Construction of an insertional–inactivation cloning vector for *Escherichia coli* using a *Rhodococcus* gene for indigo production

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pSLH8, an insertional–inactivation cloning vector for *Escherichia coli* has been constructed by inserting a pigment gene (probably encoding an indole dioxygenase) from *Rhodococcus* sp. ATCC 21145 into pUC18. Wild-type *E. coli* colonies containing pSLH8 produce insoluble indigo and turn dark blue on unsupplemented LB agar. Insertion of DNA fragments into the unique *Bam*HI, *Eco*RI, *Eco*RV, *Hind*III, *Pst*I, *Sph*I and *Sst*I polylinker cloning sites disrupts the reading frame of the fully sequenced pigment gene and results in unpigmented colonies. pSLH8 may be an attractive alternative to pUC18 and similar plasmids because it does not require specifically mutated host strains or an expensive substrate for colour development.

Introduction

Hill *et al.* (1989) discovered a DNA fragment from a *Rhodococcus* species which stimulated the production of an insoluble blue pigment in wild-type *Escherichia coli* strains. Hart *et al.* (1990) found that a single open reading frame encoding a hypothetical protein of 42560 Da was necessary and sufficient for pigment production. A database search identified no similar protein sequence and gave no clues about the possible enzymic activity specified by the cloned *Rhodococcus* gene. The pigment has been identified as indigo (blue) and a smaller amount of indirubin (red), both of which are probably derived from indole, a degradation product of tryptophan (Hart *et al.*, 1992). The cloned *Rhodococcus* gene probably encodes an indole dioxygenase.

Here we describe a cloning vector derived from pUC18 (Vieira & Messing, 1982) in which a modified 'indigo gene' replaces *lacZα* as the reporter gene.

Methods

Bacterial strains and plasmids. These are listed in Table 1. LB agar (Maniatis *et al.*, 1982) with ampicillin (Ap) (100 µg ml⁻¹) or chloramphenicol (Cm) (25 µg ml⁻¹) was used for all experiments. Blue to blue-grey pigment-producing *E. coli* colonies were observed after overnight incubation at 37 °C followed by 8–20 h incubation at room temperature. Addition of tryptophan or indole (0.5–1 mM) to the medium resulted in increased pigment production (Hart *et al.*, 1992).

Abbreviations: Ap, ampicillin; *bla*, β-lactamase; Cm, chloramphenicol.

Pigment production was also enhanced by the addition of lactose (0.2%, w/v) or IPTG (0.3 mM) and was repressed by the addition of glucose presumably because of catabolite repression of tryptophanase (Hart, 1991).

Preparation of plasmid DNA. Plasmid DNA was prepared from *E. coli* strains by the alkaline lysis method of Ish-Horowitz & Burke (1981). Large-scale plasmid preparations were purified by CsCl equilibrium gradient centrifugation (Maniatis *et al.*, 1982). Plasmid preparations for DNA sequencing were treated with RNAase A (Maniatis *et al.*, 1982) followed by a phenol/chloroform extraction and ethanol precipitation (Maniatis *et al.*, 1982).

Subcloning procedures. Restriction endonucleases (Boehringer-Mannheim, Anglian Biotechnology or BRL) were used as described by Maniatis *et al.* (1982). DNA fragments were gel-purified from low-melting-point agarose (Seaplaque) gels and separated from the agarose with 'GeneClean' (Bio 101) according to the manufacturer's instructions. Blunt ends were generated by filling in with T4 DNA polymerase I (Maniatis *et al.*, 1982). The DNA products of ligation reactions (Maniatis *et al.*, 1982) were transformed into competent cells (Chung & Miller, 1988). Transformants were selected on LB agar plates containing the appropriate antibiotic.

Oligonucleotide primers. Primers were obtained from Professor D. P. Botes (Department of Biochemistry, University of Cape Town, South Africa). A 17-mer synthetic primer with the sequence 5'-CGAAGGCCGTTACCAGG-3', complementary to nucleotide positions 849–832 at the 5'-end of the pigment gene (Hart *et al.*, 1990) was designed for nucleotide sequencing into the region upstream of the pigment gene to verify constructs from subcloning and mutagenesis procedures.

DNA sequencing. DNA sequencing was done by the dideoxy chain-terminating method of Sanger *et al.* (1977) with a 'Sequenase' kit (US Biochemicals Corp.) following the manufacturer's instructions. The DNA was radiolabelled with [³⁵S]dATP (>1000 Ci mmol⁻¹; Amersham).

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype*	Source or reference
<i>E. coli</i> 3.300	<i>lacI22 λ⁻ relA1 spoT1 thi-1</i>	Pardee <i>et al.</i> (1959)
<i>E. coli</i> LK111	<i>lacI lacZ ΔM15 lacY⁺</i>	Zabeau & Stanley (1982)
<i>E. coli</i> WK6	<i>Δ(lac-proAB) galE strA/F'laqI^{ts} lacZ ΔM15 proA⁺B⁺</i>	Stanssens <i>et al.</i> (1989)
<i>E. coli</i> WK6 mutS	<i>Δ(lac-proAB) galE strA mutS::Tn10/F'laqI^{ts} lacZ ΔM15 proA⁺B⁺</i>	Stanssens <i>et al.</i> (1989)
pKS3	Ap ^r Lac ⁻ Pig ⁺	Hart <i>et al.</i> (1990)
pKSmut	Ap ^r Lac ⁻ Pig ⁺	This study
pMa3	Ap ^r Cm ^s	This study
pMa5-8	Ap ^r Cm ^s	Stanssens <i>et al.</i> (1989)
pMc5-8	Ap ^s Cm ^r	Stanssens <i>et al.</i> (1989)
psLH1	Ap ^r Lac ⁻ Pig ⁺	This study
psLH4	Ap ^r Lac ⁻ Pig ⁺	This study
psLH8	Ap ^r Lac ⁻ Pig ⁺	This study
pSmu25	Ap ^s Cm ^r	This study

* Pig, pigment production.

Results and Discussion

Introduction of an *SphI* site at the start codon of the indigo gene by site-directed mutagenesis

This procedure was performed as described by Stanssens *et al.* (1989) using the phasmids pMa5-8 and pMc5-8 in a gapped-duplex approach to site-directed mutagenesis (Kramer *et al.*, 1984). pMa5-8 and pMc5-8 contain, in addition to a ColE1-type origin of replication, the f1 filamentous phage origin of replication. Superinfection with the helper phage makes it possible to package one specific strand of the phasmids into phage rods. Phasmid pMa5-8 confers resistance to Ap and contains an amber mutation in the *cat* gene. The complementary vector pMc5-8 confers resistance to Cm and contains an amber mutation in the β -lactamase (*bla*) gene. *E. coli* WK6 was used as a recipient for the phasmids and their derivatives. *E. coli* WK6mutS is a mismatch-repair-deficient strain that is unable to suppress amber mutations. Strains WK6 and WK6mutS were regularly inoculated onto minimal medium to select for the *pro* marker present on the F' episome.

The fragment of DNA between the *SmaI* site located within the pigment gene in the plasmid pKS3 and the *HindIII* site of the multiple cloning cassette of pKS3 was subcloned into the *SmaI/HindIII* sites in the multiple cloning site of pMa5-8 (Ap^r Cm^s) to produce the phasmid pMa3 (Fig. 1), and transformed into *E. coli* WK6. Gapped-duplex DNA was formed by hybridizing pMa3 ssDNA with denatured pMc5-8 (Ap^s Cm^r) DNA which was digested with *HindIII* and *SmaI*. A 34-mer oligonucleotide (5'-ACA CCG GAG GAA **AGC** ATG CAC

Fig. 1. Construction of pKSmut by site-directed mutagenesis. pMa3 was made by subcloning the *HindIII/SmaI* fragment of pKS3 (750 bp) into the plasmid pMa5-8. A new *SphI* site was formed at the start of the 1161 bp coding sequence (black box) in pSmu25. pKSmut was made by replacing the *SphI/SmaI* fragments of pKS3 with the restriction fragment between the newly-created *SphI* site and the *SmaI* site of pSmu25.

ATC ACC CGC ACC G-3') complementary to positions 778–808 of the pigment gene (Hart *et al.*, 1990), containing three mismatches (bold type) and a *SphI* site (underlined) was used as the mutagenic primer. This primer enabled the creation of an *SphI* site overlapping the ATG start codon of the pigment gene at position 791–793 [numbering of Hart *et al.* (1990)]. After annealing of the pMa3–pMc5-8 heteroduplex with the mutagenic primer, the gap was filled in, ligated and the DNA transformed into *E. coli* WK6mutS. The strands of the hybrid plasmid were allowed to segregate by two

(a)

pUC18 *lacOP*--*EcoRI* *SstI* *KpnI* *SmaI* *BamHI* *XbaI* *SalI* *PstI* *SphI* *HindIII*--pSLH1 *lacOP*--*EcoRI* *SstI* *KpnI* *SmaI* *BamHI* *XbaI* *SalI*-C TGC AGG CGA TAA GCT TGC ATG CAC--
PstI HindIII SphIpSLH4 *lacOP*--*EcoRI* *SstI* *KpnI* *SmaI* *BamHI* *XbaI* *SalI*-C CAC--mcc1 5'- CT CTG CAG CTG GAT CCG GAT ATC CA -3'
mcc2 3'-TCG AGA GAC GTC GAC CTA GGC CTA TAG GTT CGA-5'pSLH8 *lacOP*-ATG ACC ATG ATT ACG AAT TCG AGC TCT CTG CAG CTG GAT CCG GAT ATC CAA GCT TGC ATG CAC--
EcoRI SstI PstI BamHI EcoRV HindIII SphI
M T M I T N S S S L Q L D P D I Q A C M H

Fig. 2. (a) Cloning cassette regions of the plasmids pUC18, pSLH1, pSLH4 and pSLH8 showing restriction sites and relevant codons. pUC18; underlined restriction sites occur in the pigment gene. pSLH1; *Clal/XbaI* fragment of pKSmut (Fig. 1) containing the pigment gene, blunted and subcloned into the blunted *SphI/HindIII* sites of pUC18, showing rare codons (underlined), stop codon (*) and start codon (>) of the pigment gene coding sequence. pSLH4; *PstI/SphI* fragment of pSLH1 deleted and the vector blunted and religated to remove rare codons and nonsense codon. mcc1/mcc2; synthetic multiple cloning cassette with 5' *SstI* and 3' *HindIII* compatible overhangs. pSLH8; mcc1/mcc2 subcloned into *SstI/HindIII*-digested pSLH1. Restriction enzymes with single cutting sites in the multiple cloning cassette of pSLH8 are: *BamHI*, *EcoRI*, *EcoRV*, *HindIII*, *PstI*, *SphI* and *SstI*. Restriction enzymes with single cutting sites in the pigment gene of pSLH8 are: *AccI*, *BalI*, *BdrI*, *BspMI*, *DrdII*, *EcoBI*, *EcoKI*, *EcoRI*241, *MluI*, *NruI*, *PpuMI*, *SalI*, *SfiI*, *SgrAI*, *SmaI*, *StuI*, *StyI* and *StySPI*. (b) pSLH8 insertional-inactivation cloning vector showing *Rhodococcus* DNA insert (hatched box), pigment gene coding sequence (←), remains of *lacZ'*-fragment (*lacZ*), *lacI*, *PlacZ* (P), *ori* and *Ap^r* genes, and cloning cassette (open box).

rounds of replication and transformation. *Cm^r* transformants were selected and the presence of the mutation in one of the transformants, designated pSmu25 (Fig. 1), was confirmed by restriction endonuclease digestion and nucleotide sequencing using the 17-mer synthetic primer. The restriction fragment between the newly created *SphI* site and the internal *SmaI* site of the pigment gene fragment in pSmu25 was used to replace the 750 bp *SmaI/SphI* fragment of pKS3 to produce pKSmut (Fig. 1). pKSmut produced normal amounts of pigment.

Construction and characterization of cloning vectors pSLH1 and pSLH4

pSLH1 (Fig. 2) was generated by ligating the *Clal/XbaI* fragment of pKSmut (Fig. 1) into *SphI*- and *HindIII*-digested pUC18. All ends were blunted with T4 DNA polymerase I. Plasmid pSLH1 was designed so that the coding sequence of the pigment gene was in-frame with the *lacZ'* coding sequence of pUC18. pSLH4 was generated by deletion of the *PstI/SphI* fragment in the

multiple cloning site of pSLH1 to remove the in-frame termination codon and rare codons (Fig. 2).

The prototype insertional-inactivation cloning vector pSLH1 contained an in-frame TAA termination codon adjacent to the *Hind*III site in the multiple cloning cassette as well as the rarely used codons AGG and CGA (underlined in Fig. 2). The presence of these codons, however, did not prevent pigment production in *E. coli* LK111(pSLH1) although the level of production appeared to be lower than that of other pigment-producing strains such as *E. coli* LK111(pKS3). In contrast to *E. coli* LK111(pSLH1), *E. coli* 3.300(pSLH1) cells produced only slight amounts of pigment even after several days incubation at room temperature. Production of pigment in LK111 may be due to translational coupling (Schümperli *et al.*, 1982) or suppression. The nonsense and rare codons from the coding sequence in pSLH1 are deleted in pSLH4 (Fig. 2) but the reading frame is maintained, resulting in an improved level of pigment production by both *E. coli* LK111 and 3.300. pSLH4, however, contained only three unique cloning sites, *Eco*RI, *Sac*I and *Bam*HI in the multiple cloning cassette (Fig. 2).

Construction and characterization of the insertional-inactivation cloning vector, pSLH8

For the construction of pSLH8, a multiple cloning cassette was made with two complementary oligonucleotides, mcc1 and mcc2, which are shown in Fig. 2 in the annealed form with ssDNA overhangs at either end which were complementary to overhangs generated by *Hind*III or *Sst*I, respectively. The nucleotide sequence of the cloning cassette was designed to maintain the same reading frame between the upstream *lacZ'* coding sequence and the downstream pigment gene when subcloned into the *Sac*I/*Hind*III sites of pSLH1 to create pSLH8. The reading frame of the multiple cloning cassette sequence was selected to contain codons which occur frequently in highly expressed genes of *E. coli* (Wada *et al.*, 1990).

Annealing of the complementary oligonucleotides mcc1 and mcc2 was carried out in a mixture with approximately 250 pmol of each oligonucleotide, 2 µl 1.5 M-KCl/0.1 M-Tris/HCl, pH 7.5, and water to a final volume of 20 µl. The annealing mixture was heated to 60 °C for 5 min then allowed to cool slowly to room temperature. The dsDNA molecule generated was ligated into pSLH1 in an equimolar ratio of multiple cloning cassette to vector and transformed into competent cells of *E. coli*. Plasmid DNA was prepared from transformants and analysed for the presence of the multiple cloning cassette by restriction endonuclease analysis on agarose gels.

The vector pSLH8 produced high levels of pigment and had a multiple cloning cassette with seven unique restriction sites (Fig. 2). The size of pSLH8 was determined from the compiled nucleotide sequence (pigment gene, pUC18, 'Bluescript' KS, synthetic cloning cassette) to be 4007 bp. Large-scale DNA preparations of pSLH8 from *E. coli* gave yields similar to pUC plasmids suggesting a high-copy-number of about 200 plasmid molecules per cell.

A trial insertional-inactivation experiment was performed by randomly subcloning *Pst*I-digested λ DNA into the unique *Pst*I site in the multiple cloning cassette of pSLH8 (Fig. 2). Transformants were a mixed population of clearly identifiable blue and white colonies on LB Ap agar plates. Plasmid DNA was prepared from six of each type of transformant and digested with *Pst*I. Agarose gel analysis of the digestion products showed that all of the blue-pigmented transformants contained unmodified pSLH8 plasmid DNA whereas all of the plasmids from white transformants contained λ DNA inserts. This demonstrated that pSLH8 was functioning as an insertional-inactivation cloning vector.

pSLH8 fulfills the basic requirements for cloning vectors in that it is small, has many unique restriction sites and an easily selectable phenotype. One of the advantages of this system is the ability of *E. coli* cells containing pSLH8 to produce the indigo pigment on commonly used LB Ap agar plates without the requirement for any further media substrate supplementation (there is always a possibility, of course, that a DNA fragment cloned in-frame could generate a functional pigment-producing enzyme).

All *E. coli* host strains transformed with pSLH8 should be capable of indigo production provided that they produce tryptophanase. Tryptophanase is expressed at a high level in most strains of *E. coli* although it is very sensitive to catabolite repression by glucose in the growth medium. Pigment production was repressed by glucose but enhanced by indole and tryptophan. It is not yet known if the pigment gene will function as a marker in other bacteria.

Alternative insertional-inactivation vectors using the melanin operon as a marker system (Altenbuchner, 1988; Tseng *et al.*, 1990) have the disadvantage that melanin diffuses into the media and may make detection of recombinants difficult if large numbers of transformed colonies are close together on the surface of the agar. The indigo produced by the pSLH series of plasmids is almost entirely intracellular (Hill *et al.*, 1989). In addition, a modified medium supplemented with tyrosine and Cu²⁺ ions was required for melanin production, which was detectable after 2 d (Tseng *et al.*, 1990). In contrast, the indigo marker functions on LB agar and is detectable within 8 h with near maximal colouration after 20 h.

pSLH8 has properties which make it suitable for use as an expression vector. The plasmid has a high-copy-number and promoterless inserts may be expressed from the IPTG-inducible *lacOP* promoter, which drives pigment gene expression. This vector would also be useful for nucleotide sequencing. The high-copy-number facilitates plasmid purification and the known vector sequence ensures that it is not mistaken for the test sequence. The cloning cassette contains seven commonly used restriction sites, three of which generate 3' overhangs and three 5' overhangs which would facilitate shortening of the DNA insert with exonuclease III (Henikoff, 1984). The M13 reverse sequencing primer and the 17-mer primer described in this paper can be used for sequencing cloned DNA from both ends.

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