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Analysis of the promoters for the two immunity genes present in the ColE3-CA38 plasmid using two new promoter probe vectors

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ABSTRACT

We have constructed two new promoter probe vectors which carry a polylinker derived from plasmid pUC19 proximal to the 5′ end of a promoter-less galactokinase gene. Using these two vectors we have demonstrated that the ColE3imm gene and the ColE8imm gene present on the ColE3-CA38 plasmid have their own promoters, independent of the SOS promoter of the colicin E3 structural gene. The activity of two terminators, one located proximal to the 5′ end of the ColE8imm gene, the other located proximal to the 5′ end of the lys gene, were shown by a comparison of the galactokinase activity conferred by several of the recombinant plasmids.

INTRODUCTION

The E colicins are a group of plasmid-coded, antibacterial proteins which use the same cell-surface receptor (1), the product of the Escherichia coli chromosomal gene btuB, to bind to sensitive cells (2). This group has been subdivided into colicins E1 to E9 on the basis of immunity tests (3,4). Each E colicin plasmid codes for the production of a specific immunity protein, which protects colicin-producing cells against colicin of the same type. E.coli K12 strains carrying an E colicin plasmid are MC sensitive compared to plasmid-free isogenic strains, due to the induction of a plasmid-coded lysis gene which may be involved in colicin release from the producing cell (5-10). Since immunity is constitutive, whilst colicin production and MC sensitivity are inducible by DNA damage (11), the regulatory control of these three genes is of considerable interest.

Experiments with transposon mutagenesis of the ColE3-CA38 plasmid have demonstrated that the colicin E3 structural gene and the lysis gene are transcribed from a common promoter, located proximal to the structural gene, whilst the ColE3imm gene , which is located between these two genes, must have its own promoter (8,9). Analysis of the nucleotide sequence of this

region of the ColE3-CA38 plasmid has revealed a gap of only nine bases between the C-terminus of the colicin structural gene and the ColE3imm gene (12). Whilst this gap is insufficient for a promoter sequence, a potential promoter sequence has been identified, from the sequence data, within the C-terminus of the colicin structural gene but no evidence that this promoter is functional has been presented. We have identified a second unrelated immunity gene, to colicin E8 (8), located in tandem with the ColE3imm gene, the nucleotide sequence of which has subsequently been reported (13). We were unable to confirm whether the ColE8imm gene is transcribed from the ColE3imm gene promoter, or whether it has its own promoter. We have investigated the promoters present in this region of the ColE3-CA38 plasmid by using two new promoter probe vectors pKC86 and pKC87.

MATERIALS AND METHODS

Strains and media

E.coli W3110 [ColE3-CA38] was obtained from the Plasmid Reference Centre, Stanford University, California. E.coli W3110 [ColE8-J] (4) was used to prepare extracts of colicin E8. E.coli JC3272 (his, lys, trp, lacY, galK, strA) was used as the host in all transformations. For selection of recombinant plasmids carrying promoter fragments we used LB agar (14) containing Amp (50μg/ml), Gal (1% w/v), and Tzo (20μg/ml). On this medium recombinant plasmids containing promoters yield white or pink colonies, whilst the vector gives rise to red colonies.

Enzymes and reagents

Restriction enzymes, T4 DNA ligase and polynucleotide kinase were purchased from Anglian Biotechnology, Bethesda Research Labs. Inc., PL Biochemicals or Boehringer Mannheim. MC (Sigma) was used as previously described to test the MC sensitivity of cultures containing recombinant plasmids (8,10).

Colicin production and immunity tests

Production of colicin and immunity testing were as previously described (4).

Plasmid isolation, restriction, ligation and transformation

Plasmids pKO1, pKO4 and pED101 were obtained from P.M. Mullineaux, John Innes Institute, Norwich, UK, whilst plasmid pUC19 was a kind gift of J. Messing, University of Minnesota, St. Paul, USA. Plasmids were prepared by a modification of the method of Birnboim and Doly (15). Digestion of DNA

with restriction endonucleases, ligation of DNA fragments, filling-in of sticky-ends, insertion of ClaI linker and transformation of competent cells were carried out as previously described (16).

Electrophoresis of DNA and purification of fragments

Electrophoresis of DNA fragments was performed as previously described (8). Specific fragments were recovered from gels using the method of Dretzen et al.(17).

Galactokinase assay

The activity of promoters present in recombinant plasmids was determined by a previously described method (18,19) using C-14 galactose (Amersham International). In the induction experiments with MC, cultures containing recombinant plasmids were grown to an OD650 of 0.4 before the addition of MC (0.5μg/ml). Duplicate 1ml samples were removed after various times of incubation in the presence of MC for galactokinase assay.

RESULTS AND DISCUSSION

Construction of promoter probe vectors

In order to analyse fragments of the colicin E3 operon for promoter activity it was necessary to construct new promoter probe vectors which had suitable ClaI, KpnI, EcoRI and blunt-ended sites. Our strategy was to transfer a modified polylinker from plasmid pUC19 (20) into two existing promoter probe vectors, pKO1 and pED101 (18), so that the multiple cloning sites of the polylinker are present in both orientations proximal to the 5′ end of the promoter-less galactokinase gene. A ClaI linker was inserted into the HincII site of the polylinker of plasmid pUC19 (Figure 1). In order to mobilize the 59bp EcoRI - HinDIII polylinker from the resulting plasmid pRJ142 into pKO1 and pED101, we had to overcome the technical problem of the absence of any selectable marker. We inserted an 8kb BamHI fragment from plasmid pP5a (Cooper and James, manuscript in preparation), into the BamHI site of pRJ142, yielding plasmid pKC82 (Figure 1). The BamHI fragment of plasmid pP5a encodes colicin E6 immunity and has no internal sites for any of the enzymes which cut in the polylinker of pUC19. This allows the purified 8.06kb EcoRI - HinDIII fragment of pKC82 to be readily transferred to any plasmid which has EcoRI and HinDIII sites and the intact 59bp polylinker to be regenerated by restriction with BamHI.

Plasmid pED101 contains unique HincII, BamHI, HinDIII, ClaI and EcoRI sites located proximal to the 5′ end of a promoter-less

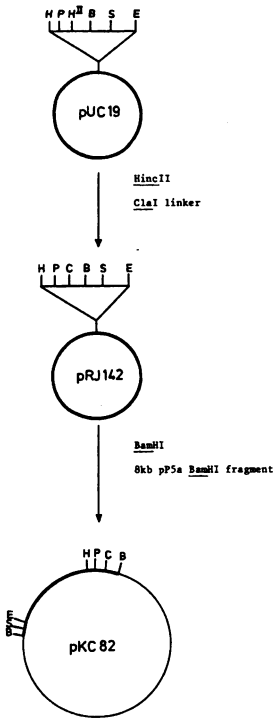

Figure 1. Construction of plasmid pKC82. Details of the construction of plasmid pKC82 are given in the text. The restriction sites present are abbreviated; H, HinDIII; P, PstI; H^II, HinCII; B, BamHI; S, SmaI; E, EcoRI; C, ClaI.

galactokinase gene (Figure 2). The BamHI site of pED101 was destroyed by restriction with BamHI, filling in the ends and re-ligation, yielding plasmid pKC83. The 8.06kb EcoRI - HinDIII fragment of plasmid pKC82 was ligated with EcoRI/HinDIII digested pKC83, using colicin E6 immunity as a selection. The resulting plasmid was digested with BamHI to release the colicin E6 immunity encoding BamHI fragment, resulting in plasmid pKC86, which has unique cloning sites upstream of the promoter-less galactokinase gene (Figure 3). In order to obtain the modified polylinker from pRJ142 in the opposite orientation with respect to the 5′ end of the promoter-less galactokinase gene, we inserted a ClaI linker into the SmaI site of plasmid pKO1, yielding plasmid pRJ141 (Figure 2). The 8.06kb EcoRI - HinDIII fragment of pKC82 was then transferred to EcoRI/HinDIII digested pRJ141 and the colicin E6 immunity encoding fragment removed by BamHI digestion, yielding pKC87 (Figure 4). An appreciable advantage of plasmids pKC86 and pKC87, compared with pKO1 especially, is that the length of untranslated RNA arising from a promoter within a cloned fragment can only vary by some 50bp, instead of the

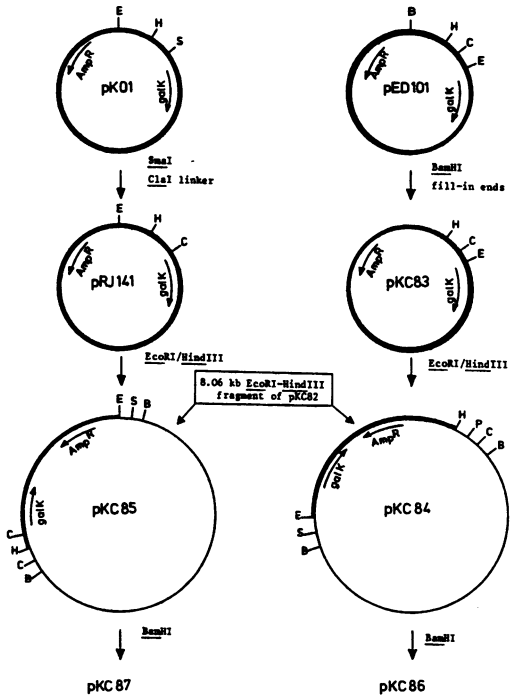

Figure 2. Construction of plasmids pKC86 and pKC87. Details of the construction of plasmids pKC86 and pKC87 are given in the text. The restriction sites present are abbreviated as in Figure 1.

300bp possible in pKO1, due to the replacement of a 300bp EcoRI - HinDIII fragment of pKO1 by the 59bp polylinker. In addition, with the exception of the ClaI site, any two enzymes which cut in the polylinker region of pKC86 or pKC87 can be used to transfer promoter fragments into M13 mp18 and M13 mp19 for DNA sequencing (20).

Sub-cloning of ColE3-CA38 fragments

Fragments of the colicin operon region of the ColE3-CA38 plasmid were sub-cloned into vectors pKC86 or pKC87 (Table 1). The orientation of the fragments cloned, with respect to the galactokinase gene , together with the phenotype conferred by the recombinant plasmids and the strength of any promoters present are shown in Figure 4. The results clearly show that there is a weak promoter present in the ClaI- KpnI fragment present in recombinant plasmid pKC106. From the previously published nucleotide sequencing data of several groups which we have collated in Figure 5, this fragment is shown to cover 75 amino-acids of the C-terminus of the colicin

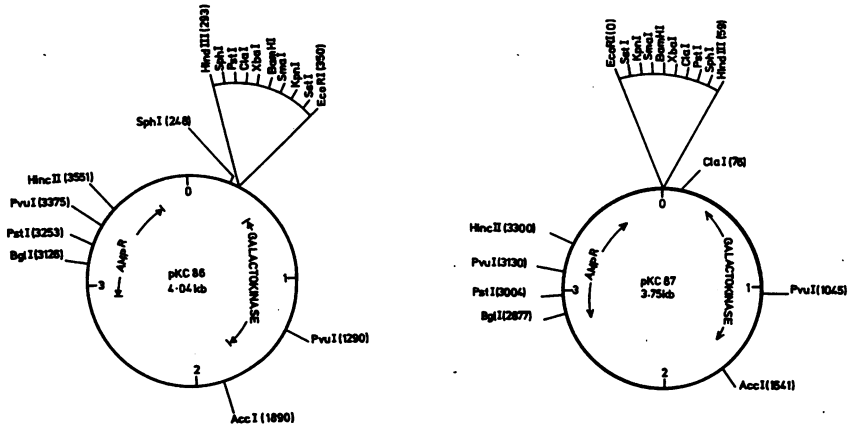

Figure 3. Restriction maps of plasmids pKC86 and pKC87. The polylinker region of both plasmids is shown in an enlarged form for clarity. The zero map positions of the two recombinant plasmids are the same as those for plasmids pED101 and pK01 respectively.

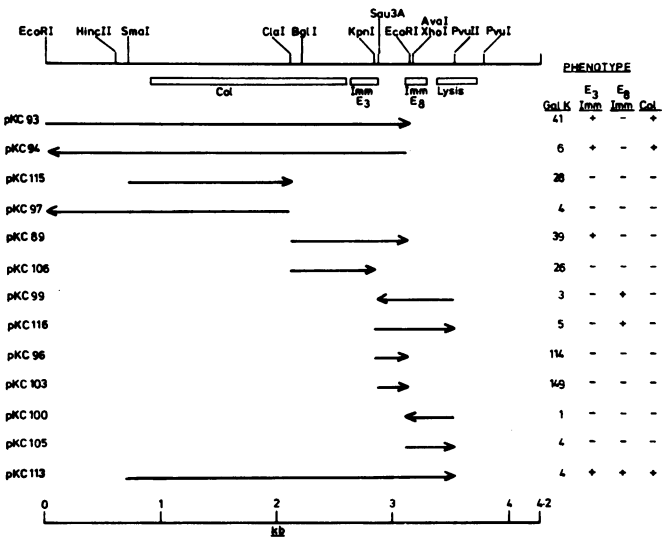

Figure 4. Sub-clones of the ColE3-CA38 plasmid. The restriction fragments present in the plasmids listed at the left are indicated, together with the phenotypes conferred. The orientation of the fragments with respect to the galactokinase gene are indicated by the arrow-heads; fragments with arrow-heads pointing to the right are located in that orientation proximal to the galK gene, whilst those with arrow-heads pointing to the left are in the reverse orientation with respect to the galK gene. The approximate locations of the colicin E3 structural gene, the ColE3imm gene, the ColE8imm gene and the lys gene are shown under the restriction map of the relevent portion of the ColE3-CA38 plasmid.

TABLE 1 A description of the phenotypes conferred, the sizes and the derivations of recombinant plasmids used in this work

Plasmid	Phenotype, size, derivation
pRJ142	Amp^R; 2.7kb. A ClaI linker was ligated into the HinCII site of pUC19
pKO1	Amp^R; 3.98kb. Promoter probe vector derived from pBR322.
pRJ141	Amp^R; 3.98kb. A ClaI linker was ligated into the SmaI site of pKO1
pED101	Amp^R; 4.01kb. Derived from pKO1 with the EcoRI and HinDIII restriction sites in reverse orientation with respect to the promoter-less galactokinase gene
pKC86	Amp^R; 4.04kb. Derived from pED101 by replacing the HinDIII - EcoRI fragment with a 59bp polylinker from pRJ142
pKO4	Amp^R;4.04kb .Derived from pKO1 with EcoRI and BamHI restriction sites proximal to the promoter-less galactokinase gene
pKC87	Amp^R; 3.75kb. Derived from pRJ141 by replacing the EcoRI - HinDIII fragment with a 59bp polylinker from pRJ142
pKC54	Amp^R, $galK^-$; 4.36kb. The 0.375kb EcoRI - BamHI fragment of pBR322, which includes the tet promoter, cloned into pKO4 restricted with EcoRI and BamHI
pKC93	Amp^R, $ImmE3^+$, $ImmE8^-$, Col^+, $galK^+$;7.14kb. The 3.1kb EcoRI fragment of ColE3-CA38 cloned into the EcoRI site of pKC86
pKC94	Amp^R, $ImmE3^+$, $ImmE8^-$, Col^+, $galK^-$; 7.14kb. As pKC93 but opposite orientation with respect to the galactokinase gene.
pKC96	Amp^R, $ImmE3^-$, $ImmE8^-$, Col^-, $galK^+$; 4.36kb. The 2.8kb EcoRI - KpnI fragment was deleted from pKC93
pKC97	Amp^R, $ImmE3^-$, $ImmE8^-$, Col^-, $galK^-$; 6.14kb. The 1.02kb EcoRI - ClaI fragment was deleted from pKC94
pKC89	Amp^R, $ImmE3^+$, $ImmE8^-$, Col^-, $galK^+$; 5.06kb. The 1.02-kb ClaI -EcoRI fragment of ColE3-CA38 cloned into pKC86 restricted with ClaI and EcoRI
pKC99	Amp^R, $ImmE3^-$, $ImmE8^+$, Col^-, $galK^-$; 4.74kb. The 0.7-kb KpnI - PvuII fragment of ColE3-CA38 cloned into pKC86 restricted with KpnI and SmaI
pKC100	Amp^R, $ImmE3^-$, $ImmE8^-$, Col^-, $galK^-$; 4.42kb. The 0.32-kb KpnI - EcoRI fragment was deleted from pKC99
pKC103	Amp^R, $ImmE3^-$, $ImmE8^-$, Col^-, $galK^+$; 4.25kb. The 210-bp Sau3A - EcoRI fragment of ColE3-CA38 cloned into pKC86 restricted with BamHI and EcoRI
pKC105	Amp^R, $ImmE3^-$, $ImmE8^-$, Col^-, $galK^-$; 4.38kb. The 0.4-kb EcoRI - PvuII fragment from ColE3-CA38 cloned into pKO1 restricted with EcoRI and SmaI
pKC113	Amp^R, $ImmE3^+$, $ImmE8^+$, Col^-, $galK^-$; 6.61kb. The 2.86-kb HincII - PvuII fragment of ColE3-CA38 cloned into the SmaI site of pKC87
pKC115	Amp^R, $ImmE3^-$, $ImmE8^-$, Col^-, $galK^+$; 5.19kb. The 1.42-kb PvuII - ClaI fragment was deleted from pKC113
pKC116	Amp^R, $ImmE3^-$, $ImmE8^+$,Col^-, $galK^-$; 4.74kb. The 0.7-kb HincII - KpnI fragment was deleted from pKC113

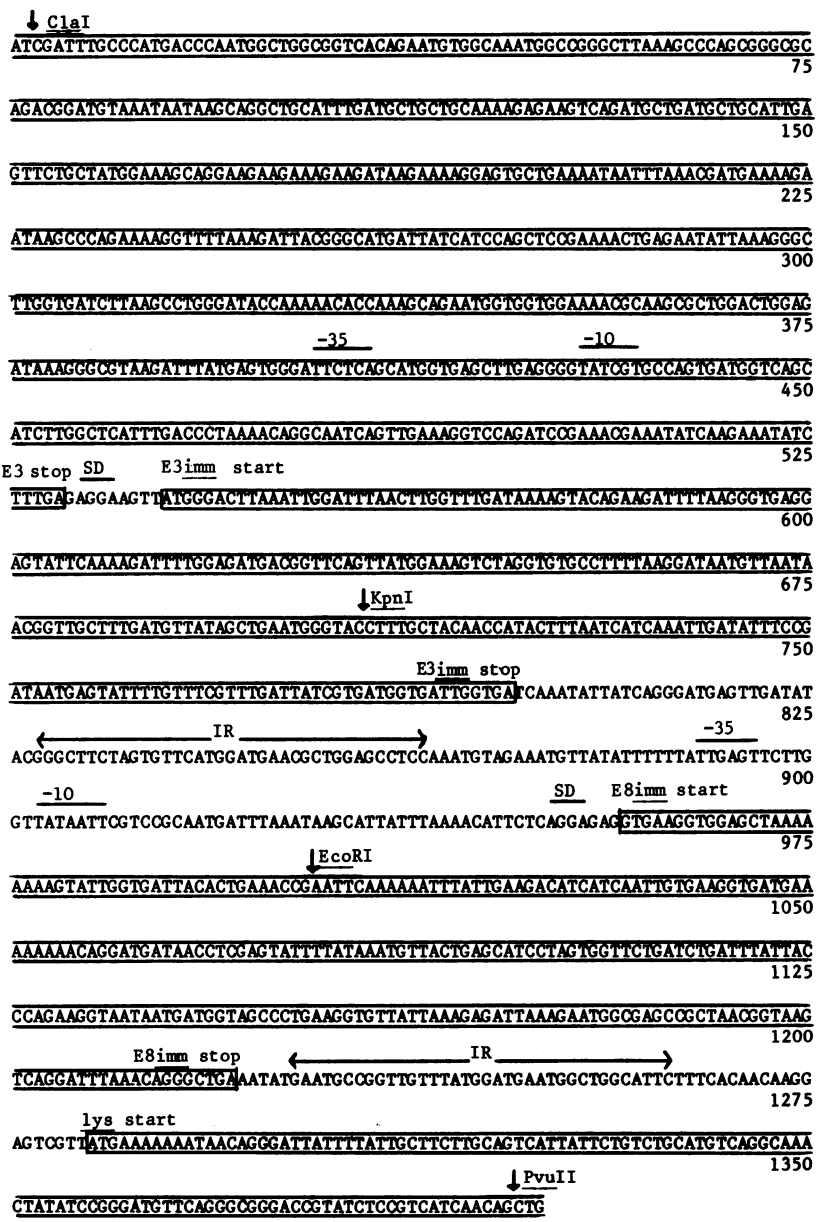

Figure 5. Nucleotide sequence of the colicin E3 operon. The sequence data shown has been assembled from previously published data (12,21-23). The start of the ClaI restriction site has arbitrarily been designated as zero. The restriction enzyme sites used in this work are indicated together with the start and stop codons of relevent genes. Presumptive regulatory sequences are boxed.

E3 structural gene and more than half of the N-terminus of the ColE3imm gene (12,21-23). A possible promoter sequence for the ColE3imm gene, located within the C-terminus of the colicin E3 structural gene (beginning at base 404 in Figure 5), has been identified (12). In the absence of transcript mapping studies, the designation of promoter sequences is an uncertain business, however, our sub-cloning and assay data confirm that the ColE3imm gene has its own weak promoter. A much stronger promoter is present in the KpnI - EcoRI fragment present in recombinant pKC96, and in the slightly smaller Sau3A - EcoRI fragment present in plasmid pKC103. This latter fragment covers the region from the stop codon of the colicin E3 immunity gene to the 14th codon of the colicin E8 immunity gene (13, see Figure 5), and therefore the galactokinase activity must represent the ColE8imm gene promoter. Since the galactokinase assays carried out with recombinant plasmids pKC106 and pKC103 effectively measure the strength of the promoters as they transcribe the ColE3imm and ColE8imm genes respectively, these results are a true measure of the relative strengths of the promoters present, uninfluenced by the presence of any terminators. For comparison purposes the tet promoter of pBR322 present in plasmid pKC54 gave a galactokinase activity of 338 units.

It is tempting to speculate that the constraints on the ColE3imm gene of having its promoter located within another gene, in contrast to the ColE8imm gene, may explain the difference in relative strengths of the two promoters. Other examples of promoters inside structural genes of E.coli have recently been reported, for example in the trp operon (24) and in the deo operon (25) and in general these are weak promoters. Our understanding of the differences in the relative strengths of the ColE3imm and ColE8imm gene promoters must await further sequencing data, especially of the ColE8-J plasmid, in order to elucidate the evolutionary origin of the ColE8imm gene on the ColE3-CA38 plasmid. If the ColE8imm gene on the ColE3-CA38 plasmid arose by a gene duplication of the ColE3imm gene and susequent divergence, then what is the origin of the ColE8imm gene promoter ?. The presence of two immunity genes which are transcribed in the same orientation as the colicin and lys genes, but have their own promoters, is unusual in comparison to other colicins. In the case of ColE1 the ColE1imm gene is located between the colicin E1 structural gene and the lys gene but is transcribed in the opposite direction (26), whereas in the case of CloDF13, the cloacin gene and the immunity gene are coordinately transcribed into mRNA (27).

A comparison of the promoter activity of recombinant plasmids pKC96 and pKC116 supports the suggestion from DNA sequencing data (Figure 5) that there is a terminator sequence located between the E8 *imm* gene and the *lys* gene (23), which would prevent significant transcription of the latter gene despite the basal level of expression of the former gene in uninduced cells. It is interesting that the promoter activity of the fragment present in plasmid pKC89 is less than the sum of the activities of plasmids pKC106 and pKC96. It is tempting to speculate that the *ClaI* - *EcoRI* fragment present in plasmid pKC89 contains a regulatory sequence which effects the transcription from the E8 *imm* gene promoter. An alternative hypothesis that the galactokinase activity of fragments cloned in the pKO based vectors is affected by the length of the fragment cloned is unlikely given the recent data of Sullivan and Donachie which showed no effect of fragment size on galactokinase activity (28).

The phenotype conferred by the *EcoRI* - *PvuII* fragment present in recombinant pKC105, a fragment covering the region from the 14th codon of the E8 immunity gene to the 37th codon of the lysis gene (23), clearly demonstrates that the lysis gene does not have its own promoter, and supports earlier findings, from transposon mutagenesis experiments, which implied that the *lys* gene is transcribed from the induced SOS promoter located proximal to the colicin E3 structural gene (8).

The phenotype conferred by recombinant plasmid pKC115 clearly locate a promoter within the *SmaI* - *ClaI* fragment of the ColE3-CA38 plasmid. This promoter is presumably the SOS inducible colicin E3 structural gene promoter since it is inducible by DNA damage with MC (Figure 6). The difference in the galactokinase activity of cultures carrying recombinant plasmids pKC115 and pKC93 supports the suggestion of Watson *et al.*, from sequence data that there is a terminator sequence located between the ColE3*imm* gene and the ColE8*imm* gene (22). A comparison between the galactokinase activity of cultures carrying plasmids pKC93 and pKC113 provides further support for the presence of a terminator proximal to the *lys* gene. There is no appreciable induction of the promoters present in recombinant plasmid pKC116 by MC, a result consistent with the hypothesis that the two immunity genes are transcribed from their own promoters. *Regulation of the colicin E3 operon* Our results clearly show that the two immunity genes present in the colicin E3 operon have their own promoter activities and thus will be constitutively synthesised at a particular basal level. It remains to be determined how the synthesis of the immunity genes

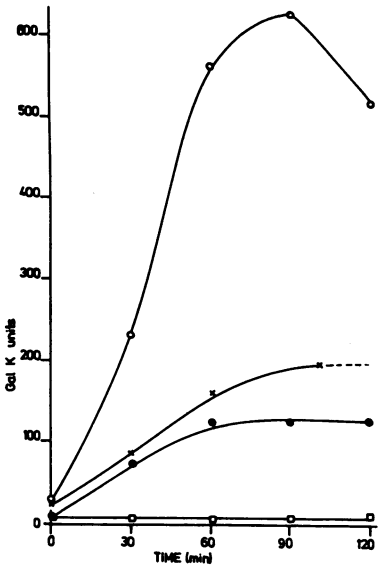

Figure 6. Kinetics of induction of galactokinase. The galactokinase activity of cultures of E.coli JC3272 carrying plasmid pKC115 (O), pKC93 (X), pKC113 (●) and pKC116 (□) were measured at the times shown after the addition of MC.

responds to the induction of the SOS promoter in the native ColE3-CA38 plasmid. It has been proposed from inspection of the DNA sequence of the C-terminus of the colicin E3 structural gene that the ColE3imm and the lys gene may be translationally coupled to that of the colicin E3 structural gene (23). An alternative hypothesis is that the SOS promoter transcribes the whole operon, including the lys gene, when induced by DNA damage. The SOS promoter is presumed to be strong enough to overcome the two terminators, located proximal to the 5′ end of the ColE8imm gene and proximal to the5′ end of the lys gene, when induction has occurred thus leading to cell lysis. From inspection of the data in Figure 6, cultures carrying recombinant plasmid pKC113 achieve a galactokinase activity of approximately 140 units within 60 min of MC induction. At this time significant cell lysis begins to occur (data not shown), therefore we propose that this is the threshold level of promoter activity for complete activation of the lys gene. The two terminators identified by Watson et al. (22) are effective in preventing this threshold level being reached in the absence of full induction of the SOS promoter. We are currently constructing terminator probe plasmids based upon plasmids pKC86 and pKC87 in order to investigate the location and strength of terminators in the colicin E3 operon.

CONCLUDING REMARKS

After completion of this work two promoter-probe vectors similar to pKC86 was reported by de Boer (29), however these vectors do not include as many unique cloning sites proximal to the promoter-less galactokinase gene and do not offer two orientations for cloning promoter containing fragments.

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ABBREVIATIONS

Amp, Ampicillin; bp, base pairs; Col ,colicin production; Gal, galactose; Imm , colicin immunity; kb, 1000bp; Lys ,mitomycin-C-induced lysis; MC, mitomycin C; Tzo, Tetrazolium; [ ], indicates plasmid-carrier state.

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