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# *kil-kor* Regulon of Promiscuous Plasmid RK2: Structure, Products, and Regulation of Two Operons That Constitute the *kilE* Locus

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**The *kil-kor* regulon of IncP plasmid RK2 is a complex regulatory network that includes genes for replication and conjugal transfer, as well as for several potentially host-lethal proteins encoded by the *kilA*, *kilB*, and *kilC* loci. While *kilB* is known to be involved in conjugal transfer, the functions of *kilA* and *kilC* are unknown. The coregulation of *kilA* and *kilC* with replication and transfer genes indicates a possible role in the maintenance or broad host range of RK2. In this work, we found that a fourth *kil* locus, designated *kilE*, is located in the kb 2.4 to 4.5 region of RK2 and is regulated as part of the *kil-kor* regulon. The cloned *kilE* locus cannot be maintained in *Escherichia coli* host cells, unless *korA* or *korC* is also present in *trans* to control its expression. The nucleotide sequence of the *kilE* region revealed two potential multicistronic operons. The *kleA* operon consists of two genes, *kleA* and *kleB*, predicted to encode polypeptide products with molecular masses of 8.7 and 7.6 kDa, respectively. The *kleC* operon contains four genes, *kleC*, *kleD*, *kleE*, and *kleF*, with predicted products of 9.2, 8.0, 12.2, and 11.3 kDa, respectively. To identify the polypeptide products, each gene was cloned downstream of the phage T7 φ10 promoter and expressed in vivo in the presence of T7 RNA polymerase. A polypeptide product of the expected size was observed for all six *kle* genes. In addition, *kleF* expressed a second polypeptide of 6 kDa that most likely results from the use of a predicted internal translational start site. The *kleA* and *kleC* genes are each preceded by sequences resembling strong $\sigma^{70}$ promoters. Primer extension analysis revealed that the putative *kleA* and *kleC* promoters are functional in *E. coli* and that transcription is initiated at the expected nucleotides. The abundance of transcripts initiated in vivo from both the *kleA* and *kleC* promoters was reduced in cells containing *korA* or *korC*. When *korA* and *korC* were present together, they appeared to act synergistically in reducing the level of transcripts from both promoters. The *kleA* and *kleC* promoter regions are highly homologous and contain two palindromic sequences (A and C) that are the predicted targets for KorA and KorC proteins. DNA binding studies showed that protein extracts from *korA*-containing *E. coli* cells specifically retarded the electrophoretic mobility of DNA fragments containing palindrome A. Extracts from *korC*-containing cells altered the mobility of DNA fragments containing palindrome C. These results show that KorA and KorC both act as repressors of the *kleA* and *kleC* promoters. In the absence of *korA* and *korC*, expression of the cloned *kleA* operon was lethal to *E. coli* cells, whereas the cloned *kleC* operon gave rise to slowly growing, unhealthy colonies. Both phenotypes depended on at least one structural gene in each operon, suggesting that the operons encode genes whose products interact with critical host functions required for normal growth and viability. Thus, the *kilA*, *kilC*, and *kilE* loci of RK2 constitute a cluster of at least 10 genes that are coregulated with the plasmid replication initiator and the conjugal transfer system. Their potential toxicity to the host cell indicates that RK2 is able to establish a variety of intimate plasmid-host interactions that may be important to its survival in nature.**

Plasmids of incompatibility group P (IncP) are well known for their extensive host range among gram-negative bacteria (14, 46, 77), but remarkably little is understood about the genetic and molecular basis of their promiscuity. Nevertheless, studies focusing on the self-transmissible IncP plasmid RK2 (27) and the closely related plasmids RP1, RP4, R18, and R68 (9, 84) have revealed a replicon of intriguing genetic and regulatory complexity (17, 18, 77).

The only RK2 determinants required for replication are *oriV*, the origin of replication (43, 72, 81), and *trfA*, a gene that encodes two polypeptides involved in initiation of replication at *oriV* (37, 48, 65). *trfA* is both necessary (4) and (with *oriV*) sufficient (59–61) for RK2 replication in diverse gram-negative species. However, RK2 derivatives based only on *trfA* and *oriV* are generally lost from growing populations of bacteria in the absence of selection for the plasmid (58, 60). Wild-type RK2, in contrast, is maintained stably in a wide variety of hosts (60). Therefore, *trfA* and *oriV* endow RK2 with the capacity to replicate in a broad range of hosts, but the survival of RK2 in nature clearly depends on auxiliary functions for stable maintenance in different bacteria. One such function is encoded by *par/mrs*, a nonessential determinant of RK2 that encodes a multimer resolution system and promotes the efficient inheritance of unstable plasmids (19, 22, 51, 57). Removal of *par/mrs* from RK2 leads to marked instability in different host species (2).

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Another mechanism that enhances the maintenance of RK2 in bacterial populations is its highly promiscuous conjugal transfer system (23). Not only is conjugal transfer an effective mechanism for horizontal spread of RK2 to other bacterial populations, but also it is likely to be important in reestablishing RK2 in rare plasmid-free daughter cells that may arise after cell division.

We have suggested that the *kil-kor* regulon of RK2 harbors additional genes for plasmid maintenance or host range (17, 18, 20). This unusual set of coregulated operons was originally discovered through the phenotypes of so-called *kil* loci (*kilA*, *kilB*, and *kilC*), whose expression is lethal to *Escherichia coli* host cells in the absence of regulation by appropriate *kor* determinants (17). In addition to the *kil* operons, the *kil-kor* regulon includes the *trfA* (61, 66), *korA* (7, 70, 89), and *kfrA* (30, 82) operons. The *trfA* operon consists of the replication initiator gene, *trfA*, and *ssb* (32), which codes for a protein functionally related to the single-stranded-DNA-binding protein of *E. coli*. The *korA* operon contains the *korA*, *korB*, and *korF* regulatory genes and *incC*, which is thought to be involved in plasmid maintenance (6, 7, 28, 35, 42, 70, 76, 80). The *kfrA* operon includes a single gene for a DNA-binding protein of unknown function (30). A striking feature of the *kil-kor* regulon is that the individual operons are negatively controlled by various combinations of *korA*, *korB*, *korC*, *korE*, *korF*, *kfrA*, and *trbA* (7, 17, 18, 28–30, 61, 66, 78, 79, 82, 87–89). Genetic evidence has indicated that the products of *korA*, *korB*, *korC*, and *kfrA* are transcriptional repressors (7, 30, 61, 66, 68, 75, 79, 89). Recent biochemical studies with purified KorB and KfrA have confirmed them to be DNA-binding proteins that interact with specific palindromic sequences (5, 30). How *korE*, *korF*, and *trbA* regulate gene expression is not yet known.

The *kilB* locus is required for the conjugal transfer of RK2 (45, 83). *kilB1* (50) and *kilD* (69) are synonymous with the *trfA* promoter (3). The functions of the *kilA* and *kilC* operons are unknown, although they are not essential for replication and transfer (17, 59). Studies of the *kilA* operon have shown that each of its three genes (*klaA*, *klaB*, and *klaC*) encodes a potentially host-lethal function (20). The unregulated expression of *klaA* has multiple effects on *E. coli* host cells: rapid inhibition of cell growth, alterations in the outer cell membrane, and inhibition of cell division (53). In addition, *klaA* overexpression specifically inhibits the synthesis of phage λ tails during lytic infection (54). Although these effects do not reveal the actual function of *klaA*, they suggest that the KlaA protein interacts with a specific host cell function, possibly a chaperonin (54). We have suggested that, under normal conditions, the *kil* functions contribute to the stable maintenance or broad host range of RK2 (17, 18).

Here we report the identification, nucleotide sequence, and analysis of a fourth *kil* locus in the *kil-kor* regulon of RK2. We found that this locus, designated *kilE*, contains two multicistronic operons: the *kleA* operon, which consists of two genes (*kleA* and *kleB*), and the *kleC* operon, which specifies four genes (*kleC*, *kleD*, *kleE*, and *kleF*). We observed the polypeptide products for all six genes and mapped the transcriptional start sites for both operons. Genetic studies showed (i) that each operon is deleterious to *E. coli* host cells in the absence of *kor* functions and (ii) that the deleterious effects of each operon are prevented by *korA* and *korC*. Transcriptional analysis demonstrated that KorA and KorC proteins act as repressors in the control of the *kleA* and *kleC* operons. We discuss the possible significance of these operons in the maintenance of RK2.

## MATERIALS AND METHODS

**Nomenclature.** Coordinates of the RK2 physical map are defined as the distance in kilobases from the unique *Eco*RI site (e.g., kb 2.4 to 4.5 region). If a relevant plasmid gene is not present in a bacterial strain, the genotype is indicated with a superscript 0 (e.g., $kor^0$). The genes of the *kilE* locus have been named according to the genetic nomenclature previously adopted for the *kil* loci of RK2 (3, 20). This system uses a three-letter genetic designation consisting of "*kl*" (for *kil*) followed by a letter indicating the specific *kil* locus. Accordingly, the genes in the *kilE* locus use the prefix *kle* (pronounced "klee") and are named *kleA*, *kleB*, etc.

**Bacteria, bacteriophages, and plasmids.** *E. coli* MV10 (26), RP1894 (87), JM107 (86), and BL21(DE3) (73) were used in this study. RP1894 ($korA^+$ $korB^+$ $korC^+$ $korE^+$ $korF^+$ $bla^+$ $trpE^+$) is a derivative of MV10 that contains a portion of RK2 integrated into the chromosome. JM107 was the host for the construction and propagation of bacteriophage M13 derivatives. BL21(DE3) contains the gene for bacteriophage T7 RNA polymerase in the chromosome under control of the inducible *lacUV5* promoter. M13 vectors used for cloning and sequencing were M13mp18, M13mp19, M13hc4, M13hc5, M13pz1, and M13pz2 (35, 36, 83, 86). Plasmids used in this study are described in Table 1.

M13 constructs containing the *kleA* and *kleC* promoters were made as follows: M13jk309, insertion of the 240-bp *kleC* promoter-containing *Sau*3AI fragment (RK2 kb 3.63 to 3.87) from pRK2473 into the *Bam*HI site of M13mp18; and M13jk346, insertion of the 161-bp *kleA* promoter-containing *Sau*3AI fragment (RK2 kb 4.31 to 4.47) from pRK2086 into the *Bam*HI site of M13mp18.

Previously unpublished plasmids were constructed as follows: pRK2094, insertion of multiple *Bam*HI DNA linkers into the *Hin*cII site (RK2 kb 2.36) of pRK2086; pRK2096, insertion of a single *Bam*HI DNA linker into the *Hin*cII site (RK2 kb 2.36) of pRK2086; pRK2208, replacement of the *Eco*RI-*Bam*HI fragment (RK2 kb 0 to 2.36) of pRK2094 with the $Tp^r$-encoding *Eco*RI-*Bam*HI fragment of pLB2 (87); pRK2459, deletion of the *Not*I fragment (RK2 kb 4.26 to 5.16) of pRK2096; pRK2473, deletion of the *Not*I fragment of pRK2208, conversion of the remaining *Not*I site to an *Eco*RI site by blunting the ends and adding *Eco*RI linkers, and insertion of the *Eco*RI-*Bam*HI fragment (RK2 kb 2.36 to 4.26) into pHSS6 (63); pRK2791, insertion of the *Hin*PI fragment containing the *kleC* promoter into the *Acc*I site of M13mp18 to yield M13jk641, ligation of the *Hin*dIII-*Acc*I fragment of M13jk641 with the *Acc*I-*Bam*HI fragment of pRK2473, and insertion of the resulting *Hin*dIII-*Bam*HI fragment (RK2 kb 2.36 to 3.83) into pHSS6; pRK2792, insertion of the *Eco*RI-*Bam*HI fragment (RK2 kb 2.36 to 3.83) of pRK2791 into pCH1; pRK2793, digestion of pRK2086 with *Hin*fI and *Bss*HII, blunting of the ends, ligation with *Eco*RI DNA linkers, purification of the *kleA* operon-containing fragment (RK2 kb 3.76 to 4.61), and insertion into pCH1; pRK2795 and pRK2796, insertion of the *Eco*RI-*Hin*dIII fragments of M13jk346 and M13jk309, respectively, into pCH1; pRK2799, insertion of the *Eco*RI-*Aha*II region (RK2 kb 3.77 to 4.26) from pRK2473 into *Eco*RI- and *Acc*I-cleaved M13mp18 to yield M13jk649, followed by insertion of the *kleB*-containing *Eco*RI-*Hin*dIII fragment of M13jk649 into pT7-5B; pRK2800, purification of the *kleD*-containing *Bss*HII fragment (RK2 kb 3.13 to 3.56) from pRK2086 and insertion into pT7-5B; pRK2843, digestion of pRK2791 with *Fnu*4HI, blunting of the ends, ligation with *Hin*dIII DNA linkers, digestion with *Hin*dIII and

TABLE 1. Plasmids

| Plasmid | Selective marker(s)[a] | Relevant genotype | Description | Reference or source |
|---|---|---|---|---|
| pCH1 | $Ap^r$ | | Cloning vector with pMB1 replicon and *rrnB* transcription terminators downstream of a polylinker region | 20 |
| pGP56 | $Ap^r$ *trpE*$^+$ | Φ(*blap-korB*$^+$) | pSM1 replicon with *korB* expressed constitutively from the *bla* promoter | 88 |
| pMK20 | $Km^r$ | | Cloning vector with ColE1 replicon | 33 |
| pRK2086 | $Km^r$ | *kleA*$^+$*B*$^+$*C*$^+$*D*$^+$*E*$^+$*F*$^+$ *korC*$^+$*E*$^+$ *klaA*$^+$*B*$^+$ | pMK20 with kb 0 to 5.96 region of RK2 (Fig. 1) | 17 |
| pRK2096 | $Km^r$ | *kleA*$^+$*B*$^+$*C*$^+$*D*$^+$*E*$^+$*F*$^+$ *korC*$^+$*E*$^+$ *klaA*$^+$*B*$^+$ | pRK2086 with a *Bam*HI DNA linker at the *Hin*cII site (RK2 kb 2.36) (Fig. 1) | This study |
| pRK2208 | $Km^r$ $Tp^r$ | *kleA*$^+$*B*$^+$*C*$^+$*D*$^+$*E*$^+$*F*$^+$*korC*$^+$*E*$^+$ | pMK20 with kb 2.36 to 5.96 region of RK2 (Fig. 1) | This study |
| pRK2260 | $Km^r$ | *klaA*$^+$*B*$^+$ | pMK20 with kb 0 to 2.44 region of RK2 (Fig. 1) | 87 |
| pRK2292 | $Tp^r$ | Φ(*catp-korA*$^+$) | pSM1 replicon with *korA* expressed constitutively from the *cat* promoter | 3 |
| pRK2411 | $Km^r$ | *klaA*$^+$*B*$^+$ | pRK2260 with a *Bam*HI DNA linker at the *Hin*cII site (RK2 kb 2.36) (Fig. 1) | 89 |
| pRK2459 | $Km^r$ | Δ(*korC-kleA*) *kleB*$^+$*C*$^+$*D*$^+$*E*$^+$*F*$^+$ *korE*$^+$ *klaA*$^+$*B*$^+$ | pRK2096 with a deletion of the kb 4.26 to 5.16 region of RK2 (Fig. 1) | This study |
| pRK2462 | $Cm^r$ | *korC*$^+$ | P15A replicon with kb 4.26 to 5.16 region of RK2 | 36 |
| pRK2473 | $Km^r$ | *kleB*$^+$*C*$^+$*D*$^+$*E*$^+$*F*$^+$ *korE*$^+$ | ColE1 replicon with kb 2.36 to 4.26 region of RK2 (Fig. 1) | This study |
| pRK2634 | $Ap^r$ | Φ(T7 φ10*p-korC*$^+$) | pT7-5B with *korC* downstream of the phage T7 φ10 promoter | 36 |
| pRK2659 | $Tc^r$ | *korA*$^+$*B*$^+$*C*$^+$*E*$^+$ *kleA*$^+$*B*$^+$*C*$^+$*D*$^+$ *E*$^+$*F*$^+$*klaA*$^+$*B*$^+$*C*$^+$ | pSC101 replicon with kb 50.4 to 56.4 and kb 0 to 5.96 regions of RK2 | 36 |
| pRK2792 | $Ap^r$ | *kleC*$^+$*D*$^+$*E*$^+$*F*$^+$ | pCH1 with the *kleC* operon | This study |
| pRK2793 | $Ap^r$ | *kleA*$^+$*B*$^+$ | pCH1 with the *kleA* operon | This study |
| pRK2795 | $Ap^r$ | *kleAp* | pCH1 with the *kleA* promoter | This study |
| pRK2796 | $Ap^r$ | *kleCp* | pCH1 with the *kleC* promoter | This study |
| pRK2799 | $Ap^r$ | Φ(T7 φ10*p-kleB*$^+$) | pT7-5B with *kleB* downstream of the phage T7 φ10 promoter | This study |
| pRK2800 | $Ap^r$ | Φ(T7 φ10*p-kleD*$^+$) | pT7-5B with *kleD* downstream of the phage T7 φ10 promoter | This study |
| pRK2843 | $Ap^r$ | Φ(T7 φ10*p-kleE*$^+$) | pT7-5B with *kleE* downstream of the phage T7 φ10 promoter | This study |
| pRK2844 | $Ap^r$ | Φ(T7 φ10*p-kleF*$^+$) | pT7-5B with *kleF* downstream of the phage T7 φ10 promoter | This study |
| pRK2845 | $Ap^r$ | Φ(T7 φ10*p-kleA*$^+$) | pT7-5B with *kleA* downstream of the phage T7 φ10 promoter | This study |
| pRK2846 | $Ap^r$ | Φ(T7 φ10*p-kleC*$^+$) | pT7-5B with *kleC* downstream of the phage T7 φ10 promoter | This study |
| pRK2856 | $Ap^r$ | *kleA*$^+$*B*$^+$*C*$^+$*D*$^+$*E*$^+$*F*$^+$ | pCH1 with kb 2.36 to 4.61 region of RK2 | This study |
| pRK2999 | $Ap^r$ | *korAp* | pMB1 replicon with the *korA* promoter | 20 |
| pRK21258 | $Ap^r$ | *kleAp* | pCH1 with the *kleA* promoter | This study |
| pRK21408 | *trpE*$^+$ | Φ(*trcp-korB*$^+$) *lacI*$^q$ | R6K replicon with *korB* expressed from the *trc* promoter | 83 |
| pRK21471 | $Ap^r$ | Φ(T7 φ10*p-korA*$^+$) | pT7-5B with *korA* downstream of the phage T7 φ10 promoter | This study |
| pT7-5B | $Ap^r$ | T7 φ10*p* | pMB1 replicon with the phage T7 φ10 promoter | 36 |

[a] $Ap^r$, $Cm^r$, $Km^r$, $Tc^r$, and $Tp^r$, resistance to ampicillin, chloramphenicol, kanamycin, tetracycline, and trimethoprim, respectively.

*Bss*HII, purification of the *kleE*-containing *Bss*HII-*Hin*dIII fragment (RK2 kb 2.75 to 3.13), and insertion into pT7-5B; pRK2844, digestion of pRK2792 with *Bgl*I, blunting of the ends, digestion with *Hin*dIII, purification of the *kleF*-containing fragment (RK2 kb 2.36 to 2.77), and insertion into *Sma*I- and *Hin*dIII-cleaved pT7-5B; pRK2845, purification of the *kleA*-containing *Aha*II fragment (RK2 kb 3.93 to 4.53) from pRK2086 and insertion into the *Cla*I site of pT7-5B; pRK2846, digestion of pRK2086 with *Hga*I, blunting of the ends, purification of the *kleC*-containing fragment (RK2 kb 3.42 to 3.77), and insertion into the *Sma*I site of pT7-5B; pRK2856, purification of the *Acc*I-*Not*I fragment (RK2 kb 3.61 to 4.26) of pRK2086, the *Not*I-*Pvu*I fragment (containing RK2 kb 4.26 to 4.61 and the pMB1 plasmid replicon) of pRK2793, and the *Acc*I-*Pvu*I fragment (containing RK2 kb 2.36 to 3.61 and a portion of the plasmid vector) of pRK2792, followed by ligation; pRK21258, multiple steps resulting in the combination of the *kleA* promoter-containing *Aha*II-*Sau*3AI region (RK2 kb 4.31 to 4.53; see nucleotides 27 to 244 in Fig. 2) and the *Bam*HI-*Hin*dIII polylinker region of pUC19 (86) inserted between the *Cla*I and *Hin*dIII sites of pCH1; and pRK21471, polymerase chain reaction amplification of *korA* from pRK2292 by using primers KORA1 (5′-GGAATTCTAAGGAGGTTTAAATGAAGAAACG GCTTACCGAAAGCC-3′) and KORA2 (5′-CAAGCTTGC CGACGCCGCCTTTCTGG-3′), blunting of the ends, cleavage with *Eco*RI, and insertion into *Sma*I- and *Eco*RI-cleaved pT7-5B, with subsequent confirmation of the nucleotide sequence of the cloned fragment. All plasmids were made in the presence of *korA*, *korB*, and *korC*.

**Media.** Media and appropriate antibiotic supplements used for growth and selection of bacteria have been described previously (36). Soft agar overlays containing isopropyl-β-D-thiogalactopyranoside (IPTG) at 1 mM and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) at 40 μg/ml were used to identify DNA fragment insertions into the *lac* region of the M13 vectors (41). For induction of the *trc* promoter, media were supplemented with 1 mM IPTG.

**DNA methodology.** Preparation of plasmid DNA, agarose gel electrophoresis, and polyacrylamide gel electrophoresis have been described previously (33). DNA manipulations with restriction endonucleases, T4 DNA ligase, and synthetic DNA linkers were done according to published procedures (1). DNA fragments with protruding 3′ ends were

made blunt by digestion with T4 DNA polymerase (1). The Klenow fragment of DNA polymerase I was used to blunt DNA fragments containing protruding 5′ ends (1). [α-$^{32}$P]dATP was included in the Klenow reaction mixture to radiolabel DNA fragments for protein-DNA binding studies. Amplification of DNA by the polymerase chain reaction was done with *Taq* DNA polymerase (52). DNA fragments were purified from gels by electroelution (36) or by the crush-and-soak method (40). Single-stranded M13 DNA was purified as described previously (41). Transformation and transfection of *E. coli* were by the method of Cohen et al. (13).

The nucleotide sequence of the kb 2.36 to 4.26 region of RK2 was determined for both strands by using 59 unique M13 clones that contain various overlapping DNA fragments. The construction and propagation of the M13 clones were done in JM107 containing pRK2659. The nucleotide sequence was determined by the dideoxynucleotide chain termination method (56), as described previously (20, 36). The computer programs of BIONET (71) and the Genetics Computer Group (15) were used for DNA sequence analysis.

**RNA analysis.** RNA transcripts initiating from the *kleA* and *kleC* promoters were analyzed by the primer extension method (1). Each promoter was inserted into pCH1, which contains transcription terminators downstream of the polylinker region. The terminators allow the stable maintenance of the plasmids (pRK2795 and pRK2796) in the absence of *kor* regulatory functions, thus permitting high-level synthesis of mRNA from the derepressed promoters. Total cellular RNA was isolated from strains MV10(pRK2795) and MV10(pRK2796). The synthetic DNA primer (35-mer) is complementary to the *Hin*dIII-*Bam*HI region of the polylinker immediately downstream of the cloned promoters (83). End labelling of the primer with [γ-$^{32}$P]ATP and T4 polynucleotide kinase, annealing of the primer to RNA, and extension of the primer with avian myeloblastosis virus reverse transcriptase were done according to published procedures (1). The extended products were analyzed by electrophoresis through a polyacrylamide sequencing gel followed by autoradiography (1, 55). To map the 5′ ends of the transcripts, nucleotide sequencing reaction products were included on the gel to generate a reference nucleotide sequence. The sequencing products were synthesized by using the same 5′-end-labelled primer and M13 constructs (M13jk346 and M13jk309) that contain the same promoter fragments.

To test for regulation of the *kleA* and *kleC* promoters, RNA was extracted from *kor*$^0$ cells and cells carrying *korA*, *korC*, and *korA* plus *korC*. The constitutively expressed *bla* transcript from the plasmid vector was used as an internal control to standardize the amount of total RNA in each sample. The abundance of *bla* transcripts was monitored by primer extension analysis using a *bla*-specific primer, as described elsewhere (83).

**Polypeptide analysis.** The bacteriophage T7 RNA polymerase-dependent expression system (73, 74) was used to express and identify the polypeptide products of the genes in the *kilE* region. Each gene was cloned individually downstream of the T7 φ10 promoter in pT7-5B. The host strain was BL21(DE3), which carries the gene for T7 RNA polymerase. Strains containing pRK2845 (*kleAp-kleA*$^+$) and pRK2846 (*kleCp-kleC*$^+$) also carried plasmids pRK2163 (*korA*$^+$) (61) and pRK2462 (*korC*$^+$) to prevent deleterious expression of the cloned genes from the *kleA* and *kleC* promoters. Radioactive labelling of polypeptides, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and autoradiography were performed as described previously (36). $^{14}$C-protein molecular weight markers were obtained from Amersham Corp. (Arlington Heights, Ill.).

**DNA binding studies.** The bacteriophage T7 RNA polymerase-dependent expression system was used to express *korA* (pRK21471) and *korC* (pRK2634) as described previously (36). The cells were pelleted, washed, and resuspended in cold lysis buffer (31). Extracts were prepared by sonication and stored at −70°C as described elsewhere (8). A 275-bp DNA fragment containing the *korA* promoter was generated by *Xba*I and *Bam*HI cleavage of pRK2999. A 225-bp fragment with the *kleA* promoter (encompassing nucleotides 27 to 244 in Fig. 2) was released from pRK21258 by digestion with *Eco*RI and *Bam*HI. The fragments were gel purified and labelled with $^{32}$P, as described above. The labelled DNA fragments were incubated with bacterial extracts in DNA-binding buffer (34). The reaction mixtures were 20 μl in volume and contained the following: 250 to 333 ng of protein from the appropriate bacterial extract, 20 mM Tris (pH 7.4), 100 mM NaCl, 7 mM $MgCl_2$, 1 mM disodium EDTA, 1 mM dithiothreitol, 25 μg of poly(dI-dC) per ml, 3.5% glycerol, and approximately 3,000 cpm of $^{32}$P-labelled DNA fragment. After incubation at 20°C for 15 min, the reaction mixtures were subjected to electrophoresis through an 8% nondenaturing polyacrylamide gel in 0.25× TBE buffer (25 mM Tris, 25 mM boric acid, 0.5 mM EDTA, pH 7.5). The gels were dried and autoradiographs were prepared as described previously (1).

**Nucleotide sequence accession number.** The nucleotide sequence and amino acid sequence data reported here have been submitted to the GenBank data bases under accession number L18919.

## RESULTS

**Genetic identification of *kilE*.** Plasmid pRK2260 contains the kb 0 to 2.44 region of RK2, which encodes the promoter and first two genes (*klaA* and *klaB*) of the *kilA* operon (Fig. 1) (20). Previous studies have shown that unregulated expression of either *klaA* or *klaB* from the *kilA* promoter is lethal to *E. coli* host cells (20). Thus, pRK2260 can be maintained only in *E. coli* cells that carry the appropriate *kor* functions to repress the *kilA* promoter (Table 2) (87). When the *kilA* promoter was inactivated by insertion of a DNA linker at the *Hin*cII site (kb 2.36) as in pRK2411 (Fig. 1), the lethal phenotype was abolished and *kor* functions were not required for maintenance of the plasmid (Table 2) (89).

Plasmid pRK2086 contains the kb 0 to 5.96 region of RK2, which encodes *korC* (36, 87), *korE* (88), and the same portion of the *kilA* operon that is present in pRK2260 (Fig. 1). Not surprisingly, it was found to require the same *kor* functions for maintenance in the cell as did pRK2260 (Table 2) (87). However, insertion of a DNA linker at the *Hin*cII site in the *kilA* promoter of pRK2086 did not completely abolish the deleterious phenotype (pRK2096, Fig. 1 and Table 2). Transformant colonies of the *kor*$^0$ strain were considerably smaller than those containing pRK2411. This phenotype conferred by pRK2096 (defined as Kil$^{+/-}$) was not observed in strains containing *korA* or *korC* on a high-copy-number plasmid (Table 2).

We deleted the *kilA* region (kb 0 to 2.36) from pRK2096 and found that the resulting plasmid (pRK2208; Fig. 1) produced a Kil$^-$ phenotype, i.e., all transformants were healthy (Table 2). Thus, the Kil$^{+/-}$ phenotype conferred by pRK2096 results from expression of *kilA* genes at a nonlethal, but deleterious, level. Because the *kilA* promoter is inactivated in pRK2096, the *kilA*-dependent phenotype

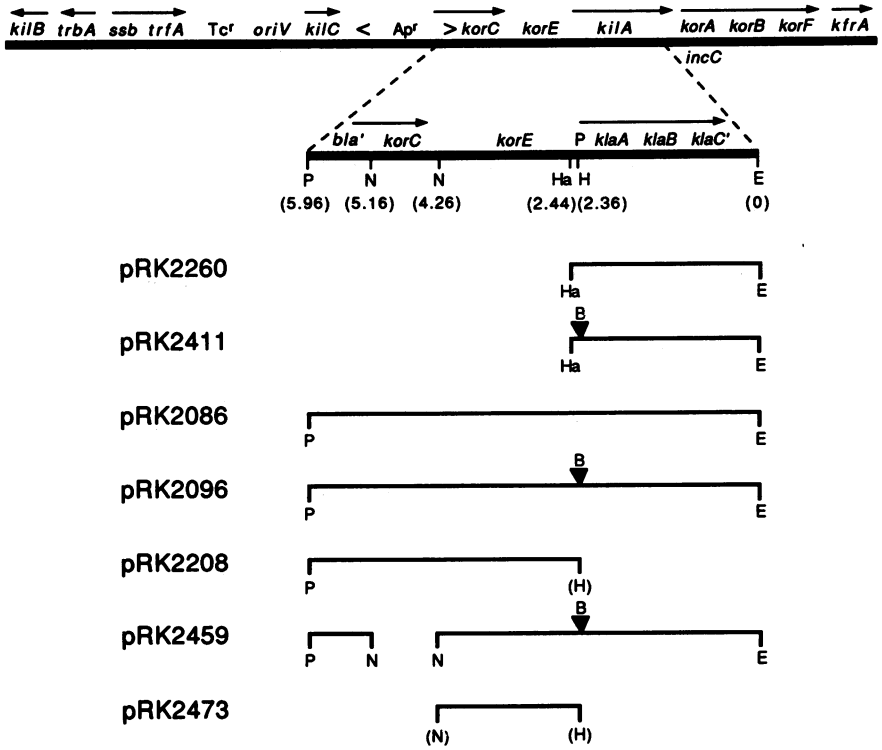


FIG. 1. Plasmids used to identify the *kilE* locus of RK2. A genetic map of an approximately 25-kb portion of RK2 is shown on top. Ap$^r$ shows the position of transposon Tn*1*, which contains the *bla* gene responsible for ampicillin resistance (11, 25). Tc$^r$ indicates the region encoding resistance to tetracycline (85). All other RK2 loci are described in the text. Arrows indicate the directions of transcription. The kb 0 to 5.96 region of RK2 is expanded below, and segments of this region present in the various plasmids are indicated. P shows the promoter for the *kilA* operon, which contains *klaA*, *klaB*, and *klaC*. *klaC'* indicates a truncated *klaC* gene; *bla'* indicates the 3' end of *bla*. Relevant restriction endonuclease cleavage sites are designated E (*Eco*RI), H (*Hin*cII), Ha (*Hae*II), N (*Not*I), and P (*Pst*I). Sites enclosed by parentheses were modified in the plasmids. Filled triangles labelled B show insertions of *Bam*HI DNA linkers at the *Hin*cII site in the *kilA* promoter. Numbers in parentheses refer to RK2 map coordinates, which represent the distance in kilobases from the unique *Eco*RI site located in *klaC*.

shows that the upstream region (kb 2.36 to 5.96) contains a promoter that can express *kilA*. Furthermore, the deleterious phenotype shown by pRK2096 was controlled by *korA* or *korC*. Therefore, the upstream region contains either a promoter regulated by both *korA* and *korC* or two promoters, one of which is controlled by *korA* and the other of which is controlled by *korC*.

If *korC* regulates an upstream promoter, then *kilA* expression in pRK2096 should increase if *korC* is removed from the plasmid. We deleted the *korC*-containing *Not*I fragment (kb 4.26 to 5.16) from pRK2096 to produce pRK2459 (Fig. 1). As predicted, pRK2459 could not be maintained in a *kor*$^0$ strain and therefore conferred a Kil$^+$ phenotype (Table 2). We reasoned that loss of *korC* allowed higher expression of *kilA* from the upstream promoter and caused a more severe phenotype. The Kil$^+$ phenotype exhibited by pRK2459 was regulated not only by *korC* in *trans* but also by *korA*, indicating that both repressors act on the same promoter.

We expected that deletion of the *kilA* region would change the Kil$^+$ phenotype conferred by pRK2459 to Kil$^-$. Instead, we found that plasmid pRK2473 (Fig. 1), which lacks the *kilA* region (kb 0 to 2.36) and the region upstream of *korC* (kb 5.16 to 5.96), still expressed a Kil$^{+/-}$ phenotype typified by slowly growing, sick colonies in a *kor*$^0$ strain. This phenotype was controlled by *korA* and by *korC* (Table 2). We concluded that the kb 2.36 to 4.26 region encodes a *kil*-like

TABLE 2. Relative transformation efficiencies of plasmids carrying DNA from the kb 0 to 5.96 region of RK2[a]

| Transforming plasmid[b] | Relative transformation efficiency[c] of recipient strain with relevant genotype of: | | | | |
|---|---|---|---|---|---|
| | *kor*$^0$ | *korA*$^+$ | *korB*$^+$ | *korC*$^+$ | *korA*$^+$*B*$^+$*C*$^+$ |
| pMK20 | + | + | + | + | + |
| pRK2260 | − | + | − | − | + |
| pRK2411 | + | + | + | + | + |
| pRK2086 | − | + | − | − | + |
| pRK2096 | (+) | + | (+) | + | + |
| pRK2208 | + | + | + | + | + |
| pRK2459 | − | + | − | + | + |
| pRK2473 | (+) | + | (+) | + | + |

[a] Recipient strains were transformed with each plasmid, and kanamycin-resistant colonies were selected. The vector pMK20 was used to determine the relative competence of each strain as described previously (17). Efficiencies of transformation were adjusted for competence differences and normalized to that of the *korA*$^+$*B*$^+$*C*$^+$ strain.

[b] See Fig. 1 for physical and genetic maps.

[c] +, the ability to form normal, healthy colonies at a frequency similar to that of the *korA*$^+$*B*$^+$*C*$^+$ strain (defined as a Kil$^-$ phenotype); −, a decrease by at least a factor of $10^3$ in the ability to form colonies relative to that of the *korA*$^+$*B*$^+$*C*$^+$ strain (defined as a Kil$^+$ phenotype); (+), the ability to form colonies at a frequency similar to that of the *korA*$^+$*B*$^+$*C*$^+$ strain, but with colonies that are considerably smaller (defined as a Kil$^{+/-}$ phenotype). Recipient strains were the following: MV10 (*kor*$^0$), MV10(pRK2292) (*korA*$^+$), MV10(pGP56) (*korB*$^+$), MV10(pRK2462) (*korC*$^+$), and RP1894 (*korA*$^+$*B*$^+$*C*$^+$).

determinant that is deleterious to *E. coli* host cells and is controlled by *korA* and *korC*. We designated this locus *kilE*.

**Nucleotide sequence of the *kilE* region.** We determined the nucleotide sequence of the region between the *Hin*cII site at kb 2.36 and the *Not*I site at kb 4.26 (Fig. 2). In previous studies, we presented the nucleotide sequences of the kb 0 to 2.39 *kilA* region (20) and the kb 4.26 to 5.96 *korC* region (36) of RK2. Figure 2 shows the complete nucleotide sequence of the region from the 3′ end of *korC* (kb 4.56) to the 5′ end of the *kilA* operon (kb 2.26).

The nucleotide sequence of the *kilE* region was searched for possible promoter sequences and potential regulatory sites for the KorA and KorC proteins. Two regions showed strong similarity to the consensus sequence for *E. coli* $\sigma^{70}$ promoters (Fig. 2). Both regions include the same two operatorlike palindromic sequences. One palindrome (A in Fig. 2) occurs immediately upstream of the −35 regions and is identical to a palindrome overlapping the −10 regions of the promoters for the *trfA*, *kilA*, and *korA* operons (68, 89). This sequence is believed to be the binding site for KorA protein (75, 89). The other palindrome (C in Fig. 2) overlaps the −10 regions. We considered this palindrome to be a possible target for the KorC protein.

We also examined the nucleotide sequence for possible protein-encoding genes. Six open reading frames (ORFs) have potential translational start codons (ATG or GTG) preceded by good Shine-Dalgarno sequences for ribosome binding (Fig. 2). RK2 genes typically exhibit a distinctive pattern of codon usage, with a strong preference for codons having a G or C residue in the third position (20). The six identified ORFs of the *kilE* region all have a high percentage of codons ending in G or C, suggesting strongly that these ORFs encode proteins. In accordance with our previously established nomenclature for genes of the *kil* loci (20) (see Materials and Methods), the ORFs were designated *kleA* (nucleotides 252 to 485), *kleB* (nucleotides 534 to 749), *kleC* (nucleotides 902 to 1132), *kleD* (nucleotides 1148 to 1366), *kleE* (nucleotides 1478 to 1801), and *kleF* (nucleotides 1829 to 2143). *kleD* has two possible translation initiation codons separated by 15 bp: an ATG at nucleotide 1148 and a GTG at nucleotide 1166. *kleF* contains a potential internal translational start at nucleotide 1973.

**Polypeptide products of the *kleABCDEF* genes.** The predicted molecular masses of the polypeptide products of the *kle* ORFs are 8,652 Da (KleA), 7,599 Da (KleB), 9,202 Da (KleC), 8,027 Da (KleD), 12,182 Da (KleE), 11,305 Da (KleF), and 6,038 Da (KleF initiated from the potential internal start). To identify the predicted polypeptides, each ORF was individually expressed in the bacteriophage T7 RNA polymerase-dependent expression system. Each *kle* ORF was found to express a polypeptide with an observed mass that corresponds closely to the calculated mass of the predicted product (Fig. 3). In addition, we observed a polypeptide corresponding to the use of the potential internal start of *kleF*. We conclude that the *kleABCDEF* ORFs are protein-encoding genes.

**Transcription initiation from the *kleA* and *kleC* promoters.** The *kilE* region contains two sequences that are related to the consensus sequence for $\sigma^{70}$ promoters. The first putative promoter (*kleA* promoter) is located immediately upstream of *kleA*, and the second (*kleC* promoter) is located between *kleB* and *kleC* (Fig. 2). Fragments containing each putative promoter were cloned, and the 5′ ends of any transcripts initiated from these regions were determined by primer extension analysis (Fig. 4). We found that transcription initiated from both predicted promoters. The start site for the *kleC* promoter corresponded to the expected +1 position, and transcription from the *kleA* promoter initiated at three consecutive nucleotides around the expected +1 position.

**Phenotypes conferred by the *kleA* and *kleC* operons and their regulation by *korA* and *korC*.** We constructed a plasmid (pRK2856) that contains the complete *kilE* region with all six *kle* genes and both promoters. This plasmid contains transcriptional terminators downstream of the *kilE* region to prevent possible disruption of plasmid maintenance by unregulated transcription, as has been observed for the *kilA* and *trfA* promoters (3, 20). We found that pRK2856 displayed a $Kil^+$ phenotype in *E. coli* cells lacking *korA* and *korC* (Table 3). Thus, expression of the complete *kilE* locus is lethal to host cells in the absence of *kor* regulatory functions.

Our results have shown that the promoters and genes in the *kilE* locus are arranged in two multicistronic operons: the *kleA* operon (*kleA* and *kleB*) and the *kleC* operon (*kleC*, *kleD*, *kleE*, and *kleF*). To test the individual roles of each operon in the host-lethal phenotype expressed by the *kilE* region, we constructed plasmids that carry only the *kleA* operon (pRK2793) or the *kleC* operon (pRK2792). In $kor^0$ cells, the *kleA* operon (pRK2793) conferred a $Kil^+$ phenotype, while the *kleC* operon (pRK2792) was responsible for a $Kil^{+/-}$ phenotype, evident as slowly growing transformants (Table 3). This is consistent with the phenotype displayed by pRK2473 (Table 2), which we now know from the nucleotide sequence is lacking the *kleA* promoter. Plasmids containing the *kleA* and *kleC* promoters, but none of the structural genes of the *kleA* and *kleC* operons (pRK2795 and pRK2796), were found to confer a $Kil^-$ phenotype in cells lacking *korA* and *korC* (Table 3). Thus, unregulated expression of at least one gene from each operon is deleterious to host cells in the absence of *kor* regulatory functions. However, the effect of the *kleA* operon is more severe than that of the *kleC* operon.

The Kil phenotypes expressed by the *kleA* operon (pRK2793) and the *kleC* operon (pRK2792) allowed us to test their regulation by *kor* genes. We found that the phenotypes expressed by both operons were prevented by strains containing *korA* or *korC*, whereas *korB* had no effect (Table 3). Therefore, *korA* and *korC* control both the *kleA* and *kleC* operons.

**KorA and KorC act as repressors of the *kleA* and *kleC* promoters.** Because *korA* is thought to be a transcriptional repressor of other RK2 promoters, it seemed likely that *korA* and *korC* negatively regulate transcription initiated by the *kleA* and *kleC* promoters. To test this possibility, we used primer extension analysis to examine the relative abundance of RNA transcripts originating in vivo from the *kleA* and *kleC* promoters in the presence and absence of *korA* and *korC*. We found that *korA* and *korC* each reduced the quantity of transcripts initiated from the *kleA* and *kleC* promoters (Fig. 5). In addition, the presence of *korA* and *korC* together caused a marked reduction in the levels of *kleA* and *kleC* transcripts beyond that observed for *korA* or *korC* alone.

The *kleA* and *kleC* promoter regions both contain two operatorlike palindromic sequences that are primary candidates for KorA and KorC binding sites. Using a gel mobility shift assay, we tested the ability of KorA- and KorC-containing extracts to bind to DNA fragments containing the palindromes. To distinguish between possible binding activities for each palindrome, we used DNA fragments that contain either the *kleA* or *korA* promoter. The palindromic

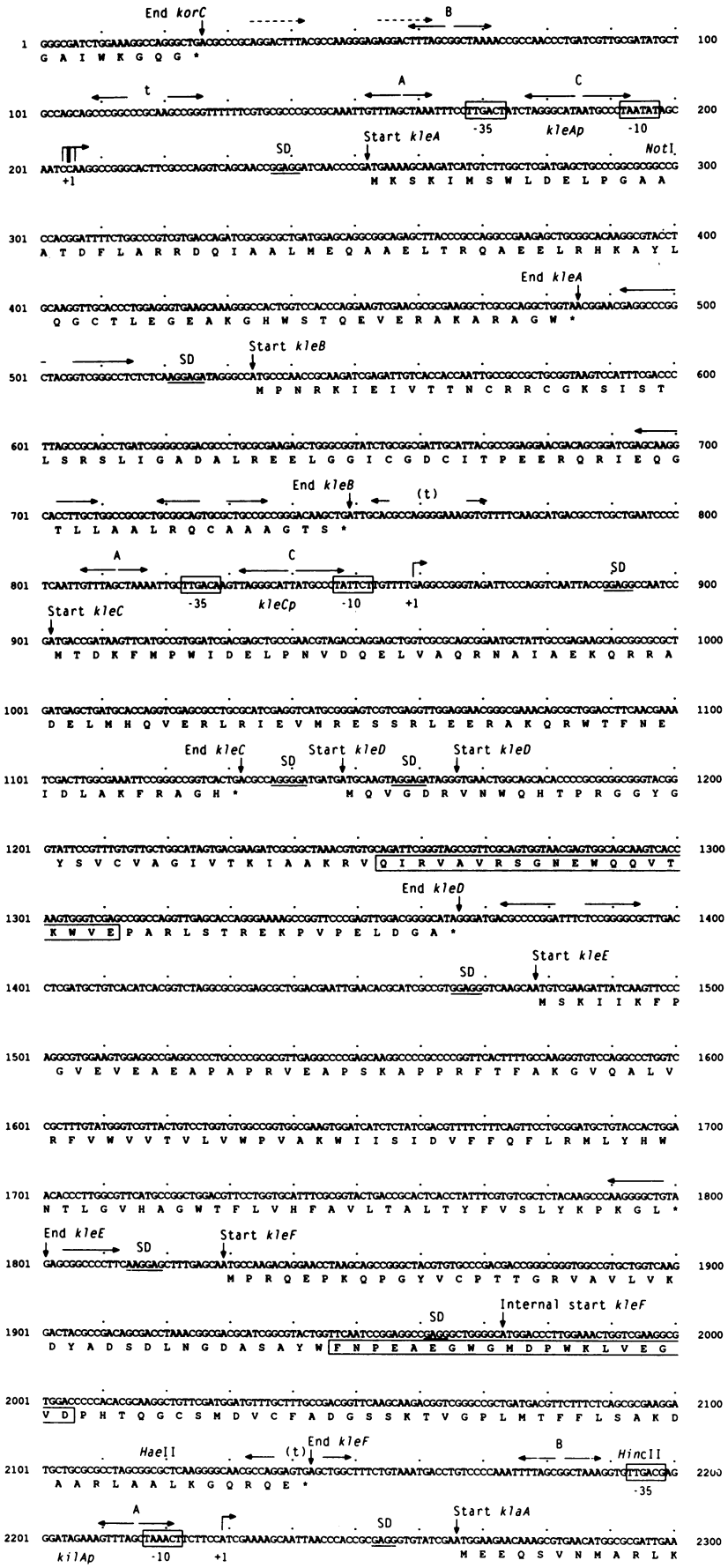
End korC
B
t
A
C
-35
kleAp
-10
Start kleA
SD
NotI
+1
End kleA
Start kleB
End kleB
(t)
Start kleC
End kleC
Start kleD
End kleD
Start kleE
End kleE
Internal start kleF
HaeII
HincII
kilAp
Start klaA

FIG. 2. Nucleotide sequence of the *kilE* region (GenBank accession number L18919). The nucleotide sequence of the kb 2.26 to 4.56 region of RK2 is shown. Numbers refer to nucleotide positions, and pertinent restriction sites are indicated. Nucleotides 1 to 300 and 2170 to 2300 were previously reported as part of the *korC* (36) and *kilA* (20) sequences, respectively. *kleAp*, *kleCp*, and *kilAp* indicate the positions of the promoters for the *kleA*, *kleC*, and *kilA* (20, 89) operons. The −10 and −35 regions are boxed, and mRNA start sites are indicated by +1 and angled arrows. The pairs of divergent arrows labelled A and C show operatorlike palindromic sequences that are the predicted targets for KorA and KorC proteins, respectively. The palindrome labelled B is the target for KorB protein (5). The predicted amino acid sequences are shown below the nucleotide sequences of *kleABCDEF*, the 3′ end of *korC*, and the 5′ end of *klaA*. Potential Shine-Dalgarno sequences for ribosome binding (64) are underlined and labelled SD. Putative helix-turn-helix regions (12) in the KleD and KleF polypeptides are boxed; these regions have similarity to the consensus pattern of amino acids in the helix-turn-helix regions of known DNA-binding proteins (47). Dashed arrows indicate the locations of two identical 9-bp direct nucleotide sequence repeats. The t shows a sequence resembling a transcription terminator (10): a G+C-rich region of dyad symmetry followed by six thymine residues. (t) shows sequences that are possible vestiges of transcription terminators. Other sequences having dyad symmetry are indicated by pairs of divergent arrows. The nucleotide sequence differs from a partial sequence of the corresponding region of plasmid RP4 (79) at the following positions: 226 (G [RK2] versus A [RP4]), 883 (A [RK2] versus G [RP4]), and 1014 (A [RK2] versus G [RP4]).

sequence immediately upstream of the −35 region of the *kleA* promoter (palindrome A, Fig. 2) is also present in the *korA* promoter, which is regulated by *korA* and *korB* (7, 89). The palindromic sequence that overlaps the −10 region of the *kleA* promoter (palindrome C, Fig. 2) is not present in the *korA* promoter. The results show that the KorA-containing extract altered the migration of both the *kleA* and *korA* promoter fragments (Fig. 6A and B). The KorC-containing extract also shifted the *kleA* promoter fragment but showed no binding activity specific for the *korA* promoter (Fig. 6C and D). In these assays, we detected no intermediate species between the uncomplexed and fully complexed fragments. Extracts from cells lacking *korA* and *korC* had no effect on either promoter fragment (Fig. 6). In addition, the KorA- and KorC-containing extracts had no effect on the *E. coli lac* promoter (data not shown). These results support previous genetic evidence that *korA* and *korC* encode DNA-binding proteins. Furthermore, our results indicate that KorA and KorC bind specifically to DNA fragments containing palindromes A and C, respectively.

## DISCUSSION

The *kil-kor* regulon was originally detected in RK2 by the discovery of three potentially host-lethal *kil* loci (*kilA*, *kilB*, and *kilC*) that are controlled by *korA*, *korB*, and *korC* (17). Subsequent studies have identified additional RK2 operons (*trfA*, *korA*, and *kfrA*) and regulatory determinants (*korE*,

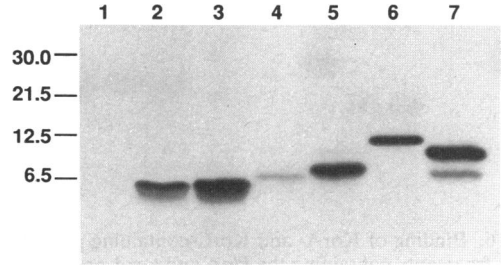


FIG. 3. Polypeptides encoded by *kleABCDEF*. Each gene was cloned individually and expressed in vivo from the bacteriophage T7 φ10 promoter. Polypeptides specified by the cloned genes were selectively labelled with $^{14}$C-amino acids, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and visualized by autoradiography. Lanes: 1, pT7-5B; 2, pRK2845 (*kleA*$^+$); 3, pRK2799 (*kleB*$^+$); 4, pRK2846 (*kleC*$^+$); 5, pRK2800 (*kleD*$^+$); 6, pRK2843 (*kleE*$^+$); 7, pRK2844 (*kleF*$^+$). Numbers on left show the positions and sizes of the following protein markers: carbonic anhydrase (30 kDa), soybean trypsin inhibitor (21.5 kDa), cytochrome *c* (12.5 kDa), and aprotinin (6.5 kDa).

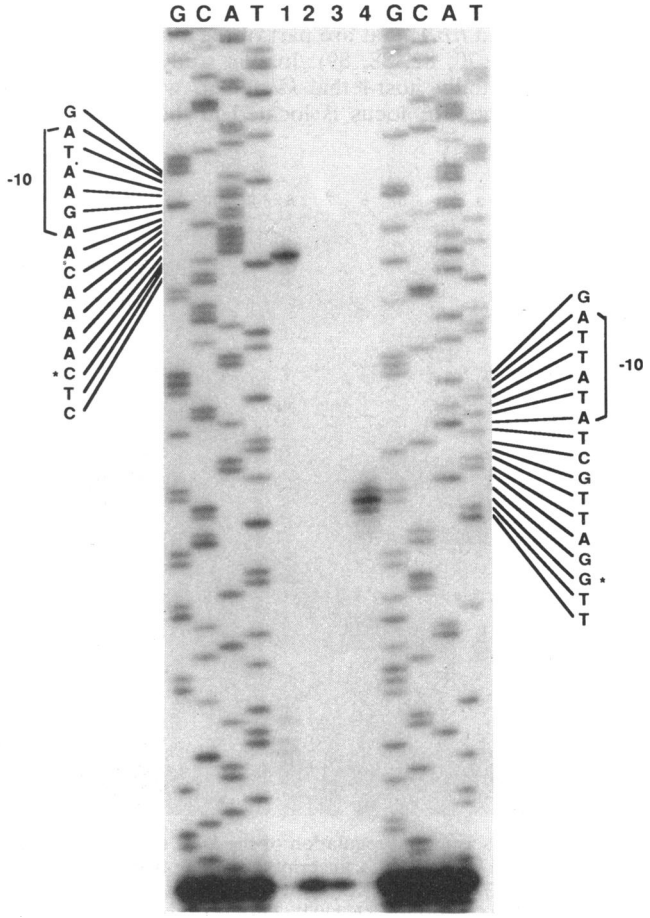


FIG. 4. Transcriptional start sites for the *kleA* and *kleC* promoters. RNA transcripts initiating from each cloned promoter (pRK2795 and pRK2796) were expressed in vivo in the absence of *kor* regulatory functions. The $^{32}$P-labelled 35-mer primer was annealed to RNA extracted from cells containing the appropriate plasmid and extended by reverse transcriptase. The labelled DNA products were separated by electrophoresis on a polyacrylamide sequencing gel and visualized by autoradiography. Reference nucleotide sequences were obtained by the dideoxynucleotide chain termination method using the $^{32}$P-labelled 35-mer primer with M13 constructs containing the same promoter fragments present in pRK2795 and pRK2796. The *kleA* promoter sequence is shown on the right; the *kleC* promoter sequence is on the left. Asterisks indicate the positions in the nucleotide sequences that correspond to the start sites for transcription. Lanes: 1, pRK2796 (*kleC* promoter); 2, pCH1 (vector); 3, untreated $^{32}$P-labelled 35-mer primer; 4, pRK2795 (*kleA* promoter).

TABLE 3. Relative transformation efficiencies of plasmids carrying portions of the *kilE* region[a]

| Transforming plasmid | Relevant plasmid genotype[b] | Relative transformation efficiency[c] of recipient strain with relevant genotype of: | | | | |
|---|---|---|---|---|---|---|
| | | *kor*$^0$ | *korA*$^+$ | *korB*$^+$ | *korC*$^+$ | *korA*$^+$*B*$^+$*C*$^+$ |
| pRK2856 | *kleA*$^+$*B*$^+$*C*$^+$*D*$^+$*E*$^+$*F*$^+$ | − | + | − | + | + |
| pRK2793 | *kleA*$^+$*B*$^+$ | − | + | − | + | + |
| pRK2792 | *kleC*$^+$*D*$^+$*E*$^+$*F*$^+$ | (+) | + | (+) | + | + |
| pRK2795 | *kleAp* | + | ND | ND | ND | + |
| pRK2796 | *kleCp* | + | ND | ND | ND | + |

[a] Recipient strains were transformed with each plasmid, and ampicillin-resistant colonies were selected. For the *korB*$^+$ strain, selection media were supplemented with IPTG to induce expression of *korB*. The relative competence of each strain was determined by transformation with vector pCH1 as described previously (17). Efficiencies of transformation were adjusted for competence differences and normalized to that of the *korA*$^+$*B*$^+$*C*$^+$ strain.

[b] All plasmids contain transcriptional terminators located immediately downstream of the cloned RK2 region.

[c] Relative transformation efficiencies are defined in Table 2, footnote *c*. ND, not determined. Recipient strains were the following: MV10 (*kor*$^0$), MV10(pRK2292) (*korA*$^+$), MV10 (pRK21408) (*korB*$^+$), MV10(pRK2462) (*korC*$^+$), and MV10(pRK2659) (*korA*$^+$*B*$^+$*C*$^+$).

*korF*, *kfrA*, and *trbA*) that are part of the *kil-kor* regulon (7, 28–30, 61, 66, 70, 82, 88, 89). In this study, we identified another potentially host-lethal *kil* locus, which we designated *kilE*. The *kilE* locus is located in the kb 2.4 to 4.5 region of RK2 and consists of two multicistronic operons: the *kleA* operon, which contains two genes (*kleA* and *kleB*), and the *kleC* operon, which specifies four genes (*kleC*, *kleD*, *kleE*, and *kleF*). We demonstrated that the genes express the predicted polypeptide products, and we confirmed that the

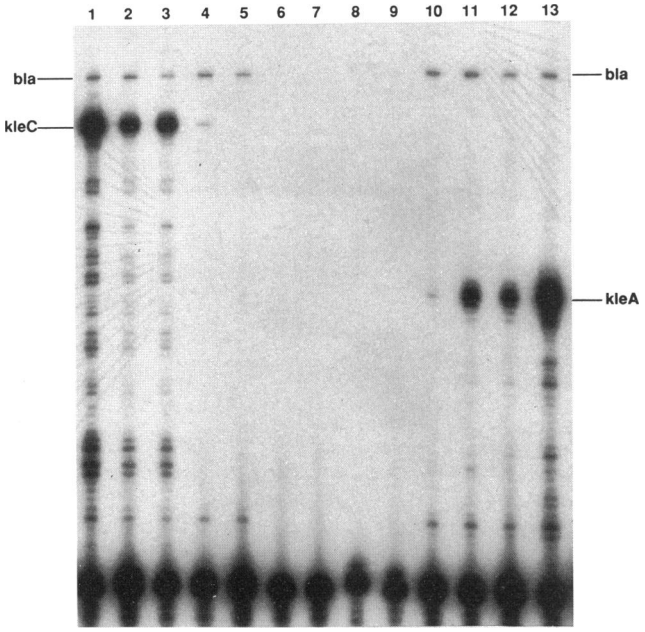


FIG. 5. Transcriptional regulation of the *kleA* and *kleC* promoters by *korA* and *korC*. RNA transcripts initiating from each cloned promoter (pRK2795 and pRK2796) were expressed in vivo in the presence of *korA*$^+$ (from pRK2292), *korC*$^+$ (from pRK2462), and *korA*$^+$ plus *korC*$^+$. The 35-mer and *bla* primers were labelled with $^{32}$P, annealed to RNA extracted from cells containing the appropriate plasmids, and extended by reverse transcriptase. The *bla* transcript from the plasmid vector was used as an internal control to standardize each sample. The labelled DNA products were separated by polyacrylamide gel electrophoresis and visualized by autoradiography. For analysis of transcripts initiated from the *kleC* promoter, RNA was isolated from strains containing pRK2796 and the following plasmids: no plasmid (*kor*$^0$) (lane 1), pRK2292 (lane 2), pRK2462 (lane 3), and pRK2292 and pRK2462 (lane 4). For analysis of transcripts initiated from the *kleA* promoter, RNA was isolated from strains containing pRK2795 and the following plasmids: no plasmid (*kor*$^0$) (lane 13), pRK2292 (lane 12), pRK2462 (lane 11), and pRK2292 and pRK2462 (lane 10). Control RNA was extracted from strains containing only the vector pCH1 (lane 5), pRK2292 (lane 6), and pRK2462 (lane 7). Lane 8, untreated $^{32}$P-labelled 35-mer primer; lane 9, untreated $^{32}$P-labelled *bla* primer.

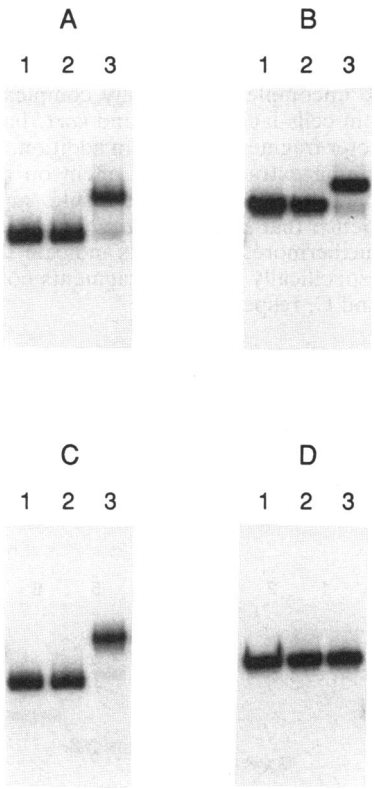


FIG. 6. Binding of KorA- and KorC-containing protein extracts to DNA fragments containing the *kleA* and *korA* promoters. KorA- and KorC-containing extracts were prepared from cells containing pRK21471 (*korA*$^+$) and pRK2634 (*korC*$^+$), respectively. Control extracts were prepared from cells containing the vector pT7-5B. $^{32}$P-labelled DNA fragments containing the *kleA* and *korA* promoters were incubated with the protein extracts, separated by polyacrylamide gel electrophoresis, and visualized by autoradiography. Top panels show binding by KorA extracts to the *kleA* promoter (A) and the *korA* promoter (B). Lanes: 1, no extract; 2, control extract; 3, extract containing KorA. Bottom panels show binding by KorC extracts to the *kleA* promoter (C) and the *korA* promoter (D). Lanes: 1, no extract; 2, control extract; 3, extract containing KorC.

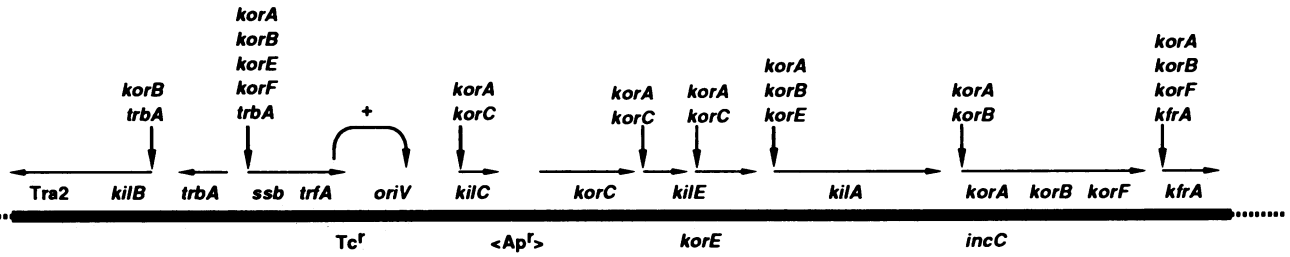


FIG. 7. The *kil-kor* regulon of plasmid RK2. Only the relevant portion of RK2 is depicted. All operons and regulatory genes are described in the text except Tra2, which contains genes involved in conjugal transfer (39). Horizontal arrows show directions of transcription. $Ap^r$ denotes the location of transposon Tn*1*, which encodes ampicillin resistance (11, 25). $Tc^r$ shows the region encoding resistance to tetracycline (85). Vertical arrows indicate negative regulatory interactions. The curved arrow shows the positive interaction of the replication initiator gene *trfA* with *oriV*, the origin of replication.

predicted promoters for each operon are indeed functional. The expression of both operons is regulated by *korA* and *korC*. Thus, the *kleA* and *kleC* operons are components of the *kil-kor* regulon, which is now known to encompass eight operons. A summary of the *kil-kor* regulon and its regulatory interactions is presented in Fig. 7.

A previous study (79) reported the nucleotide sequence of a region from the IncP plasmid RP4 that is virtually identical to a portion of the *kilE* region of RK2. The partial RP4 sequence also revealed two promoterlike sequences, which are identical to the *kleA* and *kleC* promoters described here, and four ORFs, which correspond to *kleA*, *kleB*, *kleC*, and *kleD*. That study also showed an active promoter in the RP4 region corresponding to the RK2 *kleA* promoter. However, in contrast to results presented here, the analysis revealed two clusters of transcription initiation sites, one of which corresponds to the *kleA* promoter. A study by Greener et al. (21) showed that a single cluster of three nucleotides, identical to the one identified here, is used by the RK2 *kleA* promoter in several gram-negative bacterial species.

Several lines of evidence show that the *kleA* and *kleC* operons are normally controlled by *korA* and *korC* at the level of transcription. (i) *korA* and *korC* suppressed the Kil phenotypes caused by the expression of the *kilA* operon from the *kleA* and *kleC* promoters (Table 2). (ii) The abundance of transcripts initiated in vivo from the *kleA* and *kleC* promoters was reduced in the presence of *korA* and *korC* (Fig. 5). (iii) *korA* and *korC* reduced the expression of a reporter gene fused to the *kleA* promoter homolog from RP4 (79). (iv) DNA binding studies (Fig. 6) indicated that KorA and KorC proteins bind to promoter regions containing operatorlike palindromic sequences (A and C, Fig. 2). Palindrome A, located upstream of the −35 sequences of both the *kleA* and *kleC* promoters, is also present in the *kilA*, *trfA*, *korA*, and *kfrA* promoters, all of which are regulated by *korA* (17, 61, 66, 68, 82, 89). Gel mobility shift assays showed that DNA fragments containing palindrome A are specifically retarded in the presence of cell extracts containing KorA. Palindrome C, which overlaps the −10 regions of the *kleA* and *kleC* promoters, has also been found to overlap a possible promoter sequence from the *kilC* region (79). *kilC* is the only other determinant known to be regulated by *korC* (17, 36). We showed that a DNA fragment containing palindrome C was specifically retarded by extracts from *korC*-containing cells. These results have provided the first direct evidence that KorA and KorC are DNA-binding proteins and strongly suggest that they are specific for the A and C palindromes, respectively. Additional studies are continuing with purified proteins to confirm the specificity of binding. Nevertheless, the results presented here are consistent with the model that KorA and KorC bind to the *kleA* and *kleC* promoters and act as repressors of *kleA* and *kleC* transcription.

The predicted structures of KorA (11 kDa) and KorC (9 kDa) have repressorlike characteristics. Both have a net positive charge and a helix-turn-helix domain similar to other known DNA-binding proteins (6, 36, 80). Because the distance between palindromes A and C is identical in the *kleA*, *kleC*, and putative *kilC* promoters, we suggest that KorA and KorC interact cooperatively to form a hetero-repressor complex. This model is supported by the apparent synergistic action of KorA and KorC on transcription from the *kleA* and *kleC* promoters (Fig. 5).

Both the *kleA* and *kleC* promoters are calculated by the matrix analysis of Hawley and McClure (24) to be strong promoters, as are nearly all the promoters of the *kil-kor* regulon. Thus, studies of the *kil-kor* regulon have revealed one strategy that may be important to the broad host range of IncP plasmids: the use of strong promoters that are tightly regulated by combinations of repressors. The consensuslike $\sigma^{70}$ promoters very likely guarantee at least some transcription in a wide variety of bacteria, while the multiple repressors ensure that the operons are not overexpressed in bacteria that efficiently utilize these promoters. The ability of the *kleA* (21), *korA* (21), and *trfA* (49) promoters to initiate transcription at the same nucleotide in different bacteria is consistent with this hypothesis.

Further analysis of the *kilE* nucleotide sequence (Fig. 2) has revealed several notable features. (i) A KorB binding site (5) is located 134 bp upstream of the transcriptional start site of the *kleA* promoter. While our genetic data show that *korB* is not sufficient to inhibit the $Kil^+$ phenotype expressed by the *kleA* operon (Table 3), it is nevertheless possible that KorB contributes to the regulation of the *kleA* promoter. There is precedent for the ability of KorB to act at a distance. We have shown that a KorB binding site located 183 bp upstream of the transcriptional start site of the *kilB* promoter participates in the regulation of *kilB* (83). (ii) Overlapping the KorB binding site upstream of the *kleA* promoter are two 9-bp direct repeats separated by one turn of the DNA helix (11 bp). It is conceivable that these direct repeats are sites for binding of another regulatory protein, which may affect the binding of KorB and provide an additional level of control on the *kleA* promoter. (iii) We observed no obvious relatedness of the *kle* gene products to other known proteins of non-RK2 origin. However, the predicted amino acid sequences of KleA and KleC show significant relatedness to each other, with 40% identical

residues and 59% similar residues. This relationship has been noted previously, and it has been suggested that *kleA* and *kleC* represent a gene duplication (79). It will be of interest to determine whether KleA and KleC proteins have related or redundant functions. (iv) The predicted amino acid sequences of the KleD and KleF polypeptides indicate the potential to form helix-turn-helix domains similar to those of known DNA-binding proteins, suggesting possible sequence-specific interactions with DNA. (v) The translational start of *kleD* is ambiguous. At the 5′ end of the ORF is a reasonable Shine-Dalgarno sequence followed by three consecutive ATG codons. We have selected the third ATG as the initiation site because it has better spacing to the Shine-Dalgarno sequence. However, six codons downstream of the third ATG is a GTG codon that is preceded by a Shine-Dalgarno sequence, and it seems equally likely that translation of *kleD* could be initiated here. (vi) In the middle of the *kleF* sequence is a good Shine-Dalgarno sequence followed by an ATG codon, suggesting a possible internal translational start. Indeed, overexpression of *kleF* revealed two polypeptide products (Fig. 3). One was the expected full-length *kleF* product; a second, smaller polypeptide corresponded well with the predicted 6-kDa polypeptide that would arise from use of the internal translational start. The functions of the two *kleF* products are not known, but it may be significant that only the larger KleF polypeptide contains the predicted helix-turn-helix structure. Three other genes of RK2, *trfA* (37, 65), *incC* (7, 37, 80), and *traC* (38, 44), specify two polypeptide products. In the case of *trfA*, the two polypeptide products may have importance with respect to host range. While both TrfA polypeptides are able to initiate replication at *oriV*, the larger polypeptide appears to be preferred in *Pseudomonas aeruginosa* (16, 67). (vii) Our studies have shown that genes of the *kilA* operon can be expressed by at least one (and very likely both) of the upstream *kle* promoters. Thus, *kilA* expression can be influenced by *korC*. The transcriptional readthrough is consistent with the absence of obvious transcriptional terminator sequences downstream of the *kleA* and *kleC* operons. While a potential *rho*-independent terminator occurs downstream of *korC* (36), only possible vestiges of a terminator lie downstream of *kleB* and *kleF*.

In an earlier study, we identified a regulatory determinant, *korE*, that affects the expression of *kilA* in *trans* and maps to the same region as *kilE* (88). We have since localized *korE* to the region containing the *kleC* operon (62). However, the activity of *korE* does not depend on the *kleC* promoter for expression, nor does it correspond to any of the *kle* structural genes. In addition, *korE* has no obvious deleterious effect on the growth of *E. coli* host cells. We are currently investigating its mode of action.

The region between the RK2 origin of replication (*oriV*) and the *korA* operon is occupied by three *kil* loci containing at least 10 genes whose functions are unknown (Fig. 7). They are not required for plasmid replication or conjugal transfer, yet they are part of a unique plasmid network of coregulated operons that includes genes essential for replication and transfer. This arrangement suggests that the *kil* loci have important functions in the biology of RK2. Furthermore, the lethal phenotypes of these determinants indicate that several RK2 gene products interact with vital functions in the host cell. Thus, the IncP broad-host-range plasmids appear to establish numerous intimate links with the host cell. The study of the *kil* loci and their targets may therefore reveal novel aspects of plasmid-host symbiosis.

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## REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1989. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
2. **Ayres, E. K., R. C. Roberts, D. R. Helinski, and D. H. Figurski.** Unpublished results.
3. **Ayres, E. K., S. Saadi, H. C. Schreiner, V. J. Thomson, and D. H. Figurski.** 1991. Differentiation of lethal and nonlethal, *kor*-regulated functions in the *kilB* region of broad host-range plasmid RK2. Plasmid **25**:53–63.
4. **Ayres, E. K., V. J. Thomson, G. Merino, D. Balderes, and D. H. Figurski.** 1993. Precise deletions in large bacterial genomes by vector-mediated excision (VEX): the *trfA* gene of promiscuous plasmid RK2 is essential for replication in several gram-negative hosts. J. Mol. Biol. **230**:174–185.
5. **Balzer, D., G. Ziegelin, W. Pansegrau, V. Kruft, and E. Lanka.** 1992. KorB protein of promiscuous plasmid RP4 recognizes inverted sequence repetitions in regions essential for conjugative plasmid transfer. Nucleic Acids Res. **20**:1851–1858.
6. **Bechhofer, D. H., and D. H. Figurski.** 1983. Map location and nucleotide sequence of *korA*, a key regulatory gene of promiscuous plasmid RK2. Nucleic Acids Res. **11**:7453–7469.
7. **Bechhofer, D. H., J. A. Kornacki, W. Firshein, and D. H. Figurski.** 1986. Gene control in broad host-range plasmid RK2: expression, polypeptide product, and multiple regulatory functions of *korB*. Proc. Natl. Acad. Sci. USA **83**:394–398.
8. **Brunelle, A., W. Hendrickson, and R. Schlief.** 1985. Altered DNA contacts made by a mutant AraC protein. Nucleic Acids Res. **13**:5019–5026.
9. **Burkardt, H.-J., G. Riess, and A. Pühler.** 1979. Relationship of group P1 plasmids revealed by heteroduplex experiments: RP1, RP4, R68 and RK2 are identical. J. Gen. Microbiol. **114**:341–348.
10. **Carafa, Y., E. Brody, and C. Thermes.** 1990. Prediction of Rho-independent *Escherichia coli* transcription terminators: a statistical analysis of their RNA stem-loop structures. J. Mol. Biol. **216**:835–858.
11. **Chen, S., and R. C. Clowes.** 1987. Variations between the nucleotide sequences of Tn*1*, Tn*2*, and Tn*3* and expression of β-lactamase in *Pseudomonas aeruginosa* and *Escherichia coli*. J. Bacteriol. **169**:913–916.
12. **Chou, P. Y., and G. D. Fasman.** 1974. Prediction of protein conformation. Biochemistry **13**:222–245.
13. **Cohen, S. N., A. C. Y. Chang, and L. Hsu.** 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. USA **69**:2110–2114.
14. **Datta, N., and R. Hedges.** 1972. Host ranges of R factors. J. Gen. Microbiol. **70**:453–460.
15. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387–399.
16. **Durland, R. H., and D. R. Helinski.** 1987. The sequence encoding the 43-kilodalton TrfA protein is required for efficient replication or maintenance of minimal RK2 replicons in *Pseudomonas aeruginosa*. Plasmid **18**:164–169.
17. **Figurski, D. H., R. F. Pohlman, D. H. Bechhofer, A. S. Prince, and C. A. Kelton.** 1982. Broad host range plasmid RK2 encodes multiple *kil* genes potentially lethal to *Escherichia coli* host cells. Proc. Natl. Acad. Sci. USA **79**:1935–1939.

18. **Figurski, D. H., C. Young, H. C. Schreiner, R. F. Pohlman, D. H. Bechhofer, A. S. Prince, and T. F. D'Amico.** 1985. Genetic interactions of broad host-range plasmid RK2: evidence for a complex replication regulon, p. 227–241. *In* D. R. Helinski, S. N. Cohen, D. B. Clewell, D. A. Jackson, and A. Hollaender (ed.), Plasmids in bacteria. Plenum Publishing Corp., New York.
19. **Gerlitz, M., O. Hrabak, and H. Schwab.** 1990. Partitioning of broad-host-range plasmid RP4 is a complex system involving site-specific recombination. J. Bacteriol. **172:**6194–6203.
20. **Goncharoff, P., S. Saadi, C.-H. Chang, L. H. Saltman, and D. H. Figurski.** 1991. Structural, molecular, and genetic analysis of the *kilA* operon of broad-host-range plasmid RK2. J. Bacteriol. **173:**3463–3477.
21. **Greener, A., S. M. Lehman, and D. R. Helinski.** 1992. Promoters of the broad host range plasmid RK2: analysis of transcription (initiation) in five species of gram-negative bacteria. Genetics **130:**27–36.
22. **Grinter, N. J., G. Brewster, and P. T. Barth.** 1989. Two mechanisms for the stable inheritance of plasmid RP4. Plasmid **22:**203–214.
23. **Guiney, D., and E. Lanka.** 1989. Conjugative transfer of IncP plasmids, p. 27–56. *In* C. M. Thomas (ed.), Promiscuous plasmids of gram-negative bacteria. Academic Press, Inc., San Diego, Calif.
24. **Hawley, D. K., and W. R. McClure.** 1983. Compilation and analysis of *E. coli* promoter DNA sequences. Nucleic Acids Res. **11:**2237–2255.
25. **Heffron, F., B. J. McCarthy, H. Ohtsubo, and E. Ohtsubo.** 1979. DNA sequence analysis of the transposon Tn*3*: three genes and three sites involved in transposition of Tn*3*. Cell **18:**1153–1163.
26. **Hershfield, V., H. W. Boyer, C. Yanofsky, M. A. Lovett, and D. R. Helinski.** 1974. Plasmid ColE1 as a molecular vehicle for cloning and amplification of DNA. Proc. Natl. Acad. Sci. USA **71:**3455–3459.
27. **Ingram, L. C., M. H. Richmond, and R. B. Sykes.** 1973. Molecular characterization of the R factors implicated in the carbenicillin resistance of a sequence of *Pseudomonas aeruginosa* strains isolated from burns. Antimicrob. Agents Chemother. **3:**279–288.
28. **Jagura-Burdzy, G., J. P. Ibbotson, and C. M. Thomas.** 1991. The *korF* region of broad-host-range plasmid RK2 encodes two polypeptides with transcriptional repressor activity. J. Bacteriol. **173:**826–833.
29. **Jagura-Burdzy, G., F. Khanim, C. A. Smith, and C. M. Thomas.** 1992. Crosstalk between plasmid vegetative replication and conjugative transfer: repression of the *trfA* operon by *trbA* of broad host range plasmid RK2. Nucleic Acids Res. **20:**3939–3944.
30. **Jagura-Burdzy, G., and C. M. Thomas.** 1992. *kfrA* gene of broad host range plasmid RK2 encodes a novel DNA-binding protein. J. Mol. Biol. **225:**651–660.
31. **Johnson, A. D., C. O. Pabo, and R. T. Sauer.** 1980. Bacteriophage λ repressor and cro protein: interactions with operator DNA. Methods Enzymol. **65:**839–856.
32. **Jovanovic, O. S., E. K. Ayres, and D. H. Figurski.** 1992. The replication initiator operon of promiscuous plasmid RK2 encodes a gene that complements an *Escherichia coli* mutant defective in single-stranded DNA-binding protein. J. Bacteriol. **174:**4842–4846.
33. **Kahn, M., R. Kolter, C. Thomas, D. Figurski, R. Meyer, E. Remaut, and D. R. Helinski.** 1979. Plasmid cloning vehicles derived from plasmids ColE1, F, R6K, and RK2. Methods Enzymol. **68:**268–280.
34. **Klemenz, R., D. J. Stillman, and E. P. Geiduschek.** 1982. Specific interactions of *Saccharomyces cerevisiae* proteins with a promoter region of eukaryotic tRNA genes. Proc. Natl. Acad. Sci. USA **79:**6161–6195.
35. **Kornacki, J. A., P. J. Balderes, and D. H. Figurski.** 1987. Nucleotide sequence of *korB*, a replication control gene of broad host-range plasmid RK2. J. Mol. Biol. **198:**211–222.
36. **Kornacki, J. A., R. S. Burlage, and D. H. Figurski.** 1990. The *kil-kor* regulon of broad-host-range plasmid RK2: nucleotide sequence, polypeptide product, and expression of regulatory gene *korC*. J. Bacteriol. **172:**3040–3050.
37. **Kornacki, J. A., A. H. West, and W. Firshein.** 1984. Proteins encoded by the *trans*-acting replication and maintenance regions of broad host range plasmid RK2. Plasmid **11:**48–57.
38. **Lanka, E., R. Lurz, M. Kroger, and J. P. Fürste.** 1984. Plasmid RP4 encodes two forms of a DNA primase. Mol. Gen. Genet. **194:**65–72.
39. **Lessl, M., D. Balzer, W. Pansegrau, and E. Lanka.** 1992. Sequence similarities between the RP4 Tra2 and the Ti VirB region strongly support the conjugation model for T-DNA transfer. J. Biol. Chem. **267:**20471–20480.
40. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labelled DNA with base-specific chemical cleavages. Methods Enzymol. **65:**499–560.
41. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101:**20–78.
42. **Meyer, R., and M. Hinds.** 1982. Multiple mechanisms for expression of incompatibility by broad-host-range plasmid RK2. J. Bacteriol. **152:**1078–1090.
43. **Meyer, R. J., and D. R. Helinski.** 1977. Unidirectional replication of the P-group plasmid RK2. Biochim. Biophys. Acta **478:**109–113.
44. **Miele, L., B. Strack, V. Kruft, and E. Lanka.** 1991. Gene organization and nucleotide sequence of the primase region of IncP plasmids RP4 and R751. J. DNA Seq. Map. **2:**145–162.
45. **Motallebi-Veshareh, M., D. Balzer, E. Lanka, G. Jagura-Burdzy, and C. M. Thomas.** 1992. Conjugative transfer functions of broad-host-range plasmid RK2 are coregulated with vegetative replication. Mol. Microbiol. **6:**907–920.
46. **Olsen, R. H., and P. Shipley.** 1973. Host range and properties of the *Pseudomonas aeruginosa* R factor R1822. J. Bacteriol. **113:**772–780.
47. **Pabo, C. O., and R. T. Sauer.** 1984. Protein-DNA recognition. Annu. Rev. Biochem. **53:**293–321.
48. **Pinkney, M., R. Diaz, E. Lanka, and C. M. Thomas.** 1988. Replication of mini RK2 plasmid in extracts of *Escherichia coli* requires plasmid-encoded protein TrfA and host-encoded proteins DnaA, B, G, DNA gyrase and DNA polymerase III. J. Mol. Biol. **203:**927–938.
49. **Pinkney, M., B. D. Theophilus, S. R. Warne, W. C. Tacon, and C. M. Thomas.** 1987. Analysis of transcription from the *trfA* promoter of broad host range plasmid RK2 in *Escherichia coli*, *Pseudomonas putida*, and *Pseudomonas aeruginosa*. Plasmid **17:**222–232.
50. **Pohlman, R. F., and D. H. Figurski.** 1983. Essential genes of plasmid RK2 in *Escherichia coli*: *trfB* region controls a *kil* gene near *trfA*. J. Bacteriol. **156:**584–591.
51. **Roberts, R. C., R. Burioni, and D. R. Helinski.** 1990. Genetic characterization of the stabilizing functions of a region of broad-host-range plasmid RK2. J. Bacteriol. **172:**6204–6216.
52. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. I. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:**487–491.
53. **Saltman, L. H., K.-S. Kim, and D. H. Figurski.** 1991. The *kilA* operon of promiscuous plasmid RK2: the use of a transducing phage (λp*klaA*-1) to determine the effects of the lethal *klaA* gene on *Escherichia coli* cells. Mol. Microbiol. **11:**2673–2683.
54. **Saltman, L. H., K.-S. Kim, and D. H. Figurski.** 1992. Inhibition of bacteriophage λ development by the *klaA* gene of broad-host-range plasmid RK2. J. Mol. Biol. **227:**1054–1067.
55. **Sanger, F., and A. R. Coulson.** 1978. The use of thin acrylamide gels for DNA sequencing. FEBS Lett. **87:**107–110.
56. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
57. **Saurugger, P. N., O. Hrabak, H. Schwab, and R. M. Lafferty.** 1986. Mapping and cloning of the par-region of broad-host-range plasmid RP4. J. Biotechnol. **4:**333–343.
58. **Schmidhauser, T. J., D. H. Bechhofer, D. H. Figurski, and D. R. Helinski.** 1989. Host-specific effects of the *korA-korB* operon and *oriT* region on the maintenance of miniplasmid derivatives

of broad host-range plasmid RK2. Plasmid **21**:99–112.
59. **Schmidhauser, T. J., M. Filutowicz, and D. R. Helinski.** 1983. Replication of derivatives of the broad host range plasmid RK2 in two distantly related bacteria. Plasmid **9**:325–330.
60. **Schmidhauser, T. J., and D. R. Helinski.** 1985. Regions of broad-host-range plasmid RK2 involved in replication and stable maintenance in nine species of gram-negative bacteria. J. Bacteriol. **164**:446–455.
61. **Schreiner, H. C., D. H. Bechhofer, R. F. Pohlman, C. Young, P. A. Borden, and D. H. Figurski.** 1985. Replication control in promiscuous plasmid RK2: *kil* and *kor* functions affect expression of the essential replication gene *trfA*. J. Bacteriol. **163**:228–237.
62. **Schreiner, H. C., O. S. Jovanovic, C. Young, and D. H. Figurski.** Unpublished data.
63. **Seifert, H. S., E. Y. Chen, M. So, and F. Heffron.** 1986. Shuttle mutagenesis: a method of transposon mutagenesis for *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **83**:735–739.
64. **Shine, J., and L. Dalgarno.** 1975. Determination of cistron specificity in bacterial ribosomes. Nature (London) **254**:30–34.
65. **Shingler, V., and C. M. Thomas.** 1984. Analysis of the *trfA* region of broad host-range plasmid RK2 by transposon mutagenesis and identification of polypeptide products. J. Mol. Biol. **175**:229–249.
66. **Shingler, V., and C. M. Thomas.** 1984. Transcription in the *trfA* region of broad host range plasmid RK2 is regulated by *trfB* and *korB*. Mol. Gen. Genet. **195**:523–529.
67. **Shingler, V., and C. M. Thomas.** 1989. Analysis of nonpolar insertion mutations in the *trfA* gene of IncP plasmid RK2 which affect its broad-host-range property. Biochim. Biophys. Acta **1007**:301–308.
68. **Smith, C. A., V. Shingler, and C. M. Thomas.** 1984. The *trfA* and *trfB* promoter regions of broad host range plasmid RK2 share common potential regulatory sequences. Nucleic Acids Res. **12**:3619–3630.
69. **Smith, C. A., and C. M. Thomas.** 1983. Deletion mapping of *kil* and *kor* functions in the *trfA* and *trfB* regions of broad host range plasmid RK2. Mol. Gen. Genet. **190**:245–254.
70. **Smith, C. A., and C. M. Thomas.** 1984. Molecular genetic analysis of the *trfB* and *korB* region of broad host range plasmid RK2. J. Gen. Microbiol. **130**:1651–1663.
71. **Smith, D. H., D. Brutlag, P. Friedland, and L. H. Kedes.** 1986. BIONET™: national computer resource for molecular biology. Nucleic Acids Res. **14**:17–20.
72. **Stalker, D. M., C. M. Thomas, and D. R. Helinski.** 1981. Nucleotide sequence of the region of the origin of replication of the broad host range plasmid RK2. Mol. Gen. Genet. **181**:8–12.
73. **Studier, F. W., and B. A. Moffatt.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189**:113–130.
74. **Tabor, S., and C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82**:1074–1078.
75. **Theophilus, B. D. M., M. A. Cross, C. A. Smith, and C. M. Thomas.** 1985. Regulation of the *trfA* and *trfB* promoters of broad host range plasmid RK2: identification of sequences essential for regulation by *trfB*/*korA*/*korD*. Nucleic Acids Res. **13**:8129–8142.
76. **Theophilus, B. D. M., and C. M. Thomas.** 1987. Nucleotide sequence of the transcriptional repressor gene *korB* which plays a key role in regulation of the copy number of broad host range plasmid RK2. Nucleic Acids Res. **15**:7443–7450.
77. **Thomas, C. M., and D. R. Helinski.** 1989. Vegetative replication and stable inheritance of IncP plasmids, p. 1–25. *In* C. M. Thomas (ed.), Promiscuous plasmids of gram-negative bacteria. Academic Press Inc., San Diego, Calif.
78. **Thomas, C. M., and A. A. K. Hussain.** 1984. The *korB* gene of broad host range plasmid RK2 is a major copy number control element which may act together with *trfB* by limiting *trfA* expression. EMBO J. **3**:1513–1519.
79. **Thomas, C. M., J. P. Ibbotson, N. Y. Wang, C. A. Smith, R. Tipping, and N. M. Loader.** 1988. Gene regulation on broad host range plasmid RK2: identification of three novel operons whose transcription is repressed by both KorA and KorC. Nucleic Acids Res. **16**:5345–5359.
80. **Thomas, C. M., and C. A. Smith.** 1986. The *trfB* region of broad host range plasmid RK2: the nucleotide sequence reveals *incC* and key regulatory gene *trfB*/*korA*/*korD* as overlapping genes. Nucleic Acids Res. **14**:4453–4469.
81. **Thomas, C. M., D. M. Stalker, and D. R. Helinski.** 1981. Replication and incompatibility properties of segments of the origin region of replication of the broad host range plasmid RK2. Mol. Gen. Genet. **181**:1–7.
82. **Thomas, C. M., B. D. M. Theophilus, L. Johnston, G. Jagura-Burdzy, W. Schilf, R. Lurz, and E. Lanka.** 1990. Identification of a seventh operon on plasmid RK2 regulated by the *korA* gene product. Gene **89**:29–35.
83. **Thomson, V. J., O. S. Jovanovic, R. F. Pohlman, C.-H. Chang, and D. H. Figurski.** 1993. Structure, function, and regulation of the *kilB* locus of promiscuous plasmid RK2. J. Bacteriol. **175**:2423–2435.
84. **Villarroel, R., R. W. Hedges, R. Maenhaut, J. Leemans, G. Engler, M. Van Montagu, and J. Schell.** 1983. Heteroduplex analysis of P-plasmid evolution: the role of insertion and deletion of transposable elements. Mol. Gen. Genet. **189**:390–399.
85. **Waters, S. H., P. Rogowsky, J. Grinsted, J. Altenbuchner, and R. Schmitt.** 1983. The tetracycline resistance determinants of RP1 and Tn*1721*: nucleotide sequence analysis. Nucleic Acids Res. **11**:6089–6105.
86. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.
87. **Young, C., D. H. Bechhofer, and D. H. Figurski.** 1984. Gene regulation in plasmid RK2: positive control by *korA* in the expression of *korC*. J. Bacteriol. **157**:247–252.
88. **Young, C., R. S. Burlage, and D. H. Figurski.** 1987. Control of the *kilA* gene of the broad-host-range plasmid RK2: involvement of *korA*, *korB*, and a new gene, *korE*. J. Bacteriol. **169**:1315–1320.
89. **Young, C., A. S. Prince, and D. H. Figurski.** 1985. *korA* function of promiscuous plasmid RK2: an autorepressor that inhibits expression of host-lethal gene *kilA* and replication gene *trfA*. Proc. Natl. Acad. Sci. USA **82**:7374–7378.