The construction of a reporter system and use for the investigation of \textit{Clostridium perfringens} gene expression

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Abstract

A reporter system was constructed to enable the study of gene expression in \textit{Clostridium perfringens}. The system was based on plasmid shuttle vector pJIR410, which contained the \textit{C. perfringens} erythromycin resistance gene. The vector was modified by the introduction of a DNA fragment comprising the open reading frame of the \textit{C. perfringens} chloramphenicol acetyltransferase gene and flanking transcriptional terminators. The presence of a unique restriction site, engineered into the extreme 5' end of the open reading frame enabled a promoter region to be inserted to form an in-frame transcriptional fusion with \textit{catP}. The system was tested by inserting the promoter region of the alpha-toxin gene of \textit{C. perfringens}. The production of chloramphenicol acetyltransferase in \textit{C. perfringens} was monitored during growth and the pattern of expression was shown to reflect levels of \textit{plc} mRNA and alpha-toxin in the parent strain.

Keywords: \textit{Clostridium perfringens}; Recombinant DNA; Chloramphenicol acetyltransferase; Shuttle vector; Toxin

1. Introduction

\textit{Clostridium perfringens} produces a wide spectrum of toxins which play a role in the pathogenesis of many diseases in man and animals [1]. The genes encoding some toxins have been isolated and their nucleotide sequences determined [2–7], but the mechanisms regulating their expression are not well understood. In view of the wide range of toxins which may be produced by the bacterium, and the association of these toxins with different diseases [1], it is likely that toxin production is regulated in vivo. Recently it has been reported that two different regulatory proteins bind to the alpha-toxin gene (\textit{plc}) of \textit{C. perfringens} [8] and also that the expression of \textit{plc} in an \textit{Escherichia coli} is negatively regulated by DNA bending of upstream sequences [9]. The theta-toxin gene (\textit{pfo}), when expressed in \textit{E. coli}, is positively regulated by an upstream sequence [10]. To further characterise these, and other mechanisms of gene regulation, it will be necessary to perform studies in \textit{C. perfringens}.

Techniques have been described for the introduction of plasmid DNA into \textit{C. perfringens} by electroporation [11] and several \textit{E. coli}/\textit{C. perfringens} shuttle vectors have been reported. The aim of this study was to construct a reporter system, based on one of these shuttle vectors (pJIR410; [12]) and the chloramphenicol acetyltransferase gene (\textit{catP}) of \textit{C. perfringens}. This reporter system could be used to
investigate the activity and regulation of a variety of gene promoters in *C. perfringens*.

2. Materials and methods

2.1. Chemicals and enzymes

Chemical and enzymes were obtained from the Sigma Chemical company or from BCL Limited unless otherwise stated.

2.2. Bacterial strains and growth

*C. perfringens* NCTC 8237 was cultured in TPYG broth [3]. 100-ml volumes of TPYG broth were inoculated with cells from 5 ml of an 18-h culture. The cultures were incubated at 37°C in an anaerobic incubator (80% N₂, 10% CO₂, 10% H₂; Don Whitley Scientific). Growth medium for Strain 13 containing plasmid pHB1 also contained erythromycin (50 µg ml⁻¹). At intervals the optical density of the culture (600 nm) was measured.

2.3. Preparation of DNA

Large- and small-scale plasmid isolations from *E. coli* were performed as described by Sambrook et al. [13]. Oligonucleotides were prepared using a Applied Biosystems DNA synthesiser (model 392).

2.4. Nucleotide sequencing

Both strands of DNA were sequenced using the dideoxy chain termination method using T7 DNA polymerase (Sequenase Version 2.0, United States Biochemical Corp.) according to the manufacturer’s instructions.

2.5. Electroporation of bacteria

*C. perfringens* strain 13 was transformed using plasmid DNA which had been isolated from *E. coli* DH5α. Electroporation conditions have been described previously [11].

2.6. Enzyme and protein assays

Extracellular alpha-toxin activity in culture supernatant fluid was measured using a microtitre tray assay with egg yolk emulsion [3]. Chloramphenicol acetyltransferase (CAT) activity was determined as described by Rodriguez and Tait [14]. Total protein was measured using the method of Bradford (Bio-Rad protein assay).

2.7. mRNA isolation and quantitation

Total RNA was isolated from 100-ml cultures of *C. perfringens* using a simplified version of the hot phenol method described by Janzon et al. [15]. Before analysis, the RNA was denatured by the addition of formaldehyde and formamide [13]. For slot blots, 1 µg and 5 µg total RNA samples were loaded directly onto a Nylon filter (Hybond N, Amersham) and fixed by baking. The RNA was detected using an SspI-HindIII restriction endonuclease fragment labelled with [α-³²P]dCTP (Amersham; 6000 Ci mmol⁻¹) to a specific activity of > 10⁸ dpm µg⁻¹ [13]. The probe corresponded to the *plc* structural gene [3]. Pre-hybridisation and hybridisation were carried out at 42°C in buffer containing formamide [13]. For the quantitation of mRNA levels, autoradiographs were scanned using a laser densitometer (Pharmacia-LKB). These experiments were performed twice.

2.8. Construction of reporter system

The *E. coli* transcriptional terminator sequence *rrnBT₁* was isolated from pKK232-8 (Pharmacia) after digestion with *EcoRI*. The DNA fragment was used in two ways: firstly, plasmid pHLY3 was generated after cloning the *rrnBT₁* cassette into pBluescript II SK⁻ (Stratagene) which had been restricted with *EcoRI* (Fig. 2). Secondly, after cloning the *rrnBT₁* cassette into *EcoRI*-digested pGEM7Zf(−) (Promega) it was excised as a *KpnI*/*XhoI* fragment which was cloned into suitably digested plasmid pHLY1 to generate pHLY2. After digestion of plasmid pHLY2 with *SmaI* and *KpnI* the fragment of DNA which contained *catP* and the downstream *rrnBT₁* was isolated. This was ligated with plasmid pHLY3, which had been digested with *EcoRV* and *KpnI*, to generate the reporter cassette contained within pBluescript II SK (pHY4). Digestion of pHLY4 with *SmaI* and *KpnI* allowed the isolation of a DNA fragment encoding *catP*, flanked by the
rrnBT₁ transcriptional terminator sequences. This fragment was cloned into pJIR410 which had been digested with PvuII and KpnI, to generate the shuttle reporter vector pTCATT. A DNA fragment which contained the plc promoter was generated using the PCR with primers containing appropriate restriction endonuclease sites at the 5' ends (5'-CAATATGATCGATATGCATAGTGTAGCCATGCTGACATAC-CTTCACCTATT-3' and 5'-ACGCGTATCGAGTTTTTCTAAATACCATTTTATCCCGGTTA-3'). Plasmid pTCATT was transformed into E. coli DH5α [16] and C. perfringens strain 13 by electroporation [13].

3. Results and discussion

3.1. Cloning and modification of catP from C. perfringens

The catP gene was selected for the construction of a reporter system as it was derived originally from the C. perfringens plasmid pIP401 [17] and codon usage effects should not limit gene expression. The gene product can be easily quantified [14]. The polymerase chain reaction (PCR) was used to amplify the open reading frame of catP, from vector pJIR418 [12]. The DNA fragment encoding CAT was cloned into pBluescript II SK to generate plasmid pHLY1. The upstream oligonucleotide primer used in the PCR (sequence: 5'-ATGTTATTGTGAAAAATCGAT-3') was designed to introduce a unique ClaI restriction endonuclease recognition site located 15 bp downstream of the 5' terminus of the open reading frame. The introduction of the ClaI site did not alter the amino acid sequence of CAT. When creating promoter fusions using this restriction enzyme site the DNA nucleotide sequence of catP upstream of the ClaI site must be regenerated. When the nucleotide sequence of catP was determined [12], a number of differences were found on comparison with the previously reported sequence. These differences have also been reported by other workers [12].

Fig. 1. DNA nucleotide sequence of the reporter cassette within pTCATT. The DNA nucleotide sequence of the reporter cassette within pTCATT, and the deduced amino acid sequence of CAT, is shown at A. ClaI restriction endonuclease recognition sequences are underlined. The previously reported nucleotide sequence [17], where different, is shown below the main sequence. The DNA nucleotide sequence of rrnBT₁ is not shown as the orientation of the transcriptional terminators was not determined. The creation of a promoter fusion is illustrated at B. The fidelity of the nucleotide sequence of the entire promoter region (P) and ribosome binding site (SD) is conserved to the ATG translational start codon. Oligonucleotides used in the amplification of the promoter should reconstitute the first 15 bp of the catP open reading frame and should include ClaI restriction sites for cloning purposes.
Fig. 2. Construction of the C. perfringens/E. coli shuttle reporter plasmid (pTCATT).
3.2. Construction of pTCATT reporter plasmid

Plasmids pHLY1 and pJIR410* were used for the construction of a reporter shuttle vector (Fig. 2; pTCATT). Plasmid pJIR410* , which was derived from pJIR410 [12] by the removal of a ClaI restriction site, contained the E. coli origin of replication from pUC18, the origin of replication from the C. perfringens plasmid pIP404 and the C. perfringens ermBP gene, which encoded erythromycin resistance. A single copy of the E. coli transcriptional terminator rrrnBT1 was introduced upstream and downstream of catP to prevent expression of catP from other sequences within the shuttle vector and to stabilise the plasmid.

A reporter system using lux has been described previously [18], but expression of a gene derived from Vibrio fischeri (54–60% A + T) may be constrained in C. perfringens (73–76% A + T). The reporter system described here also included transcriptional terminator sequences, a feature which was absent in the catP reporter system constructed by Matsushita et al. [19]. The terminator sequences should prevent read-through from other plasmid encoded genes. In addition, the use of the ClaI site for fusion of the promoter and the reporter gene ensures that the authenticity of the nucleotide sequence of the promoter, ribosome binding site and the region to the start of the coding sequence is retained. Other reporter systems described [18,19] incorporate the Shine-Dalgarno site and associated regions which are native to the catP gene.

When creating promoter fusions using the ClaI restriction enzyme site, the DNA nucleotide sequence of catP upstream of the ClaI site and the ClaI recognition sequence can be incorporated into oligonucleotides used for PCR amplification of promoter regions to ensure that the complete catP is regenerated.

3.3. Cloning of the plc gene promoter into the reporter plasmid

The reporter system was tested using the promoter for the C. perfringens NCTC 8237 plc [3]. The location of the transcript start site for this gene has been determined [20]. An 0.8-kb fragment containing the promoter was amplified using the PCR and cloned upstream of catP into the ClaI site in plasmid pTCATT. The authenticity and orientation of the DNA fragment cloned into pHBL was confirmed by determining the nucleotide sequence of this fragment. The plasmid was transformed into C. perfringens strain 13 by electroporation. This plasmid was isolated and transformed into E. coli for the retrospective confirmation of its structure by examining the restriction endonuclease digestion profile.

Fig. 3. Comparison of alpha-toxin and CAT production from the C. perfringens NCTC8237 alpha-toxin promoter. C. perfringens strain 13 containing plasmid pHBL (a) or strain NCTC8237 (b) were cultured in TPYG broth. At intervals the optical density (600 nm) of the culture (A) was measured. Extracellular phospholipase C (O) or CAT specific activity (D) produced between consecutive time points was calculated. The level of plc mRNA within cells of strain NCTC8237 (●) was determined at the times shown.
3.4. Analysis of expression of the plc/catP transcriptional fusion

The pattern of expression of catP from the strain NCTC 8237 cpa promoter was determined after electroporation of plasmid pHB1 into strain 13. Attempts to transform strain NCTC 8237 with plasmid pHB1, using a variety of techniques, were not successful and other workers have found that plasmid DNA could be transferred only into some strains of *C. perfringens* [11]. The production of CAT in *C. perfringens* strain 13 containing pHB1 was measured during growth of the bacterium (Fig. 3) and compared with the production of alpha-toxin by strain NCTC 8237. It is known that CAT and alpha-toxin are both stable in cultures of *C. perfringens* and therefore it was possible to calculate the amount of enzyme produced between consecutive time points. The maximum level of CAT activity was detected after 3 h of growth, which corresponded to the late exponential phase. In strain NCTC 8237, the plc mRNA levels increased throughout the exponential phase of growth, reaching a maximum level after 3 h, which corresponded to the late exponential phase of growth. The level of production of alpha-toxin exported into the culture medium reached a maximum after 3 h. These results indicate that the strain NCTC 8237 plc promoter, within the genetic background of strain 13, reflects the level of intracellular plc mRNA or extracellular alpha-toxin in strain NCTC 8237.

The use of the reporter vector will be simplified if ClaI restriction endonuclease sites are absent in the promoter to be cloned; the recognition sequence for the ClaI enzyme is absent in the upstream regions of the genes encoding *C. perfringens* perfringolysin O (pfo; [2]), enterotoxin gene (cpe; [4]), beta-toxin (cpb; [5]), iota-toxin (1a, 1b; [6]) and epsilon-toxin (etxB, etxD; [7]).

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References


