Construction of Multicopy Expression Vectors for Regulated Over-production of Proteins in *Klebsiella pneumoniae* and Other Enteric Bacteria

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A number of expression vectors have been constructed to allow over-production of selected gene products in *Klebsiella pneumoniae* and other enteric bacteria. The plasmids use the strong hybrid *trp-lac* (*tac*) promoter for gene expression, which is regulated by the *lacI^Q* allele of the *lac* repressor carried on the vector. This provides very tight regulation of gene expression, which is important for over-production of proteins which may be detrimental to cell growth. The vectors carry the standard mp18 cloning nest in which all the restriction sites are unique to the plasmid (with the exception of *EcoRI* in pDK7). Derivatives were constructed carrying kanamycin, chloramphenicol or ampicillin resistance as selectable markers, the first two of which are advantageous in *K. pneumoniae* due to the high inherent β -lactamase activity of this organism.

INTRODUCTION

In recent years a number of plasmid vectors have been developed for the purpose of over-producing particular gene products in *Escherichia coli* (Pouwels *et al.*, 1985). These vectors have several potential applications, including the identification of gene products, studies on gene regulation, and synthesis of large amounts of a protein as an aid in its purification. In most situations the gene of interest is placed downstream of a strong promoter in order to generate high levels of the required transcript. Constitutive high-level expression is often undesirable as it can be detrimental to the host cell and frequently results in loss of the plasmid or selection of mutant plasmids which fail to express the product. Consequently many vectors have utilized either the *lac* promoter or variants of it (Amann *et al.*, 1983) or the λ p_L promoter (Remaut *et al.*, 1981), both of which can be regulated. In the case of λ p_L these vectors must be propagated in a host containing a thermolabile repressor protein (encoded by *lcI857*) and induction of gene expression is achieved by a temperature shift (usually 28 °C to 42 °C) which inactivates the repressor. Expression from vectors carrying the *lac* promoter has been achieved by using host strains which overproduce the *lac* repressor, i.e. *lacI^Q* mutants (Müller-Hill *et al.*, 1968), and induction is achieved by addition of the *lac* inducer IPTG (Amann *et al.*, 1983).

We have recently been interested in the application of these approaches to over-production of gene products in *Klebsiella pneumoniae*. As a result we have developed a number of new expression vectors which have certain advantages for work in this organism and potentially have wider application for over-production of proteins in other enteric bacteria. As at least one of the proteins we are interested in is temperature sensitive we have chosen to base our vectors on the hybrid *tac* promoter (Amann *et al.*, 1983) and we have incorporated the *lacI^Q* gene into the vectors so that their use is not restricted to specific *E. coli* strains. Furthermore, as nearly all previous expression vectors carry the *bla* (Ap^r) gene as the selectable antibiotic resistance, and as *K. pneumoniae* is significantly more resistant to penicillin and its derivatives than *E. coli*, we

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Abbreviations: dNTP, deoxynucleoside triphosphate; X-gal, 5-bromo-4-chloro-3-indolyl β -D-galactoside; IPTG, isopropyl β -D-thiogalactopyranoside.

have constructed alternative vectors carrying the *cat* (Cm^r) or *kan* (Km^r) genes. In this paper we describe the construction of these vectors and their application for over-production of certain gene products in *K. pneumoniae*.

METHODS

Strains and plasmids. These are listed in Table 1.

DNA isolation and manipulation. Plasmid DNA was prepared by the method of Holmes & Quigley (1981). Restriction endonucleases were obtained from commercial sources and used according to the manufacturer's instructions. Other DNA manipulations were carried out according to Maniatis *et al.* (1982). Transformation of plasmid DNA was carried out by the freeze/thaw procedure of Merrick *et al.* (1987).

Construction of expression vectors. Plasmid pMM40 was based on the *ptac* expression vector pKK223-3 (Pharmacia Ltd; Amann *et al.*, 1983), which is derived from pBR322 and which carries the *tac* promoter followed by the mp8 cloning nest and a strong transcription terminator from the *E. coli rrnB* operon. In order to facilitate the use of this vector in *K. pneumoniae*, the *lacI*^Q gene was isolated as an *Asp*718-*Pst*I fragment from pMJR1560 (Stark, 1987) and cloned between the *Sph*I and *Xma*III sites of pKK223-3 (Fig. 1) after all the ends had been rendered blunt using Klenow polymerase and dNTPs. The ligation products were transformed into *E. coli* strain 5K, which is Lac⁺, and clones carrying *lacI*^Q were selected as white colonies on plates containing the indicator X-gal. The orientation of *lacI*^Q was subsequently determined by restriction mapping.

Plasmid pMM63 was constructed in an analogous manner to pMM40 but in this case the *lacI*^Q fragment was cloned between the *Sph*I and *Pvu*II sites of pKK223-3 (Fig. 2). pDK5 was derived from pMM63 in two steps.

Fig. 1. Restriction map of pMM40. The *lacI*^Q gene was cloned on an *Asp*718-*Pst*I fragment into pKK223-3 cut with *Sph*I and *Xma*III; all ends were rendered blunt prior to ligation. Abbreviations for restriction sites in this and following Figures are: A, *Asp*718; Ah, *Aha*III; B, *Bam*HI; E, *Eco*RI; H, *Hind*III; Ha, *Hae*II; K, *Kpn*I; M, *Mlu*I; P, *Pst*I; Pv, *Pvu*II; S, *Sal*I; Sa, *Sac*I; Sm, *Sma*I; Sp, *Sph*I; X, *Xba*I; Xm, *Xma*III. Hybrid sites are shown in parentheses.

Table 1. *Bacterial strains and plasmids*

Relevant characteristics		Source or reference
<i>E. coli</i> strains		
5K	<i>thi thr-1 leuB lacI⁺ Y1 tonA21 supE44 hsdR</i>	B. Spratt, University of Sussex, UK
MC1061	<i>araD139 Δ(ara, leu) 7697 ΔlacZ4 galU galK strA hsdR</i>	Casadaban & Cohen (1980)
RB791	<i>lacI^Q lacL8 hsdR</i>	Amann <i>et al.</i> (1983)
Plasmids		
pKK223-3	<i>ptac rrnBT1T2 Ap^r</i> ; derivative of pBR322	Pharmacia Ltd
pMJR1560	<i>lacI^Q Ap^r</i> ; derivative of pUC18	Stark (1987)
pBGS18	Km ^r derivative of pUC18	Spratt <i>et al.</i> (1986)
pACYC184	Km ^r Cm ^r	Chang & Cohen (1978)

Fig. 2. Construction of pDK5. Plasmid pMM63 is analogous to pMM40 (Fig. 1) but *lacI^Q* is inserted between *SphI* and *PvuII* in the vector and is in the opposite orientation. pDK5 was derived by filling-in *BamHI*(2) and replacing the cloning nest with that from pBGS18. Abbreviations for restriction sites are given in the legend to Fig. 1.

Fig. 3. Restriction map of (a) pDK6 and (b) pDK7. The *bla* (Ap) gene of pDK5 was deleted using *AhaIII* and replaced with either the *kan* (Km) gene from pBGS18 or the *cat* (Cm) gene from pACYC184 to create pDK7. Abbreviations for restriction sites are given in the legend to Fig. 1.

Firstly the *BamHI* site outside the nest was removed by partial digestion with *BamHI*, filling in and religation followed by screening of individual clones to identify those in which the correct *BamHI* was removed. The cloning nest was then replaced by restricting with *EcoRI* and *HindIII*, and inserting the polylinker from pBGS18 (Spratt *et al.*, 1986). The resultant plasmid pDK5 is approximately 4.3 kb in size.

pDK6 and pDK7 were derived from pDK5 by restricting with *AhaIII* to remove the majority of the *bla* gene, and cloning in either the 'filled-in' *MluI* fragment carrying the *kan* gene from pBGS18 to create pDK6, or the *HaeII* fragment (rendered blunt with Klenow polymerase) carrying the *cat* gene from pACYC184 to create pDK7 (Fig. 3). pDK6 and pDK7 are 5.1 kb and 4.8 kb in size respectively.

Induction of high-level gene expression. Cultures (20 ml) of strains carrying the expression vectors were grown to exponential phase at 32 °C in Luria broth containing the appropriate selective antibiotic (carbenicillin 100 µg ml⁻¹ for *E. coli* or carbenicillin 200 µg ml⁻¹ + ampicillin 200 µg ml⁻¹ for *K. pneumoniae*; kanamycin 15 µg ml⁻¹ for *E. coli* or 30 µg ml⁻¹ for *K. pneumoniae*; chloramphenicol 15 µg ml⁻¹ for *E. coli* or 40 µg ml⁻¹ for *K. pneumoniae*). For induction of *ptac* expression IPTG was added to a final concentration of 1 mM and incubation was continued at 32 °C. To analyse total cellular proteins 1 ml samples were taken at 1 h intervals. Cells were pelleted and resuspended in 100 µl Laemmli sample buffer, boiled for 5 min and analysed by SDS-PAGE using Coomassie brilliant blue R stain.

RESULTS

Our initial experiments on protein over-production were concerned with the *ntrA* gene of *K. pneumoniae*. This gene encodes an alternative RNA polymerase sigma factor (σ^{54}) (Merrick & Gibbins, 1985; Hunt & Magasanik, 1985) and consequently over-production of this protein might be expected to be detrimental to cell growth. When *ntrA* was cloned into pKK223-3 and the resultant plasmid was propagated in *E. coli* strain RB791, which carries a chromosomal *lacI^Q* gene, over-production of σ^{54} could be obtained after induction with IPTG but the plasmid was very unstable. Fast-growing sectors occurred at high frequency in colonies growing on Luria agar plates and mutant forms of the plasmid arose which could no longer be induced to over-produce. Subsequent experiments using multicopy plasmids carrying the *lacZ* gene confirmed that a single chromosomal copy of *lacI^Q* was insufficient to repress transcription completely from multiple copies of the *lac* promoter (data not shown). Consequently even in non-inducing conditions some over-production can occur and in the case of σ^{54} this is sufficient to inhibit cell growth.

In order to overcome this problem and to allow regulated high-level expression in any strain of *K. pneumoniae* or *E. coli* the *lacI^Q* gene was cloned into pKK223-3 to generate pMM40 (Fig. 1). Plasmids carrying *lacI^Q* inserted in either orientation were obtained and studies of the efficiency of *ptac* repression indicated that the level of repression was not affected by the orientation of *lacI^Q* (data not shown). This type of plasmid was used to achieve regulated over-production of both the *ntrA* product (on pMM38) and the *ntrB* product (on pSA7) in response to IPTG induction in either *K. pneumoniae* or *E. coli* (Austin *et al.*, 1987). After 2–3 h growth in the presence of IPTG the desired product constituted 1–2% of the total cell protein.

Plasmid pKK223-3 contains two *Bam*HI sites, one in the cloning nest and one in the truncated *tet* gene still present in the plasmid (Fig. 1); both these sites are retained in pMM40. In order to improve pMM40 the *Bam*HI site outside the nest was removed and the mp8 cloning nest was replaced by the mp18 nest (Fig. 2). In the resultant plasmid pDK5, all the restriction sites in the cloning nest are unique. pDK5 is also smaller than pMM40 because more of the pKK223-3 vector was deleted when *lacI^Q* was inserted to produce pMM63, the predecessor of pDK5.

The complete DNA sequence of pDK5 could be determined from its component parts and a detailed restriction analysis of the plasmid indicated that approximately 120 bp were deleted at the *Asp*718/*Pvu*II junction during construction of pMM63. This deletion explains the absence of the *Asp*718 site which was predicted to be retained at this junction. With this exception the restriction analysis of pDK5 is entirely consistent with the expected sequence.

Although as mentioned previously pMM40 has been successfully used in *K. pneumoniae* for protein over-production, selection of ampicillin or carbenicillin resistance is difficult in many strains of *Klebsiella* owing to the high intrinsic level of β -lactamase produced by the host. Consequently alternative selectable markers are preferable for work in *Klebsiella* and for this reason derivatives of pDK5 were constructed. Plasmids pDK6 and pDK7 carry the *kan* (K^m) and *cat* (Cm^r) genes respectively cloned in the place of *bla* (Fig. 3) although the *bla* promoter and the first 212 bp of the *bla* coding sequence are retained in both plasmids. All the unique sites in the cloning nest are retained with the exception of *Eco*RI in pDK7, which is also present in the *cat* gene.

The utility of these pMM40 derivatives has been demonstrated by over-production of the *K. pneumoniae nifM* product using plasmid pDK7. The *nifM* gene has been sequenced recently (Paul & Merrick, 1987) and shown to encode a 30.6 kDa polypeptide. Both the *bla* gene and the *kan* gene encode polypeptides with molecular masses similar to that of NifM; β -lactamase (Bla) has a molecular mass of 31.5 kDa and neomycin phosphotransferase (Kan) a molecular mass of 31.0 kDa. By comparison, chloramphenicol acetyltransferase (Cat) has a molecular mass of 25.7 kDa and is therefore unlikely to confuse SDS-PAGE analysis of NifM synthesis. For these reasons *nifM* was cloned on a 0.9 kb *Sst*I–*Hpa*I fragment between the *Sst*I and *Sma*I sites in the cloning nest of pDK7. The resultant plasmid, pWP12, was transformed into *E. coli* strain MC1061 and high-level expression of NifM was induced as described in Methods. After 1–3 h induction a polypeptide of the correct molecular mass for NifM was synthesized and constituted more than 1% of total cellular protein (Fig. 4). A second polypeptide was also produced at high

Fig. 4 Induction of NifM synthesis using plasmid pWP12 (based on pDK7) in *E. coli* strain MC1061. Cultures were grown and induced as described in Methods and total cell proteins were analysed by SDS-PAGE. Track 1, prior to IPTG induction; tracks 2 and 3, 1 h and 3 h respectively after IPTG induction; track 4, MC1061 alone 3 h after IPTG induction; track 5, molecular mass standards. The NifM polypeptide is marked with an arrow.

levels in these strains even in the absence of induction. This polypeptide has the molecular mass expected of the *cat* product, and the high level of expression observed under all conditions in these experiments is probably due to the tandem arrangement of the *bla* and *cat* promoters in this plasmid.

DISCUSSION

The expression vectors described in this paper have a number of advantages for overproduction of specific gene products in enteric bacteria. The use of the *tac* promoter rather than the λp_L promoter to direct high levels of transcription provides a method of induction of gene expression (namely addition of IPTG) which is often preferable to the temperature shift required for λp_L . Firstly, whilst a temperature shift is easily achieved on a small scale, i.e. cultures of 5–500 ml, it is far more difficult to obtain a comparable rapid shift when working on a large scale, e.g. 100–400 l. By comparison, addition of IPTG is simple and can effect efficient rapid induction. In large-scale cultures (e.g. 100 l) effective induction can be achieved with a final concentration of 100 μ M-IPTG (S. Austin & R. Dixon, personal communication). Secondly, if the activity of the protein being synthesized is temperature sensitive, induction with IPTG is clearly preferable to a temperature shift.

The location of the *lacI^Q* gene on the expression vector has two distinct advantages. Firstly, as observed by ourselves and others (Amann *et al.*, 1983; Stark, 1987) a single copy of *lacI^Q* is insufficient to repress expression from multiple copies of the *tac* promoter completely. Consequently without *lacI^Q* on the plasmid there is always a significant level of expression from *ptac* and, as we found with the σ^{54} product of *ntrA*, this level of expression can be very

detrimental to cell growth if the over-produced protein interferes with normal cellular metabolism. By contrast, the location of *lacI^Q* in *cis* to *ptac* provides tight regulation of the inserted gene. Secondly the plasmid location of *lacI^Q* allows the expression vector to be used in any chromosomal background including other genera, e.g. *K. pneumoniae* as described here.

The use of the *lacI^Q* gene on multicopy expression vectors has also recently been described by Stark (1987), who constructed a similar series of plasmids based on the pUC vectors. These vectors (designated pTTQ) have many features in common with the pDK vectors described here in that they utilize the *tac* promoter and the *rrnB* transcription terminator. The pTTQ vectors also contain the *lacZ* α -complementing region which potentially allows the use of a blue-white colony colour screen (in the presence of X-gal and IPTG) to differentiate recombinant and non-recombinant clones. However, this selection is often not possible since plasmids which express a desired gene at high levels may be difficult or even impossible to isolate under conditions of induction (Stark, 1987) and it is normally preferable to select transformants in non-inducing conditions. All the pTTQ vectors carry the *bla* (Ap^r) gene as a selectable marker and as discussed earlier this particular marker is not ideal for use in *Klebsiella* due to the intrinsic β -lactamase activity in this organism. In this respect pDK6 and pDK7 offer the advantage of using Km^r or Cm^r as selectable markers which may well be of benefit in other organisms besides *K. pneumoniae*.

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