EXPRESSION SYSTEM

The present invention concerns an expression system suitable for the microbial expression of recombinant polypeptides.

T7-based perfect palindrome operator sequence-based protein expression systems are known from patent US 6,537,779. T7 based systems suffer from drawbacks in that operation of the T7 system requires phage polymerase which is commonly provided by inserting a λDE3 prophage expressing the required phage polymerase into the *Escherichia coli* host strain to create lysogenic host strains. The phage polymerase can also be delivered to the cell by infection with a specialised λ transducing phage that carries the gene for the phage polymerase (e.g. T7 RNA polymerase). The λDE3 prophage lacks the genetic elements required for the excision of the prophage to form lytic phage particles. However, λDE3 lysogenic host strains have been shown to release phage particles and thus cause undesirable infections in fermentation plants. Indeed, the use of λDE3 strains is not permitted by certain fermentation plant operators.

Expression of the heterologous protein prior to induction is not desirable because some heterologous proteins have deleterious effects on the host cell growth and plasmid stability which reduce overall productivity. To avoid this, T7-based expression systems generally control expression of heterologous proteins at two levels. First, induction of expression of the T7 RNA polymerase gene to produce T7 RNA polymerase is required to drive expression from the T7 promoter. Secondly, the T7 promoter itself also needs to be induced. This increases the complexity of operating T7-based expression systems.

There are a large number of heterologous protein expression systems with different modes of control and induction, making selection and optimisation of the expression system/fermentation process for proteins of interest a largely empirical process. This is time consuming and undesirable. Thus, there is a need for systems which can provide improved control of expression and improved levels of protein expression without the use of phage polymerase and lysogenic host strains. There is also a need for systems which can provide inducible heterologous expression in prokaryotic cells, as well as eukaryotic cells such as mammalian and yeast cells.

According to the present invention, there is provided a perfect palindrome operator sequence-based protein expression system comprising:

a) a promoter; and

b) a perfect palindrome operator sequence;

characterised in that the promoter is not T7.

Promoters which can be employed in the expression system of the present invention are commonly host RNA polymerase-based promoter systems, and preferably *E. coli* RNA polymerase-based promoter systems. Examples of promoters which can be employed include T7A1, T7A2, T7A3, λpL, λpR, lac, lacUV5, trp, tac, trc, phoA and rrnB.

Operator sequences which may be employed in the expression system according to the present invention include lac, gal, deo and gln. One or more perfect palindrome operator sequences may be employed. In many preferred embodiments, two perfect palindrome operator sequences are employed, most advantageously one operator sequence being located downstream of the promoter, and one operator sequence being located upstream of the promoter. When two operator systems are employed, the operator sequences are preferably spaced to maximise control of the promoter. In many embodiments, the spacing is from 85 to 150 base pairs apart, preferably from 90 to 126 base pairs apart, and most preferably 91 or 92 base pairs apart. In certain embodiments, an operator sequence overlaps with the transcriptional start point

It will be recognised that the operator system is commonly employed with an appropriate repressor sequence. Repressor sequences produce repressor protein, for example lacI gene sequence when using the lac operators. Other lac repressor sequences may also be used, for example the $lacI^Q$ sequence can be used to increase the level of lac repressor protein. The repressor sequence may also be provided by the host cell genome or by using an additional compatible plasmid.

The expression system may be integrated into the host cell genome, but is preferably comprised within an extrachromosomal element such as a plasmid. Alternatively, the expression system may be incorporated into phage or viral vectors and these used to deliver the expression system into the host cell system. Plasmids or expression vectors can be assembled by methods known in the art. The plasmid typically also comprises one or more of the following: a selectable marker, for example a sequence conferring antibiotic resistance, a cer stability sequence and an expression cassette. The expression system may also incorporate a signal sequence if secretion of the desired protein is required.

Expression may be induced by the addition of an inducer such as isopropyl-β-D-1-thiogalactopyranoside (IPTG), analogues of IPTG such as isobutyl-C-galactoside (IBCG), lactose or melibiose. Other inducers may be used and are described more fully elsewhere (e.g. see The Operon, eds Miller and Renznikoff (1978)). Inducers may be used individually or in combination. The construction of appropriate plasmids or expression vectors will be apparent to the scientist of ordinary skill.

The expression system of the present invention can be employed to express proteins in host cells, and especially in microorganisms. As used herein, "proteins" refers generally to peptides and proteins having more than about 10 amino acids. The host cell may be prokaryotic or eukaryotic. Examples of prokaryotic cells include bacterial cells, for example gram-negative bacterial cells, including *E. coli*, *Salmonella typhimurium*, *Serratia marsescens* and *Pseudomonas aeruginosa*, and gram-positive bacterial cells including *Bacillus subtilis*. Examples of eukaryotic cells include yeasts, such as *Pichia pastoris*, *Saccharomyces cerevisiae*, *Hansenula polymorpha*, *Kluyveromyces lactis*, *Schizosaccharomyces pombe*. Mammalian host cells which can be employed include

human cell lines, such as human embryonic kidney and PERC.6 cells; murine cell lines, such as NS0 cells; and particularly hamster cell lines such as baby hamster kidney cells and especially Chinese hamster ovary cells. Other eukaryotic host cells such as those of filamentous fungi, plant, insect, amphibian cells or ovarian species may also be employed. Preferred host cells are bacteria, particularly enterobacteriacae, preferably *E coli*, and especially B or K12 strains thereof.

The expression system of the present invention is commonly employed in the form of a plasmid, and plasmids comprising a promoter and a perfect palindrome operator sequence, wherein the promoter is not T7, form another aspect of the present invention. The plasmids may be autonomously replicating plasmids or integrative plasmids.

The expression system of the present invention is advantageously employed for the manufacture of proteins, especially recombinant proteins, by culturing recombinant cells. For the expression of proteins, it will be recognised that the promoter and operator sequence are operably linked to DNA encoding a protein to be expressed.

Accordingly, the present invention also provides a method for the production of a protein which comprises expressing an expression system comprising

a) a promoter;

b) a perfect palindrome operator sequence; and

c) an expression cassette for a protein;

characterised in that the promoter is not T7.

One or more promoters, operator sequences and expression cassettes, which may be the same or different, may be present if desired.

The expression system is expressed by methods well known in the art for the cells employed. Preferred expression methods include culturing the recombinant cells in growth medium, especially by fermentation, and then recovering the expressed protein. The term "growth medium" refers to a nutrient medium used for growing the recombinant cells. In many embodiments, a nutrient solution is employed. Suitable growth media for given recombinant cells are well known in the art.

The present invention is illustrated without limitation by the following examples.

1. Generation of pAVE series of vectors

Vectors pAVE011, pAVE012 and pAVE013

The starting vector for the generation of pAVE011 was pZT7#2.0, prepared as described in US 6,537,779. pZT7#2.0 has a pAT153 vector backbone, cer stability sequence, tet A/R, a single native lac operator sequence upstream of the gene of interest and an upstream T4 transcription terminator. A T7A3 promoter and dual perfect palindrome lac operators were cloned into this plasmid using synthetic oligonucleotide linkers by means of the Nco I, EcoR I and Xba I restriction enzyme sites.

Linker 12.1 was prepared by annealing the oligonucleotides 1 and 2.1:

Oligonucleotide 1 (SEQ ID NO 1)
5'CATGTGGGAATTGTGAGCGCTCACAATTCCAAGAACAATCCTGCACG

Oligonucleotide 2.1 (SEQ ID NO 2)
5'AATTCGTGCAGGATTGTTCTTGGAATTGTGAGCGCTCACAATTCCCA

The linker was then ligated to plasmid pZT7#2.0 and transformed into cloning host strain XL-1 Blue MR (Stratagene) as an Nco I/EcoR I fragment. Initial screening of transformants was by restriction digestion using Nco I. The sequence was confirmed by sequencing. The resultant plasmid was named pAVE012.

The T7A3 promoter cassette was then cloned into pAVE012 by annealing oligonucleotides 3 and 4:

Oligonucleotide 3 (SEQ ID NO 3)
5'AATTCAAACAAAACGGTTGACAACATGAAGTAAACACGGTACGATGTACCGGAATT
GTGAGCGCTCACAATTCCCCA

Oligonucleotide 4 (SEQ ID NO 4)
5'CTGGTGGGGGGTTGTGGGCGCTCGCGGTTCCGGTGCGTCGTGCCGT
GTTTGCTTCGTGTTGTCGGCCGTTTTGTTTG

the annealed oligonucleotides being ligated to plasmid pAVE012 and transformed into cloning host strain XL-1 Blue MR (Stratagene) as an Xba I/EcoR I fragment. Initial screening was by restriction digest of plasmid DNA. The sequence was then confirmed by sequencing. The resultant plasmid was named pAVE011.

Human TNFα gene was cloned into this plasmid as an Nde I/Xho I fragment to generate pAVE013. A plasmid map for pAVE013 is presented in Figure 18. This shows the arrangement of operators and promoter, and the restriction enzyme sites used in the construction. The operators are both perfect palindrome lac operators. RBS is the ribosomal binding site. The vector includes a pAT153 vector backbone, a cer stability sequence, an inducible tetracycline resistance gene (tet A/R), and an upstream T4 transcription terminator.

Vectors pAVE038 and pAVE041

The starting vector for the generation of pAVE038 was pZT7#2.0, prepared as described in US 6,537,779. A tac promoter and single native lac operator were cloned

into this plasmid using a synthetic oligonucleotide linker by means of the EcoR I and Xba I restriction enzyme sites.

Linker 1112 was made by annealing the oligonucleotides 11 and 12

Oligonucleotide 11 (SEQ ID NO 5)
5'AATTTTCTGAAATGAGCTGTTGACAATTAATCATCGGCTCGGATACTGTGTGGAATT
GTGAGCGGATAACAATTCCCCA

Oligonucleotide 12 (SEQ ID NO 6)
5'CTAGTGGGGAATTGTTATCCGCTCACAATTCCACACAGTATCCGAGCC
GATGATTAATTGTCAACAGCTCATTTCAGAA

The linker was then ligated to plasmid pZT7#2.0 and transformed into cloning host strain XL-1 Blue MR (Stratagene) as an Xba I/EcoR I fragment. Initial screening of transformants was by restriction digestion using Nco I. The sequence was confirmed by sequencing. The resultant plasmid was named pAVE038.

A human TNFα gene was cloned into this plasmid as an Nde I/Xho I fragment to generate plasmid pAVE041.

Vector pAVE037 and pAVE040

The starting vector for the generation of pAVE037 was pZT7#2.0 prepared as described in US 6,537,779. A tac promoter and single perfect palindrome lac operator were cloned into this plasmid using a synthetic oligonucleotide linker by means of the EcoR I and Xba I restriction enzyme sites.

Linker 1314 was made by annealing the oligonucleotides 13 and 14

Oligonucleotide 13 (SEQ ID NO 7)
5'AATTTTCTGAAATGAGCTGTTGACAATTAATCATCGGCTCGGATACTGT
GTGGAATTGTGAGCGCTCACAATTCCCCA

Oligonucleotide 14 (SEQ ID NO 8)
5'CTAGTGGGGAATTGTGAGCGCTCACAATTCCACACAGTATCCGAGCCG
ATGATTAATTGTCAACAGCTCATTTCAGAA

The linker was then ligated to plasmid pZT7#2.0 and transformed into cloning host strain XL-1 Blue MR (Stratagene) as an Xba I/EcoR I fragment. Initial screening of

transformants was by restriction digestion using Nco I. The sequence was confirmed by sequencing. The resultant plasmid was named pAVE037.

A human TNFα gene was cloned into this plasmid as an Nde I /Xho I fragment to generate pAVE040.

Vector pAVE028 and pAVE030

The starting vector for the generation of pAVE028 was pAVE012. A T7A3 promoter cassette was cloned into pAVE012 by annealing oligonucleotides 5 and 6.

Oligonucleotide 5 (SEQ ID NO 9)
5'AATTCGAAACAAAACGGTTGACAACATGAAGTAAACACGGTACGATGTACCGGAAT
TGTGAGCGCTCACAATTCCCCA

Oligonucleotide 6 (SEQ ID NO 10)
5'CTGGTGGGGGGTTGTGGGCGCTCGCGGTTCCGGTGCGTCGTGCCGT
GTTTGCTTCGTGTTGTCGGCCGTTTTGTTTCG

the annealed oligonucleotides being ligated to plasmid pAVE012 and transformed into cloning host strain XL-1 Blue MR (Stratagene) as an Xba I/EcoR I fragment. Initial screening was by restriction digest of plasmid DNA. The sequence was then confirmed by sequencing. The resultant plasmid was named pAVE028.

A human TNFα gene was cloned into this plasmid as an Nde I/Xho I fragment to generate pAVE030.

Vector pAVE007 and pAVE031

The starting vector for the generation of pAVE007 was pZT7#2.0 prepared as described in US 6,537,779. A T7A3 promoter and single perfect palindrome lac operator was cloned into this plasmid using a synthetic oligonucleotide linker by means of the EcoR I and Xba I restriction enzyme sites.

The linker containing the T7A3 promoter was made up of oligonucleotides 3 and 4.

Oligonucleotide 3 (SEQ ID NO 3)
5'AATTCAAACAAAACGGTTGACAACATGAAGTAAACACGGTACGATGTACCGGAATT
GTGAGCGCTCACAATTCCCCA

Oligonucleotide 4 (SEQ ID NO 4)
5'CTGGTGGGGGGTTGTGGGCGCTCGCGGTTCCGGTGCGTCGTGCCGT
GTTTGCTTCGTGTTGTCGGCCGTTTTGTTTG

Oligonucleotides 3 and 4 were annealed, the linker formed was then ligated to plasmid pZT7#2.0 and transformed into cloning host strain XL-1 Blue MR (Stratagene) as an Xba I/EcoR I fragment. Initial screening was by restriction digest of plasmid DNA. The sequence was then confirmed by sequencing. The resultant plasmid was named pAVE007.

A human TNFα gene was cloned into this plasmid as an Nde I/Xho I fragment to generate pAVE031.

Vectors pAVE029 and pAVE027

The starting vector for the generation of pAVE029 was pZT7#2.0 prepared as described fully in US 6,537,779. A λpL promoter and single perfect palindrome lac operator was cloned into this plasmid using synthetic oligonucleotide linker by means of the EcoR I and Xba I restriction enzyme sites.

Linker 78 was made by annealing the oligonucleotides 7 and 8

Oligonucleotide 7 (SEQ ID NO 11)
5'AATTATCTCTGGCGGTGTTGACATAAATACCACTGGCGGTGATACTGAGCGGAATT
GTGAGCGCTCACAATTCCCCA

Oligonucleotide 8 (SEQ ID NO 12)
5'CTAGTGGGGAATTGTGAGCGCTCACAATTCCGCTCAGTATCACCGCCA
GTGGTATTTATGTCAACACCGCCAGAGAT

The linker was then ligated to plasmid pZT7#2.0 and transformed into cloning host strain XL-1 Blue MR (Stratagene) as an Xba I/EcoR I fragment. Initial screening of transformants was by restriction digestion using Nco I. The sequence was confirmed by sequencing. The resultant plasmid was named pAVE029.

A human TNFα gene was cloned into this plasmid as an Nde I/Xho I fragment to generate pAVE027.

Vectors pAVE043 and pAVE044

The starting vector for the generation of pAVE043 was pAVE012. A tac promoter cassette was cloned into pAVE012 by annealing oligonucleotides 17 and 18:

Oligonucleotide 17 (SEQ ID NO 37)
5'AATTTTCTGAAATGAGCTGTTGACAATTAATCATCGGCTCGTATAATGTG
TGGAATTGTGAGCGCTCACAATTCCCCA

Oligonucleotide 18 (SEQ ID NO 38)
5'CTAGTGGGGAATTGTGAGCGCTCACAATTCCACACATTATACGAGCCG
ATGATTAATTGTCAACAGCTCATTTCAGAA

the annealed oligonucleotides being ligated to plasmid pAVE012 and transformed into cloning host strain XL-1 Blue MR (Stratagene) as an Xba I/EcoR I fragment. Initial screening was by restriction digest of plasmid DNA. The sequence was then confirmed by sequencing. The resultant plasmid was named pAVE043. .

A human TNFα gene was cloned into this plasmid as an Nde I/Xho I fragment to generate pAVE044.

Vectors pAVE034 and pAVE035

The starting vector for the generation of pAVE034 was pAVE012. A λpL promoter cassette was cloned into pAVE012 by annealing oligonucleotides 9 and 10:

Oligonucleotide 9 (SEQ ID NO 39)
5'AATTCATCTCTGGCGGTGTTGACATAAATACCACTGGCGGTGATACT
GAGCGGAATTGTGAGCGCTCACAATTCCCCA

Oligonucleotide 10 (SEQ ID NO 40)
5'CTAGTGGGGAATTGTGAGCGCTCACAATTCCGCTCAGTATCACCGCCAGTGGTATT
TATGTCAACACCGCCAGAGATG

the annealed oligonucleotides being ligated to plasmid pAVE012 and transformed into cloning host strain XL-1 Blue MR (Stratagene) as an Xba I/EcoR I fragment. Initial screening was by restriction digest of plasmid DNA. The sequence was then confirmed by sequencing. The resultant plasmid was named pAVE034.

A human TNFα gene was cloned into this plasmid as an Nde I/Xho I fragment to generate pAVE035.

Vector pAVE020 and pAVE021

The starting vector for the generation of pAVE020 was pAVE012. A λpL promoter cassette was cloned into pAVE012 by annealing oligonucleotides 7 and 8.

Oligonucleotide 7 (SEQ ID NO 11)
5'AATTATCTCTGGCGGTGTTGACATAAATACCACTGGCGGTGATACTGAGCGGAATT
GTGAGCGCTCACAATTCCCCA

Oligonucleotide 8 (SEQ ID NO 12)
5'CTAGTGGGGAATTGTGAGCGCTCACAATTCCGCTCAGTATCACCGCCA
GTGGTATTTATGTCAACACCGCCAGAGAT

the annealed oligonucleotides being ligated to plasmid pAVE012 and transformed into cloning host strain XL-1 Blue MR (Stratagene) as an Xba I/EcoR I fragment. Initial screening was by restriction digest of plasmid DNA. The sequence was then confirmed by sequencing. The resultant plasmid was named pAVE020.

A human TNFα gene was cloned into this plasmid as an Nde I/Xho I fragment to generate pAVE021.

Vectors pAVE016 and pAVE017

The starting vector for the generation of pAVE016 was pAVE012. A tac promoter cassette was cloned into pAVE012 by annealing oligonucleotides 15 and 16.

Oligonucleotide 15 (SEQ ID NO 13)
5'AATTCCTGAAATGAGCTGTTGACAATTAATCATCGGCTCGTATAATGTG
TGGAATTGTGAGCGCTCACAATTCCCCA

Oligonucleotide 16 (SEQ ID NO 14)
5'CTAGTGGGGAATTGTGAGCGCTCACAATTCCACACATTATACGAGCCG
ATGATTAATTGTCAACAGCTCATTTCAGG

the annealed oligonucleotides being ligated to plasmid pAVE012 and transformed into cloning host strain XL-1 Blue MR (Stratagene) as an Xba I/EcoR I fragment. Initial screening was by restriction digest of plasmid DNA. The sequence was then confirmed by sequencing. The resultant plasmid was named pAVE016.

A human TNFα gene was cloned into this plasmid as an Nde I/Xho I fragment to generate pAVE017.

Vector pAVE049

The starting vector for the generation of pAVE049 was pAVE017. The tac promoter cassette was not altered. To increase the spacing between the two operators

from 91 to 124 base pairs, an EcoR I linker was cloned in. This was made up of oligonucleotides 19 and 20.

Oligonucleotide 19 (SEQ ID NO 15)
5'AATTCACCGGTGTACAGTCATGTACAACCGGTG

Oligonucleotide 20 (SEQ ID NO 16)
5'AATTCACCGGTTGTACATGACTGTACACCGGTG

Initial screening was by restriction digest of plasmid DNA. The sequence was then confirmed by sequencing. The resultant plasmid was named pAVE049.

Vector pAVE046

The starting vector for the generation of secretion vector pAVE046 was pAVE027. A D1.3 Fab expression cassette (Figure 1, SEQ ID NO 17) was cloned as an Nde I-Bam HI fragment. Initial screening was by restriction digest of plasmid DNA. The sequence was then confirmed by sequencing. The resultant plasmid was named pAVE046.

Table 1: Summary of pAVE vectors

Plasmid	Promoter	Operator System	Comments
pAVE041	tac	Single native lac sequence	
pAVE017	tac	Double perfect palindrome sequences (DPPS)	Operator spacing 91 base pairs (DPPS91)
pAVE040	tac	Single perfect palindrome sequence (SPPS)	
pAVE049	tac	Double perfect palindrome sequences	Operator spacing 124 base pairs (DPPS124)
pAVE013	T7A3	Double perfect palindrome sequences	Operator spacing 91 base pairs (DPPS91)
pAVE030	T7A3	Double perfect palindrome sequences	Operator spacing 92 base pairs (DPPS92)
pAVE031	T7A3	Single perfect palindrome sequence	
pAVE021	λpL	Double perfect palindrome sequences	Operator spacing 91 base pairs (DPPS91)
pAVE035	λpL	Double perfect palindrome sequences	Operator spacing 92 base pairs (DPPS92)
pAVE027	λpL	Single perfect palindrome sequence	
pAVE046	λpL	Single perfect palindrome sequence	Secretion Vector

2. Generation of recombinant strains

E.coli strains W3110 (available from the American Type Culture Collection as strain ATCC27325) and BL21 (available from EMD Biosciences Inc, San Diego, USA) were transformed by electroporation with the plasmids as described in Table 2 below. The resultant recombinant strains were purified and maintained in glycerol stocks at -80°C.

Table 2: Recombinant strains constructed

Host	Plasmid	Description (protein:promoter:operator system)	Recombinant Designation No
ATCC27325	pAVE013	TNFα:T7A3:DPPS91	CLD018
ATCC27325	pAVE030	TNFα:T7A3:DPPS92	CLD026
ATCC27325	pAVE031	TNFα:T7A3:SPPS	CLD032
ATCC27325	pAVE041	TNFα:tac:single native lacO	CLD043
ATCC27325	pAVE017	TNFα:tac:DPPS91	CLD019
ATCC27325	pAVE040	TNFα:tac:SPPS	CLD042
ATCC27325	pAVE049	TNFα:tac:DPPS124	CLD050
ATCC27325	pAVE021	TNFα:λpL:DPPS91	CLD021
ATCC27325	pAVE035	TNFα:λpL:DPPS92	CLD038
ATCC27325	pAVE027	TNFα:λpL:SPPS	CLD030
BL21	pAVE013	TNFα:T7A3:DPPS91	CLD035
BL21	pAVE030	TNFα:T7A3:DPPS92	CLD028
ATCC27325	pAVE046	D1.3 Fab:λpL:SPPS	CLD048

Comparison 1

The starting vector for the generation of a plasmid with the T7A3 promoter without any operator was pZT7#2.0. A T7A3 promoter was cloned into this plasmid using synthetic oligonucleotide linker by means of the EcoR I and Xba I restriction enzyme sites.

Linker 2122 was made by annealing the oligonucleotides 21 and 22

Oligonucleotide 21 (SEQ ID NO 18)
5'AATTCGAAACAAAACGGTTGACAACATGAAGTAAACACGGTACGATGTACCACATG
AAACGACAGTGAGTCA

Oligonucleotide 22 (SEQ ID NO 19)
5'CTAGTGACTCACTGTCGTTTCATGTGGTACCTCGTACCGTGTTTACTTCATGTTGTC
AACCGTTTTGTTTCG

The linker was then ligated to plasmid pZT7#2.0 and transformed into cloning host strain XL-1 Blue MR (Stratagene) as an Xba I/EcoR I fragment. Initial screening was by restriction digest of plasmid DNA. The sequence was then confirmed by sequencing. Eighty-two clones were screened by restriction digest and sequencing.

No clones were identified with the correct T7A3 promoter sequence (all contained mutations in the sequence). This suggests that construction of plasmids containing this powerful constitutive promoter is problematic.

Comparison 2

The starting vector for the generation of a plasmid with the T7A3 promoter under the control of a single native Lac operator sequence was pZT7#2.0. A T7A3 promoter and native Lac operator (LacO) sequence was cloned into this plasmid using synthetic oligonucleotide linker by means of the EcoR I and Xba I restriction enzyme sites.

Linker 2324 was made by annealing the oligonucleotides 23 and 24

Oligonucleotide 23 (SEQ ID NO 20)
5'AATTCGAAACAAAACGGTTGACAACATGAAGTAAACACGGTACGATGTACCGGAAT
TGTGAGCGGATAACAATTCCCCA

Oligonucleotide 24 (SEQ ID NO 21)
5'CTAGTGGGGAATTGTTATCCGCTCACAATTCCGGTACATCGTACCGTGTTTACTTCA
TGTTGTCAACCGTTTTGTTTCG

The linker was then ligated to plasmid pZT7#2.0 and transformed into cloning host strain XL-1 Blue MR (Stratagene) as an Xba I/EcoR I fragment. Initial screening was by restriction digest of plasmid DNA. The sequence was then confirmed by sequencing. Ninety-four clones were screened by restriction digestion and sequencing. Again no clones were identified with the correct sequence. However, one clone was found to have a near intact sequence. This clone contained an additional 'G' in the sequence approximately at position -37. It is difficult to assign exact position of the mutation since the expected sequence contains –GG- in this region. Human TNFα gene was cloned into the plasmid with the near intact sequence as an Nde I/Xho I fragment. Twenty colonies from the cloning host strain XL-Blue MR (Stratagene) were screened. One was positive clone with no mutations (other than the additional 'G' described above). This plasmid was transformed into a production host (ATCC27325) and the plasmid re-sequenced.

This indicated that the plasmid contained gross mutations in both the T7A3 promoter and the human TNFα sequences indicating that the use of the T7A3 promoter, even under the control of the native lac operator sequence, results in plasmid instability.

Example 3

A vial of CLD032 was removed from the –80°C freezer and allowed to thaw. 10µl of the thawed glycerol stock was inoculated into 5ml Luria Broth (LB, 5g/L yeast extract (Oxoid), 10g/L tryptone (Oxoid), and 5g/L sodium chloride) supplemented with tetracycline (10µg/ml) and glucose (1g/L). This was incubated at 37°C in an orbital shaker for 16h. 500µl of this culture was then used to inoculate two 250ml Erlenmeyer flasks containing 50ml of Luria Broth (composition as described above). The flasks were incubated at 37°C, at 200rpm in an orbital shaker. Growth was monitored until OD_{600}=0.5-0.7. At this point one flask was induced with IPTG (isopropyl-.β.-D-1-thiogalactopyranoside) to a final concentration 0.05mM whilst the second flask was left un-induced to monitor basal expression. The incubation was continued, under the conditions described above, during which samples were taken for measurement of growth, accumulation of hTNFα within the bacterial cells. The accumulation level of hTNFα was determined using densitometry scanning of Colloidal Blue stained SDS-PAGE gels of whole cell lysates of the sampled bacteria. The results are summarised below in Table 3.

Table 3

Time (hours)	Accumulation Level of hTNFα (%TCP*)
3	2
4	18
6	25
8	33
24	42
24 (basal, no IPTG)	13

(*): TCP = Total Cell Protein

Taken together the data presented in Comparisons 1 and 2, and Example 3, show that effective control of the powerful T7A3 promoter was surprisingly achieved using a single perfect palindrome operator sequence. This was totally un-expected given that the use of the single native operator (Comparison 2) did not provide sufficient basal control to allow a stable recombinant production strain to be established. High product accumulation levels were achieved with the single perfect palindrome control system using relatively low concentration of inducer for induction. Although basal expression (in the absence of inducer) was observed it was evident only after significantly extended incubation (24h).

Example 4

Vials of CLD018 was removed from the –80°C freezer and allowed to thaw. 10µl of the thawed glycerol stock was inoculated into 5ml Luria Broth (LB, 5g/L yeast extract (Oxoid), 10g/L tryptone (Oxoid), and 5g/L sodium chloride) supplemented with tetracycline (10µg/ml) and glucose (1g/L). The seed culture was incubated at 37°C in an orbital shaker for 16h. 500µl of the seed culture was then used to inoculate 250ml Erlenmeyer flasks containing 50ml of Luria Broth (composition as described above). The flasks were incubated at 37°C, at 200rpm in an orbital shaker. Growth was monitored until OD_{600}=0.5-0.7. At this point flasks were induced with IPTG (isopropyl-.β.-D-1-thiogalactopyranoside) to a final concentration 0.05mM and 1mM. A flask was also left un-induced and the incubation of the flasks continued, under the conditions described above, during which samples were taken for measurement of growth, accumulation of hTNFα within the bacterial cells. The accumulation level of hTNFα was determined using densitometry scanning of Colloidal Blue stained SDS-PAGE gels of whole cell lysates of the sampled bacteria. The results are summarised below in Table 4.

Table 4

0.05mM IPTG Time (hours)	Accumulation Level of hTNFα (%TCP)
3	2
4	5
6	8
8	13
24	19
24 (basal, no IPTG)	Not detected

1mM IPTG Time (hours)	Accumulation Level of hTNFα (%TCP)
5	7
6	12
8	19
24	26

This data demonstrated that further control of the powerful T7A3 promoter could be realised using two perfect palindrome operator sequences spaced at 91 bp apart. Basal expression (in the absence of inducer) has been reduced significantly from that achieved using a single perfect palindrome operator to control repression. The control of basal expression achieved using the dual perfect palindrome sequences was un-expected when compared to the T7 system of US 6,537,779 where control of basal expression requires two different control elements. In this example control of basal expression was achieved in a high background of *E.coli* RNA polymerase.

Example 5

Vials of CLD026 was removed from the –80°C freezer and allowed to thaw. 10µl of the thawed glycerol stock was inoculated into 5ml Luria Broth (LB, 5g/L yeast extract (Oxoid), 10g/L tryptone (Oxoid), and 5g/L sodium chloride) supplemented with tetracycline (10µg/ml) and glucose (1g/L). This was incubated at 37°C in an orbital shaker for 16h. 500µl of this culture was then used to inoculate 250ml Erlenmeyer flasks containing 50ml of Luria Broth (composition as described above). The flasks were incubated at 37°C, at 200rpm in an orbital shaker. Growth was monitored until OD_{600}=0.5-0.7. At this point flasks were induced with IPTG (isopropyl-.β.-D-1-thiogalactopyranoside) to a final concentration 0.05mM and 0.005mM. A flask was also left un-induced and the incubation continued, under the conditions described above, during which samples were taken for measurement of growth, accumulation of hTNFα within the bacterial cells. The accumulation level of hTNFα was determined using densitometry scanning of Colloidal Blue stained SDS-PAGE gels of whole cell lysates of the sampled bacteria. The results are summarised below in Table 5.

Table 5

0.005mM IPTG induction Time (hours)	Accumulation Level of hTNFα (%TCP)
8	15
24 (basal, no IPTG)	Not detected

0.05mM IPTG induction Time (hours)	Accumulation Level of hTNFα (%TCP)
8	17

The results demonstrated that changing the spacing between the two perfect palindrome operator sequences by 1bp (from 91 to 92 bp) did not adversely influence performance both in terms of basal expression and final accumulation level achieved. Unexpectedly, reducing the IPTG concentration 10 fold (from 0.05mM to 0.005mM) did not significantly reduce induced productivity.

Example 6

Vials of CLD042 and CLD043 were removed from the –80°C freezer and allowed to thaw. 10µl of each of the thawed glycerol stock was inoculated separately into each of 2x5ml Luria Broth (LB, 5g/L yeast extract (Oxoid), 10g/L tryptone (Oxoid), and 5g/L sodium chloride) supplemented with tetracycline (10µg/ml) and glucose (1g/L). These were incubated at 37°C in an orbital shaker for 16h. 500µl of these cultures were then used to separately inoculate 250ml Erlenmeyer flasks containing 50ml of Luria Broth (composition as described above). The flasks were incubated at 37°C, at 200rpm in an

orbital shaker. Growth was monitored until OD_{600}=0.5-0.7. At this point flasks were induced with IPTG (isopropyl-.β.-D-1-thiogalactopyranoside) to a final concentration 0.5mM. Flasks containing a culture of each strain were also left un-induced and the incubation continued, under the conditions described above, during which samples were taken for measurement of growth, accumulation of hTNFα within the bacterial cells. The accumulation level of hTNFα was determined using densitometry scanning of Colloidal Blue stained SDS-PAGE gels of whole cell lysates of the sampled bacteria. The basal accumulation level of hTNFα in the un-induced cultures of CLD042 and CLD043 after 20 hours incubation was compared by Western blot analysis (using anti- hTNFα antibody) following SDS-PAGE of the sampled bacteria. The blots were scanned and the data normalised to enable comparison. The results are summarised below in Table 6.

Table 6

CLD043: tac promoter, single native lac operator - 0.5mM IPTG induction	
Time (hours)	**Accumulation Level of hTNFα (%TCP)**
3	6
12	23
20	25
Western Blot: scan intensity*	
20 (Basal, no IPTG)	1

CLD042: tac promoter, single perfect palindrome operator - 0.5mM IPTG induction	
Time (hours)	**Accumulation Level of hTNFα (%TCP)**
3	2
12	18
20	21
Western Blot: scan intensity*	
20 (Basal, no IPTG)	0.25

(*) = scan of hTNFα band on Western blot. Intensity scan data for CLD042 normalised against the intensity scan data for CLD043.

The results demonstrated that the single perfect palindrome operator sequence can be used to reduce basal expression (in the absence of inducer) four fold without adversely influencing the induced productivity of the tac promoter system.

Example 7

A vial of CLD019 was removed from the –80°C freezer and allowed to thaw. 10µl of the thawed glycerol stock was inoculated into 5ml Luria Broth (LB, 5g/L yeast extract (Oxoid), 10g/L tryptone (Oxoid), and 5g/L sodium chloride) supplemented with tetracycline (10µg/ml) and glucose (1g/L). This was incubated at 37°C in an orbital shaker for 16h.

500µl of this culture was then used to inoculate 250ml Erlenmeyer flasks containing 50ml of Luria Broth (composition as described above). The flasks were incubated at 37°C, at 200rpm in an orbital shaker. Growth was monitored until OD_{600}=0.5-0.7. At this point the flasks were induced with IPTG (isopropyl-.β.-D-1-thiogalactopyranoside) to a final concentration 0.5mM, 0.1mM, 0.05mM and 0.005mM. A flask was also left un-induced and the incubation continued, under the conditions described above, during which samples were taken for measurement of growth, and accumulation of hTNFα within the bacterial cells. The accumulation level of hTNFα was determined using densitometry scanning of Colloidal Blue stained SDS-PAGE gels of whole cell lysates of the sampled bacteria. The results are presented in Figure 2.

The data presented in Figure 2 demonstrated that the combination of the tac promoter with dual perfect palindrome operator sequences lead to a system in which the expression rate can be modulated directly by the concentration of IPTG used for induction. Such systems may be exploited to modulate expression of heterologous proteins, for example, to maximise accumulation of proteins in a soluble form or to circumvent the problem of the deleterious effect that heterologous protein secretion can have on the growth and productivity of recombinant cells.

Example 8

A vial of CLD030 was removed from the –80°C freezer and allowed to thaw. 10µl of the thawed glycerol stock was inoculated into 5ml Luria Broth (LB, 5g/L yeast extract (Oxoid), 10g/L tryptone (Oxoid), and 5g/L sodium chloride) supplemented with tetracycline (10µg/ml) and glucose (1g/L). This was incubated at 37°C in an orbital shaker for 16h. 500µl of this culture was then used to inoculate 250ml Erlenmeyer flasks containing 50ml of Luria Broth (composition as described above). The flasks were incubated at 37°C, at 200rpm in an orbital shaker. Growth was monitored until OD_{600}=0.5-0.7. At this point a flask was induced with IPTG (isopropyl-.β.-D-1-thiogalactopyranoside) to a final concentration 0.05mM whilst the other flask was left un-induced and the incubation continued, under the conditions described above, during which samples were taken for measurement of growth, accumulation of hTNFα within the bacterial cells. The accumulation level of hTNFα was determined using densitometry scanning of Colloidal Blue stained SDS-PAGE gels of whole cell lysates of the sampled bacteria. The results are summarised below in Table 7.

Table 7

Time (hours)	Accumulation Level of hTNFα (%TCP)
4	2
6	5
8	9
24	12
24 (basal, no IPTG)	Not detected

The data presented in Table 7 clearly show that control of the very powerful λpL promoter can be surprisingly achieved using a single perfect palindrome operator sequence. High product accumulation levels can be achieved using the single perfect palindrome control system.

Example 9

Vials of CLD021 and CLD038 were removed from the –80°C freezer and allowed to thaw. 10µl of each of the thawed glycerol stock was inoculated separately into 5ml Luria Broth (LB, 5g/L yeast extract (Oxoid), 10g/L tryptone (Oxoid), and 5g/L sodium chloride) supplemented with tetracycline (10µg/ml) and glucose (1g/L). These were incubated at 37°C in an orbital shaker for 16h. 500µl of this culture was then used to inoculate 250ml Erlenmeyer flasks containing 50ml of Luria Broth (composition as described above). The flasks were incubated at 37°C, at 200rpm in an orbital shaker. Growth was monitored until OD_{600}=0.5-0.7. At this point a flask was induced with IPTG (isopropyl-.β.-D-1-thiogalactopyranoside) to a final concentration 1mM whilst a second flask was left un-induced and the incubation continued, under the conditions described above, during which samples were taken for measurement of growth, accumulation of hTNFα within the bacterial cells. The accumulation of hTNFα was determined using Colloidal Blue stained SDS-PAGE gels and Western blot analysis (using anti- hTNFα antibody) following SDS-PAGE of whole cell lysates of the sampled bacteria. The data are summarised in Table 8. The Western blot analysis for strain CLD038 is presented in Figure 3.

Table 8

Analysis	hTNFα Accumulation - CLD021 (λpL:DPPS91)	hTNFα Accumulation -CLD038 (λpL:DPPS92)
Colloidal Blue SDS-PAGE (post IPTG induction)	Not detected	Not detected
Western blot (post IPTG induction)	Positive	Positive (see Figure 2)
Colloidal Blue SDS-PAGE (Basal no IPTG induction, 24h)	Not detected	Not detected
Western blot (Basal no IPTG induction, 24h)	Not detected	Not detected

These results demonstrated that the combination of dual perfect palindrome operator sequences with the λpL promoter with either the 91bp or 92bp spacing resulted in very tight repression. Western blots indicate that no basal expression of the target protein was detected. On induction low-level expression level was achieved. These results were totally unexpected given that the λpL promoter is an extremely powerful promoter. Such a system may, for example, be used to direct the expression of proteins of high toxicity to the host cell. It can be used when controlled expression is advantageous, for example, for the expression and insertion of membrane proteins.

Example 10

Vials of CLD028 and CLD035 were removed from the –80°C freezer and allowed to thaw. 10µl of each of the thawed glycerol stock was inoculated separately into each of 2x5ml Luria Broth (LB, 5g/L yeast extract (Oxoid), 10g/L tryptone (Oxoid), and 5g/L sodium chloride) supplemented with tetracycline (10µg/ml) and glucose (1g/L). These were incubated at 37°C in an orbital shaker for 16h. 500µl of these cultures were then used to separately inoculate 250ml Erlenmeyer flasks containing 50ml of Luria Broth (composition as described above). The flasks were incubated at 37°C, at 200rpm in an orbital shaker. Growth was monitored until OD_{600}=0.5-0.7. At this point flasks were induced with IPTG (isopropyl-.β.-D-1-thiogalactopyranoside) to a final concentration 1mM

and the incubation continued, under the conditions described above, during which samples were taken for measurement of growth, accumulation of hTNFα within the bacterial cells. The accumulation level of hTNFα was determined using densitometry scanning of Colloidal Blue stained SDS-PAGE gels of whole cell lysates of the sampled bacteria. The results are summarised below in Table 9.

Table 9

CLD035: T7A3 promoter, dual perfect palindrome operators with 91bp spacing	
Time (hours) post IPTG induction	**Accumulation Level of hTNFα (%TCP)**
2	7
4	14
20	27

CLD028: T7A3 promoter, dual perfect palindrome operators with 92bp spacing	
Time (hours) post IPTG induction	**Accumulation Level of hTNFα (%TCP)**
2	10
4	15
20	23

These data taken together with the data presented in Examples 4 and 5 previously indicated that both *E.coli* K-12 and B strains can be used.

Example 11

Fermentation inocula were raised by adding 200µl of glycerol stock of each of the strains described below to a 2.0L baffled shake flask containing 200mL of Luria Broth (LB, 5g/L yeast extract (Oxoid), 10g/L tryptone (Oxoid), and 5g/L sodium chloride) supplemented with 15µg/ml of tetracycline. Inocula were grown for 12h at 37°C in a shaker-incubator with an agitation of 250rpm. 200ml shake flask inoculum was used to inoculate a 15L working volume fermenter containing 10L of batch growth medium. Fermentations were carried out under the operating conditions described below. Temperature was controlled at 37°C and pH at 6.8, controlled by automatic addition of 35% (w/v) ammonium hydroxide. The dissolved oxygen tension (dOT) set point was 30% of air saturation and was controlled by automatic adjustment of the fermenter stirrer speed, from a minimum of 250rpm up to a maximum of 1500rpm, and automatic supplementation of oxygen to the inlet gas stream. Airflow to the fermenter vessel was 10 L/min throughout. Pressure in the fermenter was maintained between 50 and 200mbar.

Fermentations were performed in batch mode until depletion of the carbon source (i.e. glycerol) which occurred ca. 10h post inoculation and was characterized by a sharp

rise in dOT. Fed-batch fermentation was initiated at the point of carbon source exhaustion by the addition of a glycerol / magnesium chloride feed at a feed rate of 11g of glycerol per L of medium per h. Induction was carried out by addition of IPTG to a final concentration of 0.5mM once the biomass level in the fermentation reached OD_{600} = 50-60. The fed-batch phase was continued for 12h post induction. Samples were taken to determine biomass level (OD_{600}) and hTNFα accumulation (%TCP)/ hTNFα titre (g/L) at harvest (Colloidal Blue stained SDS-PAGE gels).

The composition of the batch growth medium is provided in Table 10.

Table 10

Component	Final concentration [g/L], mg/L] and [ml/L] of purified water
$(NH_4)_2SO_4$	14.0
Glycerol	35.0
Yeast extract (Becton Dickinson)	20.0
KH_2PO_4	2.0
K_2HPO_4	16.5
Citric acid	7.5
$MgSO_4.7H_2O$	2.47
H_3PO_4	1.5 ml/L
$CaCl_2.2H_2O$	0.294
Antifoam AF204	0.2 ml/L
Tetracycline	15 mg/L
$FeSO_4.7H_2O$	114 mg/L
$ZnSO_4.7H_20$	29 mg/L
$MnSO_4.H_20$	17 mg/L
$Na_2MoO_4.2H_2O$	9 mg/L
$CuSO_4.5H_2O$	4 mg/L
$H_3.BO_3$	12 mg/L

The composition of the glycerol / magnesium chloride feed is provided in Table 11.

Table 11

Component of Feed	Amount required [g/L] of purified water
Glycerol	714
$MgSO_4.7H_2O$	7.4

The results are summarised in Table 12. The hTNFα productivity profile for Strain CLD030 is presented in Figure 4.

Table 12

Strain	Expression vector description	OD600 at harvest	hTNFα accumulation (%TCP) at harvest	hTNFα titre (mg/L) at harvest
CLD018	T7A3 promoter, dual perfect palindrome with 91bp spacing	147	29	8400
CLD026	T7A3 promoter, dual perfect palindrome with 92bp spacing	204	34	11400
CLD032	T7A3 promoter, single perfect palindrome sequence	194	41	12500
CLD019	tac promoter, dual perfect palindrome sequence with 91bp spacing	196	22	8300
CLD030	λpL promoter with single perfect palindrome sequence	167	7	2600

The data clearly demonstrate the utility of the systems for the manufacture of heterologous proteins. High product titres were achieved using a simple generic un-optimised fermentation and induction processes. The control characteristics of plasmid pAVE027, as demonstrated by productivity profile exemplified in Figure 4, can be exploited to maximize the production of heterologous proteins, particularly proteins that require control of expression to maximize secretion.

Example 12

A vial of CLD050 was removed from the –80°C freezer and allowed to thaw. 10µl of the thawed glycerol stock was inoculated into 5ml Luria Broth (LB, 5g/L yeast extract (Oxoid), 10g/L tryptone (Oxoid), and 5g/L sodium chloride) supplemented with tetracycline (10µg/ml) and glucose (1g/L). This was incubated at 37°C in an orbital shaker for 16h. 500µl of this culture was then used to inoculate 250ml Erlenmeyer flasks containing 50ml of Luria Broth (composition as described above). The flasks were incubated at 37°C, at 200rpm in an orbital shaker. Growth was monitored until OD_{600}=0.5-0.7. At this point a flask was induced with IPTG (isopropyl-.β.-D-1-thiogalactopyranoside) to a final concentration 0.05mM whilst another flask was left uninduced and the incubation continued, under the conditions described above, during which samples were taken for measurement of growth, accumulation of hTNFα within the bacterial cells. The accumulation level of hTNFα was determined using densitometry scanning of Colloidal Blue stained SDS-PAGE gels of whole cell lysates of the sampled bacteria. The results are summarised below in Table 13.

Table 13

Time post induction (hours)	Accumulation Level of hTNFα (%TCP)
4	16
24 (basal, no IPTG)	Not detected

Surprisingly the dual perfect palindrome operator sequence worked when the spacing was increased. The spacing of the dual perfect palindrome can be altered, for example, to achieve effective control of other promoters.

Example 13

A vial of CLD048 was removed from the –80°C freezer and allowed to thaw. 10µl of the thawed glycerol stock was inoculated into 5ml Luria Broth (LB, 5g/L yeast extract (Oxoid), 10g/L tryptone (Oxoid), and 5g/L sodium chloride) supplemented with tetracycline (10µg/ml) and glucose (1g/L). This was incubated at 37°C in an orbital shaker for 16h. 500µl of this culture was then used to inoculate a 250ml Erlenmeyer flask containing 50ml of Luria Broth (composition as described above). The flask was incubated at 37°C, at 200rpm in an orbital shaker. Growth was monitored until OD_{600}=0.5-0.7. At this point the flask was induced with IPTG (isopropyl-.β.-D-1-thiogalactopyranoside) to a final concentration of 0.1mM and the incubation continued, under the conditions described above for a further 2h. The cells and residual cell free growth medium were then harvested. The harvested cells were further subjected to osmotic shock cell fractionation to isolate the cellular fraction containing proteins that had partitioned in the soluble *E. coli* periplasmic fraction. The accumulation of biologically active D1.3 Fab in the soluble

periplasmic extract and residual growth medium was estimated by determining the binding of D1.3 Fab to lysoszyme (antigen) in an ELISA assay by reference to a standard curve prepared with purified active D1.3 Fab. The accumulation of biologically active D1.3 Fab in the periplasm of *E.coli* and in the residual growth medium (due to leakage of material from the periplasm to the growth medium) is presented in Table 14. The accumulation of D1.3 Fab in the periplasm and residual growth medium was normalised as "µg active material per litre of culture per unit of biomass (OD_{600}).

Table 14

Fraction	Biologically active D1.3 Fab (µg/L culture/OD)
Residual growth medium	460
Periplasm	4020
Total (residual growth medium + periplasm)	4480

The utility of the control provided by this system to enable high level secretion of heterologous proteins particulary those requiring complex disulphide bond formation is clearly exemplified by the secretion and accumulation of high levels of biologically active D1.3 Fab in the periplasm of *E.coli*. Additionally, it will be evident to those skilled in the art how fed-batch fermentation (for example, as described previously in Example 11 or below in Example 14) can be used to manufacture such proteins at high yield.

Example 14

The fermentation process described in Example 11 was repeated using CLD048. Induction was carried out by addition of IPTG to a final concentration of 0.15mM once the biomass level in the fermentation reached OD_{600} = ca. 50. The fed-batch phase was continued for 35-45h post induction. The cells and residual cell free growth medium were then harvested. The harvested cells were further subjected to osmotic shock cell fractionation to isolate the cellular fraction containing proteins that had partitioned in the soluble *E. coli* periplasmic fraction. The accumulation of biologically active D1.3 Fab in the soluble periplasmic extract and residual growth medium was estimated by determining the binding of D1.3 Fab to lysoszyme (antigen) in an ELISA assay by reference to a standard curve prepared with purified active D1.3 Fab. The accumulation of D1.3 Fab in the periplasm and residual growth medium was normalised as "mg active material per litre of culture".

The accumulation of biologically active D1.3 Fab in the periplasm of *E.coli* and in the residual growth medium (due to leakage of material from the periplasm to the growth medium) is presented in Table 15.

Table 15

Fraction	Biologically active D1.3 Fab (mg/L culture)
Residual growth medium	525
Periplasm	57
Total (residual growth medium + periplasm)	582

High level secretion of biologically active D1.3 Fab is demonstrated using the expression system.

Example 15

A synthetic bispecific single chain tetravalent diabody (bsctDb) was designed, in which the variable light and variable heavy regions from D1.3 (anti-lysozyme) and A5B7 (anti-CEA (carcinoembryonic antigen)), were linked on a single polypeptide chain. The DNA sequence for this molecule is shown in Figure 5 (SEQ ID NO 22). This was cloned as an Nde I/Not I fragment into pAVE046 which had been digested with Nde I and Not I. Recombinant plasmids were screened by restriction digest and confirmed by sequencing. The resultant plasmid was named pAVE078. pAVE078 was transformed into *E. coli* W3110 to make CLD073, which was purified and maintained in glycerol stocks at -80°C.

A vial of CLD0073 was removed from the –80°C freezer and allowed to thaw. 10µl of the thawed glycerol stock was inoculated into 5ml Luria Broth (LB, 5g/L yeast extract (Oxoid), 10g/L tryptone (Oxoid), and 5g/L sodium chloride) supplemented with tetracycline (10µg/ml) and glucose (1g/L). This was incubated at 37°C in an orbital shaker for 16h. 500µl of this culture was then used to inoculate two 250ml Erlenmeyer flasks containing 50ml of Luria Broth (composition as described above). The flasks were incubated at 37°C, at 200rpm in an orbital shaker. Growth was monitored until OD_{600}=0.5-0.7. At this point the flasks were induced with IPTG to a final concentration of either 0.5mM or 0.1mM and the incubation continued, under the conditions described above for a further 20 hours. The cells and residual cell free growth medium were then harvested. The harvested cells were further subjected to osmotic shock cell fractionation to isolate the cellular fraction containing proteins that had partitioned in the soluble *E. coli* periplasmic fraction. The expression, secretion, folding and accumulation of biologically active D1.3-A5B7 bsctDb in the periplasmic extract and residual growth medium was estimated by determining the inhibition of binding of an anti-CEA monoclonal antibody to CEA (antigen) in a competitive ELISA assay and by the binding of an anti-lysozyme Fab antibody fragment to lysozyme (antigen) in a competitive ELISA assay.

The data obtained indicated that the majority of D1.3-A5B7 bsctDb partitioned in the residual growth medium (leakage from the periplasm) at the end of the induction.

This data (binding of bsctDb in competitive ELISA) is shown in Table 16. The data obtained demonstrates that the residual growth medium sample from the culture induced with 0.5mM IPTG completely inhibits the binding of both the anti-CEA and anti-lysozyme antibodies in the competition ELISA assays. The residual growth medium sample from the culture induced with 0.1mM IPTG shows a reduced level of inhibition indicating a lower accumulation level of biologically active D1.3-A5B7 bsctDb in this sample.

Table 16

Sample	% Inhibition in CEA Competition ELISA	% Inhibition in D1.3 Competition ELISA
Control (No D1.3-A5B7 bsctDb)	None	None
Supernatant from culture induced with 0.5 mM IPTG	100	100
Supernatant from culture induced with 0.1 mM IPTG	Partial	Partial

Using the new expression system it is possible to produce complex multi-chain heterologous proteins which have been difficult to produce using *E.coli*. This has been exemplified by demonstrating that bispecific single chain tetravalent diabodies in a biologically active form can be produced in *E.coli* using the new expression system. This further exemplifies the utility of the expression system.

Example 16

The glutathione-S-transferase-3C proteinase fusion (GST-3C) gene was cloned as an Nde I/Xho I fragment into pAVE011 digested with Nde I and Xho I. The sequence of the insert is shown in Figure 6 (SEQ ID NO 23). Recombinant plasmids were screened by restriction digest and confirmed by sequencing. The resultant plasmid was named pAVE052. pAVE052 was transformed into *E.coli* BL21 to make CLD054, which was purified and maintained in glycerol stocks at -80°C.

The human Interferon α2 (IFNα2) gene was cloned as an Nde I/Xho I fragment into pAVE011 digested with Nde I and Xho I. The DNA sequence of the insert is shown in Figure 7 (SEQ ID NO 24). Recombinant plasmids were screened by restriction digest and confirmed by sequencing. The resultant plasmid was named pAVE058. pAVE058 was transformed into *E. coli* W3110 to make CLD059, which was purified and maintained in glycerol stocks at -80°C.

The human erythropoietin (EPO) gene, which had been codon optimised for expression in *E coli*, was cloned as an Nde I/Xho I fragment into pAVE011 digested with Nde I and Xho I. The DNA sequence of the insert is shown in Figure 8 (SEQ ID NO 25). Recombinant plasmids were screened by restriction digest and confirmed by sequencing. The resultant plasmid was named pAVE061. pAVE061 was transformed into *E.coli* W3110 to make CLD060, which was purified and maintained in glycerol stocks at -80°C.

Fed-batch fermentations using CLD054, CLD059 and CLD060 were carried out using the media and process conditions described in Example 11. Fermentations were maintained at 30°C or 37°C as described in Table 19. Fermentations were performed in batch mode until depletion of the carbon source (i.e. glycerol). Fed-batch fermentation was initiated at this point by the addition of a feed containing glycerol (714g/L) and magnesium sulphate (30g/L). Induction was carried out by addition of IPTG once the biomass level in the fermentation reached OD_{600} = 50-60. The IPTG concentrations used are described in Table 17. The fed-batch phase was continued for 12-15h post induction. Samples were taken throughout the fermentations to determine biomass level (OD_{600}) and protein product ((GST-3C, IFNα2 and EPO) titre (g/L), using Colloidal Blue stained SDS-PAGE gels of whole cell lysates of the sampled bacteria).

Table 17

Strain	*E.coli* Host	Protein and Expression Vector Description	Ferm Temp °C	Induction IPTG Conc (mM)	OD600	Product Titre (g/L)
CLD 054	BL21	GST-3C T7A3:DPPS91	37	0.50	100	8
CLD 059	W3110	IFNα2 T7A3:DPPS91	37	0.10	120	9
			37	0.25	150	14
			37	0.50	160	14
CLD 060	W3110	EPO T7A3:DPPS91	37	0.10	100	>13
			30	0.50	90	>13

The data presented in Table 17 further demonstrate the utility of the systems for the manufacture of a wide range of heterologous proteins. High product titres are achieved using a simple generic fermentation process coupled with manipulation of only the

concentration of IPTG used for induction. This is particularly beneficial to reduce the process development timelines for therapeutically useful heterologous proteins.

Example 17

The L-2-haloalkanoate dehalogenase (hadL) gene from *Pseudomonas putida* was cloned using Nde I and Spe I sites that had been engineered using PCR. The gene sequence is shown in Figure 9 (SEQ ID NO 26). Plasmid pAVE011 was digested with Nde I and Spe I and the band was gel extracted. The hadL gene was digested with Nde I and Spe I and the hadL gene was gel extracted and ligated to pAVE011 to produce pAVE075. The *Pseudomonas savastanoi* origin of replication was copied using the PCR from Plasmid pCN60 (ATCC 77101; Nieto C, et al. (1990) Gene 87: 145-149).

The primers used were:

F37A: Sequence: 5' AGATCTACGCTTATGGGTGCCTTTCC (SEQ ID NO 27), and

B29a: Sequence: 5' AGATCTAATACGCAAACCGCCTCTCC (SEQ ID NO 28).

The PCR product was cloned initially into TOPO TA pCR2.1 (Invitrogen) and then into pAVE075 by Bgl II digestion. The resultant plasmid, pAVE086 was transformed into *Pseudomonas putida* NCIMB 12018, via electroporation to make CLD075, which was purified and maintained in glycerol stocks at -80°C. A vial of CLD075 was removed from a –80°C freezer and allowed to thaw. 10µl of the thawed glycerol stock was inoculated into 5ml Luria Broth (LB, 5g/L yeast extract (Oxoid), 10g/L tryptone (Oxoid), and 5g/L sodium chloride) supplemented with tetracycline (10µg/ml). This was incubated at 30°C in an orbital shaker for 16h. 500µl of this culture was then used to separately inoculate two 250ml Erlenmeyer flasks containing 50ml of Luria Broth (composition as described above). The flasks were incubated at 30°C, at 200rpm in an orbital shaker. Growth was monitored until OD_{600}=0.5-0.7. At this point one flask was induced with IPTG to a final concentration 0. 5mM whilst the second flask was left un-induced to monitor basal expression. The incubation was continued, under the conditions described above, during which samples were taken for measurement of growth and accumulation of HadL protein within the bacterial cells. The accumulation level of HadL was determined using densitometry scanning of Colloidal Blue stained SDS-PAGE gels of whole cell lysates of the sampled bacteria.

The expression and accumulation of HadL protein is presented in Figure 10. The data indicate that the T7A3:DPPS91 expression system functioned in another prokaryotic host system. Surprisingly, the expression system performed with the same efficiency in *Pseudomonas putida* as that observed when using *E.coli* as the host system. Basal expression was not detected even following 23h incubation in the absence of inducer. High level protein expression and accumulation was achieved in *Pseudomonas putida* following induction using IPTG.

Example 18

Fed-batch fermentation using *Pseudomonas putida* CLD075 was carried out using the generic *E.coli* media and process conditions described in Example 11. Fermentations were maintained at 30°C and pH 7.0 (controlled with 25% ammonium hydroxide and 10% phosphoric acid). Fermentations were performed in batch mode until depletion of the carbon source (i.e. glycerol). Fed-batch fermentation was initiated at this point by the addition of a feed containing glycerol (714g/L) and magnesium sulphate (30g/L). Induction was carried out by addition of 1mM IPTG (final concentration) once the biomass level in the fermentation reached OD_{600} = 50. The fed-batch phase was continued for 12-15h post induction. Samples were taken throughout the fermentation to determine biomass level (OD_{600}) and HadL protein accumulation ((%TCP) Colloidal Blue stained SDS-PAGE gels of whole cell lysates of the sampled bacteria). The growth of CLD075 and expression/accumulation of HadL protein following induction are presented in Figure 11.

High levels of protein expression and accumulation (>40% TCP) were achieved using the expression system in *Pseudomonas putida* even by just using a generic growth medium designed for use with *E.coli*.

Example 19

A synthetic Gal repressor gene (*E.coli*) was cloned into vector pZen042 (as described in EP 0 502 637) as a PstI fragment into the PstI site. Clones were identified with the Gal repressor gene in both clockwise and anticlockwise orientations. A clone with anticlockwise orientation was selected to generate pAVE071.

Construction of the Gal promoter and operator sequences was initiated in plasmid pZT7#2.0, prepared as described in US 6,537,779. pZT7#2.0 has a pAT153 vector backbone, cer stability sequence, tet A/R, a single native lac operator sequence upstream of the gene of interest and an upstream T4 transcription terminator. The native Gal operator sequence was modified to produce a perfect palindromic operator sequence. This was cloned into the plasmid described above using synthetic linkers by means of EcoRI and XbaI restriction enzyme sites. The linker GalB was prepared by annealing the oligonucleotides GalB1 and GalB2:

GalB1 (SEQ ID NO 29)
5'AATTCATACCATAAGCCTAATTCTACGAATTATCAGAGTTCTGGTTACCGGT
GTAAGCGCTTACACTGT

GalB2 (SEQ ID NO 30)
5'CTAGACAGTGTAAGCGCTTACACCGGTAACCAGAACTCTGATAATTCGTAGA
ATTAGGCTTATGGTATG

The linker was then ligated to plasmid pZT7#2.0 and transformed into cloning host strain XL-1 Blue MR (Stratagene) as an EcoR I/Xba I fragment. Initial screening of transformants was by restriction digestion using AgeI. The sequence was confirmed by sequencing. The hTNFα gene was cloned into this plasmid as a NdeI/XhoI fragment.

The hTNFα gene and partial Gal perfect palindromic operator sequence were cloned by digesting with XmaI and MscI and ligating into pAVE071 digested with XmnI and XmaI. Clones were screened for the presence of the hTNFα gene by restriction digestion.

Upstream perfect palindromic Gal operator and Gal promotor were each cloned into this plasmid using synthetic linkers by means of StuI and EcoRI sites. Linker GalA was prepared by annealing the oligonucleotides GalA1 and GalA2:

GalA1 (SEQ ID NO 31):
5'CAATTGTGTAAGCGCTTACACAACTTTATTCCATGTCACACTTTTCGCATCTTTGTTATGCTATGGTG

GalA2 (SEQ ID NO 32)
5'AATTCACCATCGCATAACAAGGATGCGAAAAGTGTGACATGGAATAAAGTTGTGTAAGCGCTTACACAATTG

The presence of the linker was detected with digestion with MfeI and confirmed by sequencing. This plasmid was transformed into *E.coli* strain W3110 to generate CLD085 , which was purified and maintained in glycerol stocks at -80˚C.

A vial of CLD085 was removed from the –80°C freezer and allowed to thaw. 10µl of the thawed glycerol stock was inoculated into 5ml Luria Broth (LB, 5g/L yeast extract (Oxoid), 10g/L tryptone (Oxoid), and 5g/L sodium chloride) supplemented with tetracycline (10µg/ml). This was incubated at 37°C in an orbital shaker for 16h. 500µl of this culture was then used to inoculate a 250ml Erlenmeyer flask containing 50ml of Luria Broth (composition as described above). The flask was incubated at 37°C, at 200rpm in an orbital shaker. Growth was monitored until OD_{600}=0.5-0.7. At this point the flask was induced with galactose to a final concentration 10.0mM. The incubation was continued, under the conditions described above, during which samples were taken for measurement of growth, accumulation of hTNFα within the bacterial cells. The accumulation level of hTNFα was determined using Western blot analysis (using anti- hTNFα antibody) following SDS-PAGE of the sampled bacteria. The data are presented in Figure 17. This demonstrates that using perfectly palindromic gal operator sequences in combination with a gal repressor gene leads to very tight repression of the gal promoter in the absence of inducer whilst surprisingly still maintaining the capacity for induction when the inducer galactose is added..

Example 20

A non-integrating yeast vector was constructed as follows:

1) Clone Sequence 1 (*E. coli* Lac I downstream of a *Saccharomyces cerevisiae* CYC1 promoter) as a Xho I fragment into Xho I digested pCR2.1 (Invitrogen). Clone Sequence 1 is shown in Figure 15 (SEQ ID NO 35).

2) Clone Sequence 2 (which consists of the *Saccharomyces cerevisiae* MF-α1 gene promoter with perfect palindromic lac operator sequences either side of the MF-α1 promoter region, with the gene sequence for the protein elafin with a C-terminal c-myc tag (elafin-cmyc) positioned downstream) as a Hind III fragment (made by PCR) into Hind III digested plasmid constructed in Step 1 to produce plasmid 2. Clone Sequence 2 is shown in Figure 16 (SEQ ID NO 36).

3) Clone the Spe I fragment from YEp13 (ATCC37115), containing the LEU2 (selection marker gene) and the yeast 2μ origin of replication, into SpeI digested plasmid 2 to generate pAVE091.

pAVE091 plasmid DNA was transformed into *Saccharomyces cerevisiae* XS95-6C (ATCC 204688) by electroporation and positive colonies selected on yeast drop-out medium without leucine (Kaiser C, Michaelis S and Mitchel A (Methods in Yeast Genetics, Cold Spring Harbor Laboratory Manual, 1994)). Shake flask growth studies to determine elafin-cmyc protein expression were carried out using the same medium. The flasks were incubated at 30°C, at 200rpm in an orbital shaker. The clones were grown to an OD of ~3 and induced with 0.5 mM IPTG (final concentration). The incubation was continued for a further 16h, under the conditions described above, during which samples were taken for measurement of growth and secretion of elafin-cmyc protein into the growth medium. The secretion of elafin-cmyc into the residual growth medium was determined using an elastase inhibition enzyme assay, as described in Wiedow O, et al, J Biol Chem. (1990) 265(25):14791-5. After 4 hours of IPTG induction there was 30 mg/L of active elafin protein in the growth medium. This demonstrates that the expression systems of the present invention are effective in yeasts.

Example 21

A DNA fragment was synthesised which contained the constitutive human Cytomegalovirus (hCMV) promoter flanked by dual perfect palindromic lac operator sequences. This was cloned into an expression vector, which expressed IgG Fc protein. The resulting plasmid was named pAVE081, and is derived from pCMV-Script (Stratagene) and contains the hCMV promoter flanked by dual perfect palindromic lac operator sequences on a Nde I/Nhe I fragment, with the IgG Fc DNA sequence in the multiple cloning site of the vector. The DNA sequence of the hCMV promoter and dual perfect palindromic lac operators is shown in Figure 12 (SEQ ID NO 33). The DNA sequence of the IgG Fc protein is shown in Figure 13 (SEQ ID NO 34). Transient co-

transfections of pAVE081 expressing IgG Fc protein and pCMVlacI (Stratagene) which expresses lac repressor were carried out, as is well described in the art, to determine whether IgG Fc protein could be expressed under the control an IPTG inducible hCMV promoter-dual perfect palindromic lac operator expression system.

2ml of Chinese Hamster Ovary (CHO cell line ECACC 85050302 adapted to suspension growth in serum free medium) suspension culture at 1.5 x 10^5 viable cells per ml was added to each well of 6-well tissue culture plates. The 6-well tissue culture plates were then incubated overnight (16h) in a humidified 37°C incubator with 5% CO_2 before transfection mixes were prepared containing 2µg of pAVE081 DNA with an equal quantity of pCMVlacI (Stratagene) DNA, 6µl of transfection reagent and 94µl of growth medium per well. 100µl of transfection mix was added to each well containing the CHO cells. The 6-well tissue culture plates were then incubated in humidified 37°C incubator with 5% CO_2. To determine the level of expression/secretion of IgG Fc protein into the growth medium a set of wells (day 2) were induced with 5 mM IPTG (final concentration) and set of wells left un-induced. On day three the set of wells induced with IPTG and those left un-induced were sampled (post IPTG induction and un-induced). The expression and secretion into the growth medium by the CHO cells of IgG Fc protein was determined by ELISA as is well established in the art. The data obtained are shown in Figure 14.

The data clearly demonstrates the broad utility of the expression system. The expression system can be used to control powerful constitutive promoters typically used with mammalian cell systems, such as the hCMV promoter, to express proteins in mammalian cells in a contollable, inducible manner.

Claims

1. A perfect palindrome operator sequence-based protein expression system comprising:
a) a promoter; and
b) a perfect palindrome operator sequence;
characterised in that the promoter is not T7.

2. A plasmid comprising:
a) a promoter; and
b) a perfect palindrome operator sequence;
characterised in that the promoter is not T7.

3. A plasmid according to claim 2, further comprising an expression cassette for a protein.

4. A plasmid according to claim 2 or claim 3, wherein the plasmid is an autonomously replicating plasmid.

5. A plasmid according to claim 2 or claim 3, wherein the plasmid is an integrative plasmid.

6. A host cell transformed by a plasmid as claimed in any one of claims 2 to 5.

7. A method for the production of a protein which comprises expressing an expression system comprising
a) a promoter;
b) a perfect palindrome operator sequence; and
c) an expression cassette for a protein;
characterised in that the promoter is not T7.

8. An expression system, plasmid, host cell or method according to any of claims 1 to 7, wherein the promoter is a host cell polymerase promoter.

9. An expression system, plasmid, host cell or method according to claim 8, wherein the promoter is an *E. coli* RNA polymerase promoter.

10. An expression system, plasmid, host cell or method according to any of claims 1 to 7, wherein the promoter is T7A1, T7A2, T7A3, λpL, λpR, lac, lacUV5, trp, tac, trc, phoA or rrnB.

11. An expression system, plasmid, host cell or method according to any of claims 1 to 10, wherein the operator system is lac, gal, deo or gln.

12. An expression system, plasmid, host cell or method according to any of claims 1 to 11, wherein two perfect palindrome operator sequences are employed, preferably one operator sequence being located downstream of the promoter, and one operator sequence being located upstream of the promoter.

13. An expression system, plasmid, host cell or method according to claim 12, wherein the operator sequences are spaced from 85 to 150 base pairs apart, preferably from 90 to 126 base pairs apart, and most preferably 91 or 92 base pairs apart.

14. A method for producing a protein, which comprises:
a) culturing a host cell transformed with a plasmid according to claim 3; and
b) recovering the protein.

15. A method according to claim 14, wherein the host cell is *E. coli.*

16. A method according to either of claims 14 and 15, wherein the plasmid is a plasmid as claimed in any one of claims 8 to 13.