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Regulation of the *trfA* and *trfB* promoters of broad host range plasmid RK2: identification of sequences essential for regulation by *trfB/korA/korD*

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ABSTRACT

Using a plasmid containing a transcriptional fusion in which the E.coli galK gene is expressed from the trfB promoter of broad host range plasmid RK2 we show that transcription from the trfB promoter is repressed by the products of both the trfB and korB genes as we have previously predicted from the sequence homology of the trfA and trfB promoters and the fact that the trfA promoter is regulated by trfB and korB. These loci, trfB and korB are normally transcribed from the trfB promoter. Thus the trfB incC korB operon of RK2 is doubly autogeneously regulated. In addition, we describe the isolation and characterization of a mutant trfA promoter which has become insensitive to repression by trfB as a result of a point mutation within the inverted repeat sequence previously predicted to be the trfB protein binding site. These results provide strong evidence for our previously proposed model for control of transcription from the trfA and trfB promoters.

INTRODUCTION

The plasmid RK2, which is indistinguishable from plasmids RP4, RP1, R68, R18 (1,2) belongs to the Escherichia coli incompatibility group IncP and to the Pseudomonas species group IncP-1. The molecular genetics of these indistinguishable plasmids has been investigated (for a review, see ref. 3), because of their ability to transfer themselves between and maintain themselves in almost all Gram negative bacterial species, a property which is of both fundamental and applied interest. Examination of the physical and genetic map of RK2 (Fig. 1) reveals that if the blocks of known transposable elements and antibiotic resistance genes are ignored then the plasmid genome consists of a large block of conjugal transfer functions and a second block of functions which include the origin of vegetative replication $oriV_{RK2}$ (9,10,11,12) and the trfA gene, which provides a trans-acting protein or proteins which activates $oriV_{RK2}$ (13,10,14,15). The other functions in this second block include kil genes, which if cloned on their own are either lethal to their bacterial host (kilA, B and C; 13,16) or inhibit plasmid maintenance (kilD 17; also

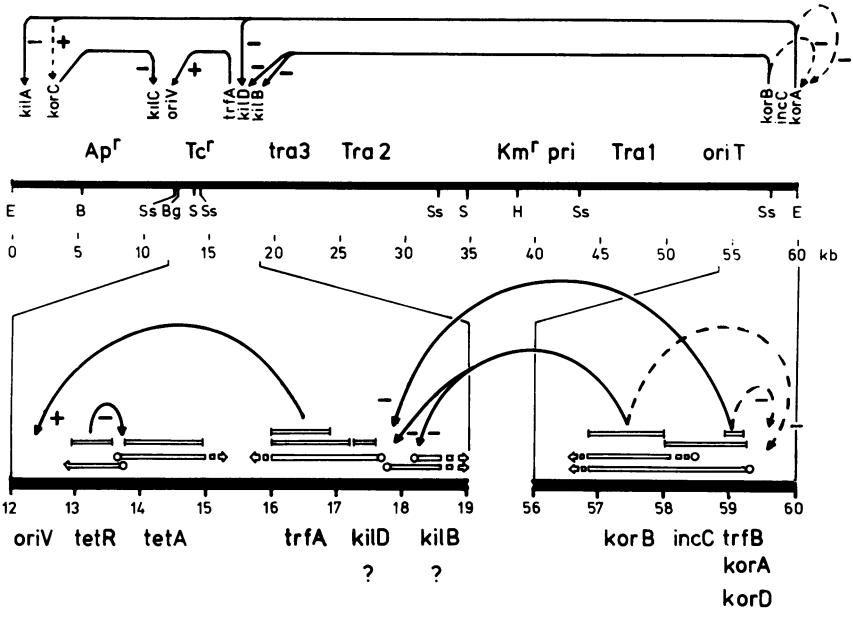

FIG. 1. Map of RK2. Key restriction sites are indicated by: E, EcoRI; B, BamHI; Bg, BglII; H, HindIII; S, SalI; Ss, SstII. Coordinates are in kilobase pairs (kb) from the EcoRI site. We have attempted to convert our map, originally estimated at 56.4kb to the 60kb estimated by Lanka et al. (4). Loci not mentioned in the text are: pri, encoding a DNA primase (5), rlx, the site of the relaxation complex and origin of conjugal transfer (6); tra3, a locus involved in transfer (7); tra1 and tra2 blocks of loci involved in transfer (8). The regions encoding the trfA, trfB and putative kilB operons are expanded to show the operon structure. Regulatory interactions are shown by arrows, continuous for known circuits, discontinuous for those which have previously only been predicted.

known as kilB1 18), kor genes which override the effect of kil genes, and at least one incompatibility locus (P. Barth and K. Ellis, personal communication; 19,20) which we have designated incC.

While it is not yet known what functions the kil and incC loci serve in the biology of RK2, recent studies have revealed that this second block of RK2 functions is organized in a series of related operons (Figure 1), suggesting that all of these functions may be involved in a common purpose, for example stable plasmid maintenance. Regulation of these operons is through repression of transcription by trans-acting proteins. We showed that the trfA operon is negatively regulated by the products of both korD and korB loci (21), a fact recently confirmed by Schreiner et al. (22). We also showed that the combination of both repressors appears to reduce expression of this operon to a level where the

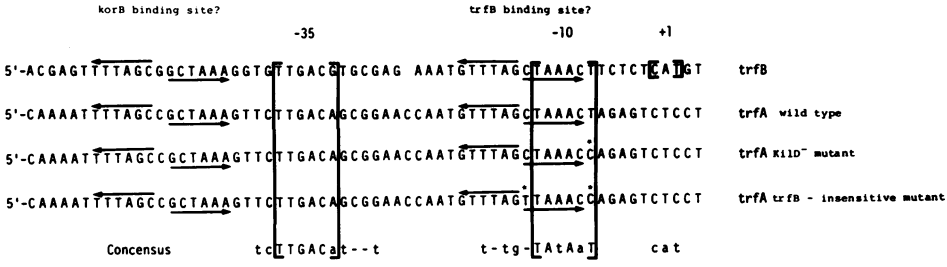

FIG. 2. Nucleotide sequence of trfA and trfB promoters. The sequences of the non-transcribed strands of the two promoters are aligned to show homology to each other and to the E.coli concensus promoter sequence (38). The putative trfB and korB repressor binding sites are shown as are the positions where the trfA promoter in pBIM1 differs from wild type and the position of the point mutation in pBIM1.1 which renders trfA expression insensitive to trfB in trans.

availability of trfA gene product(s) becomes the overriding limitation for plasmid replication (23). Originally the region encoding korD and incC was designated trfB and since korD does appear to be a trans-acting function involved in replication we still refer to this locus as trfB. This locus also seems to be identical to korA (17,24) which represses transcription of kilA (25) and which also seems to play a role in expression of korC or coregulation of kilC (26). We have also identified an operon whose transcription is negatively regulated by korB but not trfB and is likely to code for kilB (21).

DNA sequence analysis of the trfA kilD operon promoter, the trfB incC korB operon promoter and the putative kilB operon promoter (27) revealed the presence of two related but different inverted repeat sequences which could act as operator sites at which repressor proteins might bind (Fig. 2). One of these sequences is common to the two promoters (trfA kilD and kilB) which are known to be regulated by korB (21) and so is proposed as the korB product binding site while the second is proposed as the trfB (korA/korD) product binding site. On the basis of these findings we predicted that the trfB incC korB operon would be autogenously regulated by the trfB and korB products. If these proposals are correct we would expect that point mutations in the putative operator sequences should alter the regulatory properties of these promoters. In this paper we show that the trfB operon is autogenously regulated and also describe the isolation of a mutant which directly implicates the putative trfB protein operator sites in repression of transcription.

Table 1. Plasmids used in this study

Plasmid	Size (kb)	Replicon	Selective Marker	Other Properties	Reference or Source
pKO4	4.0	pMB1	Pn^r	galK promoter probe	33
pKO6	4.0	pMB1	Pn^r	galK promoter probe	33
pKM-1	4.4	pMB1	Pn^r	λt_{R1} galK promoter probe	K.McKenney
pCT654	5.05	pMB1	Pn^r	trfB promoter in pKM-1	This study (Fig. 3)
R300B	8.9	IncQ	$Su^r Sm^r$	-	7
pCT409	9.6	IncQ	Sm^r	$trfB^+$	21
pCT415	17.0	IncQ	Sm^r	$trfB^+ incC^+ korB^+$	21
pCT417	20.5	IncQ	$Sm^r TrpE^+$	$trpE^+$ $korB^+$	23
pCT201	10.35	IncP	$Pn^r Tc^r$	$oriV^+_{RK2}$ $trfA^+$	34
pBIM1	10.35	IncP	$Pn^r Tc^r$	$oriV^+_{RK2}$ $trfA^+$	This study Fig. 3
pBIM1.1	10.3	IncP	$Pn^r Tc^r$	$oriV^+_{RK2}$ $trfA^+$	This study
pBIM9	5.7	pMB1	Pn^r	pBIM1 trfA promoter in pKO6	This study Fig. 3
pBIM9.1	5.7	pMB1	Pn^r	pBIM1.1 trfA promoter in pKO6	This study

MATERIALS AND METHODS

Bacterial strains and plasmids

Escherichia coli K12 strain C600K$^-$ (*thr-1*, *leu-6*, *thi-1*, *lacY1 supE44 tonA21 galK*) from M. Rosenberg was used throughout. Plasmids used are listed in Table 1.

Growth media

L broth (28) was used as standard liquid medium and solidified with 1.5% (w/v) agar for nutrient plates. For galactokinase assays bacteria were grown in M56 medium (29) with fructose as carbon source. Bacteria were grown at 37°C. Antibiotic resistance was selected by the addition of penicillin-G for Pn^r (100 or 150μg/ml in liquid, 300μg/ml in solid medium), streptomycin sulphate for Sm^r (30μg/ml) and tetracycline hydrochloride for Tc^r (10μg/ml).

Plasmid DNA isolation, analysis and manipulation

Plasmid DNA was isolated for rapid analysis by the alkaline SDS method of Birnboim and Doly (30) with slight modifications (17). Purification on CsCl/Ethidium bromide gradients was as described previously (17). Agarose gel electrophoresis was carried out by standard techniques. Restriction

enzymes and DNA modifying enzymes were purchased from Bethesda Research Laboratories or nbl enzymes and used essentially according to the manufacturers recommendations.

DNA sequence determination

DNA sequences were determined by the method of Maxam and Gilbert (31) with minor modifications as previously described (27).

Mutagenesis of plasmid DNA

Hydroxylamine mutagenesis of plasmid DNA *in vitro* was carried out as described elsewhere (32).

Assay of galactokinase activity

Galactokinase enzyme activity was assayed essentially as described by McKenney *et al.* (33).

RESULTS

The trfB promoter is negatively regulated by trfB/korA/korD and korB

To study the regulation of transcription from the *trfB* promoter we attempted to couple expression of a gene whose product could be assayed easily to this promoter. Initially, we inserted a *Sau*3A fragment carrying the *trfB* promoter into *galK* promoter probe plasmid pKO4, using a host strain carrying a compatible plasmid, pCT407, supplying *trfB*/*korA*/*korD* in *trans*, since we thought that if the *trfB* promoter were repressed by *trfB*, this might aid the isolation of the desired plasmid. While analysis of plasmid DNA from some of the transformants obtained indicated the successful cloning of the *Sau*3A fragment containing the *trfB* promoter the resulting plasmids were very unstable and could not be introduced by transformation into a strain lacking *trfB*/*korA*/*korD*. This suggested to us that the *trfB* promoter is indeed regulated by the product of the *trfB*/*korA*/*korD* locus but that it may be a very strong promoter since it is known that in pKO4 and other pBR322 derivatives a very strong promoter in one orientation will cause sufficient readthrough into the *rom* gene and *ori* region to inhibit plasmid replication (35).

In order to overcome these difficulties the vector pKM-1 was used. In this plasmid the λt_{R1} transcriptional terminator is present between the *galK* gene and the site(s) where foreign promoters can be inserted. While expression of the *galK* gene is still proportional to the number of transcripts initiated at the foreign promoter that is inserted, more than 90% of these terminate prior to *galK* and consequently do not readthrough into the replication region of the vector. Into the *Eco*RI site of pKM-1

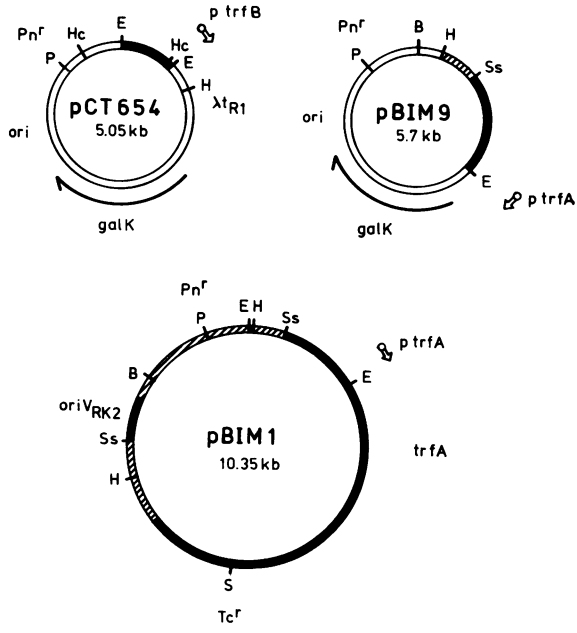

FIG. 3. Structure of plasmids not previously reported. Restriction sites are indicated as in Fig. 1 with the addition of P, PstI and Hc, HincII. The HincII sites are only shown in pCT654. Genetic symbols are as described in the text. RK2-derived DNA is shown as ▬▬▬ ; galK promoter vectors pKM-1 and pKO6 in pCT654 and pBIM9 are shown as ▭ ; while DNA of other origin in pBIM9 is shown as ▨ .

we inserted an EcoRI fragment carrying the trfB promoter. This fragment was derived from a pBR322-based plasmid, pCT143, carrying the wild type trfB region into which had been inserted Tn1725, a Cm^r transposon derived from Tn1722 whose 38bp invert repeats contain an EcoRI site 15bp from their ends (36,15). The particular pCT143::Tn1725 derivative used has the transposon inserted between the trfB promoter and the potential translational starts observed in the DNA sequence (24; C.M. Thomas and C.A. Smith, unpublished). One pKM-1 derivative, pCT654 (Fig. 3), which expressed galactokinase and whose DNA gave restriction patterns expected if the desired fragment were inserted in the correct orientation was analysed further. C600K⁻ strains containing pCT654 and one of a range of IncQ plasmids (compatible with pKM-1) carrying combinations of the genes predicted to regulate the trfB promoter were studied. Estimation of plasmid DNA levels in these strains revealed no significant variation in pCT654 plasmid copy number. Galactokinase assays showed that while R300B,

Table 2. Effect of trfB and korB on expression of galK from the trfB promoter

Coresident Plasmid	Regulatory Gene Present	Average Galactokinase Units[a]	
		Absolute	Relative
R300B	-	37.5	1.00
pCT409	*trfB*$^+$	0.96	0.026
pCT415	*trfB*$^+$ *korB*$^+$	0.31	0.008
pCT417	*korB*$^+$	15.35	0.41

C600K$^-$ containing pCT654 and a co-resident plasmid was assayed for galactokinase as described in Materials and Methods.
[a] 1 unit = 1 nmol galactose phosphorylated per min per apparent E^{1cm}_{650nm}=1.0

the IncQ vector carrying the potentially regulatory genes, had no significant effect on galactokinase levels, *trfB* on its own depressed levels 40-fold, *korB* without *trfB* 2.5 fold, and *trfB* and *korB* together about 120-fold (Table 2).

While we predicted a large effect of *trfB* on its own it was of considerable interest to find that *korB* without *trfB* had a much smaller effect. While the level of *korB* expression from the *trpE* promoter in pCT417 may not accurately represent the natural level of *korB* expression the relatively small effect of *korB* on its own is consistent with the location of the proposed *korB* repressor binding site in the *trfA* and *trfB* promoters which is centred at -46 relative to the start of transcription while the proposed *trfB* repressor binding site overlaps the -10 region and is centred at position -13/14 (Fig. 2). These results are also consistent with the 2-fold repression of the *trfA* promoter that we have observed for *korB* on its own (V.Shingler and C.M. Thomas, unpublished) and also recently reported by Schreiner *et al.* (22). It appears therefore that the regulatory properties described here for the *trfB* promoter are similar to those described previously for the *trfA* promoter (21, 22) as we had predicted from the DNA sequences of these promoters (27).

Isolation of mutants with trfA expression insensitive to trfB repression

We have previously reported that mini IncP plasmid pCT201 is lost very

rapidly if a second plasmid, carrying the trfB/korA/korD gene, is introduced into the same strain (23). Plasmid pCT201 lacks the trfB region of RK2 and in it trfA is expressed from a mutant trfA promoter which is weaker than the wild type promoter due to a T→C transition at one end of the -10 region, at position -7 relative to the start of transcription (27). This spontaneous promoter mutation was selected as a result of deleting the trfB region, thus demanding loss of the (then unknown) KilD$^+$ phenotype (14) implying that the KilD$^+$ phenotype is associated with strong transcription of the trfA operon. We predicted that the effect on pCT201 of trfB/korA/korD in trans results from repression of trfA operon transcription from this mutant promoter to a level incompatible with efficient trfA-dependent replication. If this is the case then it should be possible to isolate mutants of pCT201 which are insensitive to trfB in trans as a result of mutations within the trfB operator rendering the trfA promoter operator-constitutive with respect to trfB.

To facilitate determination of the DNA sequence of the trfA promoter region of any mutants obtained we constructed a plasmid analogous to pCT201 but carrying the trfA region from pVI108.1 (15) in which a 36bp segment containing an EcoRI site is inserted approximately 100bp from the trfA promoter as a result of transposon mutagenesis with Tn1723 (36,15) and subsequent deletion of the internal transposon EcoRI segment. This plasmid, pBIM1 (Fig. 3), exhibits the same sensitivity to trfB in trans as pCT201 (results not shown). The plasmid DNA was mutagenized in vitro with hydroxylamine and transformed into a strain containing pCT409, a Sm^r IncQ replicon-based plasmid carrying trfB. After allowing time for expression of pBIM1 genes, the transformed bacteria were diluted into a large volume of selective medium and grown overnight at 37°C to select those pBIM1 derivatives which could be maintained in the presence of pCT409.

Plasmid DNA was isolated from individual transformant clones and analysed by agarose gel electrophoresis to check that cointegration of pCT409 and pBIM1 had not occurred, and then the pBIM1 derivative was isolated by transformation into C600K$^-$ and rechecked for stability when pCT409 was introduced. One plasmid isolated in this way, pBIM1.1, has been analysed in detail. When the stability of pBIM1.1 was determined by plating on media selecting for the incoming plasmid, pCT409, following by replica plating to estimate retention of the resident plasmid, for twelve clones of pBIM1.1 the retention ranged between 82 and 100% whereas with the control of pBIM1 only 55% retention was observed. When the transformed

culture was instead grown overnight in liquid medium selecting for pCT409 only and then serially diluted and spread on selective plates to determine the proportion of bacteria containing pCT409 which still retained the resident plasmid, the control pBIM1 was present in only 2.7% of bacteria while pBIM1.1 was present in between 15 and 68% (average=41%) of bacteria. Thus pBIM1.1 is clearly much less sensitive to the presence of trfB in trans than is pBIM1. Examination of the yield of pBIM1 and pBIM1.1 DNA indicated that they had approximately the same copy number in the absence of trfB. DNA of pBIM1.1 was purified on a large scale and the DNA sequence of the trfA promoter region was determined by the method of Gilbert and Maxam (see Materials and Methods) after 5' labelling the EcoRI-cut DNA by the use of γ^{32}P ATP and T4 polynucleotide kinase followed by recutting with SstII and purification of the relevant singly end-labelled fragment. The only detectable difference between the trfA promoter regions of pBIM1 and pBIM1.1 was a C→T transition (as expected from hydroxylamine mutagenesis) in the centre of the putative trfB/korA/korD repressor binding site and just upstream of the -10 region at -13 relative to the start of transcription (Fig. 2). HinfI and AluI digests of pBIM1 and pBIM1.1 DNA showed that, while no change occurs in the HinfI digestion pattern, two AluI bands disappear to be replaced by a larger one, consistent with a loss of a single AluI site as predicted from the AGCT sequence in the trfA promoter of pBIM1 being changed to AGTT in pBIM1.1 (Fig. 2). Therefore the mutant selection regime resulted as predicted in a point mutation in the putative trfB repressor protein operator sequence within the trfA promoter region.

The mutation in the trfA promoter region abolishes regulation by trfB

In order to determine the effect of the C→T transition, in the trfA promoter region of pBIM1.1, on the regulatory properties of this promoter the EcoRI to HindIII fragments carrying the trfA promoter from pBIM1 and pBIM1.1 were each inserted into pKO6 allowing transcription from the trfA promoter across the EcoRI site to be coupled to expression of the E.coli galK gene. These plasmids, pBIM9 (Fig. 3) and pBIM9.1, were introduced into C600K$^-$ in the presence of R300B or of the R300B derivatives, pCT409, pCT415 and pCT417 which express trfB, trfB and korB, or korB respectively and the levels of galactokinase assayed (Table 3). The results show clearly that the mutation does not significantly alter promoter strength or regulation by korB but does largely abolish regulation by trfB and reduces repression by the combination of trfB and

Table 3. Effect of trfB and korB on expression of galK from the trfA promoter.

Coresident Plasmid	Regulatory Gene Present	Average Galactokinase Units[a]			
		pBIM9		pBIM9.1	
		Absolute	Relative	Absolute	Relative
R300B	-	89.6	1.00	85.7	0.96
pCT409	trfB$^+$	8.4	0.09	75.1	0.84
pCT415	trfB$^+$ korB$^+$	1.9	0.02	19.1	0.21
pCT417	korB$^+$	15.8	0.18	20.8	0.23

C600K$^-$ containing pBIM9 which has the trfA promoter of pBIM1 that is sensitive to trfB in trans, and pBIM9.1 which has the trfA promoter of pBIM1.1 that is insensitive to trfB in trans, as well as a coresident plasmid was, assayed for galactokinase as described in Materials and Methods.

[a] 1 unit = 1nmol galactokinase phosphorylated per min per apparent $E^{1cm}_{650nm}=1.0$

korB to the level obtained with korB alone. We conclude therefore that this point mutation in the trfA promoter region alters a sequence necessary for the action of the trfB repressor, presumably an operator site.

DISCUSSION

The results described in this paper provide further evidence that the inverted repeats found in the trfA and trfB promoter regions represent operator sites for the trfB and korB repressor proteins. We originally proposed this (27) because the trfA promoter region contains two inverted repeats and is regulated by trfB and korB while the putative kilB promoter contains only one of these inverted repeats and is regulated only by korB (21, 27). We predicted also that the trfB promoter, having both inverted repeats arranged as in the trfA promoter, is regulated in the same way as the latter. We have shown here that the trfB promoter is indeed repressed by the products of both trfB and korB. This means that the operon encoding trfB/korA/korD, incC and korB (24,20) is autogeneously regulated by both trfB and korB. It is not uncommon for repressor genes to be autogenously regulated but double autogenous regulation is unusual. The

trfB promoter clearly functions very efficiently in E.coli. Given the key role of trfB/korA/korD and korB in copy number control (23) and regulation of kil genes (16,17,18,21) it is probably important for these genes to be adequately expressed in all of the diverse hosts in which RK2 can be maintained. While the absolute strength of transcription from the trfB promoter region may vary from species to species autogenous regulation may allow the repressor concentration to be maintained at roughly constant levels thus possibly playing a role in the broad host range of RK2.

We also predicted that if the trfA or trfB promoters were mutated within the sequences proposed to be the operator sites for trfB and korB the regulatory properties would be altered. We have shown that this second prediction is also correct. This evidence indicates that more detailed studies to analyse the way that trfB and korB proteins interact with the trfA and trfB promoters are justified. In view of the similarity of the operator sequences proposed for trfB and korB (Fig. 2), differing only in the separation of the two halves of the inverted repeats, it will be extremely interesting to determine whether the trfB and korB proteins interact with the same set of bases in the DNA and, if so, whether there is an evolutionary relationship between the two repressor proteins.

In addition, our results shed light on another aspect of the regulation of RK2 plasmid maintenance which appears somewhat unclear in the literature. Schreiner et al. (22) report a positive role for kilB1 (kilD) in plasmid replication. Their evidence for this comes from the study of plasmids similar to those which we have studied in detail. They report that a plasmid consisting of $oriV_{RK2}$ and a wild type trfA kilB1 (kilD) region requires korA (korD/trfB) in trans (or in cis) to be able to replicate, presumably because if kilB1 (kilD) expression were unregulated the plasmid would not survive. They isolated kilB1$^-$ (kilD$^-$) mutants which did not require korA in trans and found that these mutants were now very sensitive to korA in trans, being rapidly lost in the presence of korA. They concluded that kilB1 normally plays a positive role in counteracting the effect of korA. Plasmids pCT201 and pBIM1, described in this paper, show phenotypically similar properties. The trfA region incorporated into these plasmids was unwittingly isolated as a phenotypically KilD$^-$ mutant (14) and we have shown by DNA sequence analysis (37,27; C.A. Smith and

C.M. Thomas, unpublished) that the KilD$^-$ phenotype of this mutant is due to a point mutation in the trfA operon promoter; a T→C transition in the -10 box at position -7 relative to the start of transcription (Fig. 2) reducing promoter strength by about an order of magnitude (21,27). For this reason, and because the trfA promoter is the only promoter in this region that is regulated by korD, we have argued that the KilD$^+$ phenotype is a manifestation of the trfA operon. The phenotype of the particular KilD$^-$ mutant we have studied therefore does not arise by the inactivation of some separate kilD gene but is the result of reduced transcription of the trfA operon of which kilD is some as yet undefined part. We have reported (23) that a mini RK2 replicon which contains this phenotypically KilD$^-$ trfA region (pCT201) is rapidly lost when korD (korA/trfB) is placed in trans and this has also been shown by Schreiner et al. (22) recently. In this paper we have shown conclusively that this is due to repression of trfA operon transcription by korD since the effect can be reversed by an operator point mutation abolishing repression of the trfA promoter by trfB (korA/korD).

From these results we can also see that a point mutation restoring full strength to the trfA promoter would also reverse the inhibitory effect of trfB (korA/korD) in trans because even when repressed by trfB the wild type trfA promoter would give sufficient trfA expression for replication. Such a point mutation would presumably reactivate kilD, but is reacivation of kilD necessary to overcome the effect of trfB (korA/korD) in trans? It is possible that the KilD$^+$ phenotype could be merely a manifestation either of the strength of the wild type trfA promoter which might read through into regions of the plasmid which are important for survival or of over expression of the trfA gene. However, in either of these cases we think that it would be misleading to imply that kilD is an independent locus which is required to counteract the effect of trfB/korA/korD. We do not rule out the possibility that a gene product of another cistron in the trfA operon, separate from trfA, is responsible for the KilD$^+$ phenotype. In this case our results still explain the trfB-sensitive phenotype of IncP replicons using KilD$^-$ trfA regions without requiring the product of kilD (kilB1) to play a positive role in the replication of RK2 as proposed by Schreiner et al. (22). We hope that further studies currently underway will reveal the genetical basis for the KilD$^+$ phenotype.

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REFERENCES

1. Burkardt, H.-J., Reiss, G. and Pühler, A. (1979) J. Gen. Microbiol. 114, 341-348.
2. Stokes, H.W., Moore, R.J. and Krishnapillai, V. (1981) Plasmid 8, 202-212.
3. Thomas, C.M. (1981) Plasmid 5, 10-19.
4. Lanka, E., Lurz, R. and Fürste, J.P. (1983) Plasmid 10, 303-307.
5. Lanka, E. and Barth, P.T. (1981) J. Bacteriol. 148, 769-781.
6. Guiney, D.G. and Helinski, D.R. (1979) Mol. Gen. Genet. 176, 183-189.
7. Barth, P.T. (1979) in Plasmids of Medical, Environmental and Commercial Importance, Timmis, K.N. and Pühler, A., Eds. pp. 399-410, Elsevier/North Holland, Amsterdam.
8. Barth, P.T., Grinter, N.J. and Bradley, D.E. (1978) J. Bacteriol. 133, 43-52.
9. Meyer, R. and Helinski, D.R. (1977) Biochim. Biophys. Acta 487, 109-113.
10. Thomas, C.M., Meyer, R. and Helinski, D.R. (1980) J. Bacteriol. 141, 213-222.
11. Thomas, C.M., Stalker, D.M. and Helinski, D.R. (1981) Mol. Gen. Genet. 181, 1-7.
12. Stalker, D.M., Thomas, C.M. and Helinski, D.R. (1981) Mol. Gen. Genet. 181, 8-12.
13. Figurski, D. and Helinski, D.R. (1979) Proc. Natn. Acad. Sci. USA 76, 1648-1652.
14. Thomas, C.M. (1981) Plasmid 5, 277-291.
15. Shingler, V. and Thomas, C.M. (1984) J. Mol. Biol. 175, 229-250.
16. Figurski, D.H., Pohlman, R.F., Bechhofer, D.H., Prince, A.S. and Kelton, C.A. (1982) Proc. Natn. Acad. Sci. USA 79, 1935-1939.
17. Smith, C.A. and Thomas, C.M. (1983) Mol. Gen. Genet. 190, 245-254.
18. Pohlman, R.F. and Figurski, D.H. (1983) J. Bacteriol. 156, 584-591.
19. Meyer, R. and Hinds, M. (1982) J. Bacteriol. 152, 1078-1090.
20. Smith, C.A. and Thomas, C.M. (1984) J. Gen. Microbiol. 130, 1651-1663.
21. Shingler, V. and Thomas, C.M. (1984) Mol. Gen. Genet. 195, 523-529.
22. Schreiner, H.C., Bechhofer, D.H., Pohlman, R.F., Young, C., Bordon, P.A. and Figurski, D.H. (1985) J. Bacteriol. 163, 228-237.
23. Thomas, C.M. and Hussain, A.A.K. (1984) EMBO J. 3, 1513-1519.
24. Bechhofer, D.H. and Figurski, D.H. (1983) Nucleic Acids Res. 11, 7453-7469.
25. Figurski, D.H., Young, C., Schreiner, H.C., Pohlman, R.F., Bechhofer, D.H., Prince, A.S. and D'Amico, T.F. (1985) in Plasmids in Bacteria, Helinski, D.R., Cohen, S.N. and Clewell, D.B., Eds. pp. 227-242. Plenum New York.

26. Young, C., Bechhofer, D.H. and Figurski, D.H. (1984) J. Bacteriol. 157, 247-252.
27. Smith, C.A., Shingler, V. and Thomas, C.M. (1984) Nucleic Acids Res. 12, 3619-3630.
28. Kahn, M., Kolter, R., Thomas, C., Figurski, D., Meyer, R., Ramaut, E. and Helinski, D.R. (1979) in Methods in Enzymology Vol. 68, Wu. R. Ed., pp. 268-280. Academic Press, New York.
29. Carlton, B.C. and Brown, B.J. (1981) in Manual of Methods for General Bacteriology, Gerhardt, P., Murray, R.G.E., Costilow, R.N., Nester, E.W., Wood, W.A., Krieg, N.R., Phillips, G.B. Eds., pp. 222-242. American Society for Microbiology, Washington.
30. Birnboim, H.C. and Doly, J. (1979) Nucleic Acids Res. 7, 1513-1523.
31. Maxam, A.M. and Gilbert, W. (1980) in Methods in Enzymology Vol. 65, Grossman, L. and Moldave, K. Eds., pp. 499-560. Academic Press, New York 32. Kolter, R., Inuzuka, M. and Helinski, D.R. (1978) Cell 15, 1199-1208.
33. McKenney, K., Shimatake, H., Court, D., Schmeissner, U., Brady, C. and Rosenberg, M. (1981) in Gene amplification and analysis, vol. II, Chirikjian, J.C. and Papas T.S. Eds., pp. 383-415. Elsevier/North Holland, Amsterdam.
34. Thomas, C.M., Hussain, A.A.K. and Smith, C.A. (1984) Nature 298, 674-676. 35. Stueber, D. and Bujard, H. (1982) EMBO J. 1, 1399-1404.
36. Schmitt, R., Bernhard, E. and Mattes, R. (1979) Mol. Gen. Genet. 172, 53-65.
37. Smith, C.A. and Thomas, C.M. (1984) J. Mol. Biol. 175, 251-262.
38. Hawley, D.K. and McClure, W. (1983) Nucleic Acids Res. 11, 2237-2255.