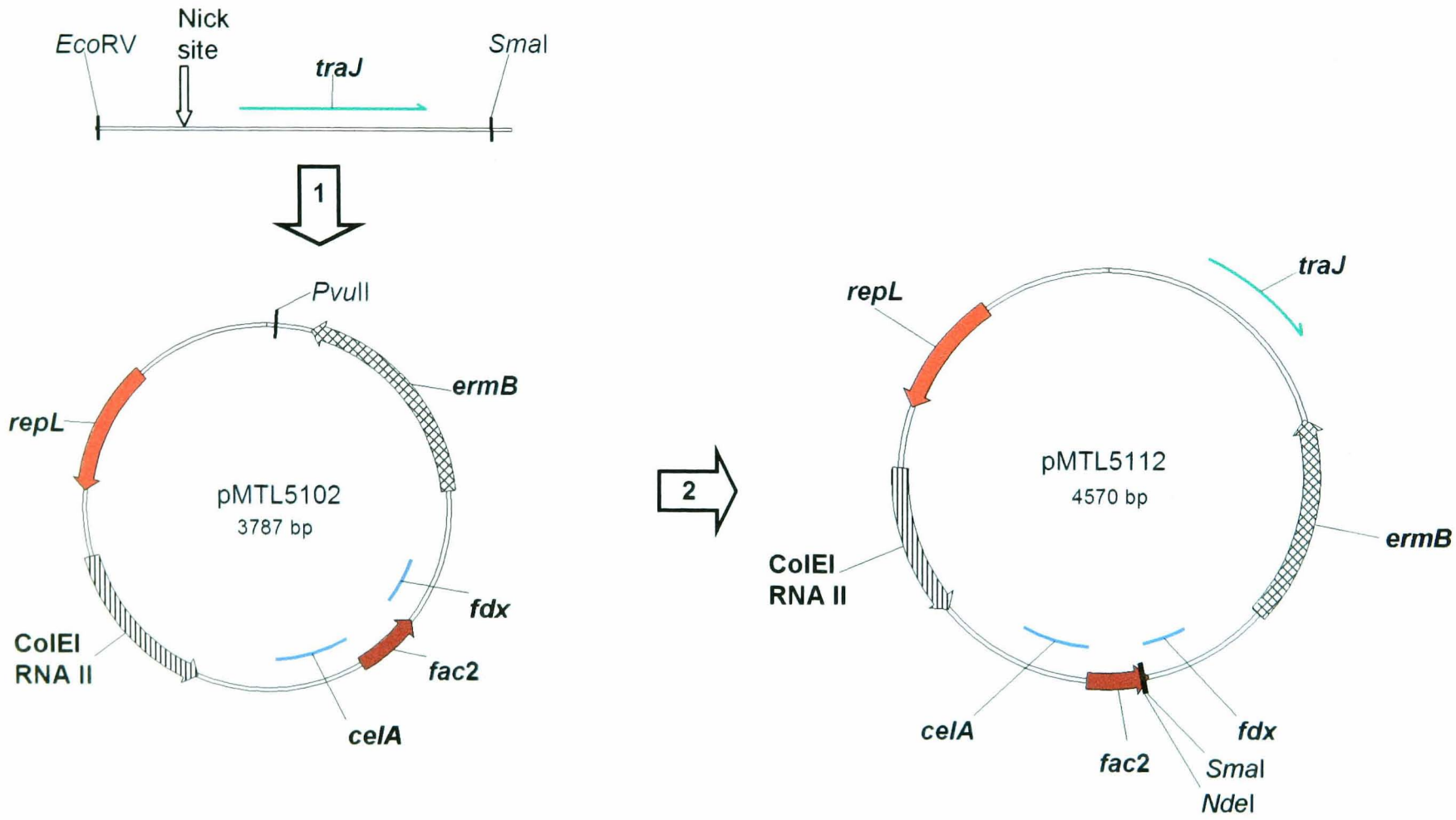


Figure 3.5: Schematic representation of the cloning strategy for the construction of pMTL5112. OriT was cloned from pEOriT as a *Eco*RV/*Sma*I fragment and cloned into pMTL5102 digested with *Pvu*II (**1**), resulting in pMTL5112 (**2**).

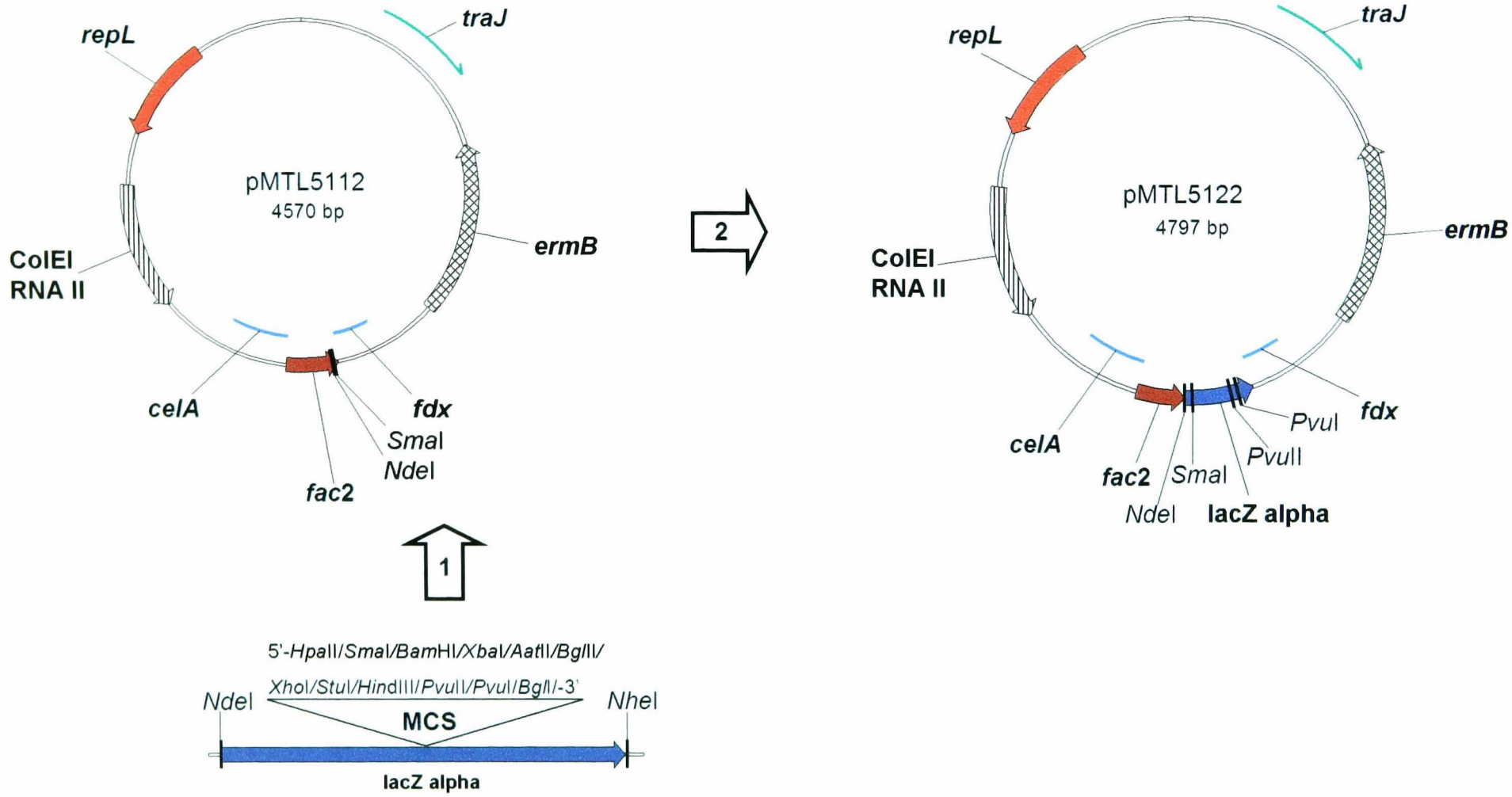


Figure 3.6: Schematic representation of the cloning strategy for the construction of pMTL5122. LacZ alpha was PCR amplified from pMTL20 and digested with *Nde*I and *Nhe*I (blunt-ended) and cloned into pMTL5112 digested with *Nde*I and *Sma*I (**1**) to generate pMTL5122 (**2**). MCS = multiple cloning site.

3.2.2 Conjugal transfer of shuttle vectors into *C. sporogenes*

Preliminary studies were performed by J. Theys (University of Maastricht, The Netherlands) at the onset of the study on the differing *in vitro* sporulation capabilities and *in vivo* tumour colonisation potential of *C. acetobutylicum* NI-4082, *C. acetobutylicum* DSM 792, *C. sporogenes* M55, and *C. sporogenes* NCIMB 10696. The *C. acetobutylicum* strains reached spore titres of 10^5-10^6 spores ml^{-1}. Much higher spore titres, in the region of 10^9-10^{10} spores ml^{-1}, were obtained with the *C. sporogenes* strains. 10^8 spores of each clostridial strain were administered to tumour bearing mice. Following colonisation of the tumours, homogenisation of the tumour mass resulted in colony counts of up to 10^9 vegetative cells per gram tumour tissue for the *C. sporogenes* strains. Colonisation by the *C. acetobutylicum* strains was several-fold lower than that obtained for *C. sporogenes*. Utilising *C. sporogenes* M55, however, resulted in severe tumour oncolysis that compromised the reliable recording of tumour measurements. This made quantification of a prodrug conversion-related effect difficult to attain. Also, occasionally, toxicity and weight loss was observed in some animals. It was therefore decided to change the clostridial delivery strain to *C. sporogenes* NCIMB 10696 as the non-desirable side-effects were not observed with this strain.

DNA transfer into *C. sporogenes* NCIMB 10696 has only recently been reported (Liu *et al.*, 2002). The electroporation method that was developed proved unreliable and irreproducible; no one outside of the author's laboratory could repeat the procedure (Minton, N.P. personal communication). One hypothesised reason for the low transformation frequency was the presence of extracellular endonucleases secreted by *C. sporogenes* (Liu *et al.*, 2002). Supplementation of the electroporation buffer with a DNase inhibitor, aurintricarboxylic acid, did improve electroporation efficiencies up to ~ 1.5×10^2 transformants per microgram plasmid DNA. Several attempts were made in this laboratory to reproduce the protocol with a variety of vectors harbouring different replicons, however all attempts failed to yield any transformants. A new DNA transfer procedure was therefore required.

Conjugal transfer of plasmids may be a way of negating the action of extracellular endonucleases due to the direct contact of the donor and recipient mating pair via the pilus during the conjugation process. Conjugative transfer has proven to be a reliable method for the introduction of plasmid DNA from an *E. coli* S17-1 donor to a variety of clostridial recipients, including *C. acetobutylicum* (Williams *et al.*, 1990), *C. botulinum* type A strains (Bradshaw *et al.*, 1998), and *C. perfringens* (Lyras and Rood, 1998). A conjugation protocol has previously been developed for transfer of plasmids from an *E. coli* CA434 (*E. coli* HB101 harbouring plasmid R702) donor to a *C. difficile* recipient (Purdy *et al.*, 2002). Currently, there are no reports of conjugative transfer of plasmid DNA into *C. sporogenes*.

Initially, pMTL5112 was introduced into *E. coli* CA434 via electroporation and then mated with *C. sporogenes* NCIMB 10696 following the method described by Purdy *et al* (2002). pMTL5112 was found to transfer into *C. sporogenes* at a frequency of approximately 1.0×10^{-7} transconjugants per donor cell. At the time, the only alternative mobilisable shuttle vector available was pMTL9301 (based on the replicon from the *C. difficile* plasmid pCD6) (Purdy *et al.*, 2002). Conjugal transfer of pMTL9301 into *C. sporogenes* occurred at a frequency of approximately 1.0×10^{-5} transconjugants per donor cell.

These initial findings indicate that conjugation is an improved method for the generation of recombinant strains of *C. sporogenes*. It has also highlighted a difference in the transfer frequency for two different plasmid replicons.

3.3 DISCUSSION

At the onset of this study there were pre-existing expression shuttle vectors available for transfer of plasmid DNA from *E. coli* to clostridia. However, these vectors were the property of CAMR, Porton Down. With the early project move from CAMR to the

University of Nottingham, these vectors could not be employed without becoming signatory to a restrictive materials transfer agreement. This imposed limitations on the consortium's freedom to exploit any process developed. Therefore, a new vector was constructed, incorporating some improved features over pre-existing plasmids.

The Gram-negative component of the vector generated was based on the pMTL4 backbone, a derivative of pBR322 (Chambers *et al.*, 1988a). This vector was chosen as in relation to the pUC series of plasmids, also based on pBR322, pMTL4 is some 293 bp smaller. The pMTL series of plasmids contain deletions relative to the pUC plasmids, thus allowing more unique restriction sites to be generated in the multiple cloning site of *lacZα*. The copy number of pMTL4-based vectors is high as the pBR322 RNAI/RNAII region was replaced with the pUC equivalent. A single base change, a G to A substitution, immediately preceding the 5′ end of the RNAI coding region in pUC is hypothesised to cause the increase in copy number (Chambers *et al.*, 1988a).

As the CDEPT host was originally planned to be *C. acetobutylicum* NI-4082, the plasmid replicon from the *B. subtilis* plasmid pIM13 was utilised to supply the Gram-positive replication functions. The minimal Gram-positive replicon from pIMP1, a derivative of pIM13, was fused to the *E. coli* vector to yield the shuttle vector pMTL5100.

Concurrent with this study, work was initiated on the identification of the most suitable CDEPT host (J. Theys, MAASTRO, University of Maastricht, The Netherlands). This resulted in a change of host from *C. acetobutylicum* NI-4082 to *C. sporogenes* NCIMB 10696. As a shuttle vector had been constructed based on the pIM13 replicon, it was opted to generate a conjugative shuttle vector due to the difficulties in reproducing the published *C. sporogenes* electroporation protocol. There has been a considerable amount of success in introducing plasmid DNA into a variety of clostridia at a high frequency by conjugation. The use of an *E. coli* donor and cloning intermediate also greatly simplified the cloning and conjugation processes. The pseudomonad IncP, RP4/RK2 type mobilisation/conjugation

system was chosen for transfer of the vectors to clostridia. This system was preferable, as it has been successfully used in the past to facilitate transfer of plasmid vectors into clostridia. Also, the availability of a RP4/RK2 compatible *E. coli* donor strain meant that the system could be utilised immediately upon construction of a suitable shuttle vector.

Prior to transfer, the ferredoxin inducible promoter expression cartridge was cloned into the vector. This then enabled conjugative transfer of the vector to be used for prodrug converting enzyme expression to be assessed for transfer into *C. sporogenes*. Conjugal transfer of pMTL5112 could be performed reproducibly. However, in comparison to the pCD6-based conjugative shuttle vector pMTL9301, transfer frequencies of pMTL5112 were 100-fold lower. This equated to tens of colonies obtained per conjugation compared to approximately 1,000 colonies obtained per conjugation with pMTL9301. This difference in transfer frequency could be attributed to the mode of replication of the two plasmids. pIM13 is known to replicate via a rolling circle mechanism (Projan *et al.*, 1987), where as it is thought that pCD6 replicates via a theta mechanism due to the homology to the theta replicating plasmid pIP404 (Purdy *et al.*, 2002). Rolling circle plasmids are in general much less segregationally stable than theta replicating plasmids, and it is this reduced segregational stability that could be affecting transfer frequency. This can be attributed to the lack of antibiotic selective pressure during the seven-hour conjugative mating process. During this time, a significant proportion of the cells will loose the plasmid dependant on the segregational stability of the replicon. The plasmid copy number of the different plasmids is not known, yet cannot be discounted as another factor influencing conjugative transfer frequencies.

Even though the transfer frequency of the pIM13 based conjugative shuttle vector is not as high as the other replicon studied, pMTL5112 appeared to be structurally stable. For this reason the pIM13-based plasmid replication functions were not changed. The segregational stability of alternative replicons could be studied at a later date, and if necessary a more stable replicon cloned.

CHAPTER 4

OVER-EXPRESSION OF PRODRUG CONVERTING ENZYMES

4.1 INTRODUCTION

In the previous chapter, a shuttle vector based on the *B. subtilis* plasmid pIM13 was constructed. An expression shuttle vector was then generated by insertion of an expression 'cartridge' based on the ferredoxin promoter from *C. pasteurianum*. Once the plasmid was generated, a conjugal transfer method was developed and transfer of two different vectors, based on different Gram-positive replicons, into *C. sporogenes* was demonstrated.

In this chapter the isolation, cloning, and expression of two prodrug converting enzymes will be described. These are carboxypeptidase G2 from *Variovorax paradoxus* ATCC 25301 and nitroreductase from *Haemophilus influenzae* Rd. Carboxypeptidase G2 from *V. paradoxus* is currently utilised in other DEPT strategies. The *H. influenzae* Rd nitroreductase is one of several novel nitroreductases isolated and characterised previously (Anlezark, G. personal communication). Kinetic and cell cytotoxicity studies of the novel enzymes, identified *H. influenzae* NTR (*Hin*NTR) to be the most effective in cell cytotoxicity assays. Kinetic studies on the conversion of CB1954 identified that *Hin*NTR only produces the toxic 4-hydroxylamine form of the drug. *Hin*NTR has a comparable Km (690 μM) compared to NfnB (682 μM), but a significantly higher Kcat of 56.2 s^{-1} compared to a Kcat of 6 s^{-1} for NfnB. It was therefore of interest to over-express this novel nitroreductase to see if it results in a significant anti-tumour effect.

4.2 RESULTS

4.2.1 Initial cloning of prodrug converting enzymes

4.2.1.1 Carboxypeptidase G2 (CPG2)

Gram-negative signal peptides are generally shorter compared to Gram-positive signal

peptides (Van Wely *et al.*, 2001). Thus, proteins from Gram-negative bacteria will not be easily secreted in Gram-positive bacteria. For efficient secretion of Gram-negative proteins in a Gram-positive host, a Gram-positive secretion signal needs to be fused to the protein. As such, the first stage in cloning and over-expressing CPG2 was to change the CPG2 signal peptide for one known to function in clostridia.

The entire CPG2 gene, including the native signal sequence, from *V. paradoxus* ATCC 25301 was initially PCR amplified from chromosomal DNA using primers CPG2F and CPG2R and cloned into pCR2.1TOPO to yield pCR2.1::CPG2. The 5′ region corresponding to the mature peptide was then amplified from pCR2.1::CPG2 in order to be fused to the endoglucanase A (EglA) signal peptide from *Clostridium saccharobutylicum* P262. For ease of cloning, the unique *Xma*I restriction site in CPG2 was utilised, thus minimising the region that would need to be re-sequenced. Primer pairs EGLASOEF/EGLASOER were used to amplify up the *eglA* signal sequence from plasmid pHZ117, creating an *Nde*I restriction site over the ATG start codon of *eglA* signal sequence. The 5′ end of EGLASOER is homologous to the 5′ end of cleaved CPG2 sequence to enable a SOEing PCR fusion of the two components. PCR primers CPG2SOEF/CPG2SOER were used to amplify the 5′ portion of CPG2, up to and including the unique *Xma*I restriction site. The amplified products were combined and a second round of SOEing PCR amplification was performed with the flanking primers. The resulting PCR amplicon was cloned into pCR2.1TOPO to yield pCR2.1::*eglA*SSCPG2SOE (see Figure 4.1). To fuse the two ends of CPG2 together, a three way ligation was performed between pMTL21 digested with *Eco*RI, the 5′ end of CPG2 fused to the *eglA* signal peptide digested with *Eco*RI and *Xma*I, and the 3′ end of CPG2 digested out of pCR2.1::CPG2 on an *Xma*I/*Eco*RI fragment. The resultant plasmid, pMTL21::*eglA*SSCPG2, contained a fully functional copy of CPG2 transcribed by the *lac* promoter. CPG2 activity can be screened for by plating on media containing folate. CPG2 converts folate to glutamate and the insoluble pteroate. CPG2 production is visualised as a precipitated yellow halo of pteroate surrounding colonies on solid media due to the

extracellular secretion of CPG2 (Minton *et al.*, 1983). The secretion of functional CPG2 from pMTL21::*eglA*SSCPG2 was confirmed on folate containing media (see Appendix II for DNA and protein sequence of CPG2).

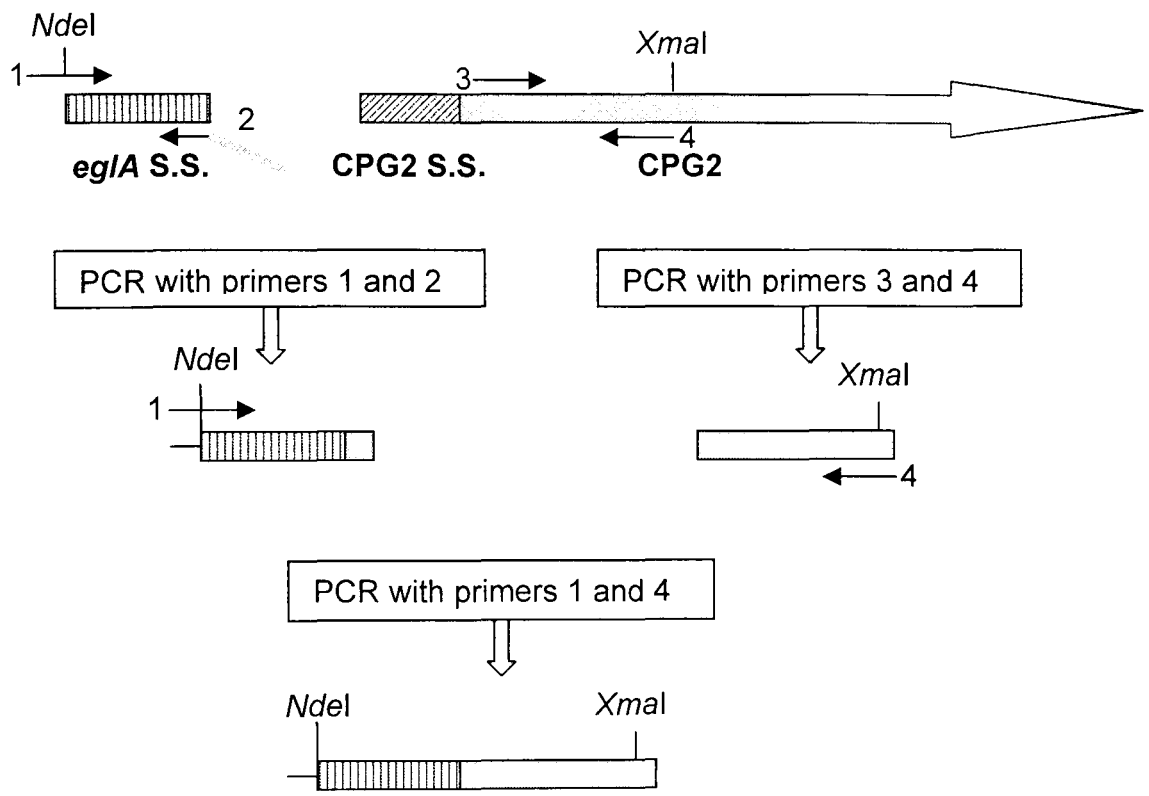


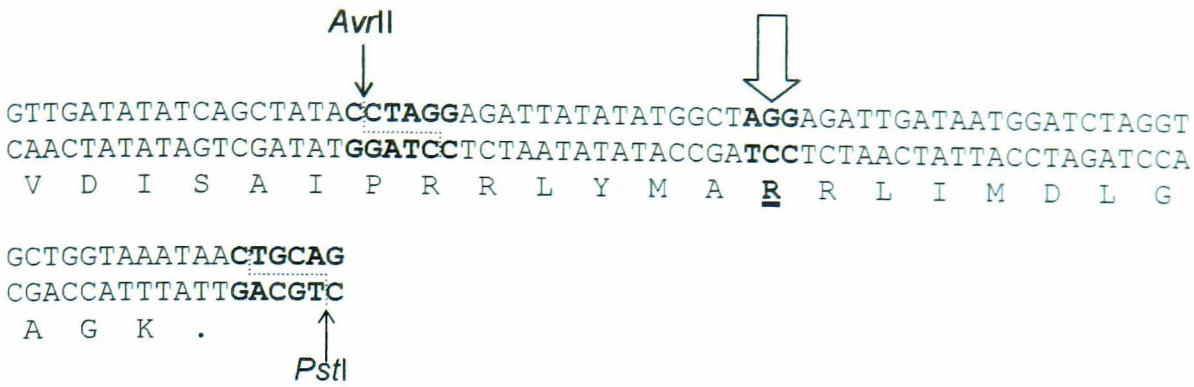
Figure 4.1: Schematic representation of SOEing PCR fusion of the *eglA* signal sequence to the 5′ end of cleaved CPG2 to generate *eglA*SSCPG2SOE. A first round of PCR was performed with primers 1+2 and 3+4, (primer 2 has a tail complementary to the 5′ end of cleaved CPG2). A second round of PCR with primers 1 and 4 was performed to generate the fusion product. S.S. = signal sequence.

For potentially improved expression, a synthetic version of CPG2 (CPG2-synth) was synthesised by Entelechon GmBH based on the amino acid sequence of GenBank entry A06774 (see Appendix II for DNA and protein sequence of CPG2-synth). The codon usage of CPG2 was optimised to the *C. acetobutylicum* codon usage table, the organism of choice for the delivery of prodrug converting enzymes at the time of the design of the gene. Unexpectedly, *E. coli* harbouring the plasmid supplied, pCR4-TOPO::CPG2-synth, did not produce the typical yellow halo seen on folate containing media. The presence of β-lactamase from the vector encoded *bla* gene is known to dramatically effect the secretion of

CPG2, possible due to the β-lactamase competing with CPG2 for translocation across the cell membrane (Chambers *et al.*, 1988b). CPG2-synth was therefore cloned into pMTL1010 (a tetracycline resistance encoding plasmid, with expression of the gene of interest driven by the *lac* promoter). However, *E. coli* recombinants harbouring pMTL1010::CPG2-synth still failed to show the expected yellow halo on folate containing media. BLAST searching the protein sequence used to generate CPG2-synth highlighted the presence of two GenBank entries for CPG2, accession numbers A06774 and M12599. Alignment of the two GenBank entries with CPG2-synth showed a single amino acid difference at position 418 where an arginine was present in CPG2-synth and A06774 and an alanine present in M12599. As the synthetic CPG2 sequence encodes a non-functional protein it was assumed that the M12599 submission was in fact the correct sequence. To ascertain if the difference at position 418 caused the observed lack of activity of CPG2-synth, the region containing the difference was changed to the alternative sequence.

In order to correct the amino acid difference, two oligonucleotides, CPG2correction1 and CPG2correction2, were synthesised with the correct DNA sequence such that when ligated together left overhangs at the 5′ and 3′ ends which are complementary to the *Avr*II and *Pst*I restriction sites (see Figure 4.2). The oligos were ligated and cloned into the *Avr*II/*Pst*I sites of pMTL1010::CPG2-synth to yield pMTL1010::CPG2-synthv2. The sequence of the resultant clones was confirmed by sequencing. Screening for the production of CPG2 on folate containing media gave the characteristic yellow coloration expected.

Original synthetic CPG2 sequence – 3′ end of GenBank entry A06774



Corrected synthetic CPG2 sequence – 3′ end correct sequence based on GenBank submission (M12599)

Figure 4.2: Correction of CPG2 synthetic by cloning an oligo in between the *Avr*II and *Pst*I restriction enzyme sites. Incorrect region excised on an *Avr*II/*Pst*I fragment and the ligated oligo cloned in its place. DNA sequence of the top strand primer (CPG2correction1) is highlighted in green, DNA sequence of the bottom strand primer (CPG2correction2) is shown in orange.

4.2.1.2 Nitroreductase - HinNTR

The nitroreductase gene from *H. influenzae* KW20 (Rd) (*Hin*NTR) (GenBank Accession number YP_249310) was PCR amplified from *H. influenzae* KW20 (Rd) chromosomal DNA (supplied by P. Langford, Imperial College London, London, U.K.). PCR primers HINNTRF and HINNTRR were used to introduce an *Nde*I restriction site over the ATG start codon, and *Stu*I/*Pst*I sites at the 3′ end of the gene after the stop codon. Once amplified, the PCR product was cloned into pCR2.1TOPO. A sequence perfect clone was generated

designated pCR2.1::*Hin*NTR (see Appendix II for DNA and protein sequence of *Hin*NTR).

A synthetic version of the gene (*Hin*NTR-synth) was also generated (synthesised by Entelechon GmbH) in which the triplet codons were altered such that they were optimised to the *C. acetobutylicum* ATCC 824 codon usage table. Restriction sites were added to the 5′ and 3′ end of the gene to aid cloning, namely, an *Nde*I site over the ATG start codon and a *Pst*I site after the stop codon, the plasmid containing the synthetic gene was designated pCR4::HinNTR-synth (see Appendix II for DNA and protein sequence of *Hin*NTR-synth).

4.2.2 Cloning prodrug converting enzymes into the expression vector

4.2.2.1 Carboxypeptidase G2 (CPG2)

The wild type sequence CPG2 was excised from pMTL21::*eglA*SSCPG2 on an *Nde*I/*Eco*RI (blunt-ended) fragment. The synthetic sequence CPG2 was excised on an *Nde*I/*Pst*I (blunt-ended) fragment from pMTL1010::CPG2-synthv2. Due to the phenotypic indicator of CPG2 expression, the expression vector pMTL5112 was used as there is no need for blue/white selection of recombinant *E. coli*. pMTL5112 was digested with *Nde*I and *Sma*I and the CPG2 wild type and synthetic genes directionally ligated to pMTL5112 to generate pMTL5112::CPG2 and pMTL5122::CPG2-synth.

4.2.2.2 Nitroreductase - HinNTR

For cloning of the *Hin*NTR wild type and synthetic genes, the over-expression vector pMTL5122 was used due to the presence of LacZα enabling blue/white selection of putative ligation transformants. The wild-type *Hin*NTR was excised from pCR2.1::*Hin*NTR on an *Nde*I/*Stu*I fragment. The synthetic *Hin*NTR was isolated from pCR4::*Hin*NTR-synth as an *Nde*I/*Pst*I (blunt-ended) fragment. Several attempts were made to clone the genes directly.

However, any clones resulting from the ligation contained severe rearrangements or insertions as seen by the restriction enzyme profile compared to the expected profile.

An alternative cloning strategy was therefore tested. The nitroreductases were digested from the same vectors as before as *Eco*RI blunt-ended fragments. These were then cloned into pMTL5122 digested with *Sma*I, the transformations being performed in the *lacI*^{q} strain of *E. coli*, *E. coli* Top10 F′. The Top 10 F′ strain over-expresses LacI, which binds to the *lac* operator present within *fac*2, the result being tighter repression of transcription from the *fac*2 promoter. Correct clones were then obtained with the nitroreductases in the correct orientation relative to the *fac*2 promoter. An *Nde*I deletion was then performed to delete the extraneous DNA between the *Nde*I restriction site of *fac*2 and that of the nitroreductase, again using *E. coli* Top 10 F′ cells. Using this strategy, correct clones were obtained for both *Hin*NTR genes, the plasmids designated pMTL5122::*Hin*NTR and pMTL5122::*Hin*NTR-synth.

4.2.3 Over-expression of prodrug converting enzymes in *E. coli*

4.2.3.1 Carboxypeptidase G2

When plasmids pMTL5112::CPG2 and pMTL5112::CPG2-synth were transformed into both *E. coli* TOP10 and the conjugation donor *E. coli* CA434, the expression of carboxypeptidase G2 was visualised by the phenotypic yellow halo surrounding colonies on folate containing media. Analysis of culture supernatants and the soluble and insoluble fraction of cell lysates by SDS-PAGE indicated no major over-expression of a 41.4 kDa protein corresponding to the size of mature CPG2, or of the 45.8 kDa preprotein (data not shown). However, CPG2 enzyme assays on the soluble and insoluble fraction of lysates, and on supernatants from 8 hour cultures showed expression of CPG2 in the soluble fraction of the lysates. The synthetic CPG2 was produced to a much higher level compared to the wild-type gene (see

Figure 4.3). It has previously been found that the wild type CPG2 does not express well in *E. coli*, possibly due to the high G+C content of the *V. paradoxus* derived gene (Minton, N.P. personal communication). One potential reason for the lack of expression in the *E. coli* culture supernatant is due to the generally lower secretion of peptides in Gram-negative bacteria compared to Gram-positive bacteria. Secondly, the EglA signal peptide employed for secretion is from a clostridial species and may well not be recognised efficiently in *E. coli*.

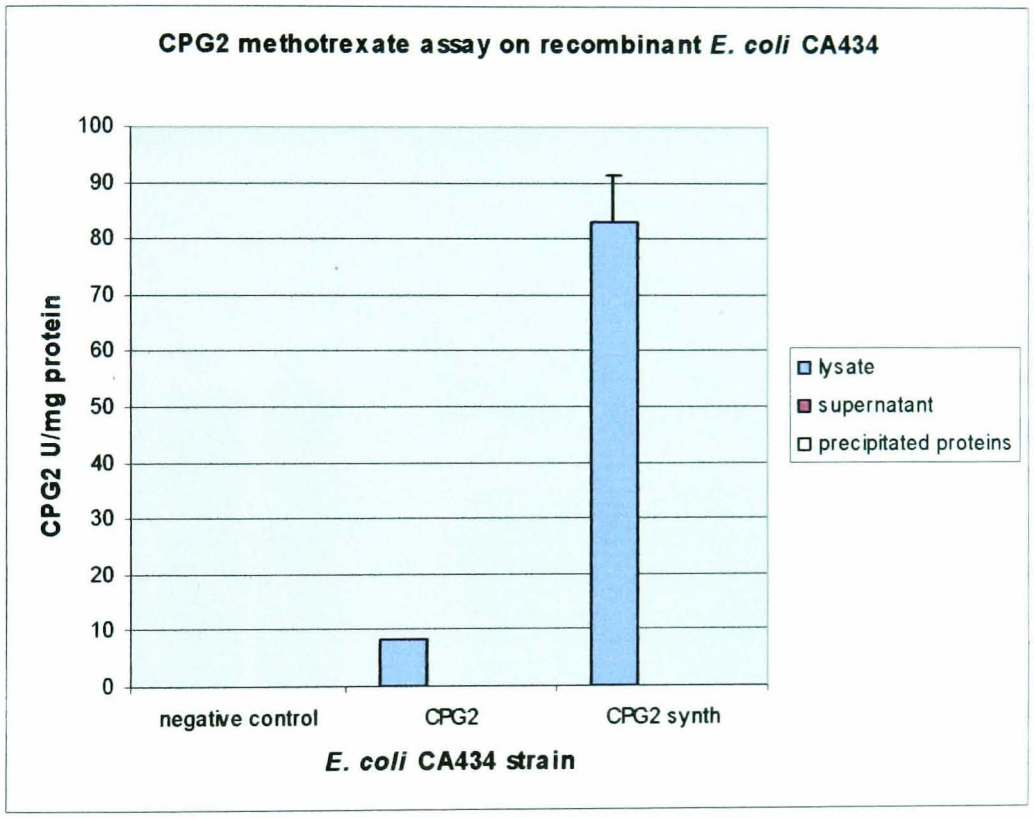


Figure 4.3: CPG2 methotrexate assay on *E. coli* CA434 donors used for conjugation into *C. sporogenes* NCIMB 10696. CPG2 activity shown in Units per mg protein of lysate (soluble fraction), supernatant, or precipitated proteins (i.e. insoluble fraction).

4.2.3.2 Nitroreductase - HinNTR

The wild type and synthetic sequence *Hin*NTR bearing shuttle plasmids were transformed into the conjugation donor *E. coli* CA434 and the levels of nitroreductase overproduced in

the lysate of the soluble fraction assessed by SDS-PAGE and menadione reductase assay. Cultures were harvested 24 hours after inoculation at an OD_{600} of approximately 2.0 and the cells lysed. The lysates of the soluble fraction were normalised to the same OD_{600} prior to analysis. SDS-PAGE of the lysates showed that there was over-expression of a protein of 25.2 kDa when the lysates of the *Hin*NTR over-expressing *E. coli* were compared to a vector only negative control lysate (see Figure 4.4). Menadione reductase assay on the same lysates indicated that a nitroreductase, presumably *Hin*NTR, was over-expressed to a level corresponding to 3-fold over background for the wild type *Hin*NTR and 4.5-fold for the synthetic *Hin*NTR (see Figure 4.4). Background levels of nitroreductase are particularly high in *E. coli* presumably due to the endogenous nitroreductase, NfnB.

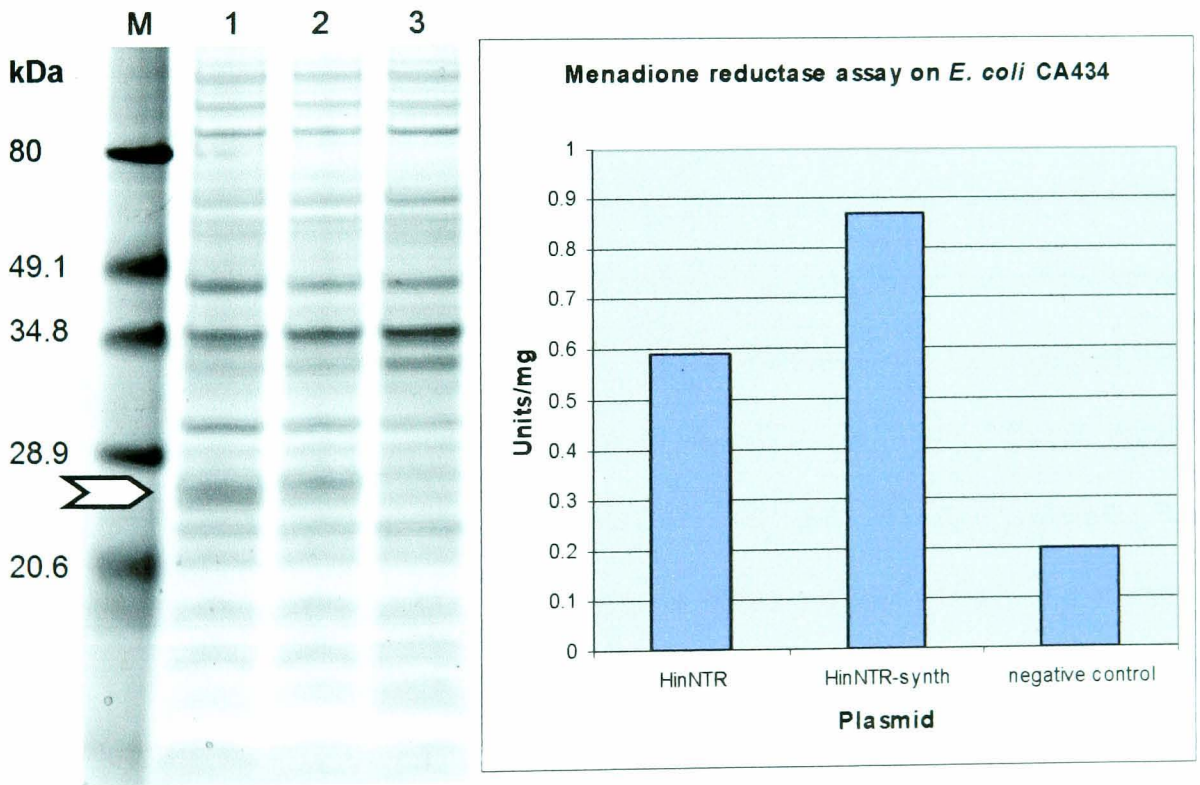


Figure 4.4: SDS-PAGE and menadione reductase assay of overnight culture soluble fraction lysates of *E. coli* CA434 harbouring pMTL5122::*Hin*NTR, pMTL5122::*Hin*NTR-synth or pMTL5122 (negative control). SDS-PAGE gel: M = BioRad Broad range protein markers, 1 pMTL5122::*Hin*NTR (25.2 kDa), 2 = pMTL5122::*Hin*NTR-synth (25.2 kDa), 3 = pMTL5122 (negative control). Arrow indicates presumptive *Hin*NTR over-expressed protein.

Based on these findings that the vectors utilised for over-expression of CPG2 and *Hin*NTR are functional in *E. coli*, studies on the over-expression in *C. sporogenes* were initiated.

4.2.4 Over-expression of prodrug converting enzymes in *C. sporogenes*

4.2.4.1 Carboxypeptidase G2

Conjugation of pMTL5112::CPG2 and pMTL5122::CPG2-synth into *C. sporogenes* NCIMB 10696 yielded transconjugants that secreted what appeared to be CPG2 due to a yellow coloration on folate containing media. The colouration was only present on the 10^0 dilution of the mating mix and was not observed on a 10^{-1} dilution of the conjugation mix. Passage of recombinants on selective media supplemented with folate resulted in the loss of yellow colouration. It was therefore assumed that the yellow coloration was possibly due to the *E. coli* donor expressed copy of CPG2 being present on the conjugation 'slurry' when the transconjugants were first plated out. The lack of expression was further confirmed by a lack of over-expressed protein in both the lysates of the soluble fraction and supernatants of 4 h and 8 h culture samples by SDS-PAGE, and by enzyme assay of lysates of the soluble fraction and supernatants (data not shown). Plasmids were checked for functionality, deletions/insertions, and/or rearrangements by isolation of the plasmids from the *C. sporogenes* recombinants. Purified clostridial derived plasmids were retransformed into *E. coli* TOP10. Restriction enzyme profiles of the plasmids had not altered compared to the parent plasmids and plating of *E. coli* TOP10 harbouring the clostridial derived plasmids gave the characteristic yellow halo on folate containing selective media.

4.2.4.2 Nitroreductase - HinNTR

The nitroreductase over-expression vectors pMTL5122::*Hin*NTR and pMTL5122::*Hin*NTR-synth were conjugated into *C. sporogenes* NCIMB 10696. Clones were verified by

retransforming plasmids derived from *C. sporogenes* recombinants back into *E. coli*. Clones of *E. coli* that were correct by restriction digest were then grown overnight and lysates of the soluble fraction screened by SDS-PAGE. A band of the expected size (25.2 kDa) was noted indicting that the vector was still functional and expressed *Hin*NTR to a high level in *E. coli* (see Figure 4.5).

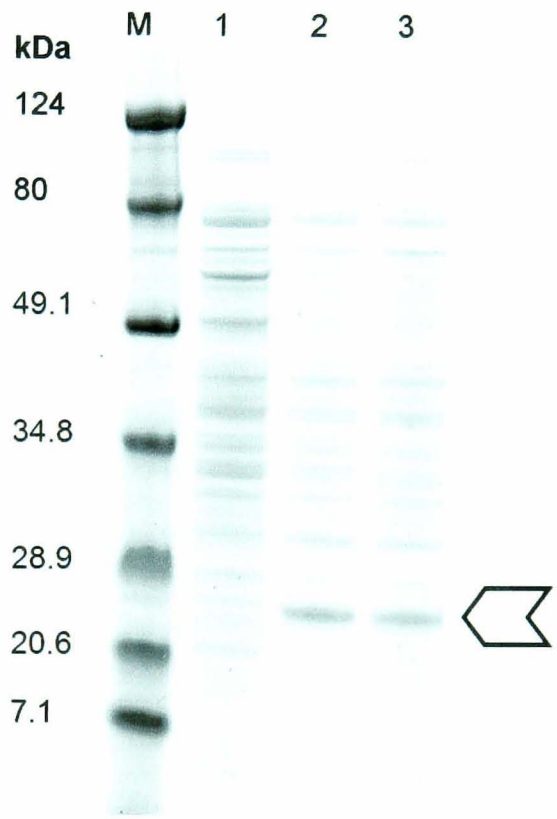


Figure 4.5: SDS-PAGE analysis of *Hin*NTR recombinants in *E. coli* CA434. M = BioRad Broad range protein markers, lane 1 = pMTL5122 (negative control), lane 2 = pMTL5122::*Hin*NTR, lane 3 = pMTL5122::*Hin*NTR-synth. Arrow represents location of *Hin*NTR.

Following successful identification of suitable erythromycin resistant recombinant clones of *C. sporogenes*, expression of *Hin*NTR was monitored by menadione assay relative to a negative control background (see Figure 4.6). Maximal expression was observed at the shoulder of exponential and stationary phase, corresponding to 6 hours post inoculation, with levels equivalent to 3-fold over background levels of endogenous nitroreductase activity.

Analysis of the lysates of the soluble fraction that gave the highest expression by assay did not give the expected over-expressed band when visualised by SDS-PAGE (data not shown).

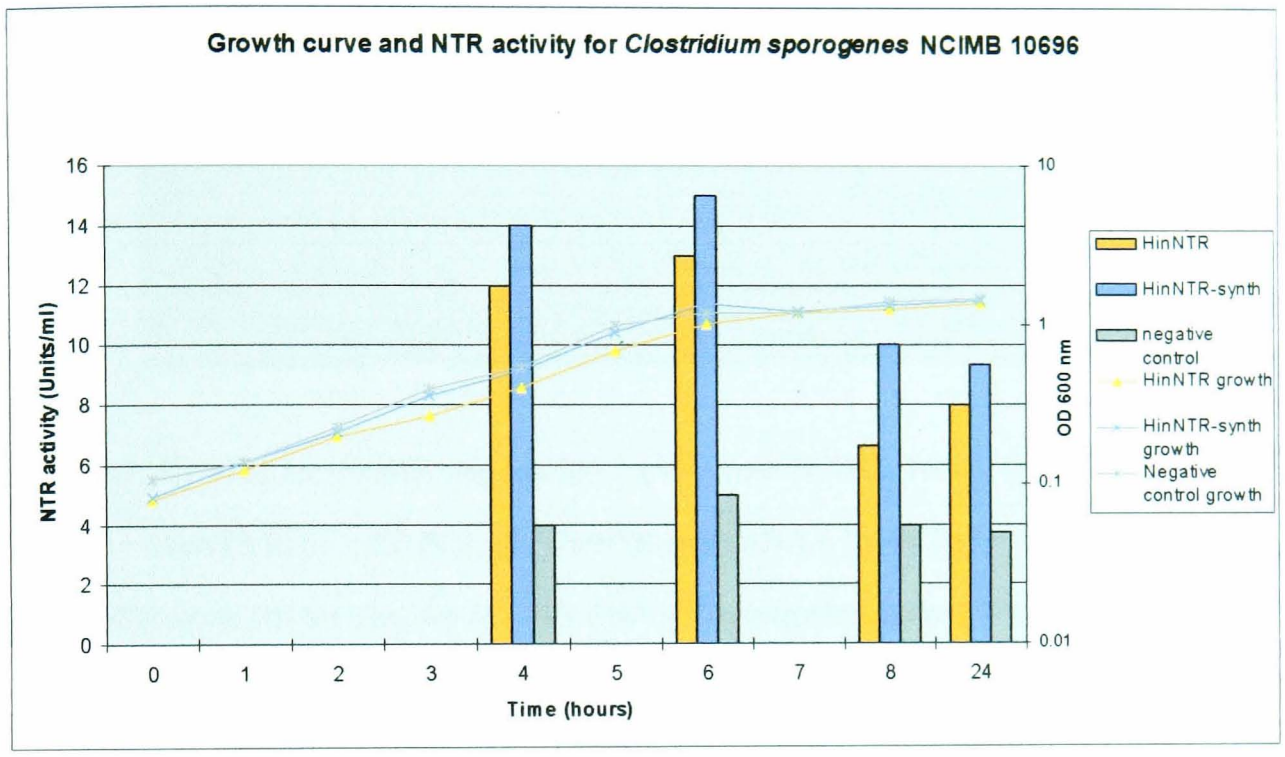


Figure 4.6: Menadione reductase assay (results represented by the bars on the graph) on *C. sporogenes* lysates collected at 4 h, 6 h, 8 h, and 24 h. A growth curve (represented by the lines on the graph) was also performed to verify that the presence of the over-expression plasmid in the host was not affecting growth.

RT-PCR was used to determine whether the relatively low-level expression of *Hin*NTR over background levels in *C. sporogenes* was due to a transcriptional or translational problem. RNA was harvested and purified from a 6 hour culture (OD_{600} = ~1.5). Following quantification of the RNA, RT-PCR was performed using primers HINWTRTF and HINWTRTR for the wild type *Hin*NTR and primers HINSYNTHRTF and HINSYNTHRTR for the synthetic *Hin*NTR. Agarose gel electrophoresis of the RT-PCR products showed a product of the expected size (663 bp) for the RNA samples and the DNA positive control (see Figure 4.7). The negative controls, RNA with no RT and water with no RT proved there was no DNA contamination of the RNA. The band intensities were of similar strength

indicating that the *Hin*NTR RNA was of sufficient proportion of the total RNA isolated.

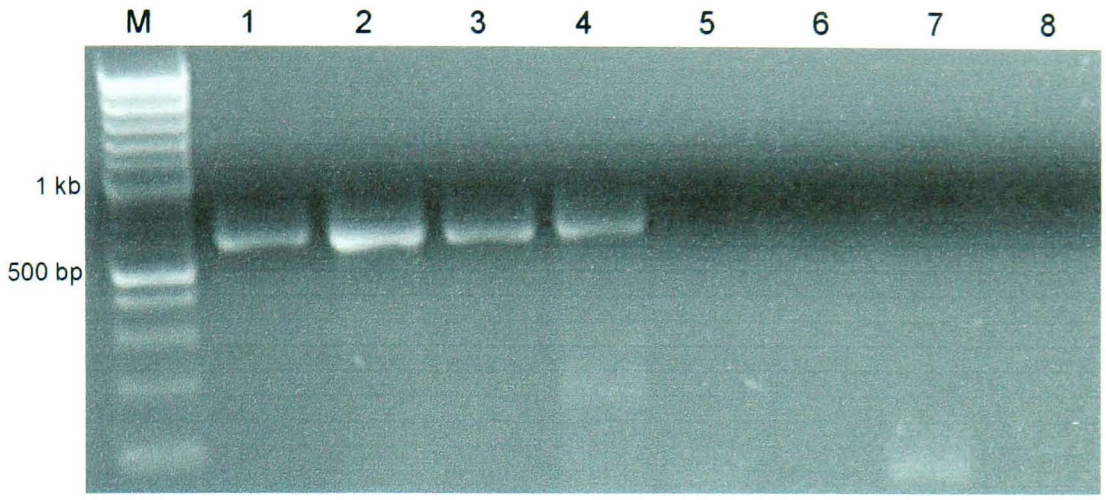


Figure 4.7: RT-PCR on *Hin*NTR expressing *C. sporogenes* NCIMB 10696. M = NEB 2 log DNA ladder, 1 = *Hin*NTR RNA + RT-PCR, 2 = *Hin*NTR-synth RNA + RT-PCR, 3 = *Hin*NTR DNA + Taq, 4 = *Hin*NTR-synth DNA + Taq, 5 = *Hin*NTR RNA + Taq (negative control), 6 = *Hin*NTR-synth RNA + Taq (negative control), 7 = negative control (H_2O + HINWTRTF+HINWTRTR primers), 8 = negative control (H_2O + HINSYNTHRTF+HINSYNTHRTR primers).

4.2.5 *In vivo* anti-tumour testing of *C. sporogenes* NCIMB 10696 expressing *Hin*NTR-synth

Having identified a level of expression from *Hin*NTR-synth 3-fold above the background level provided by genome encoded nitroreductase expression, it was of interest to test this first generation strain in an *in vivo* model. All of the work described in this section **(4.2.5)** was performed by J. Theys, MAASTRO, University of Maastricht, The Netherlands. It is included here for completeness.

4.2.5.1 Development of an in vivo model

Previous *in vivo* work at MAASTRO focused on the use of the rat rhabdomyosarcoma model. However, it was decided early on in the project to change to a mouse animal model, in particular the immunodeficient nu/nu mouse, due to more cell lines and cell lines of

human origin being available for the model. This would make it more suitable to study anti-tumoural effects on a variety of cancer cells in a later stage.

Initially, toxicity of *C. sporogenes* NCIMB 10696 spores was assessed *in vivo* by intravenous injection of 10^8 spores (in a total volume of 100 µl PBS in the tail vein of nu/nu mice) in a series of non-tumour bearing nu/nu mice. The animals showed no signs of toxicity and were normally active throughout the entire follow-up period.

To test the tumour colonisation capability of *C. sporogenes*, HCT116 colon carcinoma cells were xenografted into nu/nu mice. Growth of the tumours was followed and three weeks post injection, when the tumours had grown to approximately 400 mm^3, animals were injected via the tail vein with either 5 x 10^7 or 5 x 10^8 spores of *C. sporogenes* NCIMB 10696. After 8 days, the tumours were excised from euthanized animals and the bacterial cells quantified. Examination of the tumours revealed that up to 10^9 cfu of *C. sporogenes* were obtained per gram of tumour tissue. Ample tumour colonisation occurred using the lower spore titre, therefore the following experiments were performed using 10^7 spores.

4.2.5.2 In vivo anti-tumour assessment of HinNTR-synth expressing C. sporogenes – single cycle treatment

Having identified the superior colonisation capability of *C. sporogenes*, the ability of *Hin*NTR-synth recombinant *C. sporogenes* NCIMB 10696 to elicit an anti-tumour activity was assessed.

Spores of recombinant *C. sporogenes* NCIMB 10696 were systemically injected into nu/nu mice, once the xenografted HCT116 colorectal carcinoma cells had developed to an average tumour volume of 400 mm^3. Colonisation of the tumours was allowed to occur for 5 days prior to CB1954 prodrug administration. As controls, animals were either left untreated receiving no spores or prodrug, received CB1954 prodrug alone from day 5 (at a concentration of 15 mg kg^{-1}, intraperitonially for 5 days), or recombinant spores (at a

concentration of 5 x 10^7 spores in a volume of 100µl PBS via the tail vein) followed by prodrug vehicle only on day 5 (sham treatment). Treatment and growth of the tumours were measured by recording tumour size using callipers and recording the body weight of the mice. A level of control was also added to the experiment to quantitatively evaluate the approach and distinguish between effects due to administration of the spores and the effects resulting from *Clostridium*-mediated prodrug conversion, by utilising an antibiotic controlled experiment. The daily prodrug injections were followed by a treatment with the antibiotic Flagyl® (200 mg kg^{-1}, two times a day for nine days), which has been previously shown to eradicate clostridial spores from tumours (Theys *et al.*, 2001b).

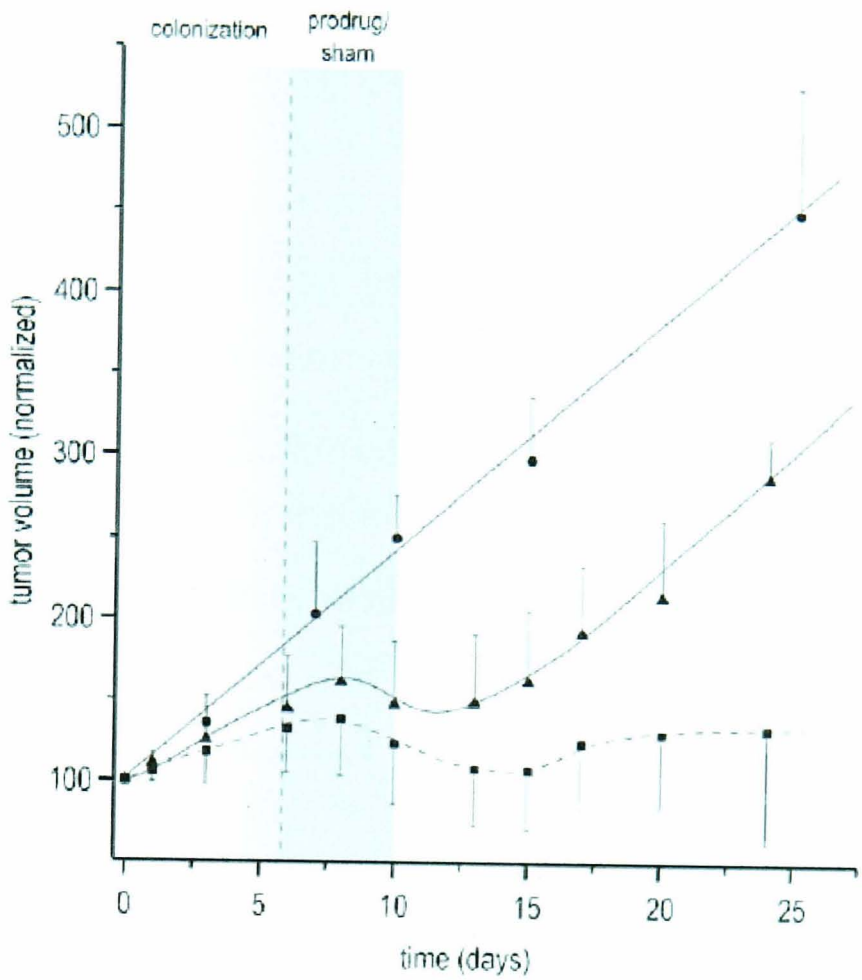
Tumour colonization following recombinant spore administration, but prior to the onset of antibiotic treatment, was quantified by performing dilution series of randomly selected tumours from spore-treated animals. All tumours screened showed colonization levels of 10^8-10^9 cfu g^{-1} tumour tissue. To confirm the specificity of the system, normal tissues were also screened for spores, and as expected no spores could be detected. CB1954 administration alone was found to have no effect on tumour volumes. Administration of spores alone caused a tumour growth delay ($p=0.09$, Student's t-test) (see Figure 4.8 (A)), which is presumed to be a consequence of moderate oncolysis and the appearance of hemorrhagic necrosis.

The most apparent effect *in vivo* was found when recombinant spores were combined with CB1954 administration. Following prodrug injection, the tumour growth rate reduced to a near static level with no significant tumour growth occurring over the 25 day study. Statistical analysis of the anti-tumour effect highlighted that the increase was significant when compared to the sham control ($p=0.016$, Student's t-test) (see Figure 4.8 (A)).

During the course of this pilot study, the evolution of the animals' body weight was followed as an indicator of the toxicity of the treatment. The data obtained indicated that the initial reduction in weight was only transient, and this weight loss was reversible with reversion to

normal body weight after 25 days (see Figure 4.8 (B)).

A



B

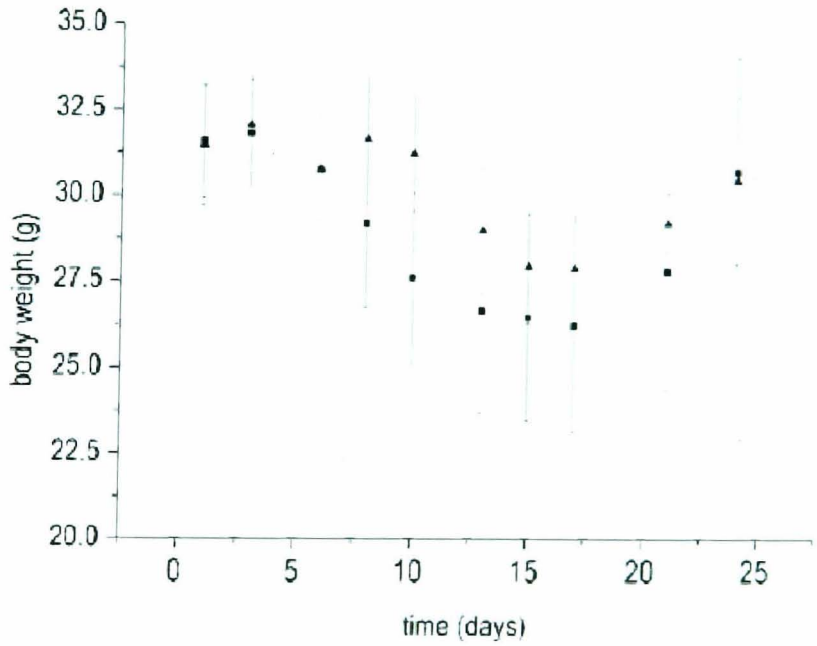


Figure 4.8: Tumour volume reduction following systemic administration of *Hin*NTR-synth recombinant *C. sporogenes* spores to nu/nu mice bearing HCT116 colon carcinoma cell line tumours. **(A)** Tumour growth as monitored after one cycle of recombinant *C. sporogenes* spore treatment with CB1954 prodrug (■) or drug vehicle only (sham) (▲) treatment. Control animals received no treatment (●). **(B)** Representative body weight evolution during a treatment cycle of prodrug (■) and

sham treated (▲) mice. Data was obtained from at least 5 mice per group. Figures kindly provided by J. Theys, MAASTRO, University of Maastricht, The Netherlands.

4.2.5.3 In vivo anti-tumour assessment of HinNTR-synth expressing C. sporogenes – repeated cycle treatment

Subsequently, it was of interest to see if repeated treatment cycles could be performed as this more closely reflects the actual clinical setting where chemotherapy is given in multiple cycles to allow recovery of the patient. To evaluate this, the basic treatment cycle as outlined above was followed by a week long recovery period, which was then followed by a second and third cycle of treatment identical to the first cycle. Sham treated animals only received two cycles as the tumours grew to a size that necessitated killing of the animals prior to a third cycle of treatment.

The tumour volume data obtained shows a reduction in tumour growth with the sham treatment (see Figure 4.9). This highlights that not only can the spores germinate and effectively re-colonise the tumour to give an anti-tumour effect, but also means that the tumour microenvironment did not change dramatically following the first treatment cycle such that re-colonisation could not occur.

Repeated treatment with the recombinant spores and CB1954 prodrug resulted in a more significant anti-tumour effect with the tumour only doubling in size over the 70 day treatment, compared to the HCT116 control which doubled every 7 days and the sham treatment which doubled every 20 days (see Figure 4.9). This reduction in tumour growth for the recombinant spore treatment allowed application of an additional third treatment cycle. Throughout the three cycles, the treatment resulted in sustained growth delay effects.

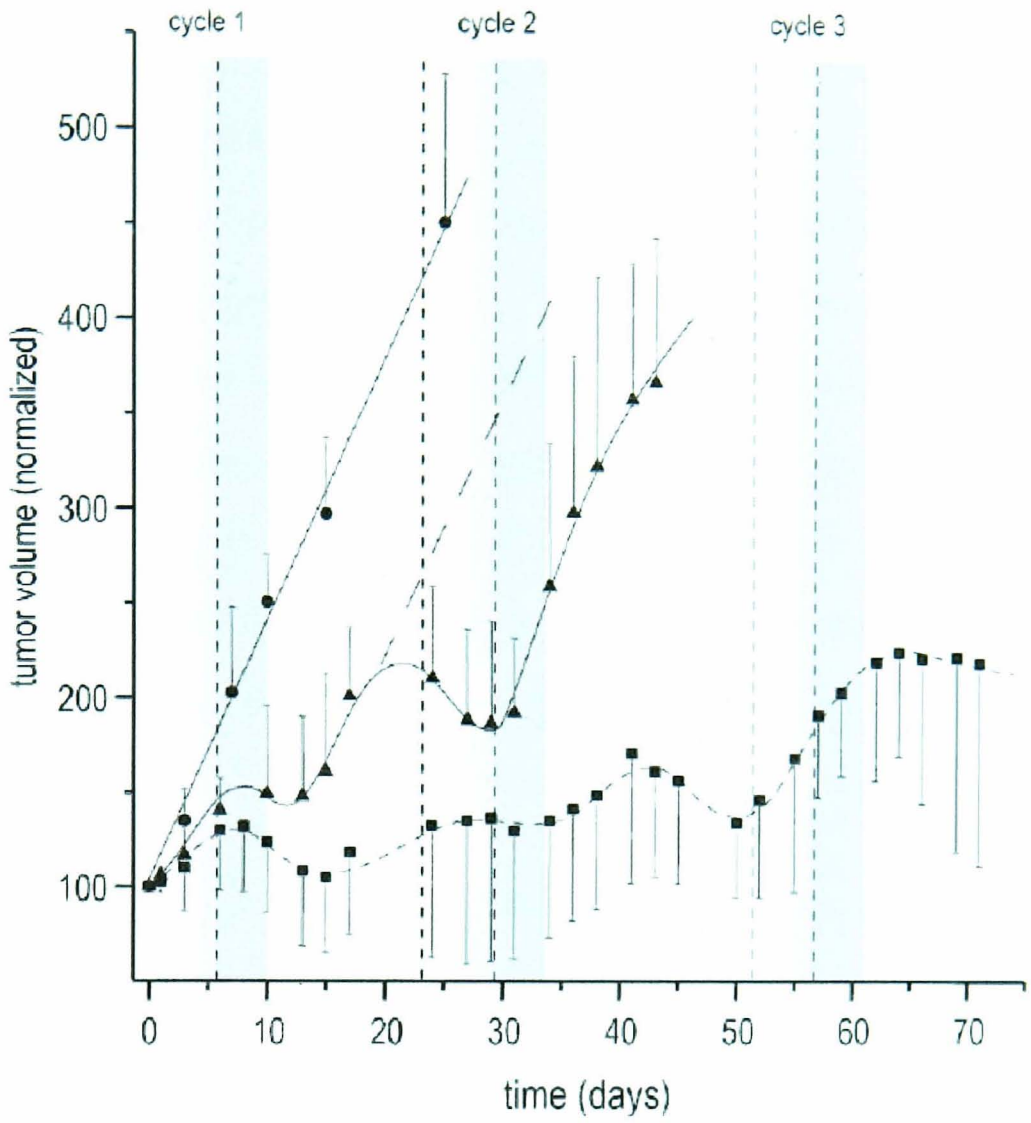


***Figure 4.9*:** Anti-tumour effect following repeated treatment cycles of *Hin*NTR-synth recombinant *C. sporogenes* in combination with CB1954 administration (■) or drug vehicle only (sham) (▲) treatment. Control animals received no treatment (●). Figure kindly provided by J. Theys, MAASTRO, University of Maastricht, The Netherlands.

4.3 DISCUSSION

The experiments presented in this chapter demonstrate that *C. sporogenes* NCIMB 10696 can be utilised for the expression of prodrug converting enzymes, in particular the nitroreductase from *H. influenzae*. Expression of CPG2 was, however, not as promising.

CPG2 enzyme activity was detectable in the soluble fraction of *E. coli* lysates but not in the

culture supernatant probably due to the general difficulties associated with secreting proteins in Gram-negative bacteria. Expression of CPG2 was not obtained in *C. sporogenes*. *C. sporogenes* is classified as a proteolytic member of the genus *Clostridium*, with high levels of extracellular proteases detectable after exponential growth (Allison and MacFarlane, 1990). Proteolysis of CPG2 by *C. sporogenes* cannot be excluded as a factor contributing to the lack of expression. Indeed, crude experiments in which late exponential *C. sporogenes* culture supernatant was incubated with purified CPG2 resulted in a marked decrease in enzyme activity (data not shown). Due to time constraints and the potential for proteolysis of secreted CPG2, efforts were focused on nitroreductase which being both intracellular and smaller than CPG2 may be more easily over produced.

Cloning of both the native sequence and synthetic *Hin*NTR into pMTL5122 resulted in over-expression in *E. coli*, which could be visualised by SDS-PAGE. However, once conjugated into *C. sporogenes*, increased expression levels could only be detected by enzyme assay. No over-expressed band was visible by SDS-PAGE of recombinant cell soluble fraction lysates. RT-PCR confirmed that *Hin*NTR mRNA was being produced. Therefore, the low level of expression as visualised by SDS-PAGE could be due to translational problems such as sub optimal codon usage.

Nitroreductase recombinant spores were delivered systemically to immunodeficient nu/nu mice harbouring xenografted HCT116 colorectal carcinoma cells. Delivery of the recombinant spores resulted in a clear sustained growth delay when combined with CB1954 prodrug. Administration of spores alone caused a significant tumour growth delay that can be attributed to moderate oncolysis and appearance of haemorrhagic necrosis. This observation following spore administration is seen when using other proteolytic clostridial species such as *C. novyii*-NT and *C. sporogenes* M55. However, when combined with CB1954 prodrug administration, the anti-tumour effect significantly increases, demonstrating the feasibility of using this process. Interestingly, the clostridial spores could also be delivered in multiple cycles, a scenario which more closely relates to clinical

chemotherapy regimes. As immunodeficient mice were used in the study, no conclusions can be drawn as to the immune response following multiple spore doses.

Since such an outstanding anti-tumour response is seen with both single and repeated cycles, it must mean that not only the hypoxic but also aerobic cells are being killed as a result of a bystander effect that is contributing to the effect seen.

Recently, an endogenous nitroreductase activity has been detected in several clostridial species (Theys, J. personal communication). Incubation of clostridial lysates with CB1954 results in conversion of CB1954 to the toxic drug as visualised by TLC. Also, incubation of CB1954 with non-toxic concentrations of clostridial lysate decreased the CB1954 LD_{50} concentration required to kill HCT116 colorectal carcinoma cells 20 to 40-fold depending on the concentration of clostridial lysate. Therefore the effect of endogenous nitroreductase activity cannot be discounted for the effect seen *in vivo*.

The only other published significant anti-tumour effect observed with recombinant *C. sporogenes* was utilising the prodrug converting enzyme cytosine deaminase (CodA) in combination with the prodrug 5-FC (Liu *et al.*, 2002). These studies followed tumour growth for 11 days following a single intravenous injection of recombinant *C. sporogenes* spores into syngenic C3H/Km mice harbouring transplanted SCCVII tumours. A tumour growth delay was observed, but resistance to the toxic drug 5-FU was observed after 7 days of daily injections of 5-FC, after which time tumour growth paralleled normal non-treated control tumour growth. In comparison to the data presented in this chapter where a significant growth delay is seen, the growth delay effect reported by Liu *et al.* was transient. This is attributed to the cancer cells developing resistance to the drug, a scenario not seen with the nitroreductase system developed here.

The potential of combining vascular targeting agents with enzyme prodrug therapy using engineered *C. sporogenes* has now been demonstrated (Liu *et al.*, 2003). For these studies

the recombinant plasmids carrying *nfnB* or *codA* that had previously been introduced into saccharolytic clostridia (Fox *et al.*, 1996; Lemmon *et al.*, 1997) were transformed into *C. sporogenes* NCIMB 10696. The resultant clones were then used in a therapy experiment in which the vascular targeting agent DMXAA (5,6 dimethylxantheone-4-acetic acid) was administered some 4 hours after spore injection. DMXAA was found to elicit a 4-fold increase in the extent of tumour colonisation. Complete tumour regression was achieved with both subcutaneously transplanted murine SCCVII and human HT29 carcinomas in C3H and nude mice, respectively. These experiments also evaluated the benefit of using more soluble derivatives of the prodrug CB1954. One of the prodrugs tested (SN24927) was shown to cause more effective tumour regression in combination with clostridial cells overproducing nitroreductase. This result further supports the findings of Wilson *et al* (2002) where SN24927 was found to provide curative activity against WiDr tumours using a multilayer tissue culture model. However, since the presentation of this data three years ago, no further data has been presented by this group on nitroreductase expressing recombinant *C. sporogenes*.

Despite the encouraging results, at this stage it is not entirely clear as to the contribution of *Hin*NTR to the observed *in vivo* effect due to the relatively low level of expression. This could be clarified by bringing about a significant improvement to expression levels.

CHAPTER 5

CHARACTERISATION OF THE SHUTTLE VECTOR AND IMPROVING EXPRESSION OF NITROREDUCTASE ENZYMES

5.1 INTRODUCTION

At this stage of the study, the level of *Hin*NTR production observed was disappointing. When it is considered that the ultimate aim of CDEPT is to have the prodrug converting enzyme integrated into the genome as a single copy this would further reduce expression levels. It became apparent that expression would need to be improved.

One factor that could affect expression levels is the segregational stability of the plasmid system utilised. An analysis of the segregational stability of the over-expression vector was therefore undertaken. The relative strength of the promoter used to drive expression of the prodrug converting enzyme is also a factor that may be effecting expression. Therefore, in parallel with the plasmid segregational stability study, the possibility of utilising an alternative promoter to *fac2* was investigated.

It is possible that there are specific gene related effects hampering expression. To study this, it was of interest to see if NfnB which has previously been expressed to high levels in *C. beijerinckii* (Minton *et al.*, 1995) and *C. sporogenes* (Liu *et al.*, 2003), could be expressed utilising the original expression vector pMTL5122.

Finally, codon usage is also known to influence expression of genes especially in bacteria with genomes of extreme high or low G+C content. Therefore, the currently available nitroreductases were analysed for codon usage. Based on this, new synthetic genes were generated with the codon usage optimised to the *C. tetani* codon usage table. A study of the completed clostridial genome codon usage tables with the preliminary data available for *C. botulinum* and *C. sporogenes* identified that the *C. tetani* codon usage table proved to be the closest match. Once synthesised, the expression of these genes was assessed.

5.2 RESULTS

5.2.1 Segregational stability of pMTL5122

To assess the segregational stability of pMTL5122 cells of *C. sporogenes* NCIMB 10696 harbouring the plasmid were grown non-selectively for 48 hours, passaging into fresh non-selective media every 12 hours. At the end of the passaging regime the cells had undergone the equivalent of 48 generations. The cells were then serially diluted and plated out onto non-selective media. Following overnight growth, 50 colonies were then picked and streaked out onto both non-selective and selective media. No growth was obtained on the antibiotic-containing selective media, relative to a positive control streak. Therefore, it can be assumed that less than 2% of the cell population are retaining pMTL5122. This indicted the segregational instability of pMTL5122 and prompted a further study of alternative clostridial replicons.

5.2.2 Segregational stability of alternative clostridial replicons

5.2.2.1 A novel clostridial plasmid

Indigenous plasmids are inherently segregationally stable in their native host. Recently, a cryptic indigenous plasmid designated pBP1 has been identified in *C. botulinum* NCTC 2916 (Davis, 1998). Due to the close phylogenetic relation of *C. botulinum* to *C. sporogenes* (Collins *et al.*, 1994) the potential of utilising this plasmid was studied.

Analysis of pBP1 translated DNA sequences and comparison to the available SwissProt database identified 8 ORF's of which a role in plasmid replication could be attributed to ORF 1 (see Figure 5.1). ORF 1 was found to bear a high degree of similarity with a group of topoisomerases with replicative origin-specific activities. This class of proteins are

implicated in initiating the replication of plasmids whose members are broadly represented by pC194, a plasmid which replicates via a rolling circle mechanism (Novick, 1989). An alignment of several replication proteins most similar to ORF 1 demonstrated that the primary sequence of ORF 1 essentially confirms to the five conserved regions described for this class of replication protein (Noirot-Gros et al., 1994).

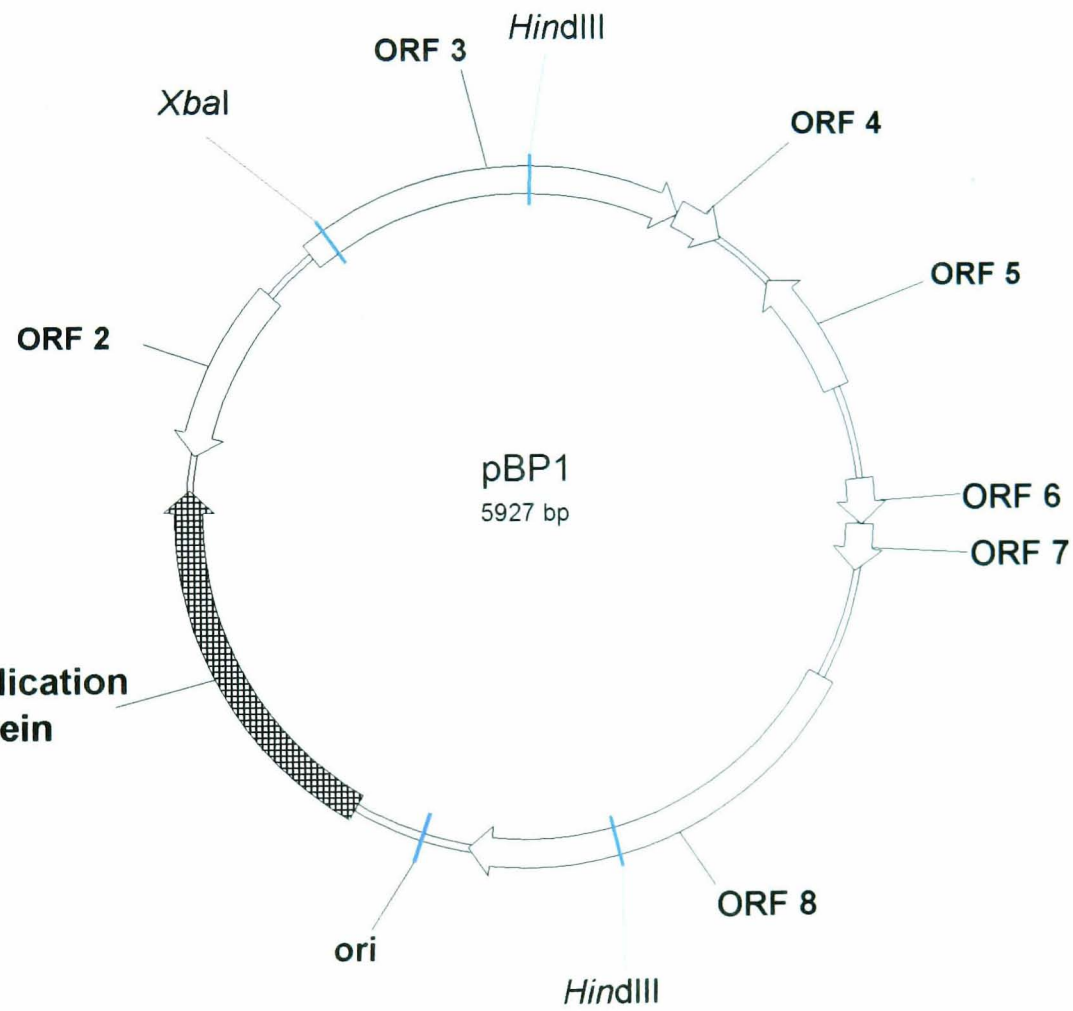


Figure 5.1: Schematic representation of pBP1. The location of all identified ORF's are highlighted as well as the replication protein and *Hin*dIII/*Xba*I restriction sites used for cloning.

Plasmid origins of replication had also been identified and two inverted repeats are found 5′ to the putative replication protein. One of these occurs 28 bp before a heptameric motif described as a double –stranded origin nick site (*dso*), characteristic of the pC194 group of plasmids (Seery *et al.*, 1993). Indeed, the occurrence of the combination of an inverted

repeat, *dso*, and pC194 type replication protein are suggestive features that this type of extrachromosomal element is a plasmid which replicates via a rolling circle mechanism. A second region of dyad symmetry is found between the *dso* and replication protein. Such features are found in other examples of this class of plasmid (Devine *et al.*, 1989; Josson *et al.*, 1990) and may also serve some function in plasmid maintenance/replication. The evidence found so far thus indicates that pBP1 replicates via a rolling circle mechanism.

5.2.2.2 Cloning of pBP1 plasmid replication protein fragment and segregational stability

Previous work was performed by T. Davis on the identification of a minimal region containing plasmid replication functions (Davis, 1998). pBP1 was first digested with *Hin*dIII into two fragments of 2.711 kb and 3.216 kb (see Figure 5.1). Cloning of these two fragments into pMTL20E yielded pMTL20EBP2 and pMTL20EBP19 respectively. The plasmids were then electroporated into *C. beijerinckii* NCIMB 8052 and transformants were only obtained with pMTL20EBP19. Thus, it was concluded that the replication functions were present on the 3.216 kb *Hin*dIII fragment of pBP1. The replicon was then further reduced by restriction digestion with *Hin*dIII and *Xba*I (see Figure 5.1). The 2.633 kb *Hin*dIII/*Xba*I fragment was cloned into pMTL20E to generate pMTL5200. The resulting plasmid could also transform *C. beijerinckii* NCIMB 8052. (Minton, N.P. personal communication). The vectors were also assessed in *C. botulinum* ATCC 3502 Hall A (Bennik, M. personal communication). Electroporation of pMTL5200 resulted in a frequency of approximately 2.0×10^3 cfu μg^{-1} plasmid DNA. The plasmid was also assessed for segregational stability and was found to be 100% stable in *C. botulinum* ATCC 3502 after four 8 hour passes in non-selective media (Bennik, M. personal communication). Based on the high segregational stability obtained in *C. botulinum* it was therefore of interest to determine if the replicon from pBP1 was functional and 100% segregationally stable in *C. sporogenes* NCIMB 10696. For conjugative mobilisation of pMTL5200 into *C. sporogenes*

NCIMB 10696, *oriT* was cloned in as an *Eco*RV/*Sma*I fragment from pEOriT into the *Sma*I site of pMTL5200 to give pMTL5210. Conjugal transfer of pMTL5210 into *C. botulinum* Hall A still resulted in a 100% segregationally stable plasmid, indicating that *oriT* was not effecting the segregational stability.

5.2.2.3 Segregational stability of clostridial replicons

During the course of these studies the following vectors based on a variety of plasmid replicons became available:

1. pMTL9301 (pMTL28 + pCD6 replicon [4133 bp *Pvu*II region] from pCD35EC + OriT from pMTL30) (Purdy *et al.*, 2002)
2. pMTL9401 (pMTL28 + pCB102 replicon [1628 bp *Csp*45I/*Hin*dIII region] from pCB102 + OriT from pMTL30) (Purdy *et al.*, 2002)
3. pMTL9611 (pMTL29 + pIP404 replicon [2544 bp *Nsp*I/*Nhe*I region] from pJIR418 + OriT from pMTL30) (Purdy *et al.*, 2002)
4. pMTL9511 (pAMβ1 based shuttle vector, M. Herbert, HPA, Porton Down, Salisbury)

Also available for segregational stability screening are pMTL5112, pMTL5210, and a conjugal version of the pIMP1 plasmid, pIMP1::OriT. pIMP1::OriT was constructed by cloning OriT as a *Sma*I/*Eco*RV fragment into *Sma*I digested pIMP1.

All the vectors were conjugated into *C. sporogenes* NCIMB 10696. Once the various recombinant strains were verified correct by PCR screening and retransforming the plasmids back into *E. coli*, the rate of plasmid loss and loss per generation was determined (see Table 5.1). Recombinant *C. sporogenes* was grown non-selectively for 48 hours passaging into fresh non-selective media every 12 hours, an equivalent of 48 generations. Once cultures had been grown for 48 generations a serial dilution of the culture was plated onto non-selective media. To calculate plasmid stability, 100 randomly picked colonies were streaked

onto media with or without antibiotic selection, the proportion of cells that grew on the plate containing antibiotic selection being taken as the representation of plasmid segregational stability. Due to the low segregational stability of pIM13-based vectors, segregational stability was calculated based on cell counts on selective and non-selective media following 48 generations of growth in non-selective media.

Plasmid	Replicon	Stability (% of cells maintaining plasmid)	Loss per generation	Method of replication
pIMP1:OriT	pIM13	0.00835 *	1.78×10^{-1}	rc
pMTL5112	pIM13	0.01087 *	1.73×10^{-1}	rc
pMTL9301	pCD6	58	1.13×10^{-2}	Theta?
pMTL5210	pBP1	73	6.54×10^{-3}	rc?
pMTL9401	pCB102	84	3.63×10^{-3}	?
pMTL9511	pAMβ1	96	8.50×10^{-4}	Theta
pMTL9611	pIP404	98	4.21×10^{-4}	Theta

Table 5.1: Plasmid segregational stability of the available clostridial replicons. * = due to low stability, data was collected from colony counts of cultures plated onto selective and non-selective media (approximately 10,000 cfu would need to be screened to isolate a single plasmid-bearing clone). rc = rolling circle, ? = not known.

Unexpectedly, the pIM13 based plasmids pMTL5112 and pIMP1::OriT were severely unstable with a plasmid loss per cell generation equating to 17%. Also, it was disappointing to find that the pBP1 replicon was not 100% segregationally stable as determined for *C. botulinum*. The most stable plasmid was pMTL9611, based on the *C. perfringens* plasmid pIP404, with 98% of the population retaining the plasmid after 48 generations, a figure that equates to a 4.21×10^{-4} loss of the plasmid per generation.

pIP404 is a small (10.207 kb), low-copy, mobilisable plasmid which encodes a UV inducible bacteriocin. Unlike other plasmids from Gram-positive bacteria, pIP404 does not appear to

replicate via a ssDNA intermediate as evidenced by a lack of signal in DNA hybridisation of crude lysates of both *C. perfringens* and *B. subtilis* harbouring either pIP404 or derivatives of pIP404 respectively (Garnier and Cole, 1988). The exact method of replication utilised by pIP404 is not yet known. It is possible that pIP404 replicates via a theta mechanism due to the lack of ssDNA intermediate and the presence of an extensive repeat region downstream of the replication protein. The increased stability of pMTL9611 could therefore be partly attributed to this alternative method of replication, as ssDNA is known to be highly recombinagenic and unstable. This is further supported by the fact that the pAMβ1-based plasmid pMTL9511, which is known to replicate via a theta mechanism, is also highly stable in *C. sporogenes*.

The minimal region of pIP404 which has been identified to confer replication proficiency is the 2.792 kb *Eco*RI/*Eco*RV fragment (Garnier and Cole, 1988). Furthermore, Bal31 digestion from the *Eco*RI site into ORF 4 (the putative copy number control ORF) increases copy number by approximately 5-fold. The region of pIP404 used in pJIR418 to provide Gram-positive plasmid replication was the 2.629 kb *Hin*dIII/*Eco*RV fragment, the restriction sites being deleted during the cloning of pJIR418 in order to free them up for introduction into the multiple cloning site. This region was further reduced during the cloning of pMTL9611 by taking a 2.544 kb *Nhe*I/*Nsp*I fragment (Purdy *et al.*, 2002).

5.2.3 Development of a segregationally stable mutant strain of *C. sporogenes* NCIMB 10696

The curing of strains harbouring pIM13-based plasmids can increase the segregational stability of these plasmids when they are reintroduced into the cured mutant (Azeddoug *et al.*, 1992). Based on the poor segregational stability of pMTL5122, it was decided to create a cured strain of *C. sporogenes* NCIMB 10696.

The *C. sporogenes* NCIMB 10696 strain harbouring pMTL5122::*nfnB* (**discussed in Section 5.2.5**) was cured of the vector by growing recombinant cells non selectively for 98 hours passaging every 24 hours, a more severe process compared to the stability study as loss of the plasmid was desired. Cultures were then plated out onto media with or without antibiotic selection in order to determine plasmid segregational stability. In total, 0.011% of the wild type *C. sporogenes* NCIMB 10696 cells harbouring pMTL5122::*nfnB* remained resistant to erythromycin, a level comparable to that obtained during the segregational stability study.

Of the colonies that grew on the non-selective media, 100 were then screened for loss of the plasmid by picking onto plates with or without antibiotic selection. Two clones that were erythromycin sensitive, designated 10696-1 and 10696-3, were screened for loss of the plasmid by colony PCR using plasmid replicon specific primers. A faint signal was present when the PCR products were visualised on a 1% agarose gel, indicating a proportion of the population of the colony still harboured the plasmid. This was resolved by passaging the strains a further two times on non-selective media and re-screening the resulting individual colonies for loss of the plasmid.

Following the generation of a cured strain the next stage was to assess any effects on the maintenance of introduced plasmids. Accordingly, pMTL5122 and pMTL5122::*Hin*NTR-synth were transferred into the cured strain 10696-1. Conjugations into the wild type strain were repeated as a control using the same donor culture for both recipients. Conjugation transfer frequencies were calculated, and an increase in transfer frequency was observed in the cured strain equivalent to an increase of 1×10^{-7} transconjugants per donor cell for the wild type bacterium to 1×10^{-5} transconjugants per donor for the cured strain. The transfer frequency of pMTL5122 in the cured strain is now comparable to the transfer frequencies observed with other clostridial replicons in the wild type *C. sporogenes* NCIMB 10696 strain.

Both the wild type and cured recombinants were cultured non-selectively for 48 hours with

passage every 12 hours, an equivalent to 48 generations. Cells were then plated out onto selective or non-selective media and the rate of plasmid loss calculated based on the number of colony forming units derived (see Table 5.2).

Plasmid	cfu ml^{-1} TYG	cfu ml^{-1} TYG+Erm	**% loss – w/t**	cfu ml^{-1} TYG	cfu ml^{-1} TYG+Erm	**% loss – cured**
pMTL5122	3.75×10^8	4.07×10^4	**99.9891**	6.30×10^8	$2.12*10^8$	**66.3492**
pMTL5122:: *Hin*NTR-synth	5.20×10^7	1.84×10^6	**96.4615**	6.30×10^8	$2.37*10^8$	**62.3810**

Table 5.2: Segregational stability of two pIM13 based shuttle vectors in the wild type and plasmid-cured derivative of *C. sporogenes* NCIMB 10696.

The plasmid losses calculated equate to a loss per generation of 1.73×10^{-1} for pMTL5122 in the wild-type bacterium and 2.24×10^{-2} for pMTL5122 in the cured strain. For pMTL5122::*Hin*NTR-synth, the loss per generation in the wild type *C. sporogenes* NCIMB 10696 was 6.72×10^{-2} , whereas in the cured strain this decreased to 2.02×10^{-2}. A significant improvement in plasmid stability was therefore seen in the cured strain of *C. sporogenes*.

5.2.4 Improving expression – assessment of available promoters using a GusA reporter vector

Reporter gene systems are an indispensable technology for characterization of promoter strength and regulation. Two reporter gene vectors have been developed in our laboratory based on either the chloramphenicol acetyltransferase gene (*catP*) of *C. perfringens* (Steffen and Matzura, 1989) or the β-glucuronidase (*gusA*) gene from *E. coli*. CatP has been previously used to study expression at the transcriptional level of the *C. perfringens plcC*

gene (Bullifent *et al.*, 1995; Matsushita *et al.*, 1994). GusA has been used previously to measure expression from the promoters of both the *cpe* gene (encoding *C. perfringens* enterotoxin) and the *nanEA* operon (encoding N-acetylmannosamine-6-phosphate epimerase and sialic acid lyase also in *C. perfringens* (Melville *et al.*, 1994; Walters *et al.*, 1999; Zhao and Melville, 1998). In *C. difficile*, GusA has also been used to study the expression of the toxin genes *toxA* and *toxB* in utilising *C. perfringens* as the host for the reporter assay (Dupuy and Sonenshein, 1998). In *C. beijerinckii* NCIMB 8052, GusA has been used as a reporter to study the solventogenesis/acidogenesis associated genes acetoacetate decarboxylase (*adc*) from *C. acetobutylicum* and phosphotransbutyrylase (*ptb*) from *C. beijerinkcii* (Ravagnani *et al.*, 2000). Finally, in *C. acetobutylicum* the thiolase (*thl*), acetoacetate decarboxylase (adc), phosphotransbutyrylase (*ptb*), hydrogenase (*hydA*) and xylose isomerase (*xylA*) promoter activities were screened using GusA as the reporter (Girbal *et al.*, 2003).

For maximal expression of the prodrug converting enzymes, the optimal promoter from a range of available promoters was sought and compared to the modified ferredoxin promoter (*fac*2) using the GusA reporter vector pMTL9361*gusA* (see Figure 5.2). The promoters available are all derived from clostridial genes and are listed in Table 5.3. The clostripain (*closI*) promoter GusA reporter vector was constructed by cloning the *closI* promoter from pKNT19*closI*$_p$*codA* on a *Hind*III/*Nco*I blunt-ended fragment and ligating to pMTL9361*gusA* digested with *Eco*RICRI/*Xba*I blunt-ended fragment (see Figure 5.2). The alternative promoter reporter vectors were constructed by G. Carter, University of Nottingham.

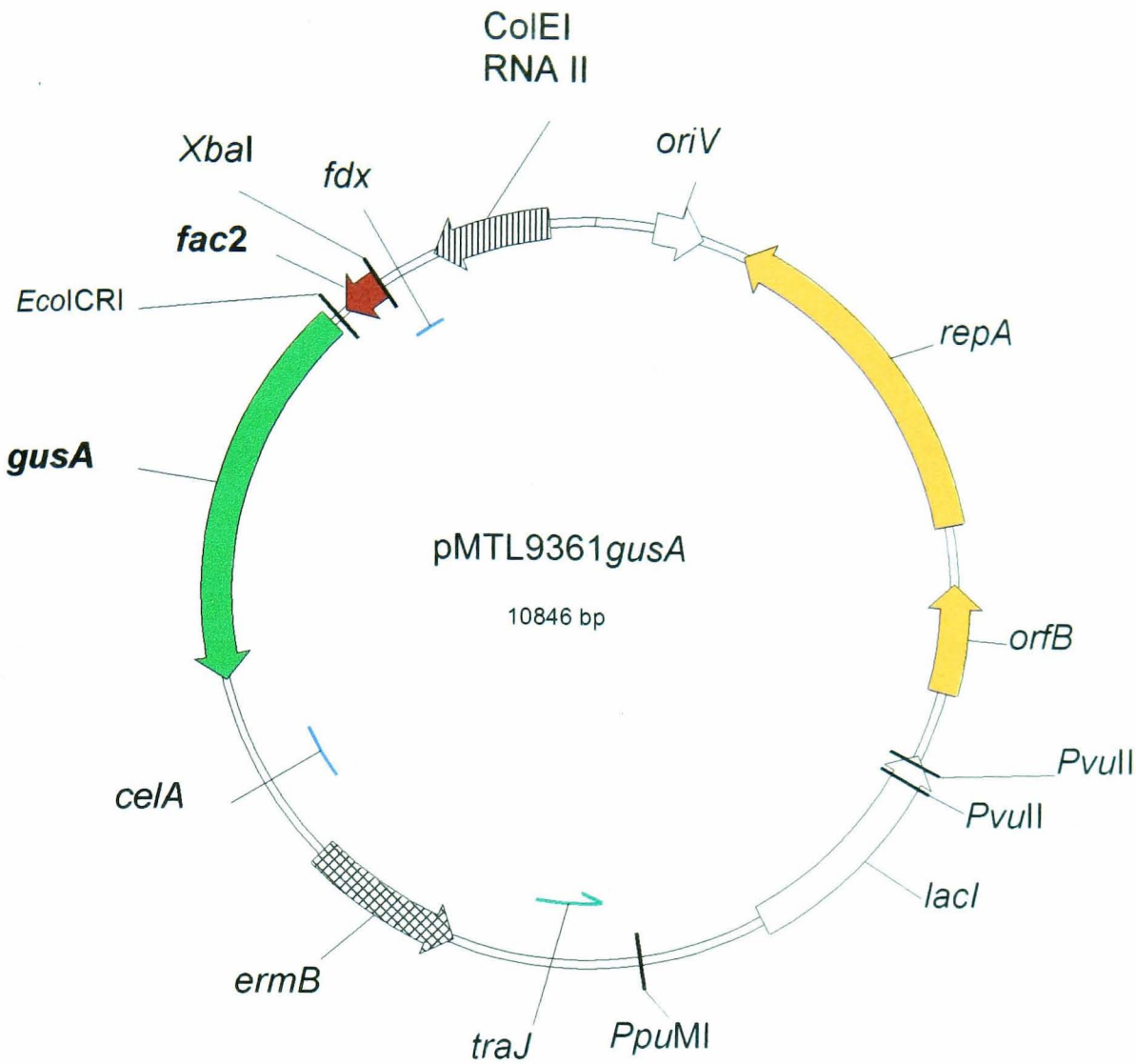


Figure 5.2: Schematic representation of the reporter vector pMTL9361*gusA*. Promoters were cloned as blunt fragments into the *XbaI*/*Eco*ICRI blunt sites of pMTL9361*gusA*. If required, the 1656 bp *Ppu*MI/*Pvu*II region encompassing the *ptb* promoter and *lacI* was deleted such that promoters containing *lac* operator sites became constitutive.

Promoter	Source	Source/Reference
eglA	β-1,4-endoglucanase from *C. acetobutylicum* P262	S. Barbé, K.U. Leuven (Zappe *et al.*, 1988)
hydA	Hydrogenase from *C. acetobutylicum* P262	(Santangelo *et al.*, 1995)
glnA	Glutamine synthetase from *C. beijerinckii* NCIMB 8052	S. Barbé, K.U. Leuven (Quixley and Reid, 2000)
closI	Clostripain from *C. histolyticum* DSM 1126	S. Barbé, K.U. Leuven (Dargatz *et al.*, 1993)

Table 5.3: Promoters available for assessment of activity in *C .sporogenes* utilising pMTL9361*gusA* reporter vector.

GusA reporter assays indicated that all the promoters are relatively similar in strength, although the clostripain promoter from *C. histolyticum* was the strongest at just over a 2.8-fold 'stronger' compared to *fac*2 (see Figure 5.3).

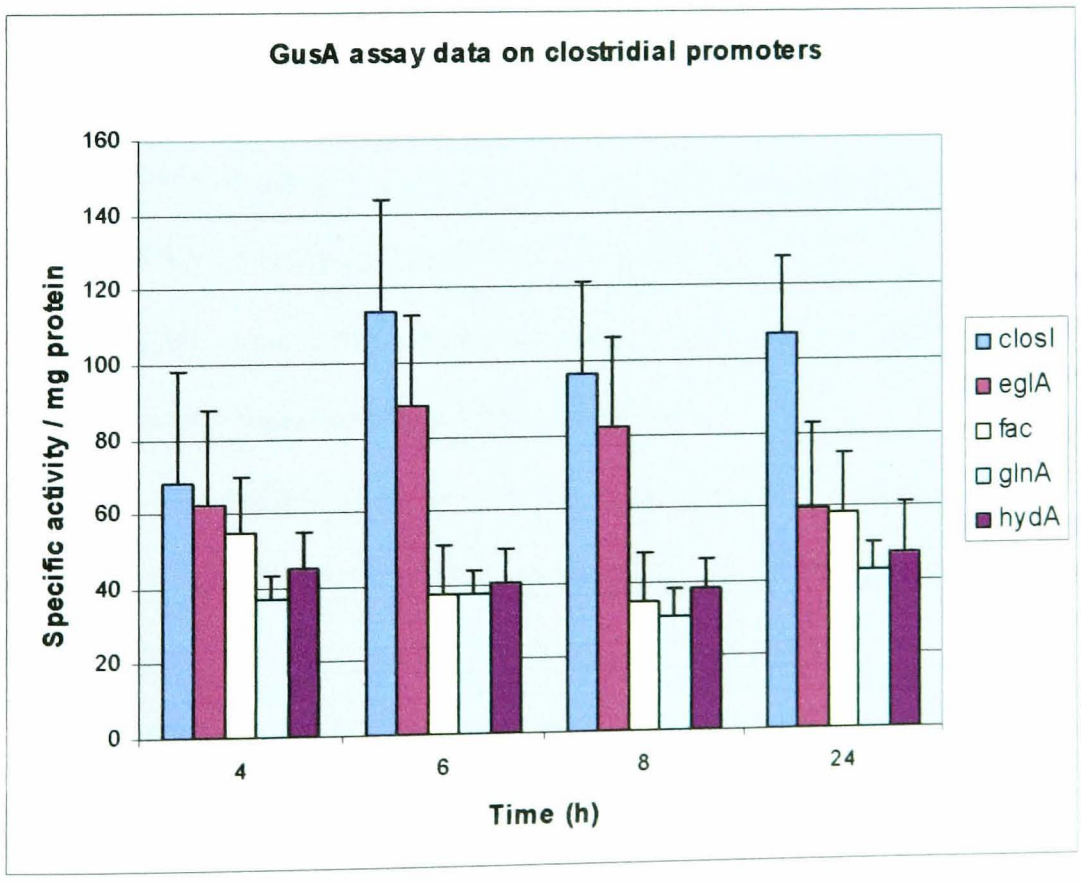


Figure 5.3: GusA reporter vector data for the clostridial promoters.

5.2.4.1 Cloning of a novel ferredoxin promoter from C. sporogenes

During the course of this study, work was published on the characterisation of a novel ferredoxin (*fdx*) promoter from *C. perfringens* strain13 and its use in the over-expression of a clostridial sialidase in *C. perfingens* strain 13 (Kaji *et al.*, 2003; Takamizawa *et al.*, 2004). The group headed by Okabe identified a homolog to the *C. pasteurianum* ferredoxin protein (Fdx) in *C. perfringens*. A region 70 bp upstream of the +1 site of the promoter was cloned and compared to the *C. pasteurianum fdx* promoter using a CatP reporter vector. Reporter assays indicated that the *C. perfringens* ferredoxin promoter is 10-fold more active compared to the *C. pasteurianum fdx* promoter (Kaji *et al.*, 2003).

Based on this result, an *in silico* analysis of the partially annotated *C. botulinum* genome (at the current time the genome sequence was only available at the private *C. botulinum* Sanger web site - http://www.sanger.ac.uk/Projects/C_botulinum/private) was performed utilising the Artemis package (Sanger Institute, Cambridge, U.K.). Screening of the genome for ferredoxin orthologues revealed three putative orthologues:

1) CBO0039 (262 amino acids, 29.8 kDa) – homology to two ferredoxins:

   a) *Clostridium tetani* putative polyferredoxin (258 amino acids) - fasta scores: E(): 2.3e-44, 50.95% homology over 263 amino acids.

   b) *Clostridium acetobutylicum* polyferredoxin (249 amino acids) fasta scores: E(): 4.7e-20, 35.65% homology over 258 amino acids.

2) CBO0059 (56 amino acids, 5.6 kDa) - homology to *Clostridium pasteurianum* ferredoxin (55 amino acids) - fasta scores: E(): 1.6e-18, 90.9% homology over 55 amino acids.

3) CBO0089 (63 amino acids, 7.0 kDa) - homology to two ferredoxins:

   a) *Thermatoga maritima* ferredoxin (60 amino acids) – fasta scores: E(): 0.00016, 38.98% homology over 59 amino acids.

   b) *Clostridium perfringens* ferredoxin (62 amino acids) – fasta scores: E(): 3.4e-07,

45.76% homology over 59 amino acids

Alignment of the three ferredoxins identified in the *C. botulinum* genome with the *C. perfringens* and *C. pasteurianum* ferredoxins highlighted that CBO0059 was the correct homologous ferredoxin from which to amplify the promoter from (see Figure 5.4). The 220 bp fragment extending from nucleotide positions -178 to +42 relative to the transcription initiation start site of bot-*fdx* was PCR amplified using primers BOTFDXF and BOTFDXR and chromosomal DNA from *C. botulinum* Hall A ATCC 3502 and *C. botulinum* ATCC 2916, plus *C. sporogenes* NCIMB 10696. The *C. sporogenes fdx* promoter was PCR amplified using three independent PCR's to avoid the introduction of PCR errors into the sequence. The 177 bp fragment extending from nucleotide positions -137 to +40 relative to the transcription start site of per-*fdx* was PCR amplified using primers PERFDXF and PERFDXR and chromosomal DNA from *C. perfringens* strain 13 and *C. perfringens* NCTC 8237 (supplied by J. Scott, Nottingham). Oligonucleotides complementary to the 5′ and 3′ ends of the promoters were used, with an *Nde*I restriction site created over the ATG start codon and a *Not*I restriction site added to the -178 end for the *C. botulinum fdx* promoter or -137 end for the *C. perfringens fdx* promoter.

```
CpastFdx MAYKIADSCVSCGACASECPVNAISQGDSIFVIDADTCIDCGNCANVCPVGAPVQE 56
CBO0059  MAYKITDACVSCGACAAECPVNAISQGDSIFDIDADTCIDCGNCANVCPVGAPVQD 56
CperfFdx MAYKILDTCVSCGACAAECPVDAISQGDTQFVIDADTCIDCGNCANVCPVGAPVQE 56
         ***** *:********:****:******: * **********************:
```

Figure 5.4: Protein alignment of clostridial Fdx homologues. "*" indicates residues or nucleotides in that column which are identical in all sequences in the alignment. ":" indicates that conserved substitutions have been observed (amino acids coloured red), no mark indicates no homology (amino acids coloured blue).

Once amplified, clones with the promoter in the correct orientation in pCR2.1TOPO (5′ end of the promoter nearest the *Xba*I site in pCR2.1), were cloned into the GusA reporter vector pMTL9361*gusA* as *Sac*I/*Xba*I fragments. Several attempts were made to clone the promoter fragments into pMTL9361*gusA* reporter vector but correct reporter vectors for all the promoters could not be obtained. To overcome the cloning difficulties, an alternative CatP reporter vector pMTL9361*catP* was utilised (see Figure 5.5). The *fdx* promoters were cloned in utilising the *Xba*I and *Sac*I sites in pCR2.1-TOPO and the *Xba*I and *Sac*I sites in the polylinker of pMTL9361*catP*. Reporter assay data from an 8 hour time point indicated that both the *C. perfringens fdx* and *C. sporogenes fdx* showed improved promoter strength compared to the *C. pasteurianum* derived *fac2*, with the *C. perfringens fdx* promoter giving 2.5-fold improvement over *fac*2 and the *C. sporogenes fdx* promoter giving a 3.5-fold improvement over *fac*2 (see Figure 5.6).

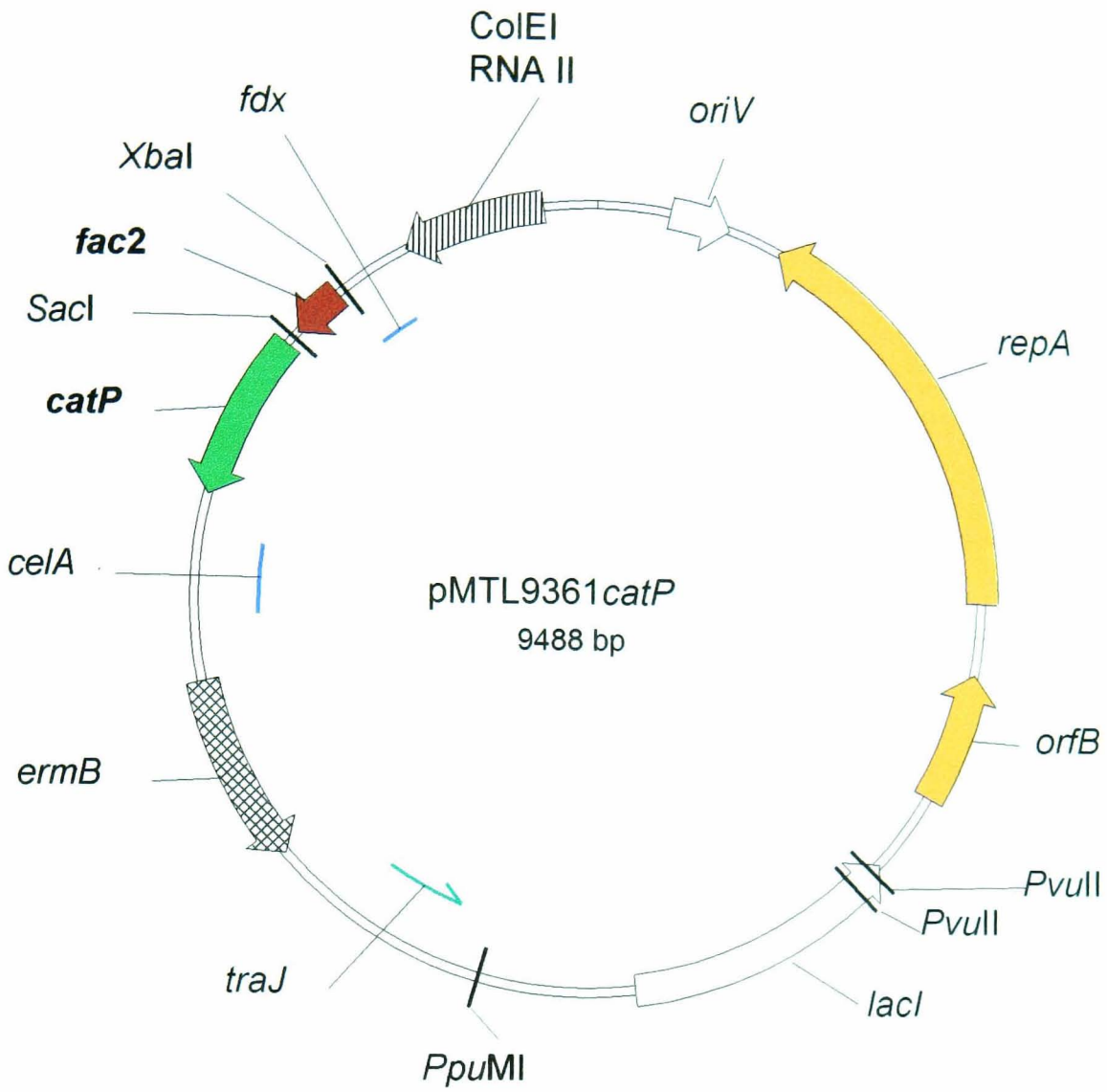


Figure 5.5: Schematic representation of pMTL9361*catP*. For cloning of promoter fragments into pMTL9361MCS*catP*, promoters were cloned in as *Xba*I/*Sac*I fragments from pCR2.1-TOPO clones into the *Xba*I/*Sac*I sites in the MCS of pMTL9361*catP*. If required, the 1656 bp *Ppu*MI/*Pvu*II region encompassing the *ptb* promoter and *lacI* was deleted such that promoters containing *lac* operator sites became constitutive.

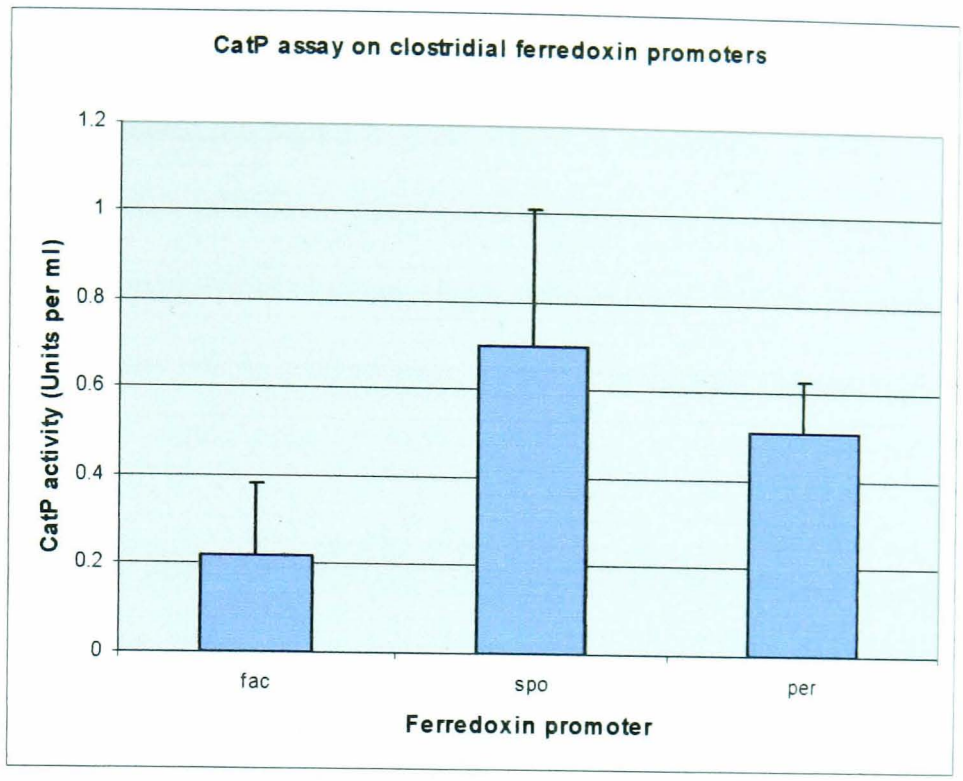


Figure 5.6: CatP promoter reporter data for clostridial ferredoxin promoters. Data collected at 8 hour time point. Samples all normalised to an OD_{600nm} of 1.0. fac = *fac*2, spo = *C. sporogenes fdx* promoter, per = *C. perfringens fdx* promoter.

Combining all the collated promoter reporter data for the clostridial promoters highlighted the optimal promoter to be the *fdx* promoter from *C. sporogenes* (*spofdx*). This was based on the relative promoter strength at the 8 hour time point of the growth cycle (see Table 5.4).

Promoter	Relative ratio*
glnA	0.89
fac2	1.00
hydA	1.10
perfdx	2.32
eglA	2.37
closI	2.80
spofdx	3.18

Table 5.4: Combined promoter data from GusA and CatP reporters. * expressed as ratios to the activity of the *fac*2 promoter in either assay.

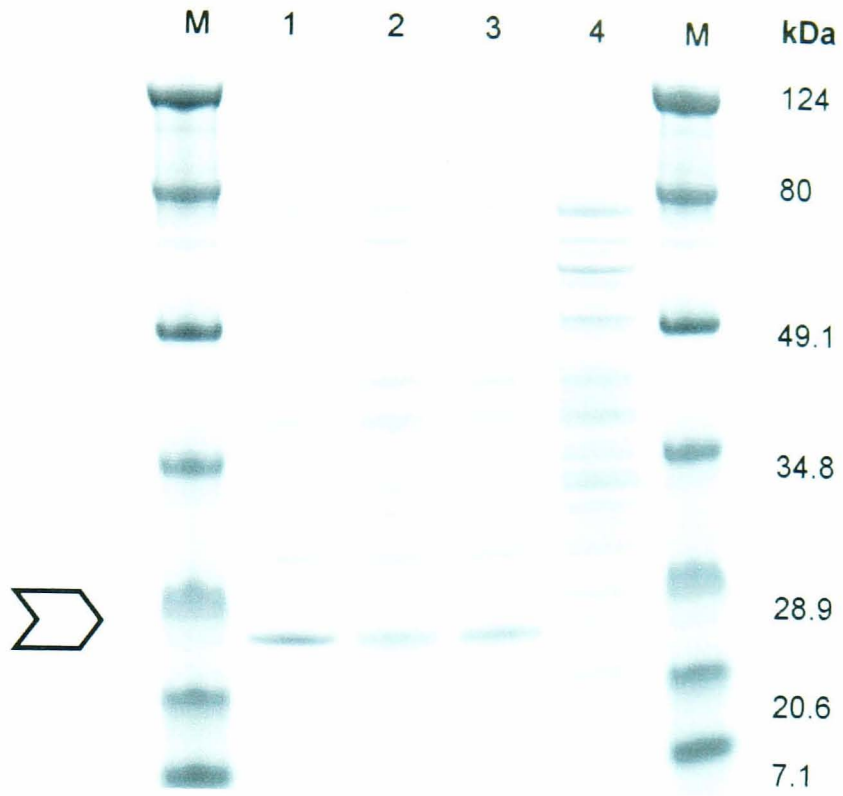
5.2.5 Cloning and expression of an alternative nitroreductase, NfnB

The *in vivo* data obtained for *Hin*NTR-synth was very promising. There was, however, no distinguishable reduction in tumour volume, just maintenance at a static level. Recently, Liu and colleagues published initial findings on the over-expression of the *E. coli* nitroreductase NfnB in *C. sporogenes* where a significant reduction in tumour volume was found (Liu *et al.*, 2003).

In order to establish if NfnB could be over-expressed, as has been previously reported (Liu *et al.*, 2003; Minton *et al.*, 1995), it was decided to replace *Hin*NTR with NfnB and repeat the expression studies using pMTL5122. This would verify if the reason for the lack of expression lies with the vector, as *nfnB* is known to be capable of being expressed in both *C. beijerinckii* and *C. sporogenes*, albeit using different expression vectors.

Primer pair NFNBF and NFNBR were used to amplify *nfnB* from pF25 (*nfnB* cloned into pTrc99A) such that an *Nde*I site was created 'over' the ATG start codon and *Xba*I/*Stu*I sites were at the 3′ end for cloning into the expression vector (see Appendix II for DNA and protein sequences of *nfnB*). pMTL5122 was digested with *Nde*I and *Pvu*II and *nfnB* was cloned in to the expression cartridge on a *Nde*I/*Stu*I fragment to give pMTL5122::*nfnB*. Expression was first verified as before in *E. coli* CA434 using SDS-PAGE and menadione reductase assay on the soluble fraction of lysates from an 8 hour culture (see Figure 5.7).

A



B

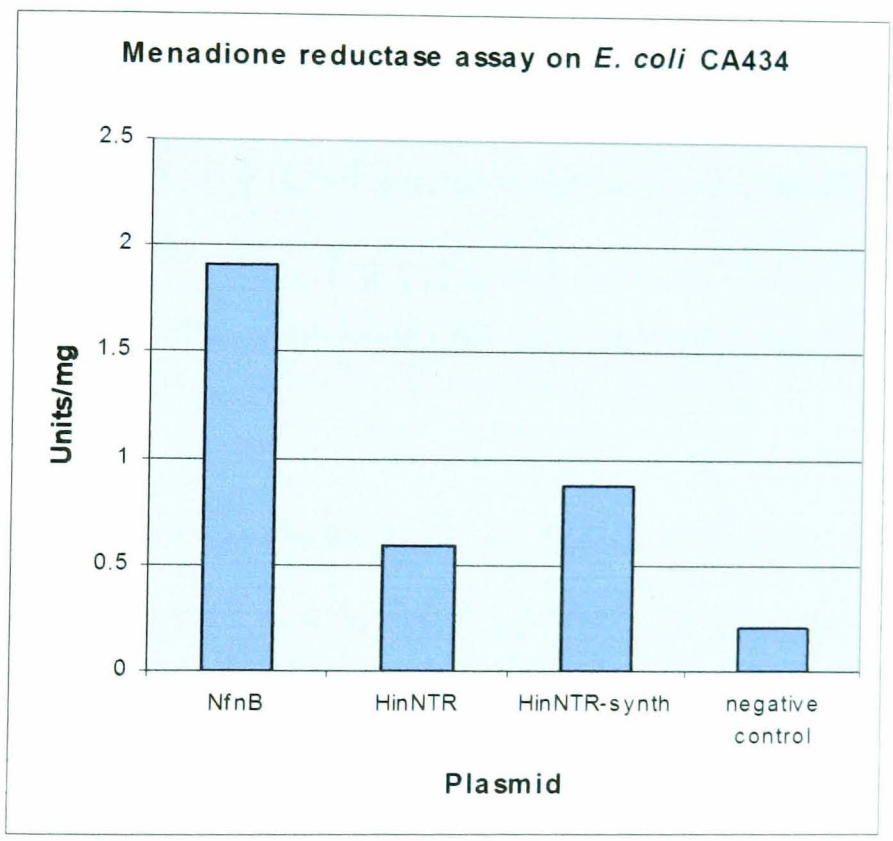


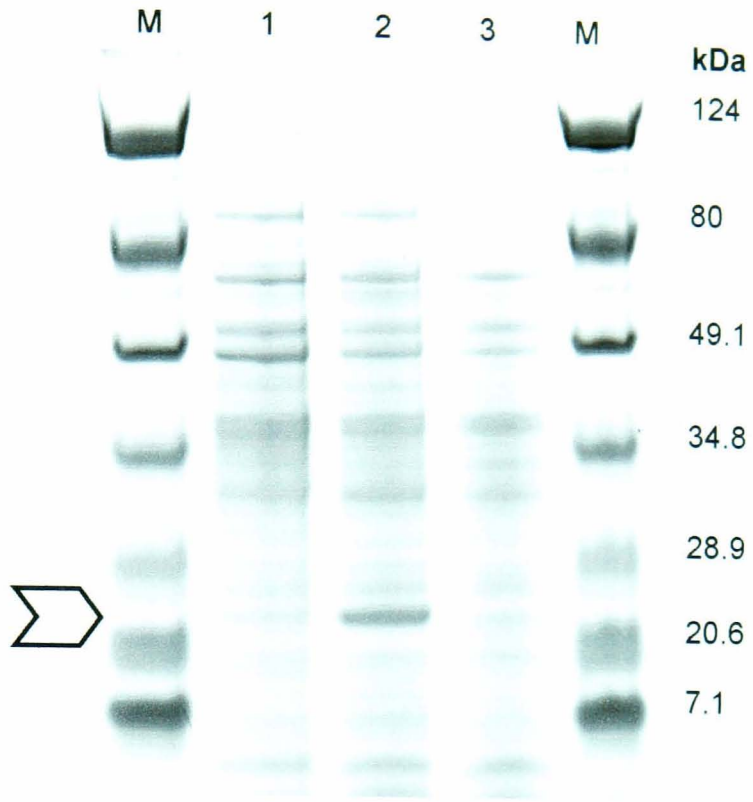
Figure 5.7: (A) SDS-PAGE of the soluble fraction of lysates from an 8 hour culture of *E. coli* over-expressing NfnB and HinNTR. M = BioRad Broad Range protein marker, lane 1 = negative control. lane2 = HinNTR-synth (9.18% soluble protein), lane 3 = HinNTR (7.46% soluble protein), lane 4 = NfnB (12.7% soluble protein). The arrow indicates the location of the over-expressed protein. (B) Menadione reductase assay on the soluble fraction of lysates from an 8 hour culture of *E. coli* CA434 over-expressing NfnB and comparison to *Hin*NTR.

NfnB was proven to have been expressed as evidenced by the over-expression of a protein of 23.9 kDa that was not evident in a vector only control. This over-expression corresponded to a value of 12.7% soluble protein by densitometric analysis of the SDS-PAGE gel. Analysis of the 8 hour soluble fraction lysates by menadione reductase assay showed that NfnB was over-expressed to a level 10-fold over background NfnB activity. This compared to a level of 3-fold over background for the *Hin*NTR (see Figure 5.7).

pMTL5122::*nfnB* was then conjugated into *C. sporogenes* NCIMB 10696 and erythromycin resistant transconjugants were verified to be correct by PCR screening for the plasmid and isolating the plasmid and retransforming back into *E. coli*. Further screening of the *E. coli* clones by SDS-PAGE proved that the plasmid had not undergone any major rearrangements whilst in *C. sporogenes*, such that expression was affected (data not shown). *C. sporogenes* soluble fraction lysates were prepared from 8 hour cultures of recombinants harbouring pMTL5122::*nfnB*. All clones screened showed an abundance of a protein of between 20.6 kDa and 28.9 kDa, which was not evident in a negative control lysate derived from cells harbouring an insert-free expression vector pMTL5122 or pMTL5122::*Hin*NTR-synth (see Figure 5.8).

Analysis of the 8 hour soluble fraction lysates by menadione reductase assay indicated a major increase in over-expression compared to the *Hin*NTR and negative control lysates, corresponding to a level of 15-fold over background levels of nitroreductase activity recorded in the negative control (see Figure 5.8).

A



B

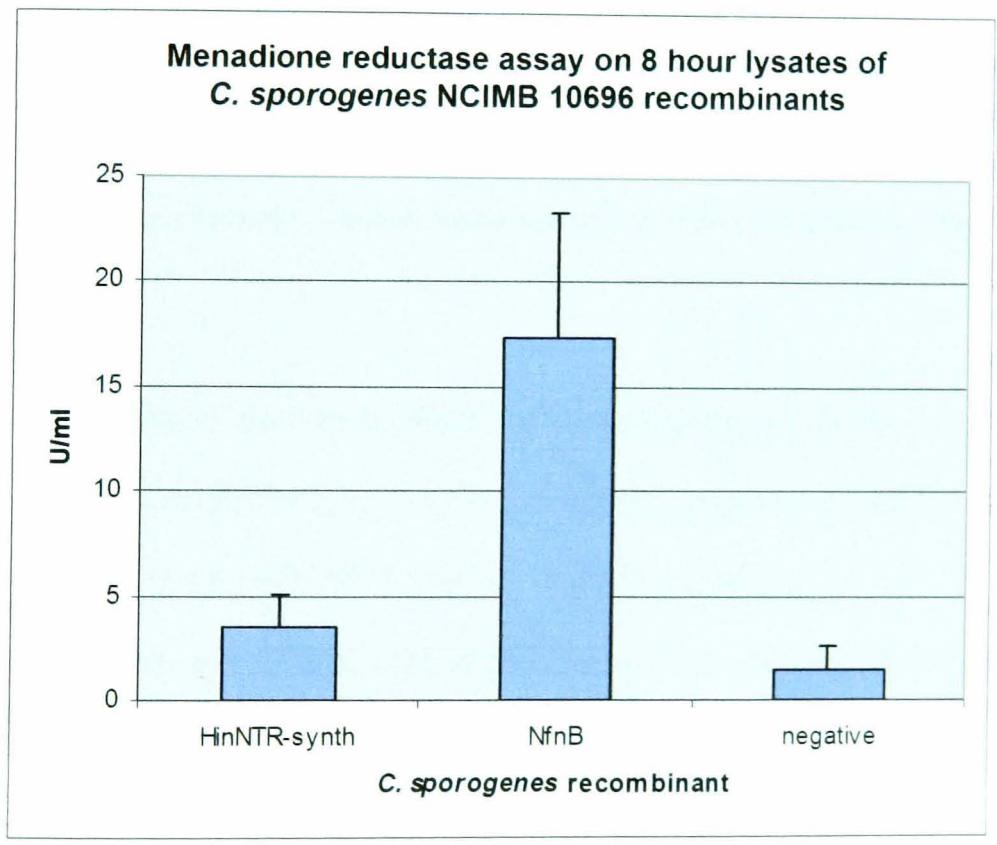


Figure 5.8: Over-expression of NfnB in *C. sporogenes* NCIMB 10696 as determined by SDS-PAGE (A) and menadione reductase assay (B), percentage soluble protein indicated in brackets. M = BioRad Broad Range protein marker; lane 1 = *Hin*NTR-synth lysate; lane 2 = NfnB lysate (7.2% soluble protein); lane 3 = negative control (pMTL5122). The arrow indicates the location of the over-expressed protein.

The data obtained for the over-expression of NfnB highlights that proteins can be over-expressed in *C. sporogenes* NCIMB 10696 utilising the expression vector pMTL5122. However, in the context of nitroreductase, in particular NfnB, the products of the reduction of CB1954 are important. As well as reducing the 4-nitro group to give the toxic 4-hydroxylamine drug, NfnB also reduces the 2-nitro group to the less potent 2-hydroxylamine derivative. Therefore either improved prodrugs or enzymes are sought for nitroreductase, a case that was thought to have been resolved using *Hin*NTR.

The first possible route to obtaining expression of *Hin*NTR was through optimisation of the over-expression vector as plasmid stability and promoter strength are two factors that can influence expression of proteins. The effect of changing the replicon and promoter was first assessed on vectors containing *nfnB* as any improvement in expression can be compared to that already obtained.

5.2.5.1 Alteration of promoter and/or replicon of the NfnB over-expressing plasmid – effect on expression

To determine the effects that the identified optimal plasmid would have on expression, variants of pMTL5122::*nfnB* were constructed. First as a negative control for expression, a pIP404 RepA version of pMTL5112 was generated by deleting out *repL* on a blunted *AatII/BamHI* fragment, and cloning in the pIP404 replicon on a blunted *NheI/NspI* fragment, to yield plasmid pMTL5612. The over-expression plasmid pMTL5122::*nfnB* was then altered to contain the pIP404 replicon using the same digest to give pMTL5622::*nfnB*. Secondly, the *C. sporogenes fdx* promoter was cloned into pMTL5122::*nfnB* as an *NdeI/NotI* fragment into an *Nde*I/*Not*I *fac*2 deleted version of pMTL5122::*nfnB* to give pMTL5123::*nfnB*. Finally, the pIP404 RepA was cloned pMTL5123::*nfnB* to yield pMTL5623::*nfnB*. The final result was four different plasmids all expressing *nfnB* driven by either the *C. sporogenes fdx* promoter or *fac*2, and with either the unstable pIM13 RepL or stable pIP404 RepA replicon. The combination of pIM13RepL and *C. sporogenes fdx* was

generated but not tested in *C. sporogenes* due to stability problems associated with the pIM13 replicon in *C. sporogenes*.

The *nfnB* variant plasmids were first transformed into *E. coli* CA434 and expression of NfnB was checked by SDS-PAGE of the lysates of the soluble fraction from overnight cultures (see Figure 5.9). All 3 plasmids expressed NfnB, indicated by over-expression of a protein of 23.9 kDa. Densitometric analysis of the SDS-PAGE gel predicted that the *fac*2-based vectors over-expressed to a level equating to 4% soluble protein, where as the *spofdx* promoter based vector over-expressed to a level equating to 15% soluble protein, indicating that the *spofdx* promoter is better recognised by *E. coli* RNA polymerase than *fac2*. This is most likely due to the spacing between the -10 and -35 sequence of the *spofdx* promoter being identical to the *E. coli* consensus of 17 bp (see Figure 5.10).

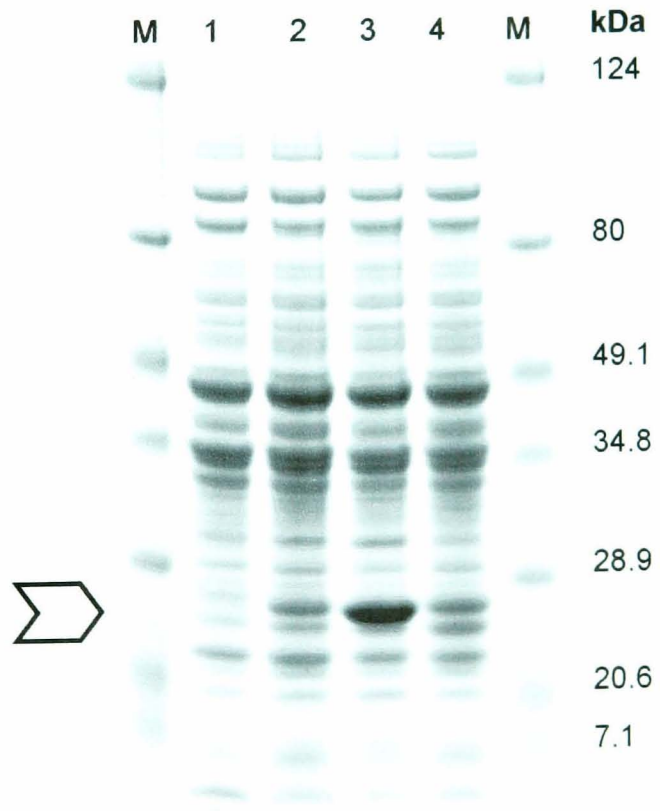


Figure 5.9: SDS-PAGE analysis of NfnB expression plasmids in *E. coli*. M = BioRad Broad range protein markers, lane 1 = pMTL5612 vector only control, lane 2 = pMTL5122::*nfnB*, lane 3 = pMTL5623::*nfnB*, lane 4 = pMTL5622::*nfnB*. The arrow indicates the location of the over-expressed protein.

	-35		-10	
fac2	ACTTACACT	**TTTAAA** AAGTTTAAAAACATGA	**TACAAT**	AAGTTA
spofdx	AAAAATTAC	**TTTAAA** AATTAATAAAAACATGG	**TAAAAT**	ATAAAT
Consensus		**TTGACA** (17 bp)	**TATAAT**	

	RBS		start
fac2	TTTA **AGGAGG**	TTAGTCAT	**ATG**
spofdx	TTTA **AGGAGG**	TGTGTTACAT	**ATG**
Consensus	**AGGAGG**	(9 bp)	**ATG**

Figure 5.10: Location and sequence of *fac*2 and the *C. sporogenes* ferredoxin promoter. Consensus = *E. coli* consensus sequence.

The plasmids were then conjugated into *C. sporogenes* NCIMB 10696. Screening of soluble fractions obtained after lysis of overnight cultures by SDS-PAGE indicated that all three vectors were capable of over-expressing NfnB, at broadly equivalent levels of 10–11% soluble protein (see Figure 5.11). Unfortunately, the expected 3-fold improvement in expression from the *C. sporogenes fdx* promoter predicted from the CatP reporter assay was not seen in the over-expression of NfnB. Based on the reporter data, an increase in expression from 10% to approximately 30% soluble protein would be anticipated.

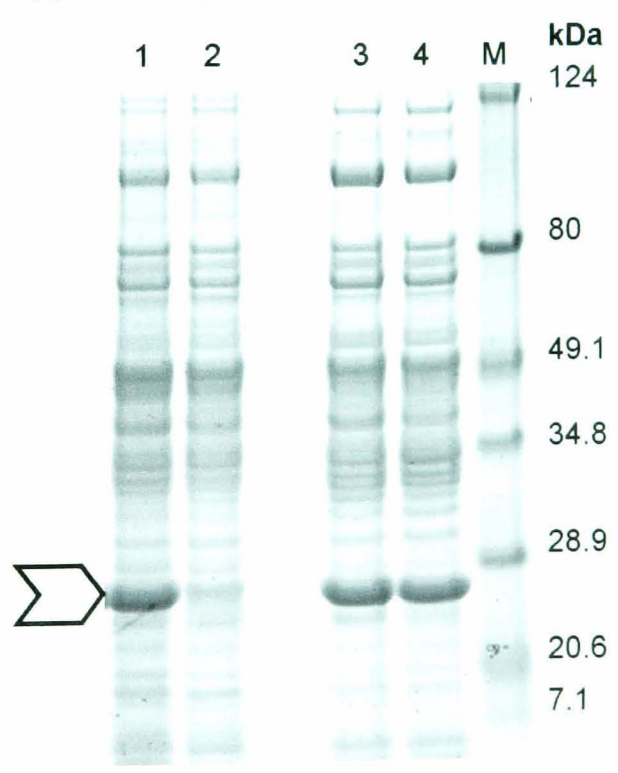


Figure 5.11: Expression of NfnB variants in *C. sporogenes* NCIMB 10696 wild type. Lane 1 = pMTL5122::*nfnB* (10.4%), lane 2 = pMTL5122::*Hin*NTR-synth, lane 3 = pMTL5622::*nfnB* (11.1%), lane 4 = pMTL5623::*nfnB* (11.0%), M = BioRad Broad range protein markers. The arrow indicates the location of the over-expressed protein.

5.2.6 Expression of nitroreductases in the cured strain of *C. sporogenes*

Having identified that the cured strain was approximately 10- to 100-fold more efficient at gene transfer and, once introduced, plasmids were several fold more stable, it was of interest to determine the effects on expression of nitroreductase enzymes.

The recombinant cured strain *C. sporogenes* NCIMB 10696 harbouring the *Hin*NTR and *Hin*NTR-synth over-expression plasmids as well as the NfnB over-expression plasmid were studied for expression levels. Lysates of the soluble fraction from 8 hour cultures were analysed by SDS-PAGE (see Figure 5.12).

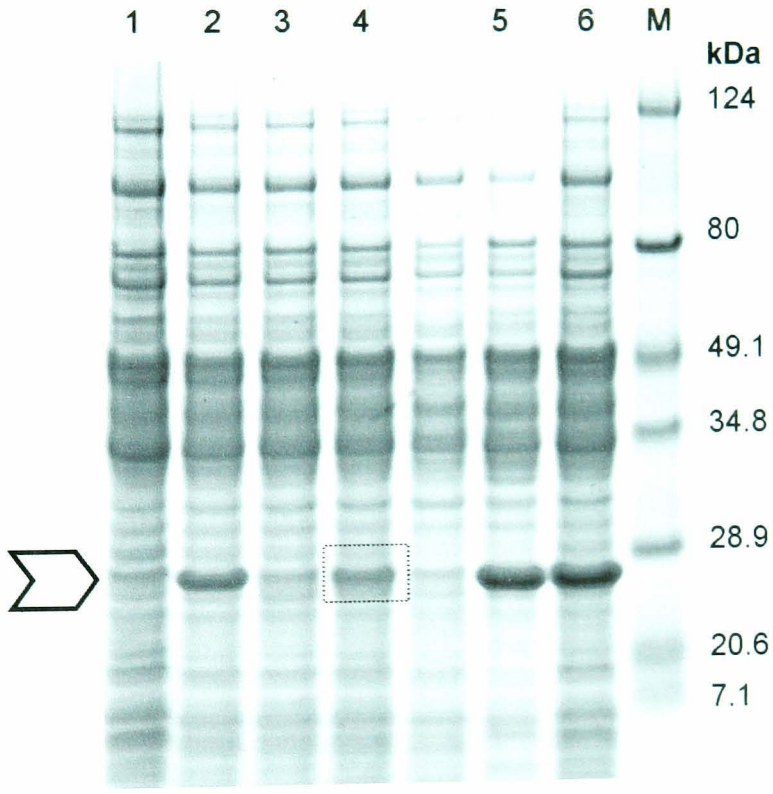


Figure 5.12: SDS-PAGE analysis of nitroreductase expressing recombinant cured strain *C .sporogenes* NCIMB 10696 soluble fraction lysates. Percentage soluble protein for each nitroreductase is shown in brackets. Lane 1 = pMTL5122, lane 2 = pMTL5122::*nfnB* (5.41%), lane 3 = pMTL5122::*Hin*NTR, lane 4 = pMTL5122::*Hin*NTR-synth (4.01%), lane 5 = pMTL5622::*nfnB* (9.82%), lane 6 = pMTL5623::*nfnB* (7.12%), M = NEB Broad range protein markers. The arrow indicates the location of the over-expressed protein.

SDS-PAGE analysis of the lysates of the soluble fraction indicated that the NfnB expressing plasmids were still functional in the cured strain. Densitometric scanning of the gel predicted levels of between 5% and 10% soluble protein at 8 hours, with the pIP404 based vectors producing the larger amount of enzyme at this time point. Of particular interest, however was the expression obtained from the synthetic *H. influenzae* nitroreductase. Over-expression of *Hin*NTR-synth could not be visualised in lysates of the soluble fraction from wild type *C. sporogenes* NCIMB 10696, however, in lysates of the soluble fraction from the cured strain recombinant harbouring pMTL5122::*Hin*NTR-synth over-expression could be detected. This was further confirmed to be the case when the soluble fraction lysates were examined using the menadione nitroreductase assay (see Figure 5.13). In comparison to NfnB expressed from pMTL5122, where a level of 22-fold over background nitroreductase activity was obtained, *Hin*NTR-synth was expressed at a level of 14-fold over background. If the levels of soluble protein produced are also taken into consideration then the units of nitroreductase per mg of total protein are comparable for the two enzymes (see Table 5.5).

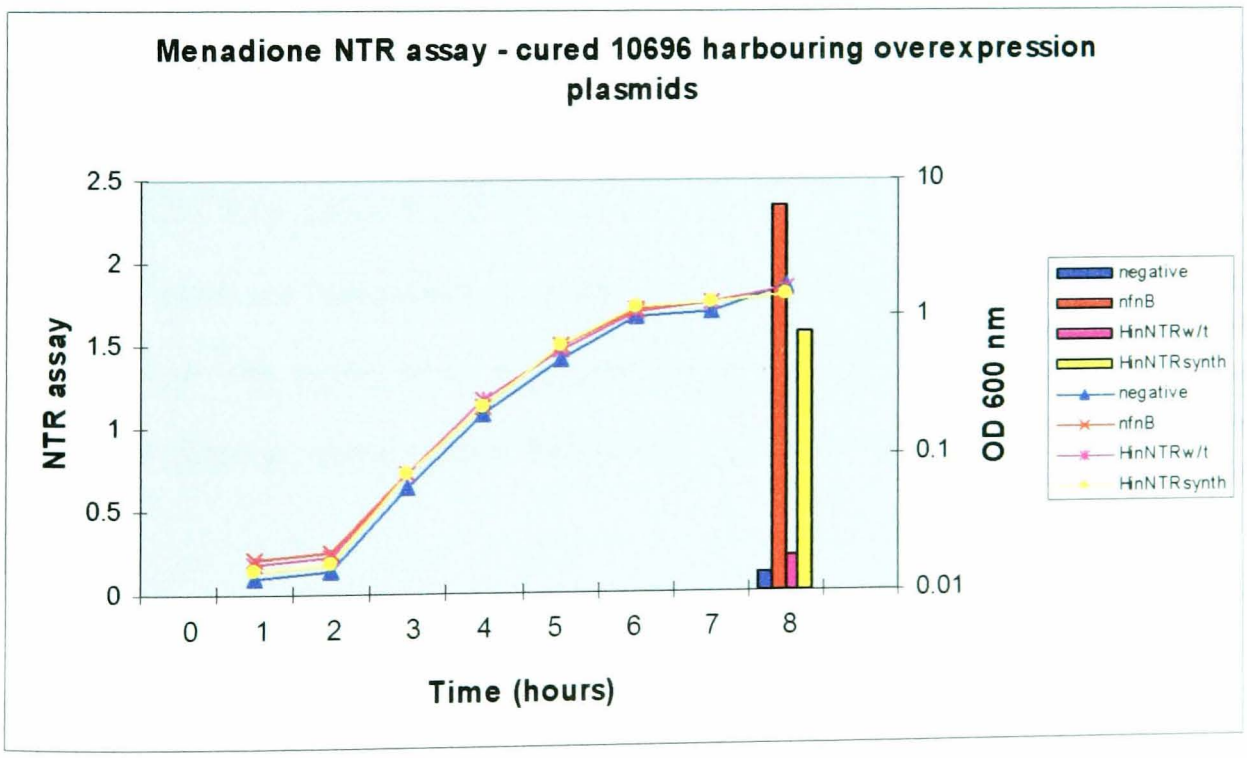


Figure 5.13: Menadione assay on the cured strain of *C. sporogenes* NCIMB 10696 over-expressing NfnB or HinNTR nitroreductases from the expression plasmid pMTL5122.

Enzyme	% soluble protein	Total soluble protein (mg ml^{-1})	Units mg^{-1} total protein	**Units mg^{-1} nitroreductase**
NfnB	5.41	1.83	2.34	**43.3**
*Hin*NTR-synth	4.01	1.37	1.57	**39.1**

Table 5.5: NTR activity calculated on units mg^{-1} protein based on densitometry scan of SDS-PAGE gel.

Segregational stability of the clones revealed that, as before, the pIM13-based clones were more stable in the cured strain compared to the wild-type strain.

5.2.7 Cloning of *Hin*NTR into a stable vector – expression in wild type and cured strains

One plausible explanation for the over-expression of *Hin*NTR-synth in the cured strain could be due to the increased segregational stability of pIM13-based plasmids in this host. To assess this, the stable pIP404 replicon was cloned into pMTL5122::*Hin*NTR-synth to yield pMTL5622::*Hin*NTR-synth. This was obtained by cloning the blunt-ended *Nhe*I/*Nsp*I pIP404 replicon from pJIR418 and cloning into pMTL5122::*Hin*NTR-synth digested with *Bam*HI and *Aat*II and blunt-ended to delete *repL*. pMTL5622::*Hin*NTR-synth was then conjugated into both strains of *C. sporogenes* NCIMB 10696 and lysates of the soluble fraction from mid-exponential cultures analysed by SDS-PAGE (see Figure 5.14).

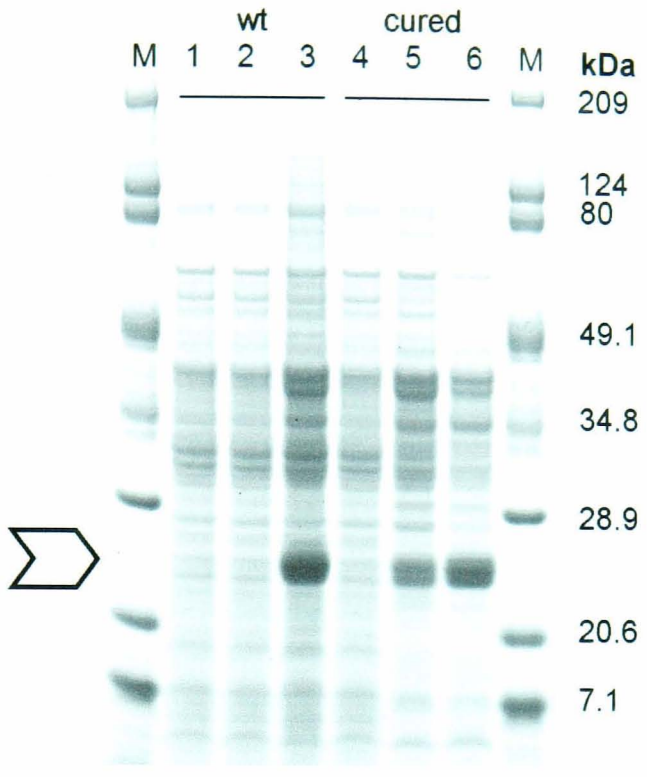


Figure 5.14: SDS-PAGE analysis of effect of plasmid stability on *Hin*NTR over-expressing clones. Lanes 1–3 are lysates of the soluble fraction from recombinant *C. sporogenes* NCIMB 10696. Lanes 4–6 are lysates of the soluble fraction from recombinant cured strain *C. sporogenes* NCIMB 10696. Percentage soluble protein determined by densitometry on over-expressed bands is shown in brackets. Lane 1+4 = pMTL5122 (negative control), lane 2+5 = pMTL5122::*Hin*NTR-synth (lane 2 = 0%, lane 5 = 7.25% soluble protein), lane 3+6 = pMTL5622::*Hin*NTR-synth (lane 3 = 8.58%, lane 6 = 13.3% soluble protein). M = NEB Broad range protein markers. The arrow indicates the location of the over-expressed protein.

Expression of *Hin*NTR-synth was obtained in the wild-type strain of *C. sporogenes* NCIMB 10696 when the gene was cloned in a segregationally stable version of the expression vector. This indicates a role for plasmid segregational stability in gene over-expression.

5.2.8 Cloning of a 'final' optimal expression vector

5.2.8.1 Characterisation of a novel improved nitroreductase

During the course of these studies another project was initiated on the *in silico* identification of new and improved nitroreductase enzymes for use in DEPT strategies (J. Heap, School of Pharmacy, University of Nottingham). One such novel nitroreductase isolated, termed NTR-N had an approximately 1- to 2-fold improvement in CB1954 specific activity compared to *Hin*NTR. An approximately 3.5- to 10-fold improvement was noted when NTR-N was compared to NfnB (Heap, J. personal communication). Additionally, NTR-N only produces the desired toxic 4-hydroxylamine drug from the CB1954 reduction. All kinetic studies performed gave the following rank order of the enzymes:

NTR-N > *Hin*NTR > NfnB

Based on this preliminary data it was of interest to obtain over-expression of this novel nitroreductase in *C. sporogenes*.

5.2.8.2 Cloning of the novel nitroreductase into the optimised vector

NTR-N was cloned from pMTL1015::NTR-N on a *Nde*I/*Pvu*I fragment and cloned into pMTL5122::*nfnB* digested with the *Nde*I and *Pvu*I to give pMTL5122::NTR-N (see Appendix II for DNA and protein sequence of NTR-N). NTR-N could not be cloned directly into pMTL5623 directly due to the presence of an internal *Nde*I restriction site present in the replication protein of pIP404. The *fac*2 promoter was then switched for the *C. sporogenes* ferredoxin promoter using *Nde*I and *Not*I to give pMTL5123::NTR-N. Finally the pIM13 replicon was excised using *Bam*HI and *Aat*II and T4 blunted, the replacement stable replicon from pIP404 was cloned in as a blunt *Nhe*I/*Nsp*I fragment. The resulting plasmid was designated pMTL5623::NTR-N.

Lysates of the soluble fraction from all the derivatives of the NTR-N over-expressing

plasmid clones (pMTL5122::NTR-N, pMTL5123::NTR-N and pMTL5623::NTR-N) in both the wild type and cured strains of *C. sporogenes* NCIMB 10696 were screened for over-expression by SDS-PAGE, however none gave the expected 24.7 kDa size over-expressed band visible on the SDS-PAGE gel. Furthermore, no activity above background levels was detectable via menadione nitroreductase assay (data not shown).

One potential cause of low level expression of genes can be poor codon usage. Therefore the next step in improving expression was to identify the codon usage of the novel nitroreductases in relation to NfnB.

5.2.9 Codon usage as a determinant of expression

Codon usage is considered to be an important contributor to setting the level of gene expression. In the two highly studied microbial species, *E. coli* and *B. subtilis*, highly expressed genes are transcribed from optimal codons that are recognised more efficiently by the most abundant tRNA (Karlin *et al.*, 1998; Kunst *et al.*, 1997). In other bacterial species codon usage is shaped more by mutational bias and, therefore, is mainly determined by the G+C content of the genome. Of the clostridial genomes sequenced so far all possess G+C contents in the region of 29% - 31% G+C (Bruggemann *et al.*, 2003; Nolling *et al.*, 2001; Shimizu *et al.*, 2002). Examination of the clostridial genomes identified a significant A+T codon bias for highly expressed genes which possessed a GC content of between 13% and 18% (Sharp *et al.*, 2005) with a significant predominance of A and T at the 'silent' third position of the triplet codon. Therefore it can be concluded that the main factor influencing codon usage is the strong mutational bias towards A and T.

The four nitroreductase genes utilised (*nfnB*, *Hin*NTR, *Hin*NTR-synth, NTR-N) were analysed for G+C content. As expected the synthetic *Hin*NTR has the lowest G+C content at 33.48%, in comparison to the wild-type *Hin*NTR that has a G+C content of 41.93%,

indicating a potential reason as to expression of the synthetic gene over the wild-type sequence gene. However, *nfnB* has a much higher G+C content at 51.53% G+C compared to the *Hin*NTR. NTR-N has a nearly identical G+C content (51.65%) compared to *nfnB* yet is not expressed. Therefore some alternative factor(s) must be influencing expression of the genes.

Codon usage of the various nitroreductases was analysed utilising the codon usage analyser GCUA (http://www.gcua.de). A partial codon usage table is currently available for *C. sporogenes* and the closely related *C. botulinum* Hall A. Codon bias may be exist when only a few coding sequences have been analysed. Therefore, a comparison of this partial data to the three full genome codon usage tables available for *C. acetobutylicum*, *C. perfringens,* and *C. tetani* was performed (see Table 5.6). This indicated that the codon usages of all clostridia are relatively similar. The *C. tetani* codon usage gave the closest match to *C. sporogenes*. (see Table 5.6).

		Clostridial species (no. of CDS's)				
		Complete genome codon usage			Partial codon usage	
Amino acid	**Triplet codon**	*C. acetobutylicum* (3945)	*C. perfringens* (2723)	*C. tetani* (2373)	*C. botulinum* (146)	*C. sporogenes* (7)
Ala	GCA	45		**45**	**47**	**44**
Ala	GCU		54			49
Arg	AGA	70	86	77	73	80
Asn	AAU	80	83	85	90	79
Asp	GAU	85	87	86	90	83
Cys	UGU	70	79	80	80	84
End	UAA	64	75	70	76	100
Gln	CAA	70	86	82	86	92
Glu	GAA	74	77	83	83	86
Gly	GGA	51	58	55	50	49
His	CAU	78	81	83	88	82
Ile	AUA	58	62	65	53	59
Leu	UUA	42	66	58	65	59
Lys	AAA	68	70	78	81	83
Met	AUG	100	100	100	100	100
Phe	UUU	85	81	88	89	83
Pro	CCA		53	**45**		**48**
Pro	CCU	45		**45**	**47**	
Ser	AGU	28	29	**29**	**31**	25
Ser	UCA	27	32			
Ser	UCU			**30**	**29**	**32**
Thr	ACA	44				
Thr	ACU		50	**44**	**47**	**55**
Trp	UGG	100	100	100	100	100
Tyr	UAU	78	84	85	90	88
Val	GUA			**51**	**47**	**56**
Val	GUU	45	52			

Table 5.6: Codon usage table for the three sequenced clostridial genomes plus 4 clostridial genes (number in brackets represents number of codong sequences used to generate codon usage). Only frequent optimal codons (27 out of 64 codons) are shown. Figures in bold indicates codons used preferentially when two or more codons are available with similar frequency.

5.2.9.1 Codon usage of currently available nitroreductase genes

GCUA analysis of the available nitroreductases (*nfnB*, *Hin*NTR, *Hin*NTR-synth, NTR-N) gave an indication as to the codon usage of each particular gene, (for the raw graphical representations see Appendix II). It was anticipated that this analysis would highlight the

correlation between the total number of low scoring codons (i.e., the tRNA occurs at less than 10% of the tRNA pool, indicated as red bars in the GCUA graphical representation), and expression of the protein. The data is presented in Table 5.7.

	% of tRNA pool for any given codon											
Gene	**1%**	**2%**	**3%**	**4%**	**5%**	**6%**	**7%**	**8%**	**9%**	**10%**	**Total <10% codons**	**% of total codons**
***nfnB* (218 aa)**	6	15	10	16	0	4	4	17	0	12	84	38.5
***Hin*NTR (221 aa)**	12	2	3	10	0	4	1	11	3	9	55	24.9
***Hin*NTR-synth**	0	0	0	1	0	0	0	0	2	0	3	1.4
NTR-N (222 aa)	13	2	7	21	0	5	5	12	13	9	87	39.2

Table 5.7: Tabulated data on the frequency of low scoring codons.

As can be seen there is no correlation between the total number of low scoring codons and expression. NfnB, which is well expressed, has 84 codons that are in less than 10% of the tRNA pool, which represents a total of 38.5% of the total number of 218 triplet codons in the entire protein. NTR-N has a similar number compared to NfnB and *Hin*NTR has nearly half the number of low scoring codons yet both are not expressed. The presence of particular exceedingly rare codons (1% of tRNA pool) does, however, correlate with expression. *Hin*NTR contains 12 rare codons and NTR-N contains 13 rare codons. The dependency on these particular rare codons (CUC [Leucine], CGC + CGG [Arginine], and UCG [Serine]) may be limiting expression even though they represent only 5% of the total number of codons. In particular, the codon CGC appears to be the most prevalent rare codon in *Hin*NTR and NTR-N. This may be the limiting factor, especially if the tRNA for CGC is actually the least abundant in *C. sporogenes*. The exception to the rule is *Hin*NTR-synth, which is not expressed, yet has only 3 low-scoring codons, none of which are rare.

Clustering of rare codons has also been suggested to limit expression levels (Varenne and Lazdunski 1986). Analysis of the nitroreductases for the clustering of low scoring codons is shown in Table 5.8.

Gene	Singlet	Doublet	Triplet	Quadruplet	Quintuplet	Sextuplet	Total <10% codons
NfnB	35	11	6	1	1	0	84
*Hin*NTR	26	13	1	0	0	0	55
*Hin*NTR-synth	3	0	0	0	0	0	3
NTR-N	35	11	6	0	0	2	87

Table 5.8: Tabulated data on the clustering of low scoring codons from GCUA analysis.

No correlation between the clustering of low scoring codons and lack of expression can be drawn. Rare codons are distributed through out the genes and clustered rare codons are present in all genes. In fact, NfnB which is expressed has up to 5 low scoring codons clustered together.

Finally, it is thought that the occurrence of low scoring codons early on in the translation of mRNA can lead to early termination of translation (Makoff *et al.*, 1989). This factor could be influencing expression of the nitroreductases. However, the GCUA analysis did not reveal any correlation as NfnB has 19 low scoring codons in the first 50 codons, the highest frequency of low scoring codons of all the nitroreductases screened.

To summarise, the presence of several rare codons appears to be influencing expression. It was of interest to see if altering the codon usage could enhance expression of *Hin*NTR and NTR-N.

5.2.9.2 Construction of second generation synthetic nitroreductase genes

It was decided to redesign the DNA sequence and re-synthesise NTR-N and *Hin*NTR such that the optimal most abundant tRNA was used at any given triplet position. This would give a definite indication of whether or not codon usage is important in obtaining over-expression. The LETO codon optimisation programme (Entelechon GmbH) was employed for the initial optimisation of both genes. LETO uses a genetic algorithm to enable the simultaneous optimisation of a large set of competing parameters such as codon usage, codon tandem repeats, mRNA secondary structure, GC distribution, long range repeats, and AT/GC stretches. Initially both genes were optimised utilising the codon usage and secondary structure optimisation criteria only. The sequence obtained for NTR-N was left as determined using LETO, however, the sequence given for *Hin*NTR was further manually optimised from the given sequence as in some cases a less than optimal codon was chosen. In addition, the sequence returned was relatively similar to *Hin*NTR-synth as Entelechon GmbH probably used LETO for the design of the first generation synthetic *Hin*NTR (*Hin*NTR-synth v1). The manual optimisation was based on the criterion that if the same amino acid was repeated in *Hin*NTR or spaced only one amino acid apart then the second most abundant tRNA was used for the second amino acid in the sequence. Both genes were synthesised by Entelechon GmbH with an *Nde*I restriction site 'over' the ATG start codon and an *Xho*I restriction site after the stop codon and supplied cloned into pCR-Script Amp SK(+) (Stratagene) (see Appendix II for DNA and protein sequences of *Hin*NTR-synth v2 and NTR-N-synth).

Both synthetic nitroreductase genes were cloned as *Nde*I/*Xho*I fragments from pCR-Script into pMTL5122 digested with *Nde*I and *Xho*I to yield pMTL5122::*Hin*NTR-synth v2 and pMTL5122::NTR-N-synth, the aim being to identify the sole effect of optimising codon usage without altering the initial plasmid. Following conjugation into *C. sporogenes* NCIMB 10696 and the cured derivative of *C. sporogenes* NCIMB 10696, lysates of the

soluble fraction from 8 hour cultures were visualised by SDS-PAGE (see Figure 5.15).

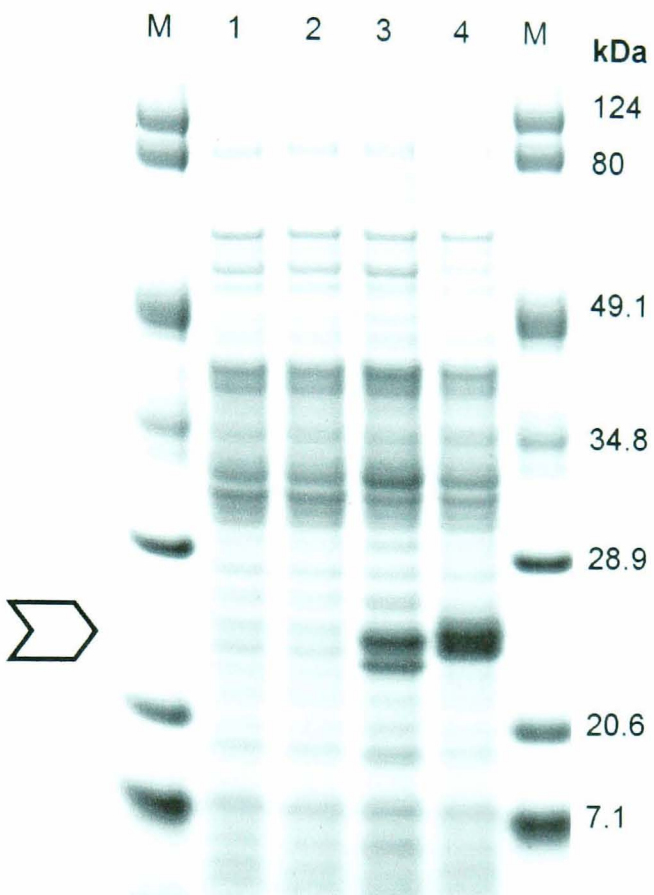


Figure 5.15: SDS-PAGE gel of soluble fraction lysates from NTR-N-synth and *Hin*NTR-synth v.2 over-expressing *C. sporogenes* NCIMB 10696. Lane 1 = pMTL5122, lane 2 = pMTL5122::NTR-N, lane 3 = pMTL5122::NTR-N-synth (9.9% soluble protein), lane 4 = pMTL5122::*Hin*NTR-synth v.2 (11.0% soluble protein). M = NEB Broad range protein markers. The arrow indicates the location of the over-expressed protein.

Both synthetic derivatives of NTR-N and *Hin*NTR were over-expressed in *C. sporogenes* NCIMB 10696 to relatively similar high levels. Expression was also obtained in the cured strain to a similar level (data not shown). The presence of a doublet for NTR-N was presumed to be due to the presence of truncated proteins caused by premature termination of translation.

5.2.10 Characterisation of the cured strain – sporulation efficiency and protease production

As a precursor to future studies, some preliminary characterisation of the *C. sporogenes* cured strain was performed to ascertain if any phenotypic changes had occurred during the curing process.

The cured strain 10696-1 was studied further for sporulation efficiency and protease production, two features of *C. sporogenes* that aid in its superior colonisation of the tumour. Spore assays were performed on 72 hour cultures grown in TYG media not supplemented with glucose. Approximately 10% of wild type *C. sporogenes* NCIMB 10696 cells were spores, compared to only 1% of the cured strain. The cured strain therefore has an approximate 10-fold reduction in sporulation efficiency compared to the wild-type bacterium. This is, however, still 100-fold higher than the sporulation efficiency of the original CDEPT host *C. acetobutylicum* (Theys, J. personal communication). Thus the reduction in spore forming capability of the cured strain still renders it a useful delivery tool compared to *C. acetobutylicum*.

Analysis of protease production by both the wild-type and cured strains of *C. sporogenes* NCIMB 10696 utilising collagen zymogram gel analysis of supernatants from overnight cultures showed that protease production, specifically collagenase secretion, was not markedly altered between the two strains for the time point studied (data not shown).

5.3 DISCUSSION

In Chapter 4, a low level of expression of *Hin*NTR was obtained, although this appeared to be sufficient to exhibit a static anti-tumour effect *in vivo*. Therefore in this chapter the expression vector was characterised and optimised for use in *C. sporogenes*. The potential

of affecting expression by altering the gene was also studied.

The segregational stability of pMTL5122 was shown to be exceedingly low in *C. sporogenes* NCIMB 10696. Studies comparing pMTL5112 to pIMP1::OriT highlighted that the pIM13 replicon is highly unstable in *C. sporogenes* NCIMB 10696, both vectors being lost at a frequency of approximately 1.7×10^{-1} per generation. This confirms that no essential plasmid replication functions of pIMP1 were deleted in the construction of pMTL5122.

pIM13-based plasmids are generally segregationally unstable in other clostridial species with losses of 2.3×10^{-2} per generation in *C. acetobutylicum* NI-4081 (Azeddoug *et al.*, 1992) and 4.7×10^{-3} per generation in *C. cellulolyticum* (Jennert *et al.*, 2000). The exceptions to this are *C. acetobutylicum* DSM 792 and *C. acetobutylicum* NI-4082 where pIM13-based plasmids are segregationally stable. The extreme low stability of pIM13-based plasmids in *C. sporogenes* prompted a segregational study of the other clostridial plasmids as segregational stability may be influencing gene over-expression.

Prior to the initiation of this study, work was performed on the isolation and characterisation of a novel cryptic plasmid, termed pBP1, from *C. botulinum* NCTC 2916. A shuttle vector was constructed based on a presumptive minimal replication region from pBP1. This vector was 100% segregationally stable in *C. botulinum* Hall A ATCC 3502. It was thought that a pBP1 replicon-based vector was likely to be segregationally stable in *C. sporogenes* even though the plasmid is thought to replicate via a rolling circle method, due to the close phylogenetic relationship of *C. sporogenes* to *C. botulinum*. This would make the pBP1 replicon an ideal candidate for the construction of a vector for use in CDEPT. Unfortunately, this expected stability was not found to be the case in *C. sporogenes*. Segregational stability studies on a variety of clostridial replicons identified pBP1 to be lost at frequency of 6.54×10^{-3} per generation. The most stable plasmid, based on the *C. perfringens* plasmid pIP404, was nearly 100% segregationally stable over 48 generations with a loss of 4.21×10^{-4} per generation. Therefore the choice of plasmid replicons appears

to be very species specific. Interestingly the most stable plasmids in *C. sporogenes* were also the plasmids that are known to replicate via the theta mechanism. This confirms to the general consensus that theta-replicating plasmids are more segregationally stable than plasmids that replicate via the rolling circle mechanism. There are situations where this general rule is not confirmed. In *C. cellulolyticum*, both the pAMβ1 (theta-replication) and pIM13 (rolling circle, ssDNA) replicons are moderately stable, with a loss per generation of approximately 7.8×10^{-3} and 4.8×10^{-3} respectively (Jennert *et al.*, 2000).

Having identified the exceedingly low segregational stability of pIM13-based shuttle vectors it was of interest to generate a cured strain of *C. sporogenes*. The aim being to see if a similar increase in segregational stability is seen as observed in *C. acetobutylicum* NI-4081 and the cured strain NI-4082 (Azeddoug *et al.*, 1992). Once generated, plasmid transfer into the cured *C. sporogenes* strain occurred at a 10- to 100-fold higher frequency. Also, the segregational stability increased from a loss per generation of 1.73×10^{-1} for the wild-type strain to 2.24×10^{-2} for the cured strain. This increase was not as severe as that observed in *C. acetobutylicum* NI-4082, however, there was still a definite improvement. The host-encoded mutation(s) in *C. acetobutylicum* NI-4082 are thought to be related to resolution of plasmid multimers as segregational stability should occur when most of the plasmid DNA is in the monomeric form. It is interesting to hypothesise that a similar mutation was selected in the curing of *C. sporogenes* NCIMB 10696. Conjugal transfer frequencies of pIM13-based plasmids were also altered in the cured strain. This increase could be attributed to the aforementioned increase in segregational stability, as the plasmid should not be lost as fast during the seven-hour non-selective stage of the conjugation procedure.

A second feature of the expression vector that was characterised was the relative strength of the *fac*2 promoter compared to other clostridial promoters. The ferredoxin promoter from *C. pasteurianum* is known to be capable of over-expressing NfnB in *C. beijerinckii* to a level equating to 8% soluble protein (Minton *et al.*, 1995). However the promoter has not been characterised in *C. sporogenes* and may behave differently in this relatively dissimilar host.

Two reporter genes, GusA and CatP, were utilised to characterise a variety of clostridial promoters. Initial studies of available promoters identified the *closI* to be the 'strongest' available. One potential reason for this could be due to the close phylogenetic relationship of *C. histolyticum* to *C. sporogenes*. Also, clostripain is present in the *C. botulinum* ATCC 3502 Hall A genome (CB01920) and is indicated to be present in *C. sporogenes* by microarray (Carter, A. personal communication). The alternative promoters are from solventogenic clostridia, which are not as closely related to *C. sporogenes* as the proteolytic *C. histolyticum.*

The identification that the *C. perfringens* endogenous ferredoxin promoter is 'stronger' than the *C. pasteurianum* ferredoxin promoter prompted a study of the ferredoxin promoters in *C. sporogenes*. The CatP reporter data obtained highlighted an approximately 3-fold increase in reporter gene expression in *C. sporogenes*. The GusA reporter and CatP reporter data was combined for all the different promoters tested and the ferredoxin promoter from *C. sporogenes* proved to be the 'strongest' with a 3.18-fold increase in reporter activity relative to *fac*2. Phased A-tracts in the *C. perfringens plc* promoter sequences have been implied to be stimulate promoter activity through a curved DNA interaction of the promoter with the α-subunit C-terminal domain of RNA polymerase (Katayama *et al.*, 1999; Katayama *et al.*, 2001). Recent data on the analysis of the *C. perfringens* ferredoxin promoter identified three upstream phased A-tracts and two downstream phased A-tracts located within the promoter (Kaji *et al.*, 2003). It is hypothesised that the phased-A tracts form a DNA curvature where the position of the bend centre appears critical for the stimulatory effect of DNA curvature on promoter activity. That the *C. perfringens* promoter was not the optimal promoter in *C. sporogenes* suggests that the DNA bend formed by the *C. sporogenes* ferredoxin promoter phased A-tracts is most suitable for interaction with *C. sporogenes* RNA polymerase.

In parallel with the above studies, the potential of using pMTL5122 to obtain over-expression of a gene, which has previously been successfully over-expressed, was studied.

Cloning *nfnB* into pMTL5122 resulted in over-expression as confirmed by SDS-PAGE and menadione assay. Conjugal transfer of the vector into *C. sporogenes* also resulted in over-expression to a level equating to 7% soluble protein, a level similar to that obtained with *C. beijerinckii*. This proves that pMTL5122 is functional and capable of over-expressing protein to a relatively high level in *C. sporogenes*. Also, this highlights that expression is gene specific, with some genes proving easier to over-express than others. Over-expression to levels equivalent of 30% of the cells soluble protein have been reported using expression systems in *E. coli* (Chambers *et al.,* 1988b). It was of interest to see what effects changing the plasmid replicon and/or promoter would have on protein expression, and to see if more elevated levels of expression could be obtained utilising the more stable pIP404 replicon and/or stronger *C. sporogenes* ferredoxin promoter. Interestingly there did not appear to be a significant increase in expression. Utilising pMTL5122, expression to a level equating to 10.4% of the cells soluble protein was obtained. If the replicon was changed for the more stable pIP404 replicon a level of 11.0% soluble protein was obtained, a similar level (11.1%) was obtained when the *C. sporogenes fdx* promoter was utilised. Based on the 3.18-fold improvement of the *C. sporogenes fdx* promoter over *fac*2 identified from the promoter reporter data, a level of expression of NfnB equating to approximately 30% soluble protein would be expected. As this was not seen, it suggests that a threshold for over-expression exists such that no greater than approximately 10% soluble protein can be over-expressed in the growth conditions tested. Alternatively, the potential toxicity associated with massively over-expressing a protein may prevent levels higher than approximately 10% soluble protein being obtained. Nevertheless, as it is an enzyme that is being expressed in the context of CDEPT current levels of over-expression may be sufficient to obtain a suitable anti-tumour effect and obtain tumour reduction.

The effect that the cured strain had on expression of the nitroreductases was then studied. All the vectors were transferred at the previously found higher frequency. Lysates of the soluble fraction from nitroreductase recombinant cured strains were then visualised by SDS-

PAGE. Expression of NfnB was apparent with higher levels in the pIP404-based plasmids. This could be attributed to a potentially higher segregational stability and/or copy number of the pIP404-based vector compared to the pIM13-based vector. Additionally, expression of *Hin*NTR was observed, albeit at a lower level (4% soluble protein) than that observed for NfnB (5 – 10% soluble protein, according to the vector used). This could be due to the increased segregational stability of pIM13-based vectors in the cured strain of *C. sporogenes* NCIMB 10696. This hypothesis was proven to be correct by the construction of a segregationally stable version of pMTL5122::*Hin*NTR-synth, pMTL5622::*Hin*NTR-synth. Once pMTL5622::*Hin*NTR-synth was transferred to the wild-type strain of *C. sporogenes* over-expression of *Hin*NTR was obtained, indicating that plasmid stability does indeed influence the probability of obtaining expression of a particular gene.

The identification of NTR-N as a novel improved nitroreductase, further added to the range of nitroreductases available for analysis. Cloning of this nitroreductase into pMTL5122 did not result in expression of NTR-N protein. Based on G+C content of the genes alone expression of NTR-N would be predicted to occur as both *nfnB* and NTR-N have almost identical G+C contents. However, as expression of NTR-N was not obtained more specific factors appear to be influencing expression.

Codon usage analysis of the nitroreductases highlighted that improvements could be made to NTR-N in order to increase the potential for obtaining expression. Also, there were some improvements that could be made to *Hin*NTR-synth (now termed *Hin*NTR-synth v1) to further improve the expression obtained in the cured strain. Once these improvements in codon usage had been generated NTR-N-synth and *Hin*NTR-synth v2 were cloned into pMTL5122. The aim being to identify the sole effect codon usage would have on expression as when the wild type NTR-N and *Hin*NTR-synth v1 were cloned into pMTL5122 no expression was obtained. Lysates from both wild type and cured strain recombinants harbouring pMTL5122::*Hin*NTR-synth v2 and pMTL5122::NTR-N-synth showed that expression of both genes could now be reliably obtained to relatively high levels in the

soluble fraction of both hosts. Thus, codon usage is also appearing to influence the potential of obtaining expression. This is confirmed by the findings of Musto *et al* (2003) where analysis of the *C. perfringens* and *C. acetobutylicum* genomes identified a strong bias towards low G+C contents. There was little variation in the mean G+C contents along the entire genome, with the exception of the ribosomal operons. For each amino acid the predominant triplet is A- and/or T-ended. This is particularly the case in highly expressed genes where the GC content is exceedingly low, in the range of 13-18%.

To summarise, a variety of features appear to influence gene over-expression:

1) Plasmid segregational stability;
2) Codon usage of the gene to be expressed;
3) Unknown features specific to each gene.

However, the studies above indicate that if the gene is expressed on a segregationally stable plasmid or the codon usage is optimised then expression should be obtainable. There is, however, a more significant effect seen by optimising codon usage compared to plasmid stability, as NTR-N was not expressed when cloned into the segregationally stable plasmid pMTL5623. Optimisation of the codon usage of NTR-N, however, resulted in expression even though the gene was cloned in a segregationally unstable vector. This indicates the importance of eliminating rare codons in a particular sequence in order to obtain over-expression.

CHAPTER 6

DEVELOPMENT OF INTEGRATIVE TOOLS FOR *C. SPOROGENES*

6.1 INTRODUCTION

Having obtained over-expression of *Hin*NTR and a novel nitroreductase NTR-N, it was of interest to develop targeted gene integration systems for *C. sporogenes*. The ultimate aim of CDEPT being to have the prodrug converting enzyme integrated into the host chromosome, such that the gene can be stably maintained without the use of antibiotic selection. In this chapter, a variety of methods for obtaining gene integrants will be assessed in *C. sporogenes* NCIMB 10696.

Obtaining mutants in clostridia is extremely difficult with current methods for gene inactivation remaining woefully inadequate. Suicide vectors remain the most common way of generating mutants in the two best-studied clostridia, *C. perfringens* and *C. acetobutylicum*. Since the initiation of these studies, suicide vectors have also been used to generate mutants by single crossover in the acidogenic *Clostridium*, *C. tyrobutyricum* (Zhu *et al.*, 2005), and by double crossover in *C. septicum*, the primary agent of spontaneous gas gangrene (Kennedy *et al.*, 2005). The ability to generate mutants, albeit rarely, using 'suicide' approaches appears relatively wide spread across the clostridial genus. In this chapter, the potential of using suicide plasmids to generate both single and double crossover targeted gene knockouts in *C. sporogenes* will be explored.

Conditionally replicating vectors are currently unavailable for use in clostridia. However, a replicative vector has been used to generate a sporulation mutant in *C. acetobutylicum* (Harris *et al.*, 2002). The basis for integration is thought to be related to the method of replication of the vector used. The vector was based on the pIM13 replicon that replicates via a rolling circle mechanism. ssDNA intermediates are generated during rolling circle replication which are highly recombinogenic. pIM13-based vectors are known to be segregationally unstable under non-selective conditions in a variety of clostridia, including *C. sporogenes* (**see Chapter 5.0**). A double crossover knockout system based on vectors containing the pIM13 replicon will be developed in this chapter.

The *E. faecalis* transposon Tn*916* has been used to generate a number of non-targeted mutants in *C. acetobutylicum*, *C. botulinum*, *C. perfringens* and *C. difficile*. However, Tn*916* has also been used to generate a targeted single crossover mutant in the sigma-factor (*sigK*) of *C. difficile* (Haraldsen and Sonenshein, 2003). Additionally, in the work of Roberts *et al* (Roberts *et al*., 2003) 50% of the *cwp66* adhesin mutants screened had in fact integrated in the genomic copy of *cwp66* (Minton, N.P. personal communication). As Tn*916* is capable of entering the genome at a variety of sites in both *C. difficile* and *C. botulinum*, it may be possible to utilise Tn*916* as an insertional mutagen in *C. sporogenes*. To identify this, in this chapter, pMTL900 will be assessed as a targeted mutational tool.

Initially, knockouts will be attempted using vectors containing antibiotic selectable crossover cassettes. However, it is planned that any methods that prove successful would be repeated replacing the antibiotic marker for the *C. sporogenes* ferredoxin promoter and nitroreductase NTR-N. Thus, resulting in the generation of the 'final' strain.

6.2 RESULTS

6.2.1 Choice of knockout targets

Two genes were identified as potential targets useful in the generation of a CDEPT integrant, either *pyrF* or *spo0A*. *pyrF* encodes the gene orotidine-5'-phosphate decarboxylase which is involved in the biosynthesis of pyrimidine ribonucleotides. PyrF converts orotidine-5′-phosphate to uridine-5′-monophosphate (UMP) with the evolution of CO_2. UMP is then further converted by other enzymes to uridine-5′-triphosphate (UTP), cytidine-5′-triphosphate (CTP) and finally cytidine-5'-diphosphate (CDP). As well as becoming uracil auxotrophs, *pyrF* knockout mutants can be positively selected for by resistance to 5-fluoroorotic acid. *pyrF* has been successfully used as a target for the generation of mutants

in *Mycobacterium smegmatis* (Husson *et al.*, 1990). An additional safety aspect is also created by the generation of a uracil auxotroph, as the germinating spores will be confined to the tumour, a location expected to contain particularly high levels of pyrimidines. Auxotrophy has been utilised as a safe guard mechanism for the delivery of prodrug converting enzymes to tumours by *Salmonella typhimurium*. A *S. typhimurium purI* auxotroph strain, strain VNP20009, has been generated that requires an external source of adenine (Clairmont *et al.*, 2000). Once systemically administered, VNP20009 auxotrophs accumulated preferentially in tumours and 41 days post-injection are cleared from all other organs screened.

The second gene target is the major sporulation initiation factor *spo0A*. One of the overall aims of the EU consortium was to generate a conditionally sporulating mutant of *C. sporogenes*. Therefore the native *spo0A* gene would need to be inactivated such that mutant containing an inducible copy of *spo0A* would only be able to sporulate *in vitro* by addition of an exogenous inducer.

6.2.2 Inactivation of *pyrF* by single crossover homologous integration

Due to a lack of conditionally replicating vectors which function in *Clostridium* species as a whole, the first attempt at generating an insertionally inactivated mutant in *pyrF* was based on a suicide plasmid, the type of which has been used to generate an assortment of mutants in a variety of clostridial species.

pMTL31 is a mobilisable suicide plasmid which carries the RP4/ RK2 *oriT* region, such that it can be transferred from a strain of *E. coli* which harbours a conjugative helper plasmid such as *E. coli* CA434 (*E. coli* HB101 carrying the IncPβ conjugative helper plasmid, R702). Also present on the plasmid are the erythromycin and ampicillin resistance markers (*ermB* and *bla* respectively), the Gram-negative ColEI replicon, and LacZα to enable blue/white

selection (Williams *et al.*, 1990). pMTL31 has previously been used to successfully generate mutants in *C. acetobutylicum, C. beijerinckii,* and *C. difficile* by conjugal transfer of the suicide plasmid from an *E. coli* donor (Liyanage *et al.*, 2001; Wilkinson and Young, 1994).

The *pyrF* integrational plasmid was generated by PCR amplifying *pyrF* from genomic DNA prepared from *C. sporogenes* NCIMB 10696. PCR primers against *pyrF* were designed based on the *C. botulinum* Hall A ATCC 3502 genome sequence (http://www.sanger.ac.uk/Projects/C_botulinum/private/). PCR amplification with primers PYRFF and PYRFR (designed to incorporate *Sph*I and *Pvu*II restriction sites at the 5' ends of the amplified fragment respectively) generated the expected product of 764 bp. The PCR product was cloned into pCR2.1-TOPO prior to cloning into the suicide plasmid pMTL31. pMTL31 was digested with *Sac*I (blunt-ended) and *Sph*I. The *C. sporogenes* NCIMB 10696 *pyrF* fragment was cloned from pCR2.1-TOPO on a *Pvu*II / *Sph*I fragment into the linearised pMTL31 fragment. The resulting plasmid was designated pMTL31::*pyrF*(Csp).

High frequency gene transfer is desirable for obtaining gene knockouts using suicide plasmids as the frequency with which a mutant will be isolated is higher. Electroporation is usually the preferred method as large quantities of DNA can be electroporated, thus easily increasing the transfer frequency. An electroporation procedure has previously been developed for *C. sporogenes* NCIMB 10696 (Liu *et al.*, 2002), however the method proved to be irreproducible in our laboratory. The development of a conjugal transfer procedure for *C. sporogenes* has negated this bottleneck in gene transfer (see **Section 3.2.2**).

Plasmid pMTL31::*pyrF*(Csp) was transferred to *C. sporogenes* NCIMB 10696 by conjugation from *E. coli* CA434. As there is no Gram-positive replicon provided in pMTL31, the only way the plasmid can be maintained is by integrating into the host genome. Integration is regarded as a rare event, therefore a positive control conjugation with a replication proficient plasmid (pMTL5112) was included during every repeat of the conjugation to confirm that the conjugation procedure utilised was successful. Plasmid

pMTL31::*pyrF*(Csp) was repeatedly conjugated into *C. sporogenes*, with 15 independent conjugations being performed in total, and an estimated 7 x 10^8 cfu of *C. sporogenes* being used. Previous work using conjugal transfer of suicide plasmids to *C. beijerinckii* NCIMB 8052 produced integrants at a frequency of 10^{-6} to 10^{-7} per recipient (Wilkinson and Young, 1994). If a similar frequency is observed in *C. sporogenes* then it would be expected that approximately 70 to 700 integrants in total would have resulted from the repeated conjugations. However, no erythromycin resistant transconjugants were observed in any of the conjugations performed.

If, however, integration in *C. sporogenes* is less efficient than in *C. beijerinckii* then a more efficient means of delivering the crossover fragment is required. In the absence of conditional vectors for the clostridial genus as a whole, the conjugative transposon Tn*916* was assessed as a suitable alternative delivery tool.

6.2.3 The conjugative transposon Tn*916*

6.2.3.1 Conjugative transfer of Tn916

The conjugative transposon Tn*916* has previously been shown to be capable of mediating its own transfer from *E. faecalis* to *C. botulinum* at a frequency of approximately 10^{-6} transconjugants per recipient (Lin and Johnson, 1991). Based on this observation, conjugative transfer of Tn*916* was attempted in *C. sporogenes* using *B. subtilis* BS34A (*B. subtilis* CU2189 harbouring a single copy of Tn*916*) as the transposon donor (Roberts *et al.*, 2003). *B. subilis* BS34A transferred the tetracycline resistance determinant, Tn*916*, at a frequency of approximately 10^{-7} transconjugants per recipient, with an estimated 1,700 tetracycline resistant *C. sporogenes* transconjugants obtained per filter mating. No spontaneous tetracycline resistant mutants of *C. sporogenes* were obtained in independent experiments involving plating of *C. sporogenes* onto filters without *B. subtilis* BS43A and

then, following incubation, plating onto TYG supplemented with cycloserine and tetracycline. Transconjugants were confirmed to be *C. sporogenes* rather than *B. subtilis* as independent experiments highlighted that cycloserine at a final concentration of 250 $\mu g\ ml^{-1}$ was completely selective against *B. subtilis* BS34A. Furthermore, PCR analysis of genomic DNA isolated from selected tetracycline resistant transconjugants with primers specific to *tetM* (TETMF and TETMR) gave a product of the expected size (2.146 kb) for *tetM*. As Tn*916* was being utilised as a delivery tool rather than a mutational tool the specific location and frequency of Tn*916* integration sites was not investigated.

6.2.3.2 Integration utilising the conjugative transposon Tn916 as a delivery tool

Following successful transfer of Tn*916* from *B. subtilis* to *C. sporogenes*, its use as a delivery tool for single crossover knockout fragments could be assessed. Plasmid pMTL900 was recently developed by Roberts *et al* (Roberts *et al.*, 2003) for the expression of Tn*916* mediated integrated antisense RNA in *C. difficile*. pMTL900 is a *B. subtilis* suicide vector which contains the Tn*916 tetM* gene, into which a polylinker and the chloramphenicol resistance gene, *catP*, from the *C. perfringens* plasmid pCW3 have been cloned. The resultant plasmid can then be used to integrate genes cloned into the polylinker of pMTL900 by homologous recombination with the genome of *B. subtilis* carrying a single copy of Tn*916* (strain BS34A). The recombinant transposon can then be transferred, by conjugation, from *B. subtilis* to the recipient of choice, in this case *C. sporogenes*.

The *pyrF* fragment utilised in pMTL31 was again exploited for the Tn*916* approach. *pyrF* was excised from pCR2.1-TOPO::*pyrF* on a *Pvu*II / *Sph*I fragment and cloned into plasmid pMTL900 also digested with *Pvu*II and *Sph*I, generating pMTL900::*pyrF*(Csp). To increase the frequency of double crossover recombination between *tetM* encoded on pMTL900::*pyrF*(Csp) and the genomic copy of *tetM* in *B. subtilis* BS34A, the plasmid was linearised with the restriction enzyme *Age*I and dephosphorylated to prevent recircularisation of the plasmid.

Approximately 1 μg of pMTL900::*pyrF*(Csp) was transformed into *B. subtilis* BS34A. Transformants were selected for on medium containing 5 μg ml^{-1} chloramphenicol. Since pMTL900 is replication deficient in *B. subtilis*, true chloramphenicol resistant clones should only arise if a double crossover event has occurred between the *tetM* gene of Tn*916* and the homologous region in pMTL900 (see Figure 6.1). After 48 h incubation at 30°C, approximately five chloramphenicol resistant clones were observed on each selective plate. Four clones were randomly selected and screened further using a colony PCR with primers PYRFF, PYRFR, TETMF and TETMR (see Table 6.1).

Forward primer	Reverse primer	Expected product size (kb)
TETMF	TETMR	4.7
TETMF	PYRFF	3.7
TETMR	PYRFR	1.6
PYRFF	PYRFR	0.76

Table 6.1: PCR amplification of pMTL900::*pyrF*(Csp)

PCR was also performed on a pMTL900::*pyrF*(Csp) plasmid DNA positive control, and a *B. subtilis* BS34A untransformed negative control.

The presence of the *pyrF* fragment in the *B. subtilis* donor was confirmed in all four randomly selected clones, and a single correct clone was used to conjugate the recombinant transposon into *C. sporogenes* NCIMB 10696. Plate filter matings were carried out between *B. subtilis* BS34A Tn*916*::pMTL900*pyrF*(Csp) and *C. sporogenes* NCIMB 10696. A positive control of *B. subtilis* BS38A (*tetM* interrupted with *catP* from pMTL900) was included. Transconjugants were selected for on 20 μg ml^{-1} thiamphenicol; the *B. subtilis* donor was counter-selected with 250 μg ml^{-1} cycloserine. Several thiamphenicol resistant

clones of *C. sporogenes* were obtained. Colony PCR's were performed with primers PYRFF and CATPR (see Figure 6.1) which generated a product of 2.4 kb proving Tn*916* had integrated into the genome. PCR screening thiamphenicol resistant clones with primers PYRCF and CATPR (see Figure 6.1) did not yield a product. PCR with PYRCF and PYRKR, however, gave the expected product size of 1.522 kb for an uninterrupted copy of *pyrF*. Thus it appears Tn*916* had integrated at an alternative genomic location.

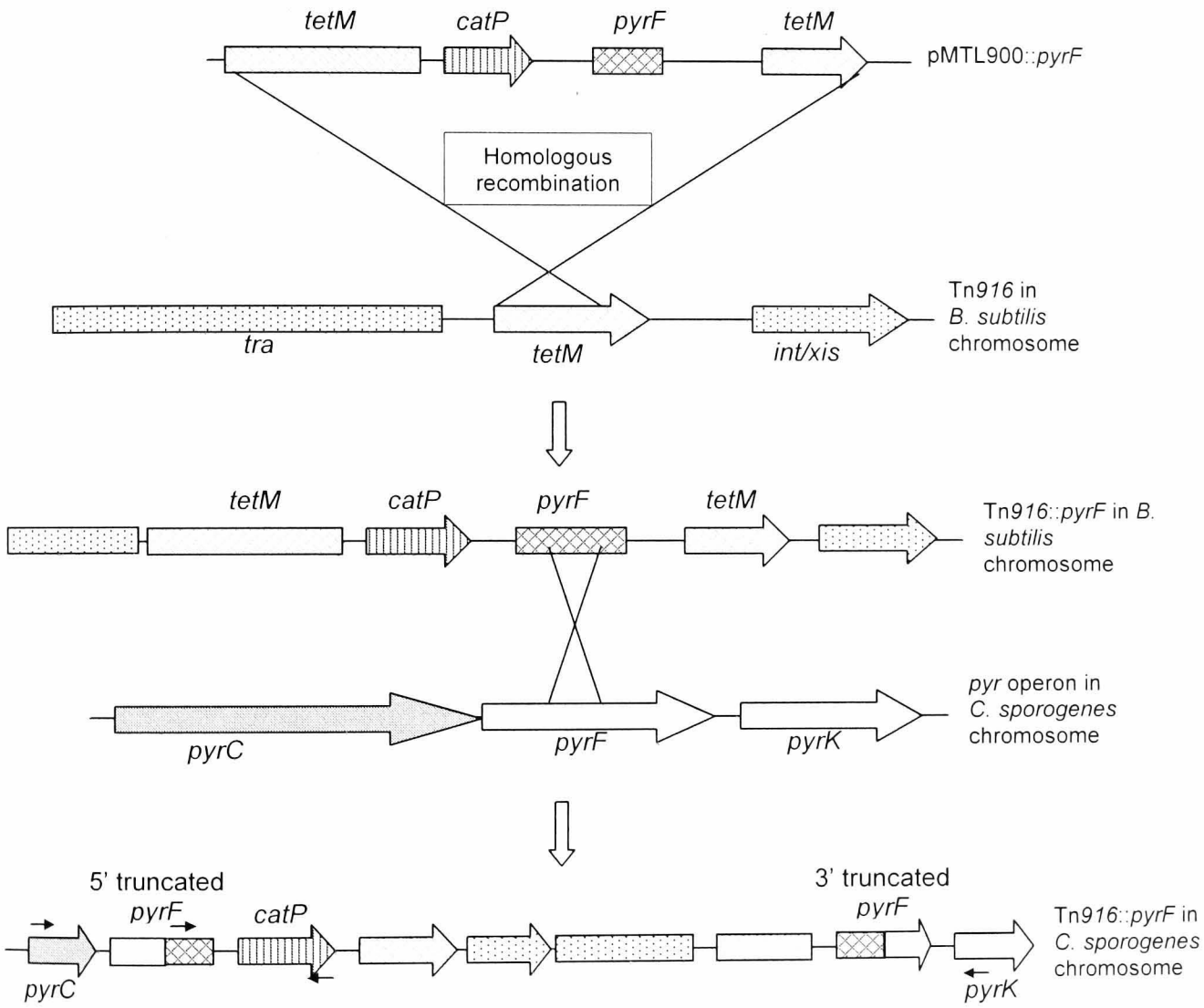


Figure 6.1: Schematic representation of Tn*916*::*pyrF* integration into *C. sporogenes* genomic *pyrF*. The first stage is integration of the internal *pyrF* fragment from pMTL900 into the genome of *B. subtilis* BS34A via double crossover integration within the *tetM* gene of Tn*916*. Subsequent transfer via conjugal transposition to the genome of *C. sporogenes* results in a single crossover recombination event within *pyrF* resulting in a non-functional copy of *pyrF*. Solid black arrows indicate the location of the primers utilised to screen putative mutants.

6.2.4 Inactivation of *spo0A* by double crossover

Since a single crossover *pyrF* mutant was not obtained using both a standard suicide vector and a transposon mediated suicide vector then the target gene was altered to *spo0A* in which mutants have previously been made in *C. acetobutylicum* and *C. perfringens* (Harris *et al.*, 2002; Huang *et al.*, 2004). Due to the innate instability of single crossover mutants and possibility of getting revertants, double crossover with *spo0A* was attempted.

The *spo0A* gene plus regions up- and down-stream were PCR amplified from *C. sporogenes* NCIMB 10696 genomic DNA using primers based on the *C. botulinum* Hall A ATCC 3502 genome sequence (work performed by C. Schwarz, University of Ulm, Germany). A 1.11 kb *spo0A* crossover cassette was constructed using primers located 237 bp downstream of *spo0A* with an *Eco*RI site generated by the primer, and a second primer located 33 bp upstream with a primer generated *Hin*dIII restriction site. The chloramphenicol resistance marker (*catP*) was then cloned into *spo0A* at a unique *Pst*I site in order to disrupt *spo0A*, thus creating a double crossover fragment. The *spo0A* double crossover fragment was then digested with *Eco*RI and *Hin*dIII and cloned into pMTL31 with the same two restriction enzymes generating plasmid pWG3 (C. Schwarz, University of Ulm, Germany).

As with previous attempts with suicide vectors, pWG3 was repeatedly transferred by conjugation into *C. sporogenes* NCIMB 10696 this time selecting for thiamphenicol resistance (encoded by *catP*), using plasmid pMTL5112 (with selection for erythromycin) as a positive control for the conjugation process. The conjugation process was repeated fifteen times however no thiamphenicol resistant clones were ever obtained indicating that a double crossover event had not occurred.

6.2.5 Development of a single stranded DNA intermediate plasmid integration system

Recently, Harris and colleagues (Harris *et al.*, 2002) developed a *spo0A* inactivated strain of *C. acetobutylicum* ATCC 824 utilising a replication proficient plasmid based on the *B. subtilis* rolling circle plasmid pIM13. Although the mutant generated was an imperfect double crossover (see Figure 6.2), it was of interest to develop a similar procedure for generating double crossover mutants in *C. sporogenes*. As has been mentioned already, pIM13 plasmids are segregationally unstable in *C. sporogenes* (see **Section 5.2.1.1.3**). It is thought that this instability combined with the generation of single stranded DNA intermediates by rolling circle replication will aid in the homologous recombination process and generation of a double crossover mutant.

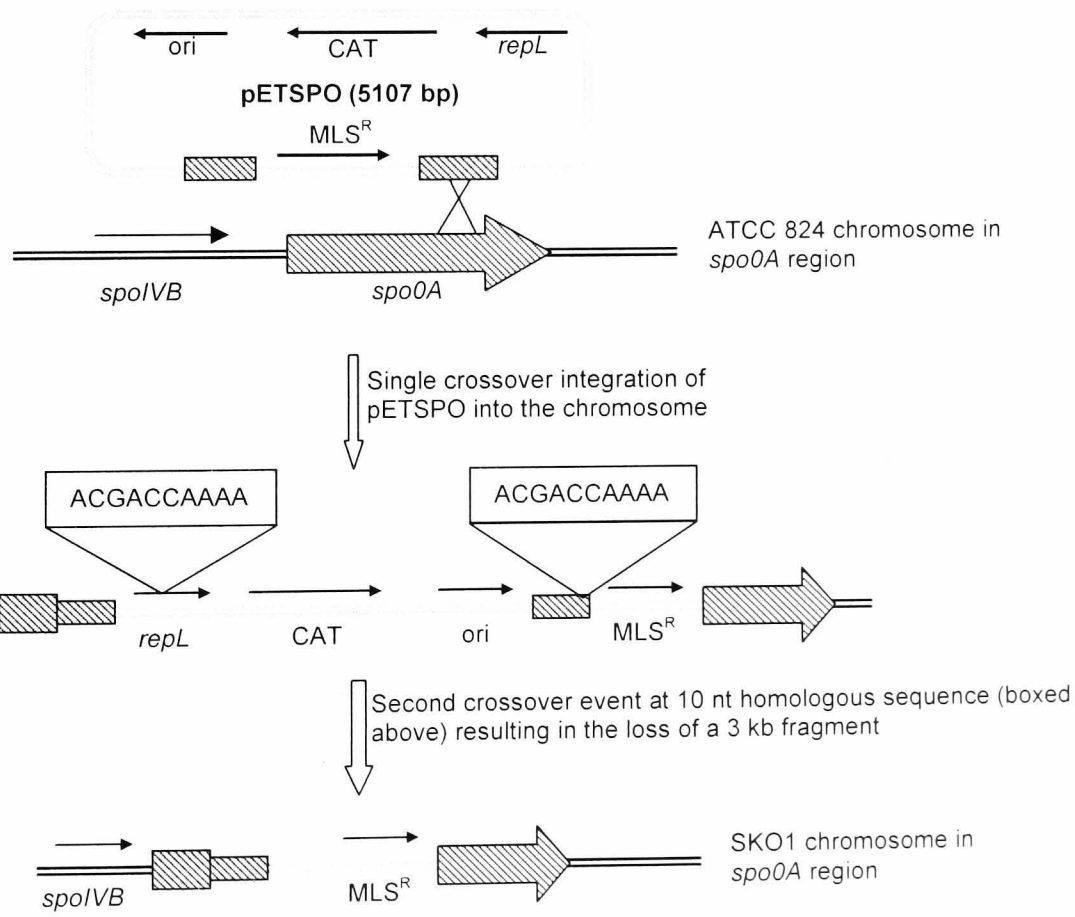


Figure 6.2: Putative crossover events during *spo0A* gene inactivation, from Harris *et al* (2002). CAT = chloramphenicol acetyl transferase antibiotic resistance marker, MLS = macrolide-lincosomide-streptogramin B antibiotic resistance marker, *repL* = pIM13 Gram-positive replicon, ori = Gram-negative origin of replication.

The segregationally unstable pIM13-based plasmids pMTL5122 and pIMP1::OriT were utilised as the delivery tools for the *spo0A* double crossover cassette previously created for use in suicide plasmids. The *spo0A* crossover fragment was cloned from pWG3 as a blunt-ended *Hin*dIII / *Eco*RI fragment and ligated to either pMTL5122 or pIMP1::OriT, both being digested with *Bam*HI (blunt-ended). The result being the *spo0A* double crossover cassette being cloned in either orientation into both delivery vectors. The plasmids were designated pMTL5122::*spo0A*:*catP* ori1 or ori2, and pIMP1::OriT::*spo0A*:*catP* ori1 or ori2. Problems were encountered conjugating the pMTL5122 derivatives into *C. sporogenes* however, transconjugants were obtained with the pIMP1::OriT derivative. These were studied further.

Correct clones containing the pIMP1::OriT *spo0A* crossover vector were initially cultured overnight in selective media. This was followed by a passaging regime of subculturing every 24 hours non-selectively for 4 consecutive days, followed by plating onto media containing either thiamphenicol to select for the crossover cassette or erythromycin to select for the plasmid backbone. It was hoped that the cell counts for the plasmid backbone would be much lower than for the crossover cassette indicating that a potential crossover event could have occurred. However, colony counts of putative integrants revealed that there was a similar number of thiamphenicol and erythromycin resistant clones (see Figure 6.3).

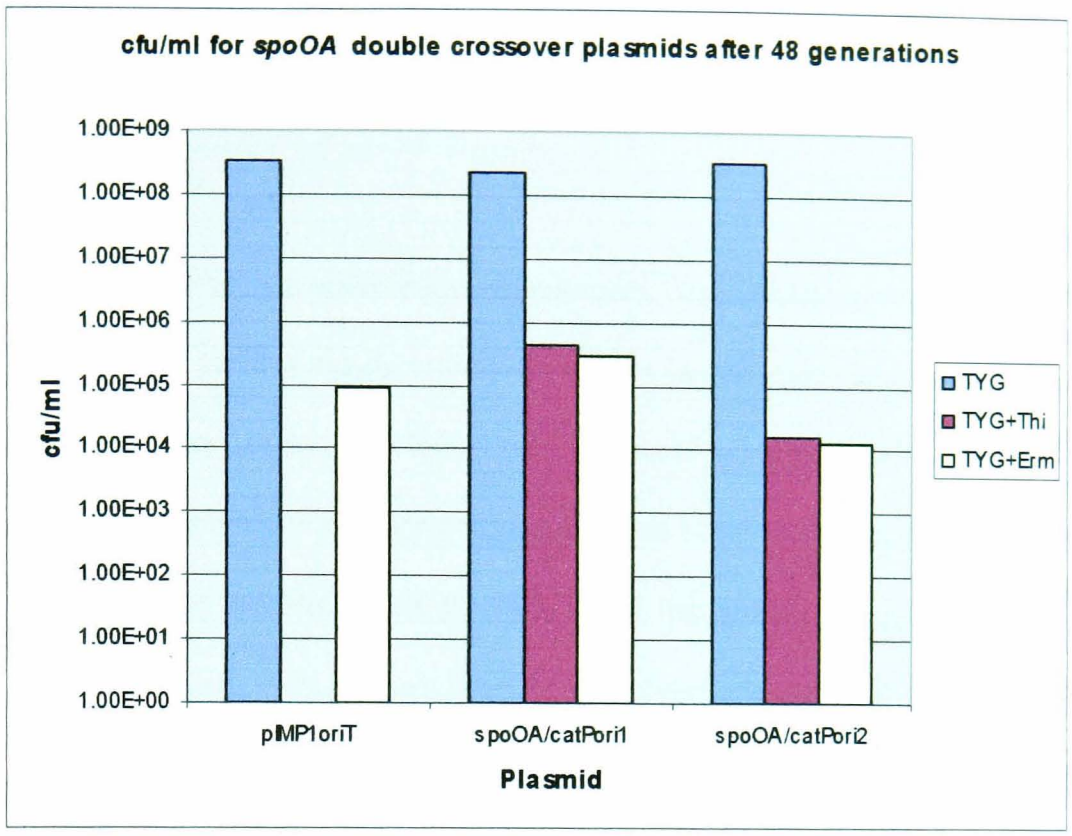


Figure 6.3: Colony counts for pIMP1oriT-based *spo0A* crossover vectors.

Potentially, all thiamphenicol resistant clones could be single crossover mutants with the plasmid backbone marker as well as the *spo0A* marker integrated into the host chromosome. 50 colonies for each plasmid were picked onto media containing erythromycin and / or thiamphenicol. All 50 colonies grew on both selective plates. Plasmid extracts from several clones revealed that no crossover event had occurred as plasmid DNA could be detected when the DNA isolated was used to retransform *E. coli*. Utilising the colony count data above the segregational stability of the plasmids was assessed and was not affected significantly by the introduction of the crossover cassette.

6.3 DISCUSSION

In this chapter the genetic tools that have previously been utilised to generate mutants in a

variety of clostridia were assessed in two different gene targets in *C. sporogenes*. Additionally, an unstable ssDNA intermediate based integrational vector was constructed and assessed.

Attempts to utilise currently available mutational tools, both suicide vector and Tn*916* based approaches, failed to yield a single crossover mutant in the *pyrF* gene of *C. sporogenes* NCIMB 10696. No erythromycin colonies were obtained using the pMTL31 suicide vector approach. Any thiamphenicol resistant colonies that were obtained with the Tn*916* approach were shown to contain the transposon by PCR using transposon specific internal primers. However, PCR screening with primers flanking the target site resulted in a product of the expected size for an intact copy of *pyrF*. Therefore, it is probable that the transposon integrated into another location other than *pyrF*. Transposition of intact Tn*916* was shown to be possible by conjugal transfer from *B. subtilis* BS34A to *C. sporogenes*. Tetracycline resistant colonies arose at a frequency of approximately 10^{-7} transconjugants per recipient. The exact location of integration of Tn*916* was not studied.

It is unclear why suicide vector systems that have proven to be capable of generating mutants in a variety of clostridia failed in *C. sporogenes*. Transfer frequency of plasmids is a determinant in the probability of mutant isolation. Transformation frequencies in the range of 3×10^5 cfu μg^{-1} plasmid DNA in *C. perfringens* (Scott and Rood, 1989), and up to 6×10^6 cfu μg^{-1} plasmid DNA in *C. acetobutylicum* (Azeddoug *et al.*, 1992) are amongst the highest reported frequencies of transfer for all clostridia. This correlates to the majority of reported mutants being obtained in *C. perfringens* and *C. acetobutylicum* using high-frequency electroporation-mediated transformation of replication deficient crossover vectors. Without a reliable electroporation protocol, vectors have to be introduced into *C. sporogenes* by conjugation. Conjugal transfer of replication deficient plasmids is estimated to occur at an approximately 100-fold lower frequency than replication proficient plasmids in *C. beijerinckii* (Young, M. personal communication). However, this is highly variable

depending on the Gram-positive replicon used as a positive control and may vary on the clostridial host as well. Taking this into consideration, the lowest transfer frequency obtained in *C. sporogenes* with pMTL5122-like plasmids typically yielded approximately 50 transconjugants per plate. If suicide plasmids transfer at a 100-fold lower frequency then recombinants would not be obtained. However, during suicide plasmid conjugations the entire conjugation 'mix' was plated out on 10 selective agar plates. If the reduced frequency were only 100-fold then it would be expected that some integrants should be obtained. As no integrants were obtained then the transfer frequency of suicide plasmids into *C. sporogenes* may be lower than 100-fold.

Transfer frequency of Tn*916* may be lower than that required in order to isolate a mutant. However, in the case of *cwp66*, 50% of thiamphenicol resistant colonies screened were *cwp66* mutants. This suggests that the Tn*916* mediated approach is highly efficient. Also, Tn*916* has been successful at generating mutants in two different genes in *C. difficile*, yet mutants in *spo0A* of *C. sporogenes* could not be obtained.

One factor known to influence the efficiency of integration into certain clostridia is the size of the gene fragment cloned into the delivery vector. This has proven to be a controlling factor as in *C. perfringens*, gene fragments in excess of 3kb have been required to obtain homologous recombination (Rood, J.I. personal communication). Secondly, double crossover mutants have in some cases been easier to obtain than single crossover mutants (Minton, N.P. personal communication). To gain insight into the affects of this a 1.11 kb fragment containing *spo0A* was PCR amplified from genes flanking *spo0A* to generate a double crossover fragment. However repeated transfer of the vector did not result in any thiamphenicol resistant mutants. Therefore in the future it may be necessary to utilise even larger fragments in order to obtain gene knockouts.

Failed attempts at using both replication deficient vectors and Tn*916* to generate integrants prompted the use of a replication proficient plasmid which would enable a larger number of

cells to be targeted. The double crossover fragment from *spo0A* was used as the target as it is larger then *pyrF* and also is cloned as a double crossover cassette. Also, *spo0A* has previously been disrupted in *C. acetobutylicum* using replication proficient plasmids (Harris *et al.*, 2002). Repeated passage of the pIMP1oriT-based vectors did result in severe loss of the plasmid, however a similar number of thiamphenicol and erythromycin resistant colonies were obtained. It appears that the plasmid had not integrated as thiamphenicol resistant clones were also erythromycin resistant, and plasmid DNA could be isolated from these clones and retransformed back into *E. coli*.

One potential alternative explanation as to the failed attempts of obtaining integrants in either *pyrF* or *spo0A* is that homologous recombination appears to be very target specific with some genes being relatively easy to inactivate. For example, in the genetically amenable *C. perfringens* certain genes known to be non-toxic if knocked out have proven impossible to inactivate (J. Rood, personal communication). This raises the question of the possible presence of specific gene targets in the form of Chi-like DNA sequences in the genome where recombination will preferentially occur (Myers and Stahl, 1994). In *B. subtilis* the gene AddAB protein complex behaves in an analogous way to RecBCD in *E. coli* (Kooistra *et al.*, 1993). *In vivo*, AddAB responds to the five nucleotide sequence 5′-AGCGG-3′, or its complement (Chédin *et al.*, 2000). RecA, AddA, and AddB have been identified in the *C. botulinum* genome and have been found to be present in *C. sporogenes* by microarray analysis, AadA is present but divergent from the *C. botulinum* gene. Therefore the requisite machinery is present to enable recombination to occur, although nothing is yet known about the relative activity of the recombination machinery of *C. sporogenes* in relation to more genetically acquiescent clostridia such as *C. perfringens*.

CHAPTER 7

SUMMARY AND GENERAL DISCUSSION

7.1 Introduction

DEPT-based cancer therapies rely on the targeted over-expression of prodrug-converting enzymes. The two main aims of this study were to obtain over-expression of prodrug converting enzymes and subsequently generate targeted mutants, in which the gene encoding the prodrug converting enzyme had been integrated into the genome. The work described here presents the first demonstrated over-expression in *C. sporogenes* NCIMB 10696 of two novel nitroreductases that have kinetic and enzymatic improvements over the nitroreductase currently used in alternative DEPT strategies, NfnB. A variety of approaches were investigated to generate targeted mutants. Unfortunately, all methods tried failed to yield a mutant in either of the designated *spo0A* or *pyrF* gene targets.

7.2 Construction of a clostridial expression system

Prior to the over-expression studies, the pMTL5100 series of clostridial shuttle vectors were constructed. These are based on the widely used Gram-positive replicon from the *B. subtilis* plasmid pIM13. This replicon was chosen due to its segregational stability in the original CDEPT host of choice, *C. acetobutylicum* NI-4082. However, after the initiation of the project, early *in vivo* data on the varying colonisation potentials of different clostridia indicated *C. sporogenes* NCIMB 10696 to be the most suitable. At the outset of this study, the only published method for introducing DNA into *C. sporogenes* was by an electroporation procedure that proved to be irreproducible outside the author's laboratory. Conjugation is thought to be a way of negating transformation barriers such as extracellular endonucleases. Utilising the vector pMTL5112, a previously published conjugal transfer method was adapted for use in *C. sporogenes* NCIMB 10696. This yielded transconjugants at a frequency of approximately 1.0×10^{-7} per donor cell. Using the pCD6-based plasmid pMTL9301, transconjugants were obtained at a frequency of 1.0×10^{-5} per donor cell. These levels of transfer frequency were similar to those obtained using a similar protocol in *C. difficile* strain CD3 (Purdy *et al.*, 2002). Higher conjugation transfer frequencies, in the

range of 10^{-3} to 10^{-4} recipients per donor, were obtained in *C. botulinum*, though a different protocol was used (Bradshaw *et al.*, 1998). Therefore, the conjugation transfer frequencies obtained in *C. sporogenes* are comparable with other reported frequencies. The observed difference in transfer frequency of pIM13- and pCD6-based vectors in *C. sporogenes* is most likely a consequence of the extreme segregational instability of pIM13-based plasmids. Experiments performed in *C. sporogenes* NCIMB 10696 showed that plasmids based on pIM13 were lost at a frequency equivalent to 17% per generation. Therefore, during the seven hour non-selective mating stage of the conjugation procedure, a significant proportion of the population will loose the plasmid due to poor segregation of the plasmid during cell division.

7.3 Over-expression of CPG2 and *Hin*NTR

Codon usage is thought to be a major contributor to obtaining expression of heterologous genes. CPG2 and *Hin*NTR were therefore synthesised based on the *C. acetobutylicum* ATCC 824 codon usage table. Once the wild type and synthetic DNA sequence prodrug converting enzymes were cloned, expression of both CPG2 and *Hin*NTR was demonstrated in *E. coli* using pMTL5100 series vectors. Interestingly, the synthetic DNA sequence CPG2 was expressed to a much higher level than the wild-type sequence gene. This could be attributed to the high G+C content of the wild-type gene, which would result in less preferential codons being used for a particular amino acid. The *Hin*NTR genes were both expressed to similar levels.

Conjugal transfer of the genes encoding both enzymes into *C. sporogenes* resulted in low-level expression of *Hin*NTR only. The synthetic sequence *Hin*NTR was expressed to marginally higher levels than the wild-type gene. *In vivo* studies on recombinant *C. sporogenes* NCIMB 10696 expressing *Hin*NTR-synth resulted in a striking anti-tumour response. This is thought to be due to an additional bystander effect where apoptosis of aerobic non-targeted cells is also occurring. This phenomenon has been reported previously

for NfnB in combination with CB1954 (Djeha *et al.*, 2000; Wilson *et al.*, 2002). The anti-tumour response observed could also be being enhanced by the identified endogenous nitroreductase activity of *C. sporogenes*. The lack of expression of CPG2 was not investigated further due to time constraints and the potential for proteolytic cleavage of CPG2.

7.4 Improving expression of prodrug converting enzymes

Having identified a level of nitroreduction by the first generation recombinant, it was thought that improving expression could lead to an even greater anti-tumour effect. Additionally, as it is the final aim to have the nitroreductase integrated into the host genome as a single copy, expression needs to be as elevated as possible. Segregational stability and codon usage were found to affect expression, although, a more noticeable effect was seen when codon usage was altered. The reason for these differences appears to be gene specific as it is unclear why *Hin*NTR-synth, which has near optimal codons, was not expressed to high levels in a segregationally unstable plasmid. The hypothesis that certain rare codons that occur only in *Hin*NTR and NTR-N, and not in NfnB, are limiting expression was proven by the re-synthesis of NTR-N.

7.5 Assessment of current integrational tools

During the course of this study a number of attempts to develop directed mutagenesis procedures for *C. sporogenes* have been made. The use of suicide vectors as a means to generate targeted mutants appears to be limited. They have been successfully used to generate mutants in a variety of clostridia including, *C. beijerinckii*, *C. perfringens*, *C. acetobutylicum*, and *C. difficile* (Awad *et al.*, 1995; Liyanage *et al.*, 2001; Wilkinson and Young, 1994; Wong and Bennett, 1996). More recently *C. septicum*, and *C. tyrobutyricum* mutants have been generated (Kennedy *et al.*, 2005; Zhu *et al.*, 2005). However, unless significant improvements in gene transfer frequency or alternatively efficient electroporation

protocols are developed, the use of suicide vectors in *C. sporogenes* has so far has not proven fruitful.

The exploitation of the conjugative transposon Tn*916* as a mutational element is intriguing. Integration of the element has been proven to be both random and non-random in a species dependent manner. However, Tn*916* has been used to obtain recombination within the *sigK* (Haraldsen *et al.*, 2003) and *cwp66* (A. Roberts personal communication) genes of *C. difficile* strains 196 and 79-685 respectively. Attempts to obtain Tn*916* mediated recombination within the *pyrF* gene of *C. sporogenes* failed to yield any correct mutants. Tn*916* was thought to have integrated into an alternative location in the genome as thiamphenicol resistant clones were proven to contain Tn*916* using internal primers. However, using primers flanking *pyrF* gave a product of a size determined for an interrupted copy of *pyrF*.

Finally, a ssDNA based integrative vector was constructed and assessed. No *spo0A* mutants were generated. Reasons for the failure of this approach, which successfully generated a *spo0A* mutant in *C. acetobutylicum*, are not known. The presence of species-specific Chi-like DNA sequences which promote integration, are hypothesised to be a potential cause for the failure to generate mutants in the gene targets chosen in *C. sporogenes*.

7.6 Latest advances of other clostridial anti-tumour therapies

This work highlights the potential of using recombinant clostridial spores to elicit an anti-tumour effect. Furthermore, the initial difficulty in obtaining over-expression of improved prodrug converting enzymes appears to have been solved. During the course of these studies work has been published on the progression of alternative clostridial therapies.

The main clostridial alternative to CDEPT is the use of a strain of *C. novyi*, *C. novyi*-NT, in a process termed combination bacteriolytic therapy (shortened to the acronym COBALT) (Dang *et al.*, 2001). Following on from these initial studies on the use of *C. novyi*-NT in

combination with conventional therapeutics, there has followed further work on reducing the toxicity observed in these initial studies. The combination of microtubule-synthesis-inhibitors resulted in a reduction in toxicity although a tumour cell specific response was still observed (Dang *et al.*, 2004). The administration of microtubule-stabilising drugs did not result in haemorrhagic necrosis but in slow regression of the tumour. When the microtubule-stabilising drugs were combined with *C. novyi*-NT this improved the duration of tumour regression to the point where a cure was observed in mice bearing HCT116 colorectal carcinoma xenografts. Recently, combination of *C. novyi*-NT with an analogue of the most promising microtubule-stabilising drug led to a complete cure in 4 out of 5 mice harbouring HCT116 colorectal carcinoma xenografts (Smith *et al.*, 2005).

An alternative to the clostridial-derived expression of prodrug converting enzymes is the production of cytokines. Intratumoural injection of recombinant *C. acetobutylicum* expressing rat interleukin-2 (rIL-2) results in a significant growth delay of the tumour in rhabdomyosarcoma-bearing rats (van Mallaert *et al.*, 2006). The rIL-2-mediated indirect activation of $CD8^+$ T lymphocytes is proposed to be responsible for the observed growth delay (van Mallaert *et al.*, 2006). This further highlights the potential of using expression of cytokines rather than prodrug converting enzymes to elicit anti-tumour effects.

7.7 Future work

Following on from these studies it is of primary interest to repeat the *in vivo* studies with both the *Hin*NTR and NTR-N over-expression clones. Additionally, any anti-tumour effect resulting from the endogenous nitroreductase activity needs to be studied, such that the effect arising specifically from the expression of the novel nitroreductase can be quantified.

It is paramount that the final strains have the prodrug converting enzyme integrated into the genome. To this aim, integrative technology needs to be developed. Indeed, it is vital to the clostridial research community as a whole. Recently, a mobile group II intron has been used

to generate an alpha toxin (*plc*) inactivated mutant in *C. perfringens* (Chen *et al.*, 2005). Mobile group II introns are site-specific retrohoming elements that are capable of targeting specific DNA sequences independently of host-encoded recombination machinery. It would be of interest to develop this method for the generation of mutants for use in CDEPT. This is due to the method being highly efficient and site-specific which avoids the random insertion of transposons, or low frequency of recombination associated with suicide plasmids. Additionally, the major clinical advantage of this system is that no antibiotic resistant genes are introduced into the chromosome making it ideal for applications such as CDEPT.

In addition, it would be interesting to attempt to solve expression of carboxypeptidase G2, as CPG2 has yet to be expressed in a bacterial DEPT strategy. Initially RT-PCR performed on the recombinant clones created in this study would highlight if the expression was related to mRNA stability. If mRNA is being produced, expression of CPG2 could be attempted in the segregationally stable vector. If no CPG2 activity could be detected at this stage then the codon usage of CPG2 could be studied. Further work could also be performed on the proteolysis of CPG2. Expression in a less proteolytic saccharolytic *Clostridium* such as *C. acetobutylicum* would highlight if the level of protease secretion by *C. sporogenes* is hampering the extracellular production of CPG2. This would limit the choice of prodrug converting enzymes to intracellular enzymes such as nitroreductase.

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APPENDIX

APPENDIX I

pMTL5 series of plasmids – *C. sporogenes* and *C. botulinum* work

pMTL5 _ _ _

1st digit = replicon	**2 digit = feature**	**3rd digit = promoter**
1 = pIM13	**0** = nothing	**0** = nothing
2 = pBP1	**1** = oriT	**1** = *fac*
3 = pCD6	**2** = oriT/lacZ	**2** = *fac*2
4 = pCB102	**3** = oriT/lacI	**3** = Cspo*fdx*
5 = pAMβ1	**4** = oriT/lacZ/lacI	**4** =
6 = pIP404	**5** =	**5** =
7 =	**6** =	**6** =
8 =	**7** =	**7** =
9 =	**8** =	**8** =
	9 =	**9** =

APPENDIX II

Prodrug converting enzyme DNA and protein sequences

eglAss:CPG2

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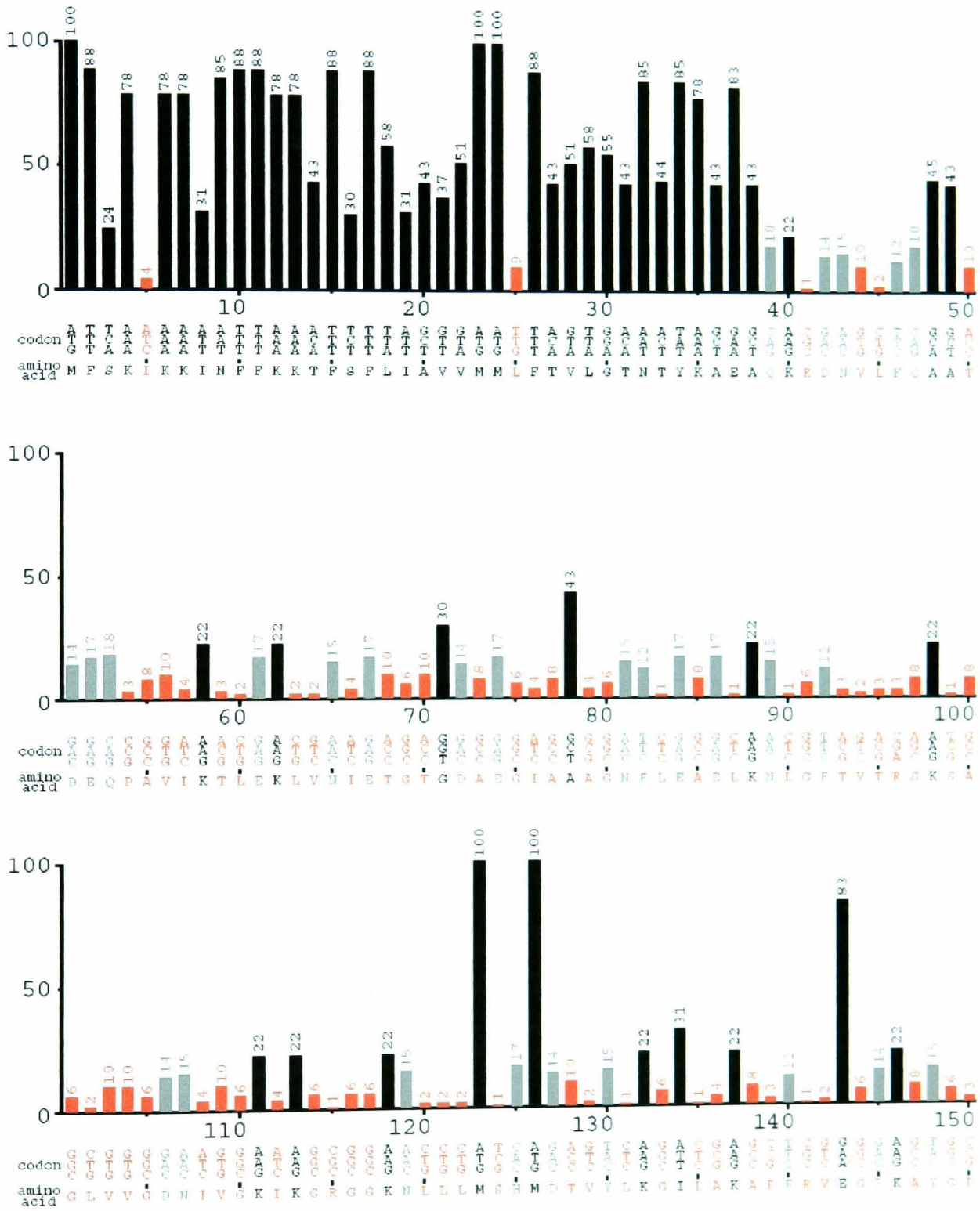
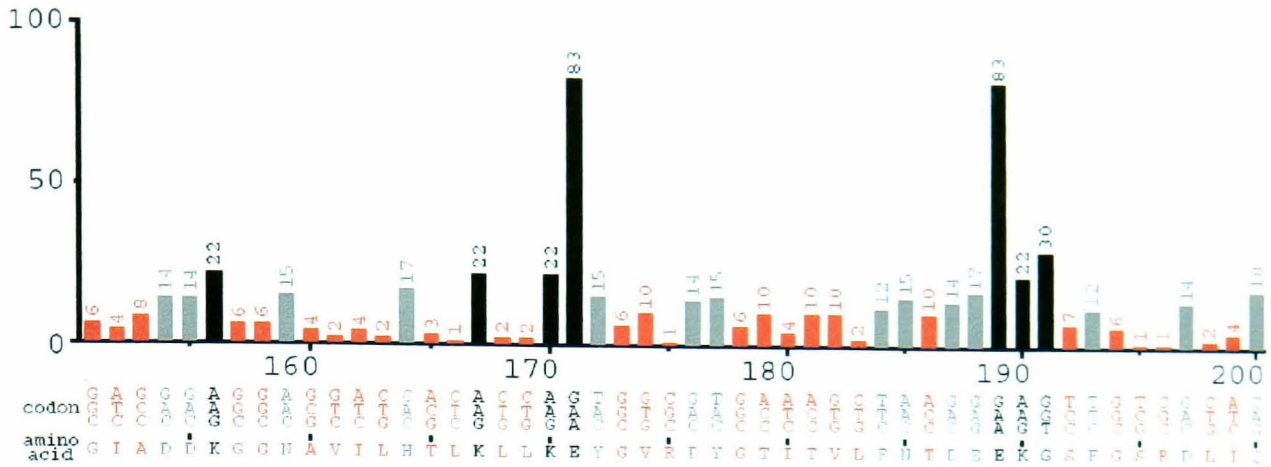
Graphical Codon Usage Analysis of prodrug converting enzymes

C. tetani codon usage table

CPG2

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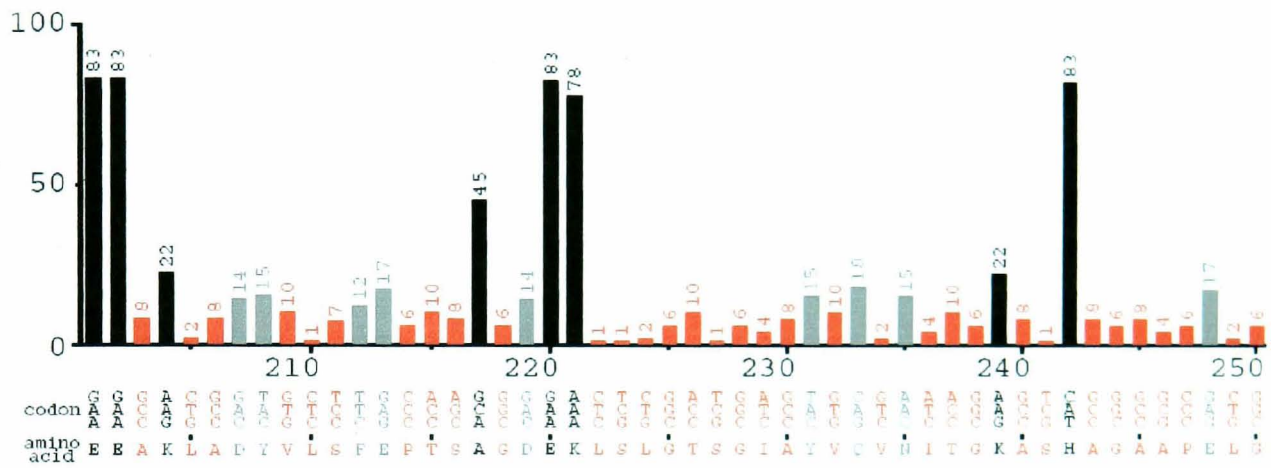
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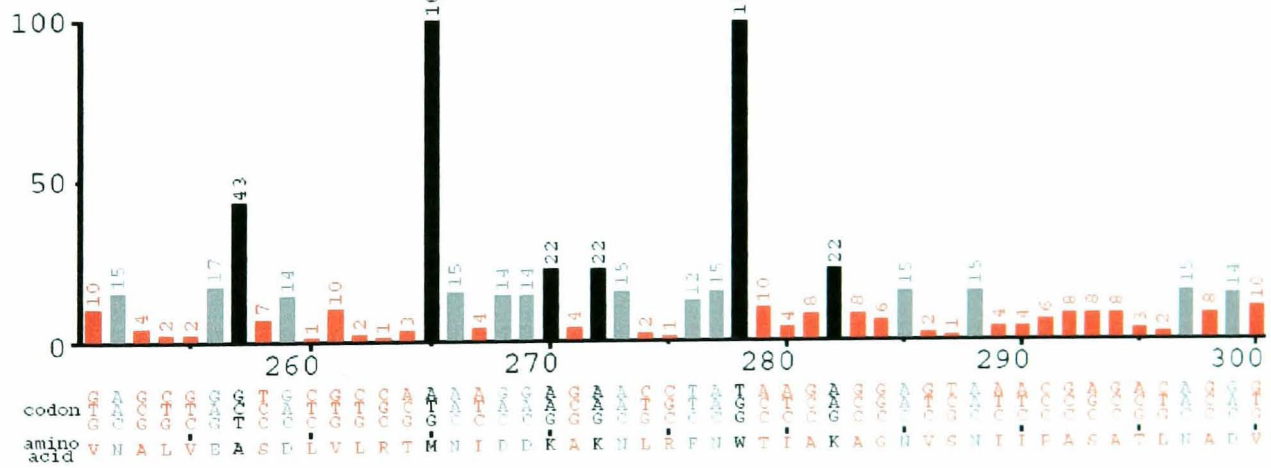
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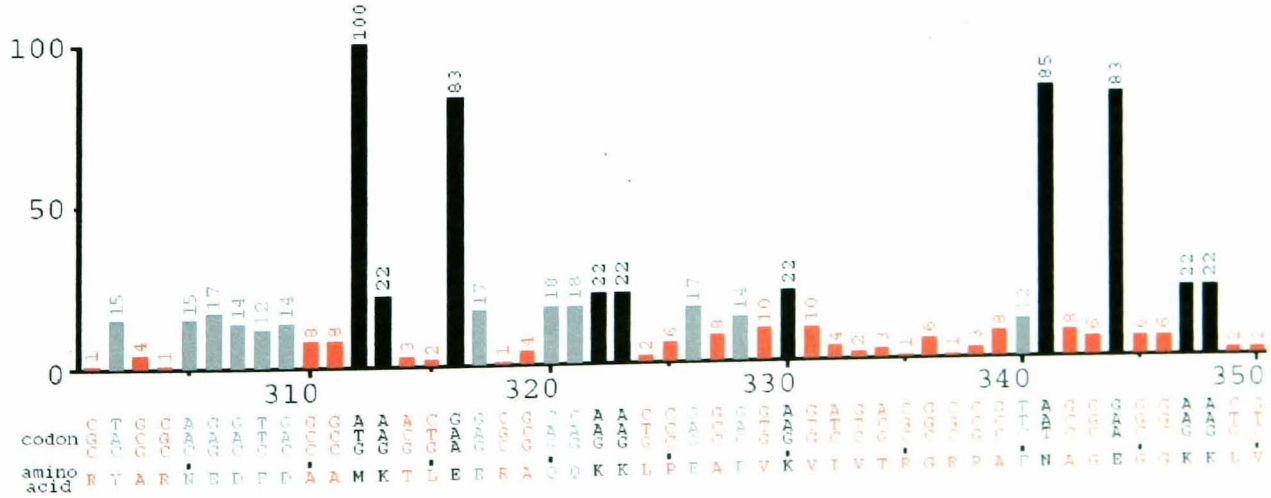
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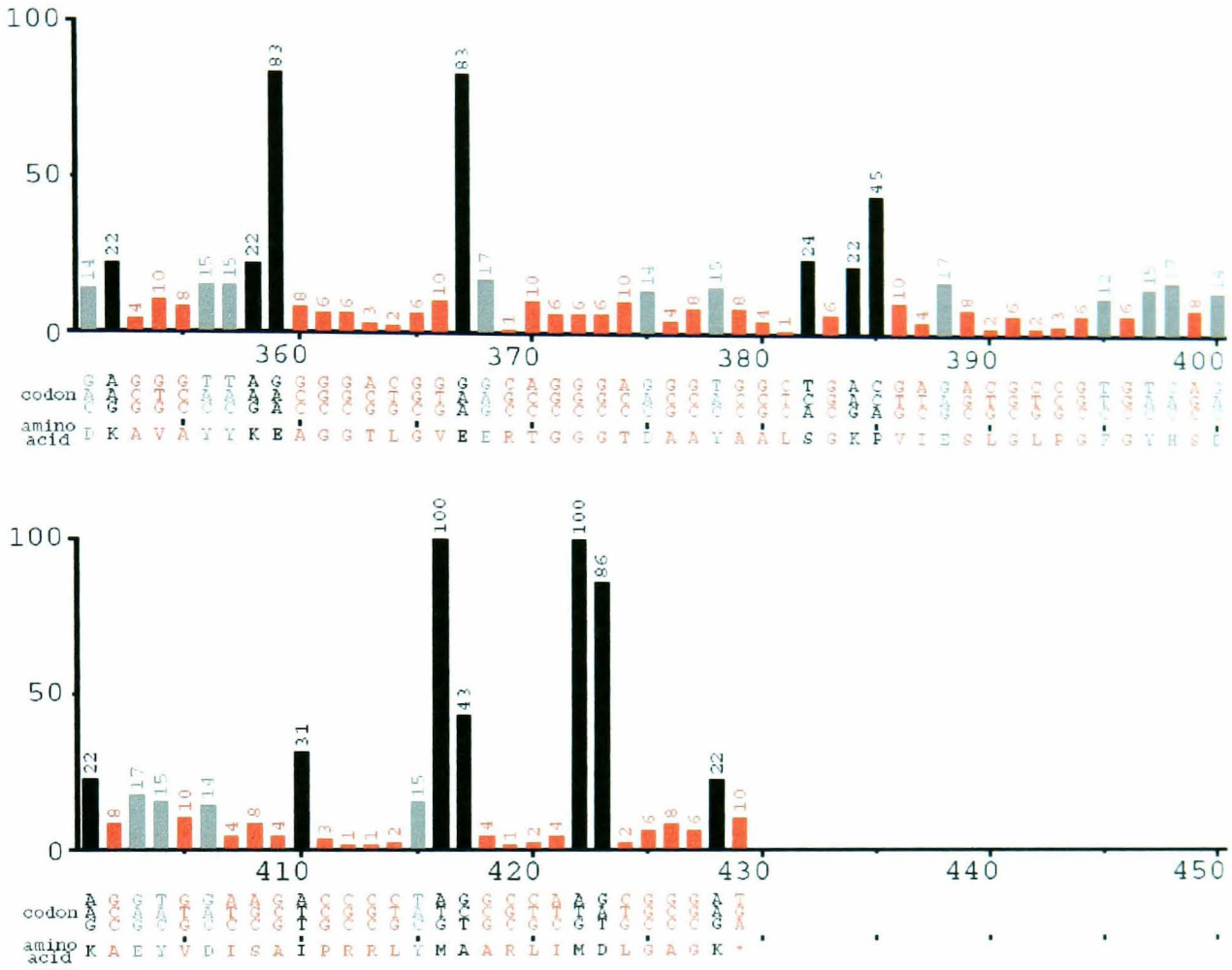
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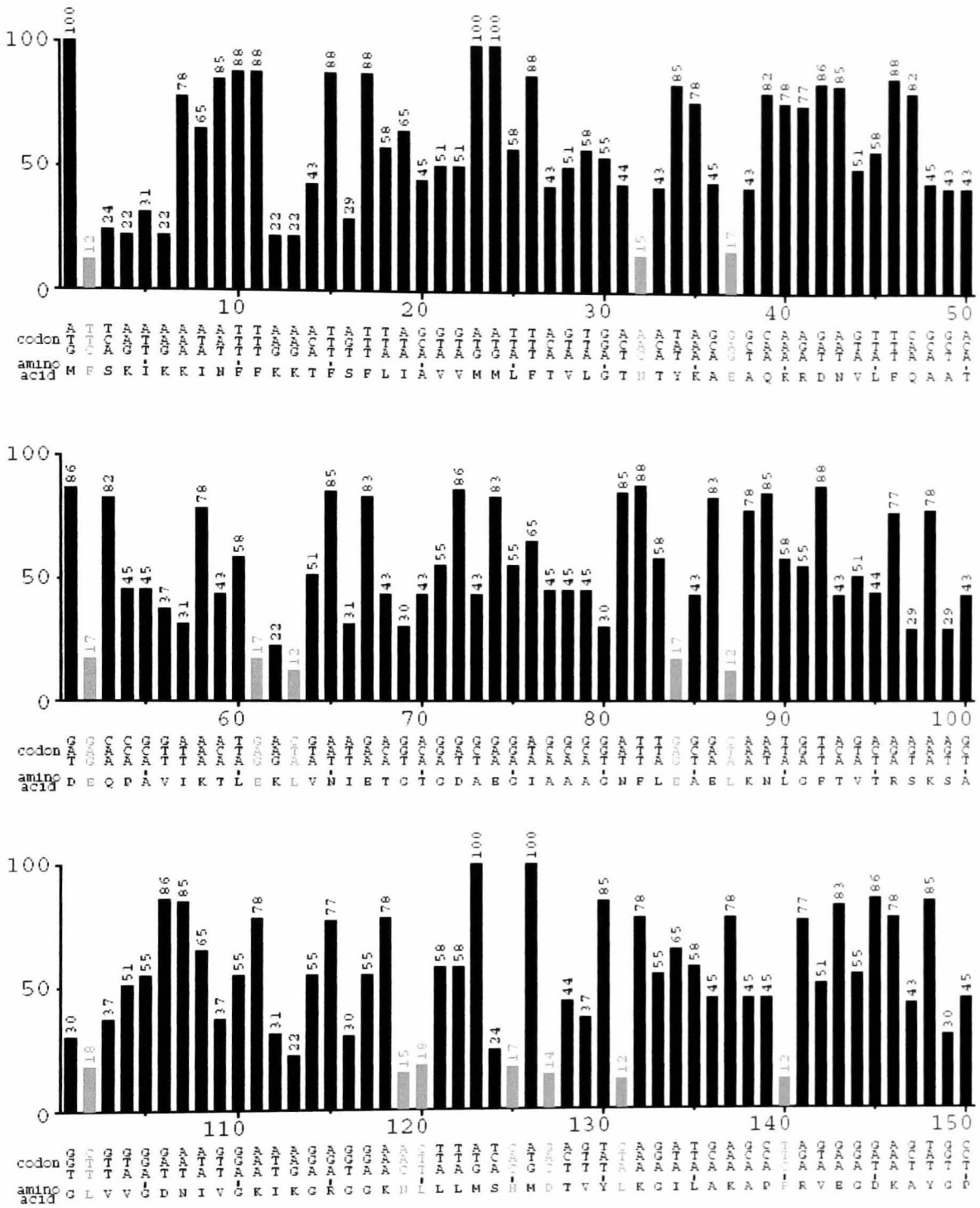
CPG2synth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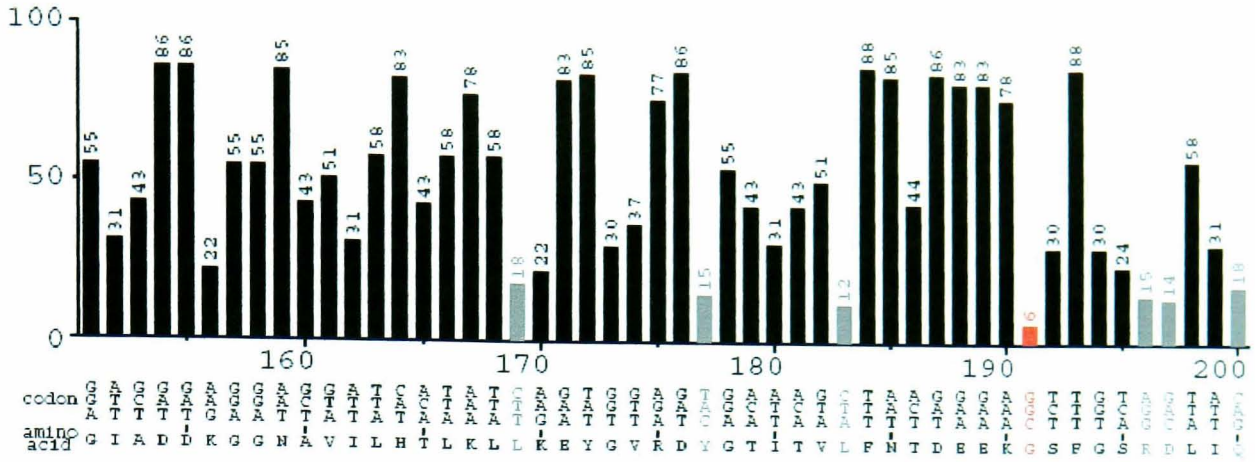
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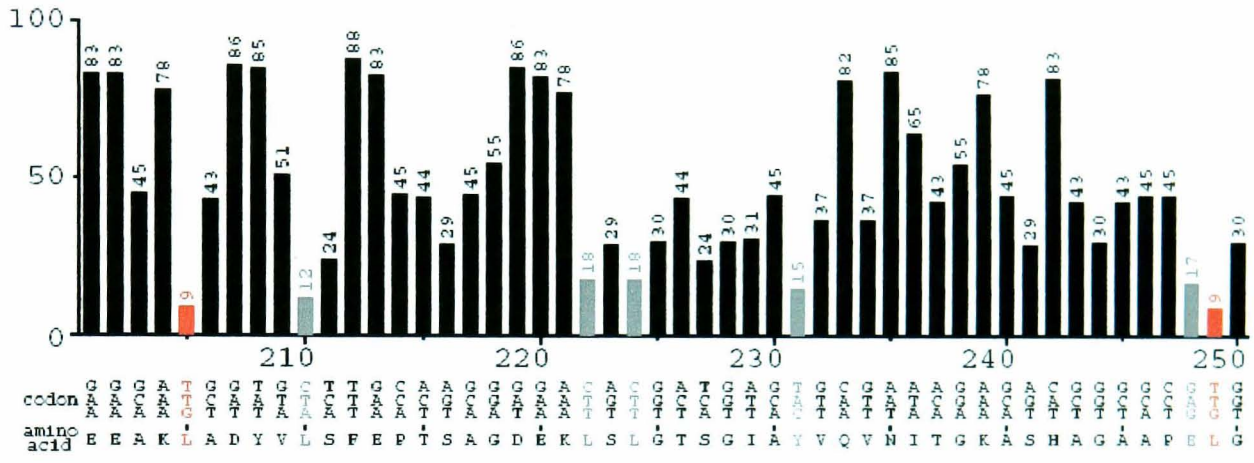
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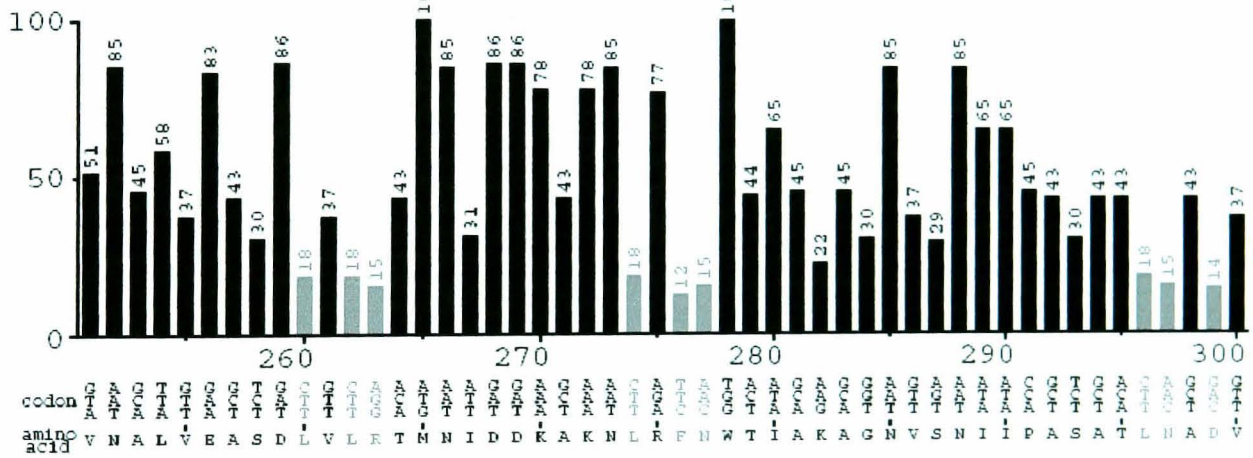
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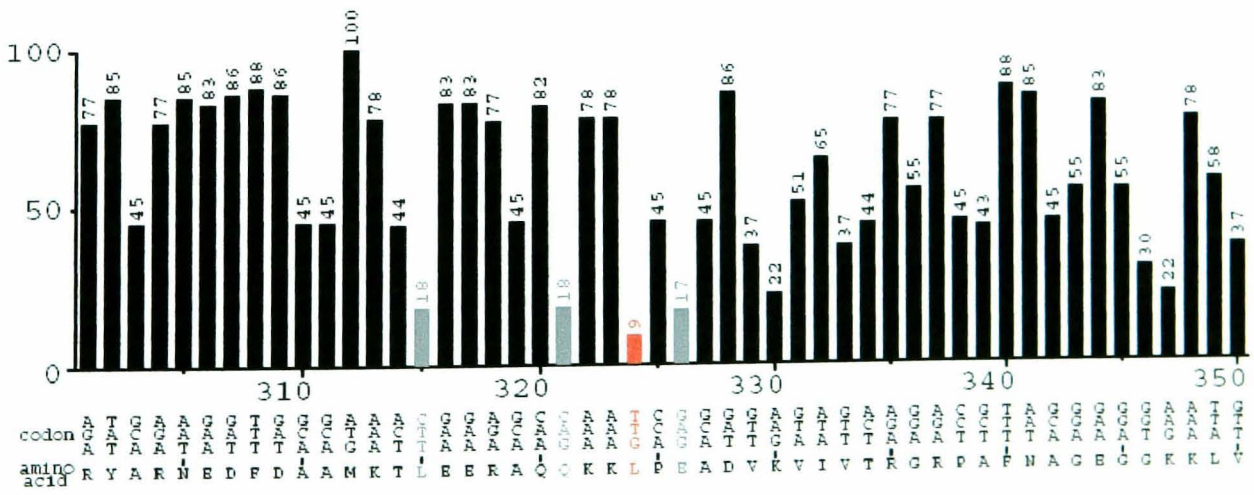
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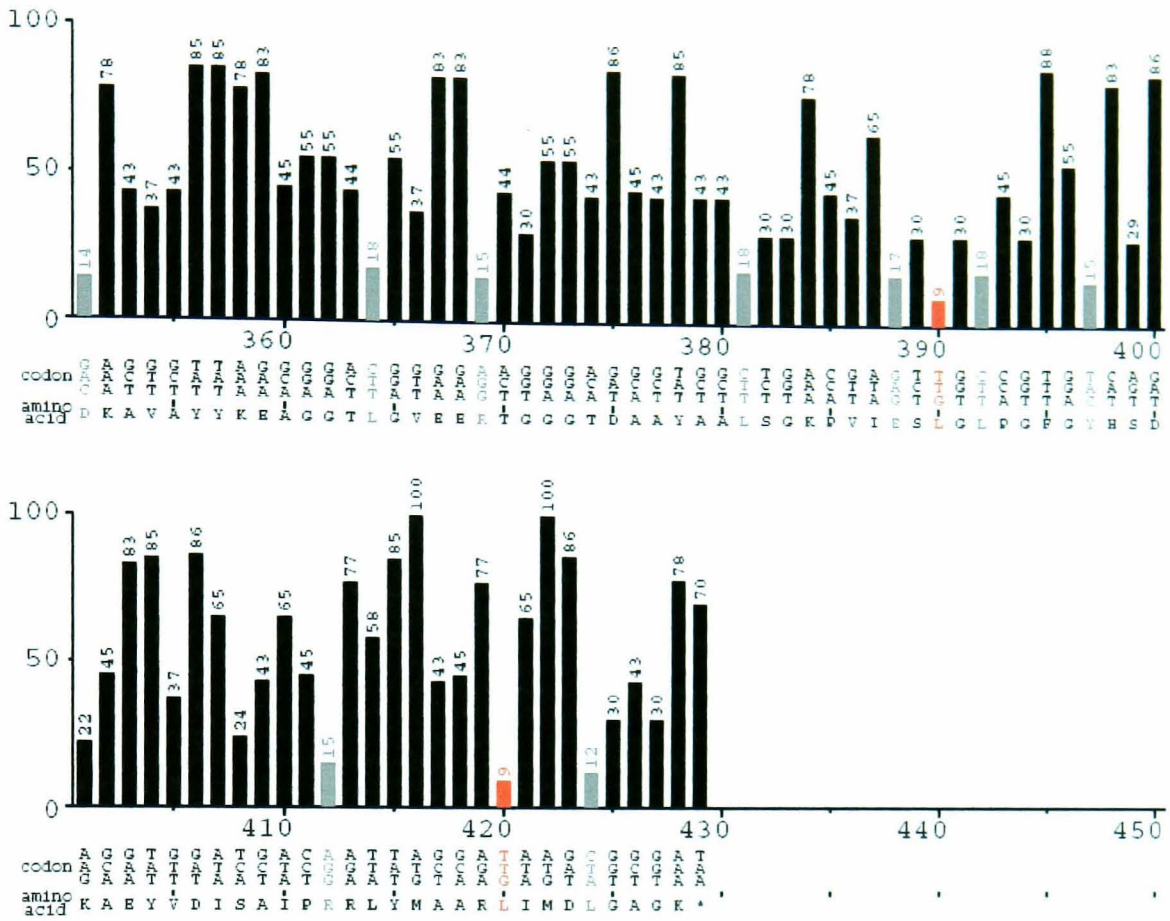
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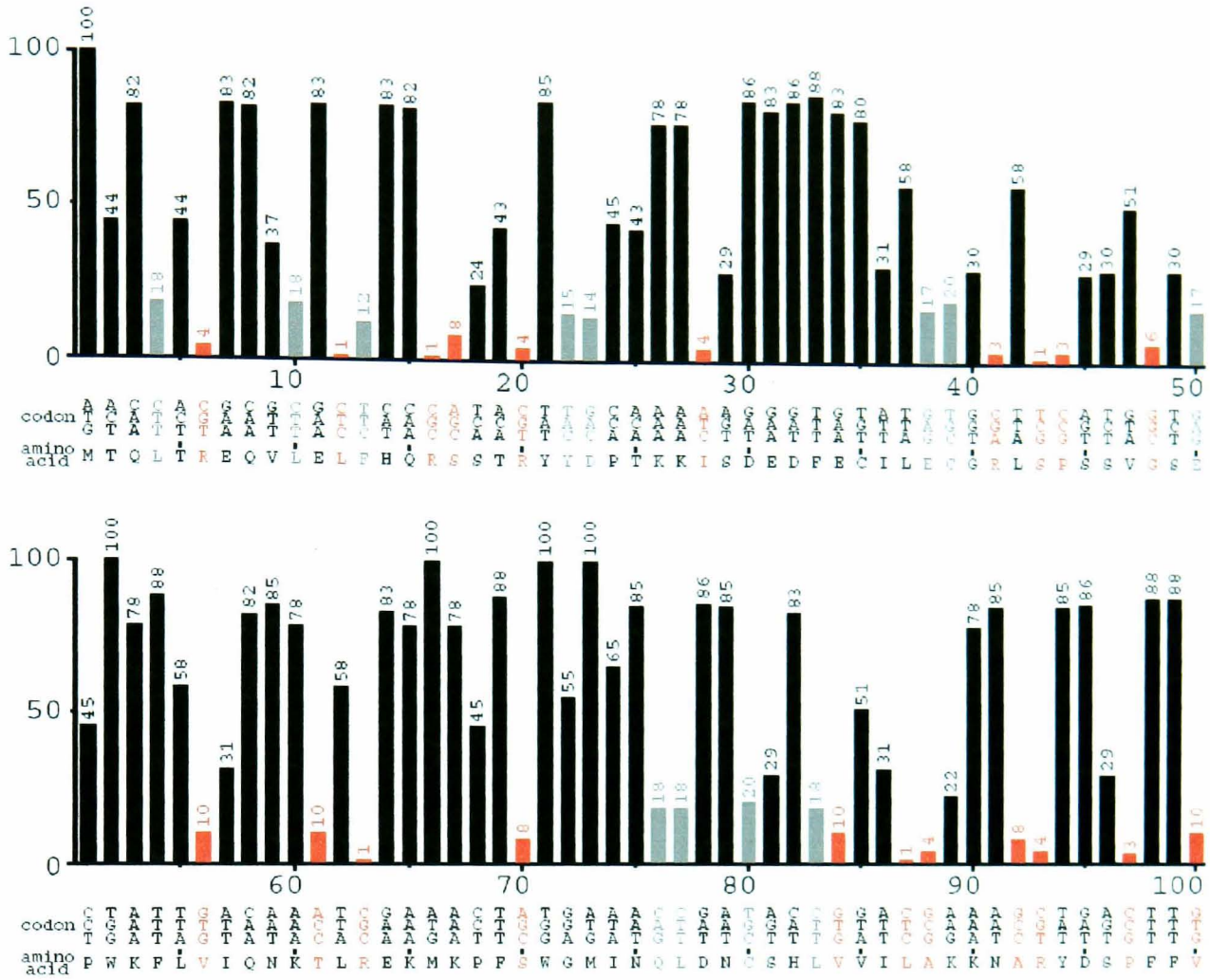
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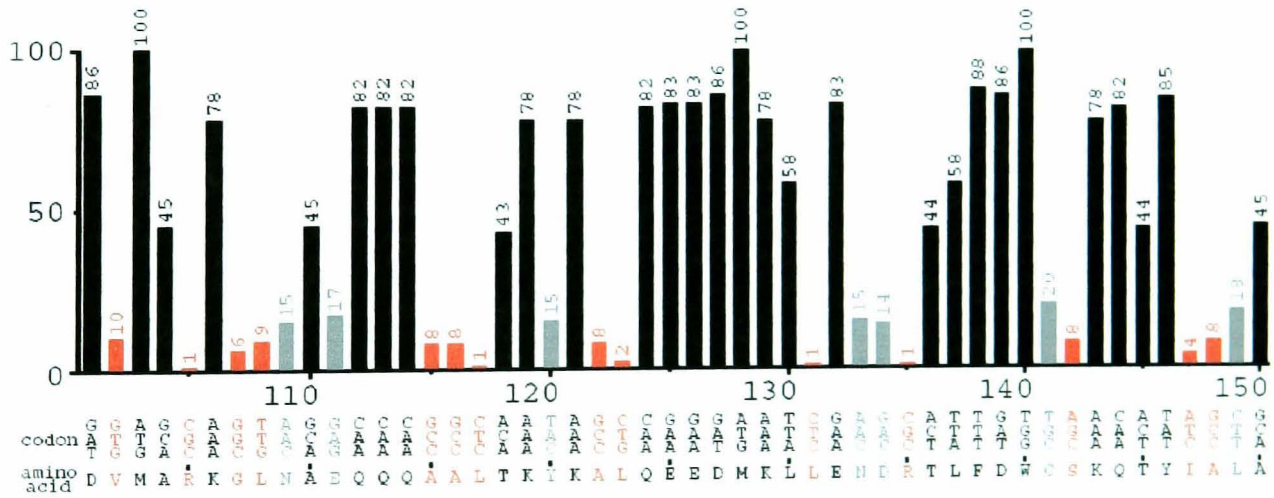
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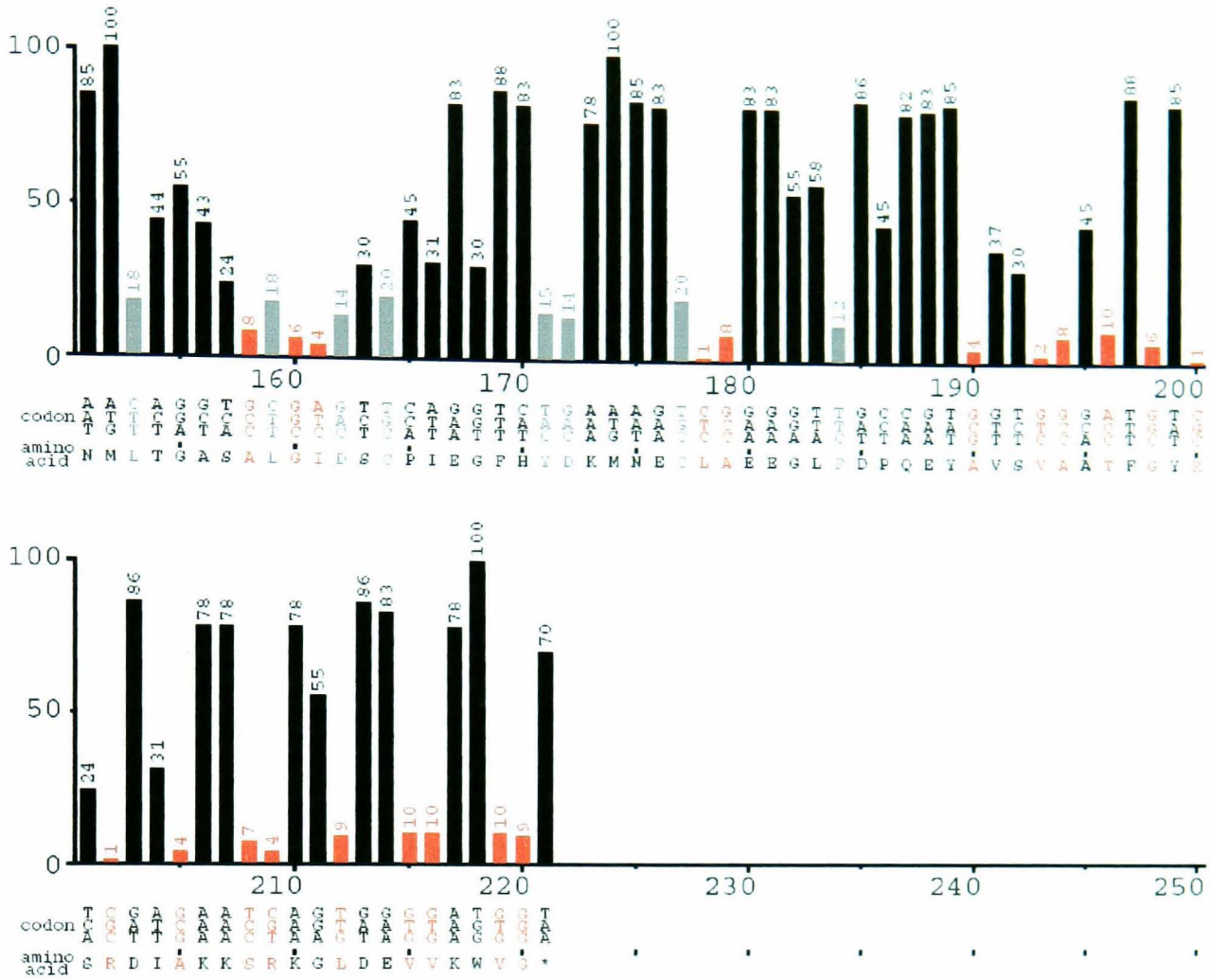
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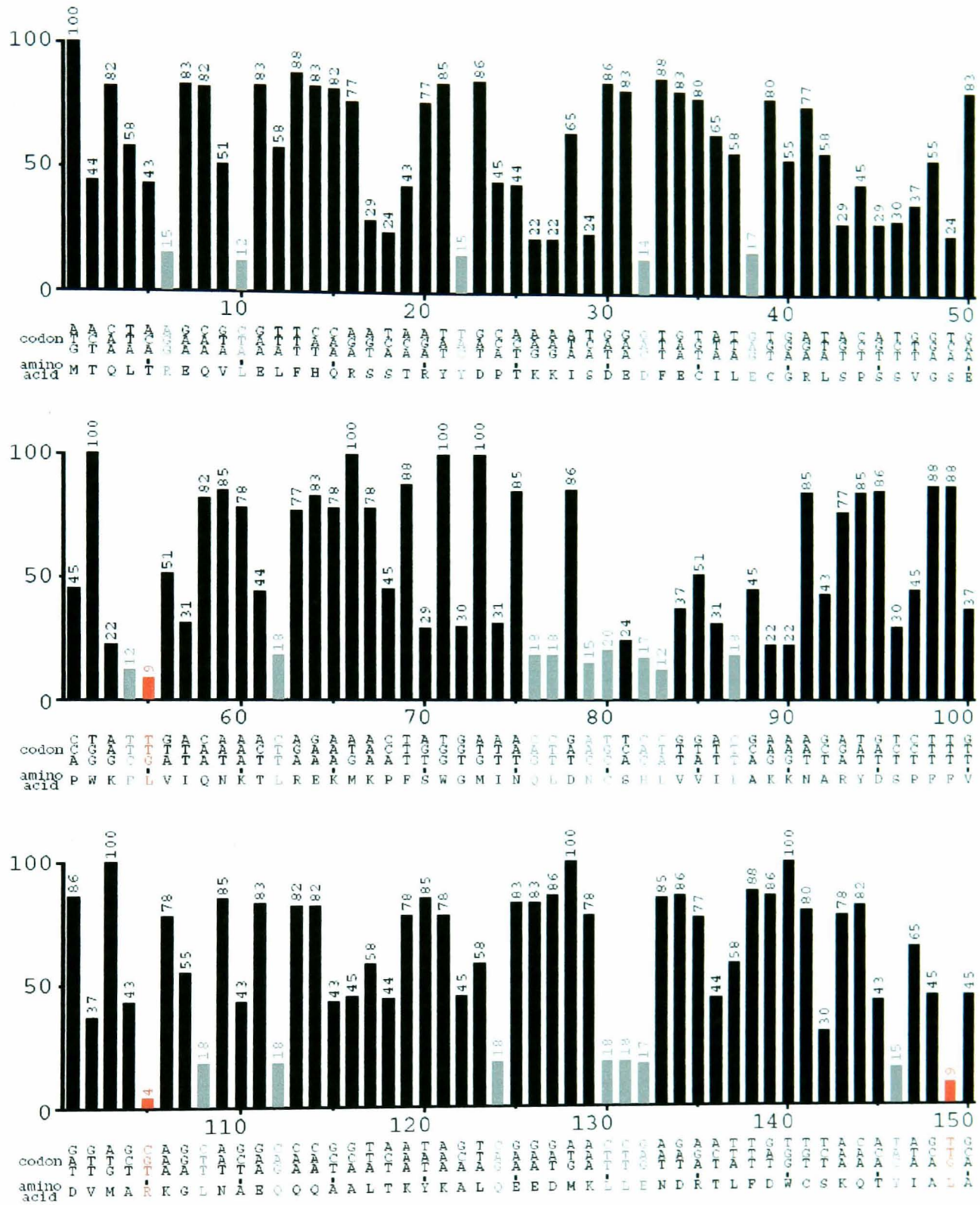

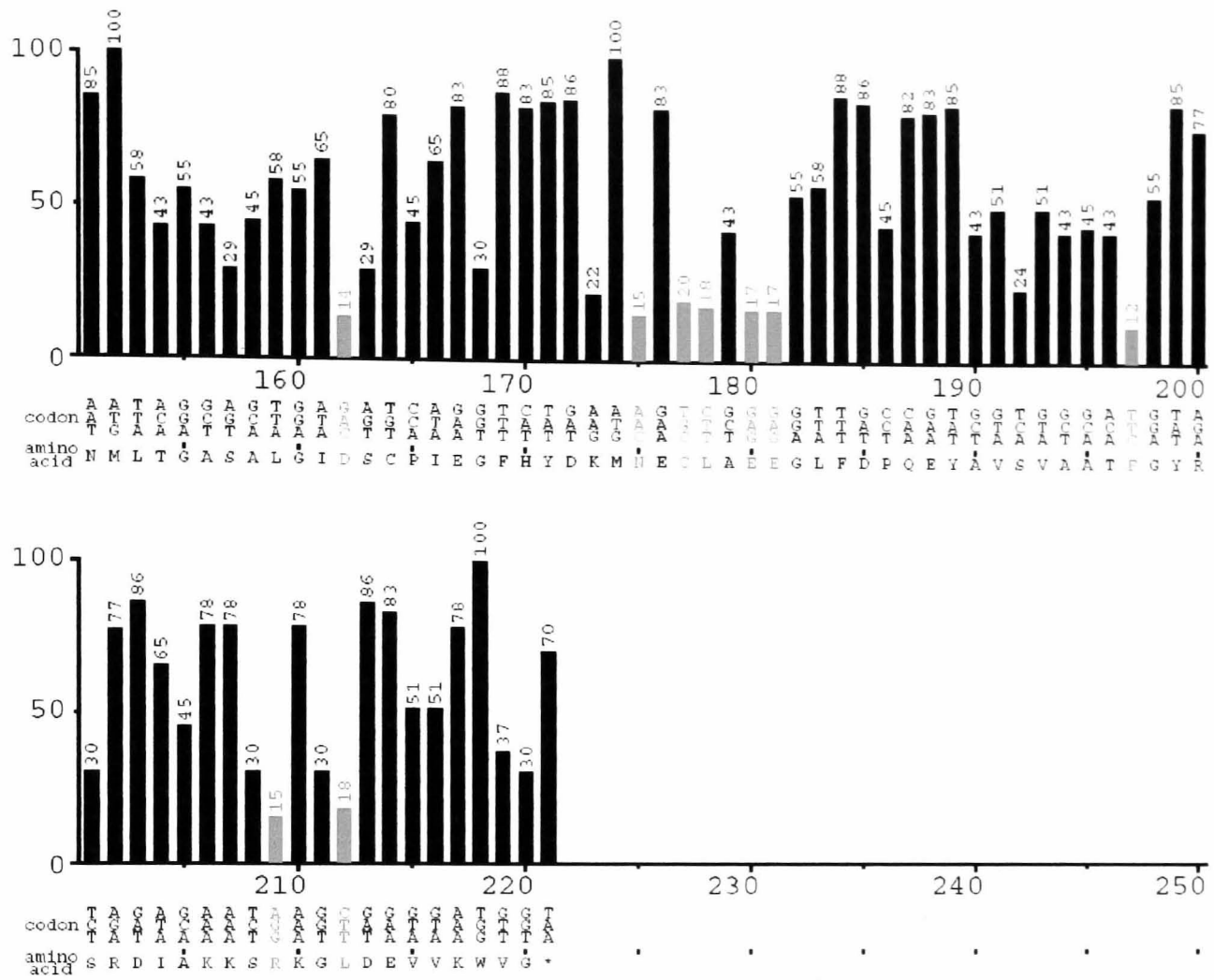
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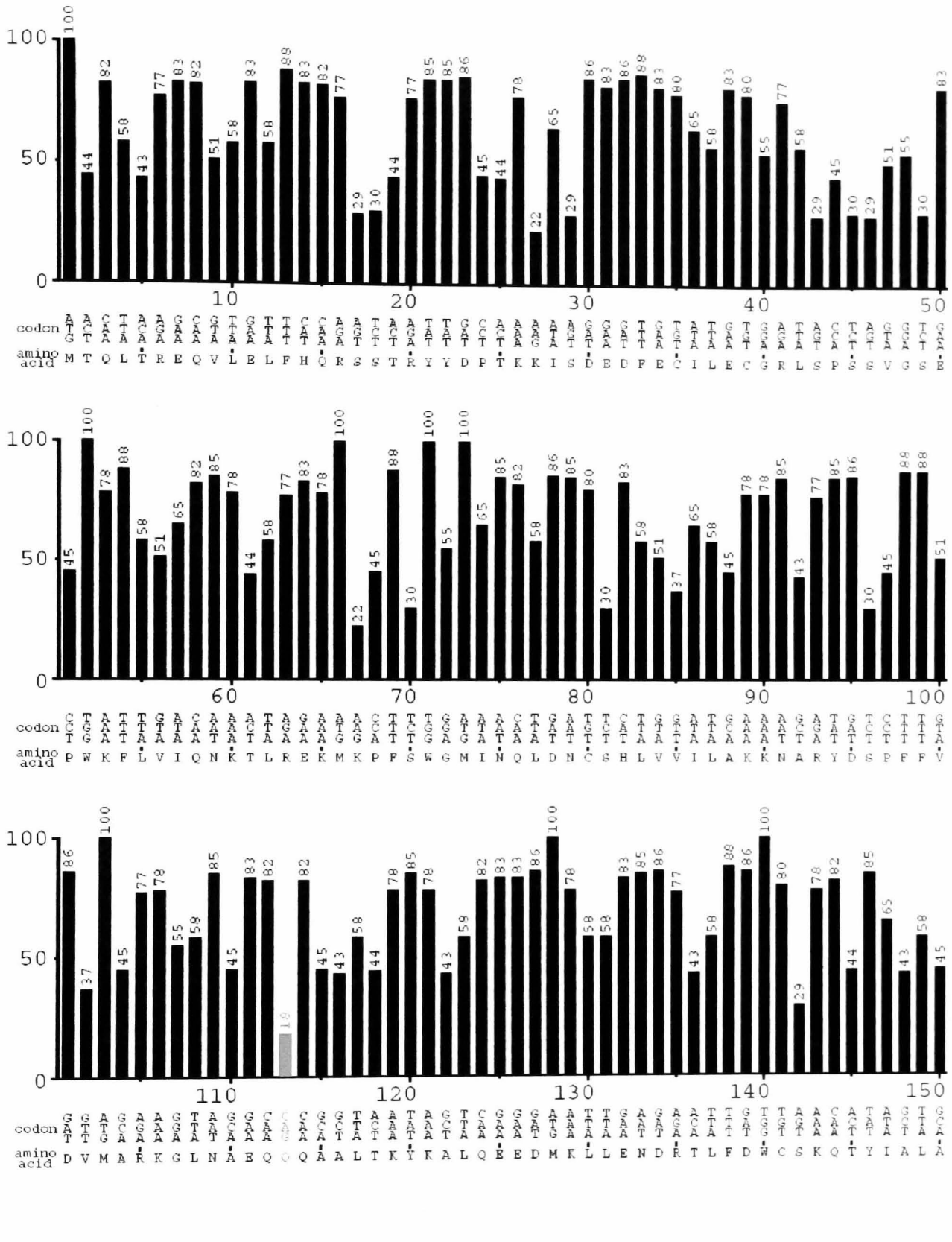

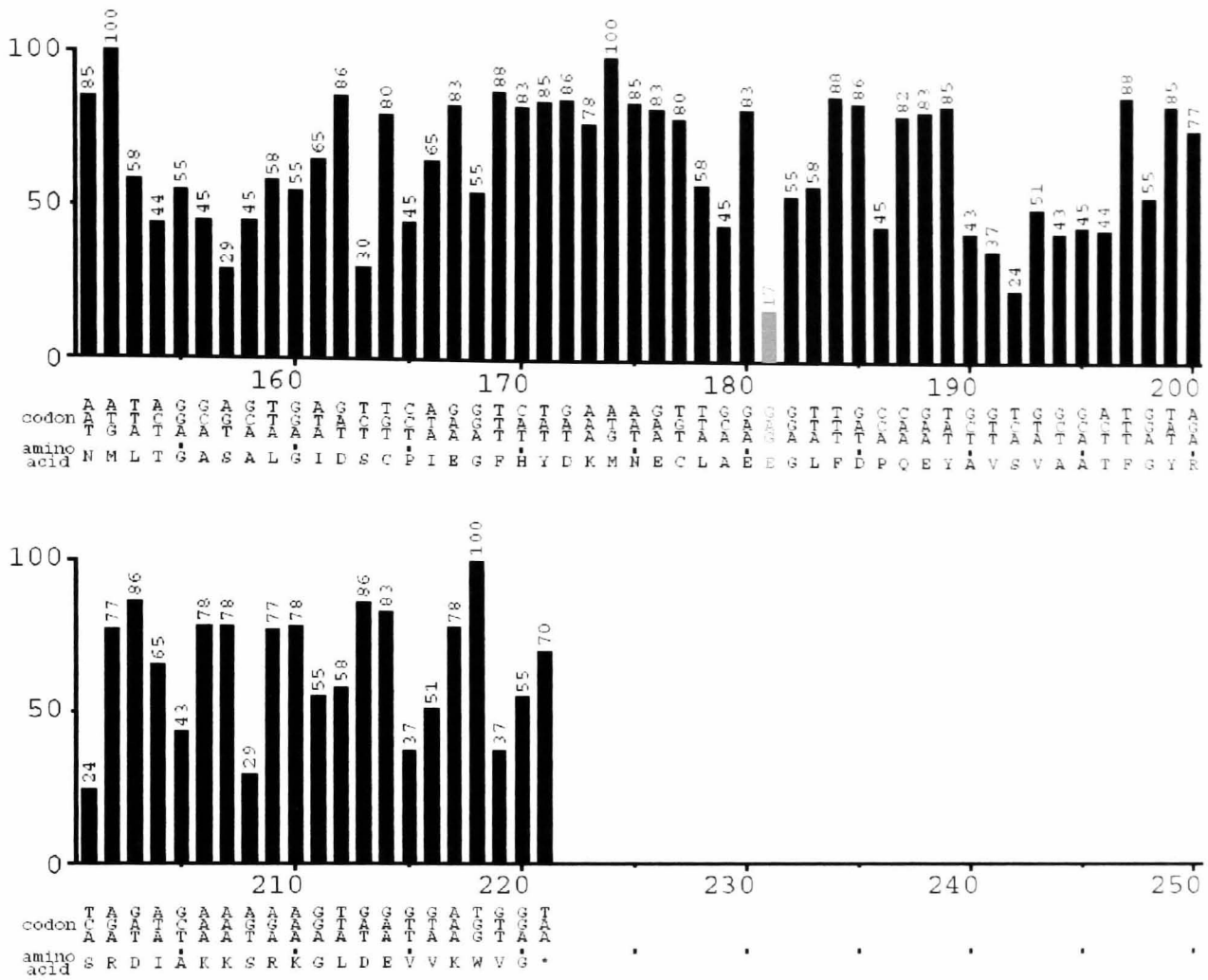
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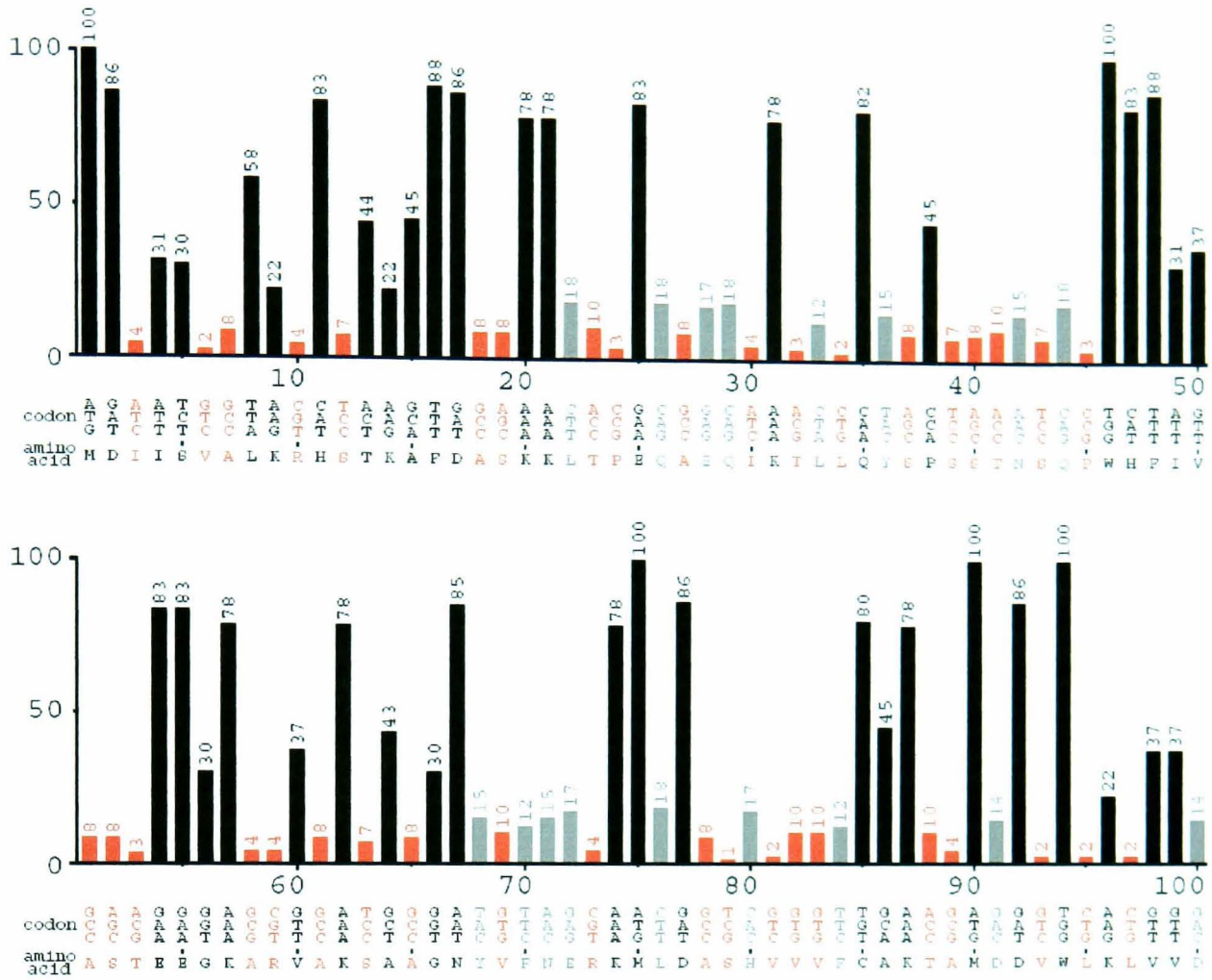
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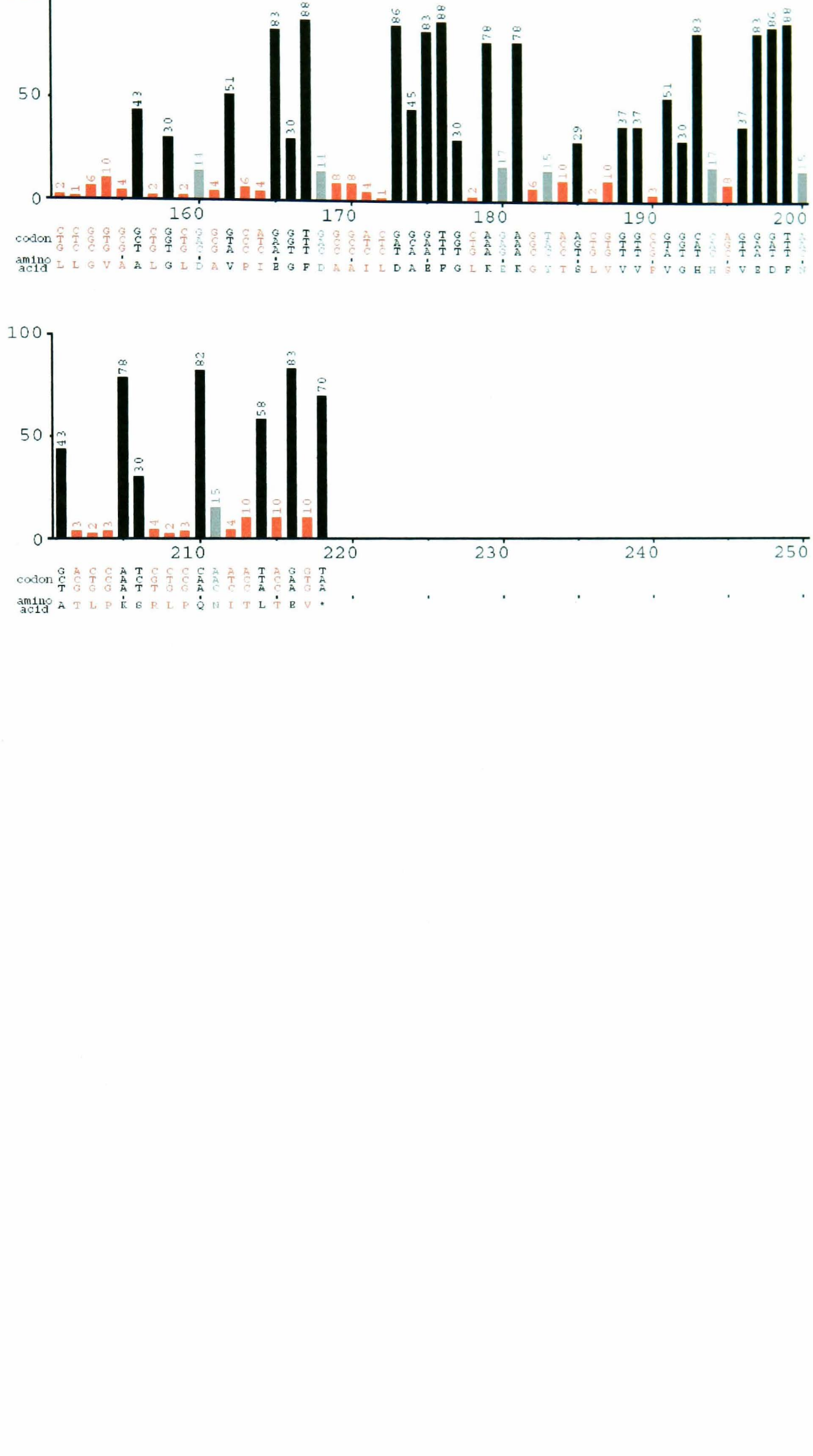
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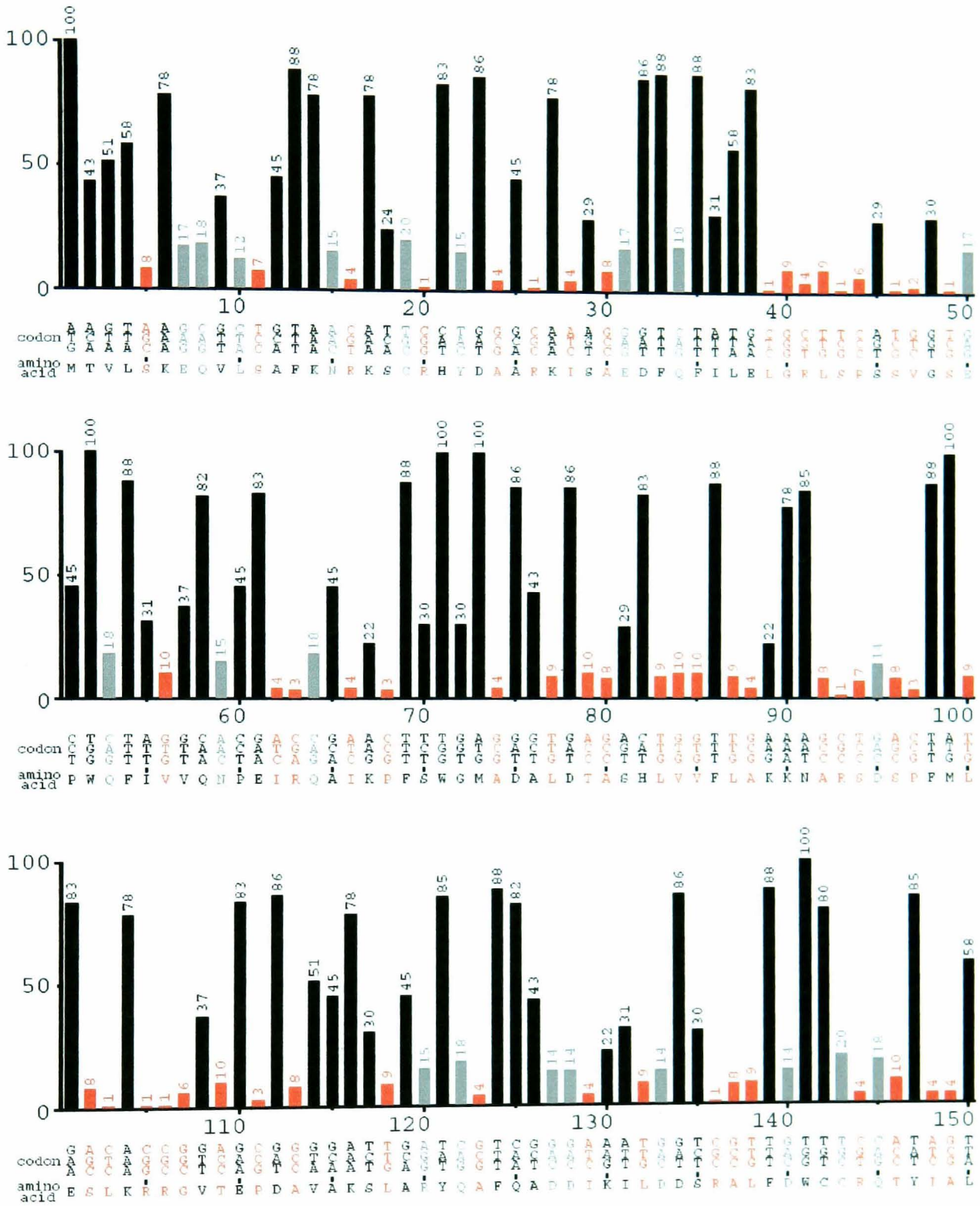
NTR-N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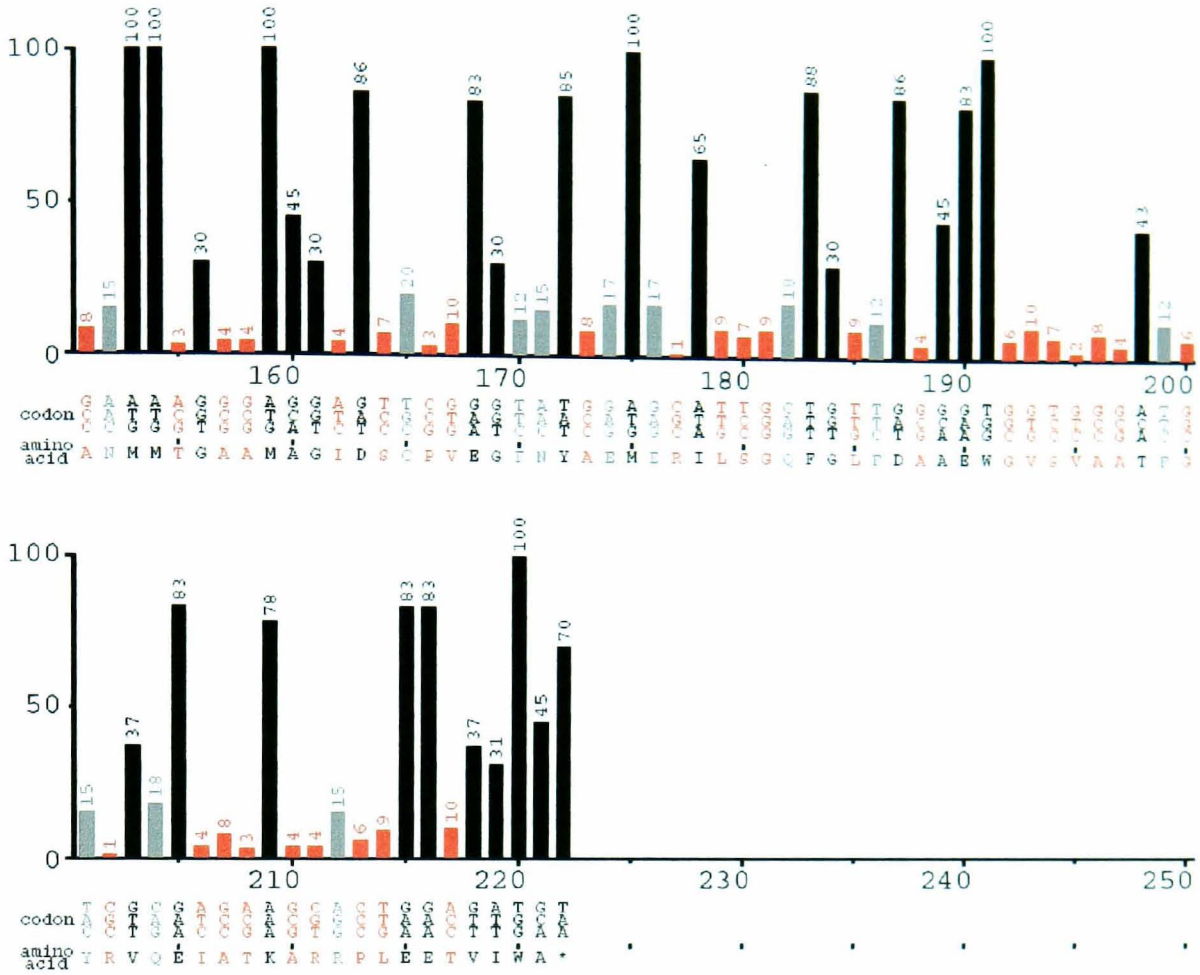
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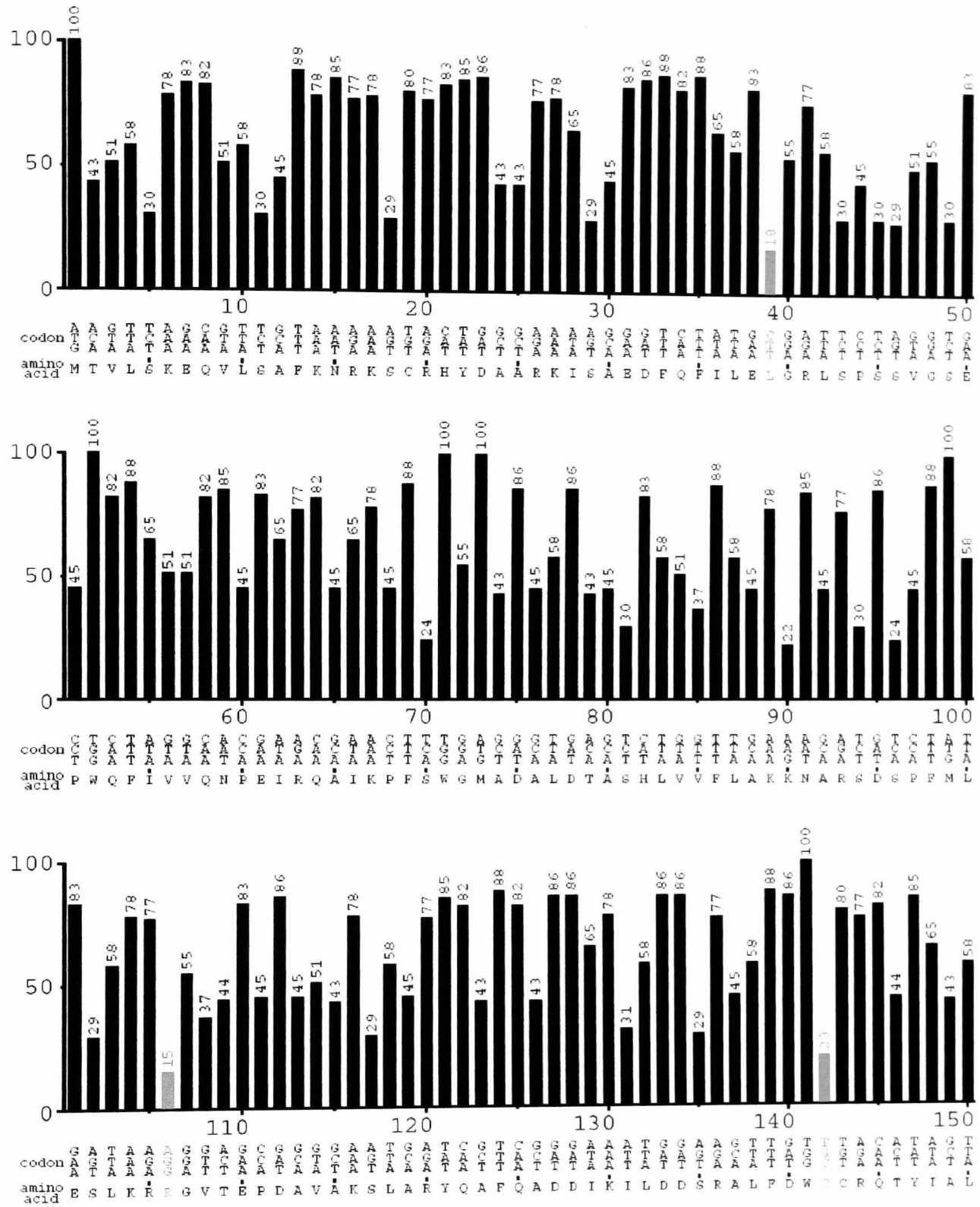
NTR-Nsynth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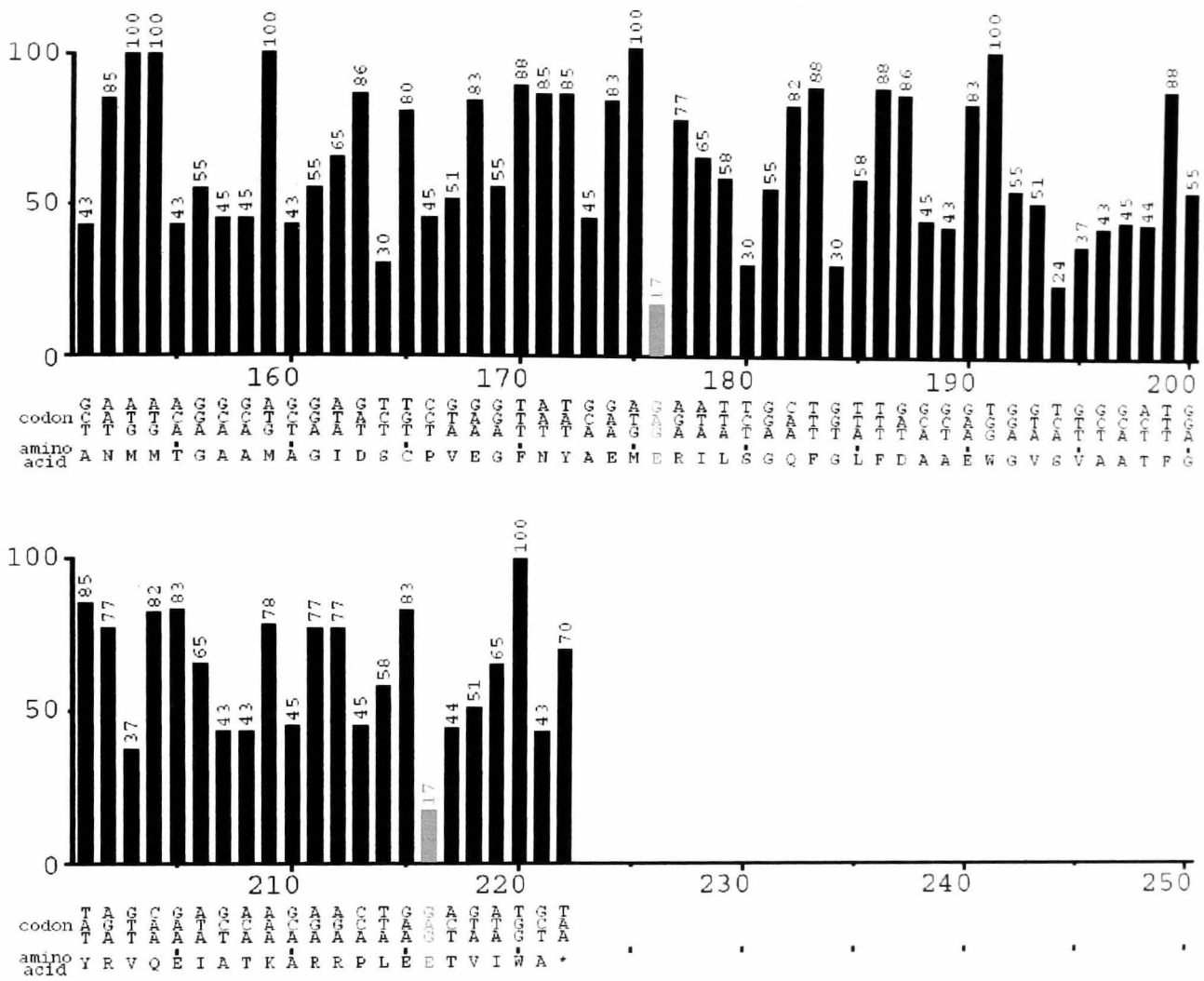
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160
170
180
190
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amino acid
210
220
230
240
250