JOURNAL OF BACTERIOLOGY, Apr. 1993, p. 2423–2435
0021-9193/93/082423-13$02.00/0

Vol. 175, No. 8

Structure, Function, and Regulation of the *kilB* Locus of Promiscuous Plasmid RK2

VALERI J. THOMSON,[1] OLIVER S. JOVANOVIC,[1] ROBERT F. POHLMAN,[2] CHUN-HUEY CHANG,[1]† AND DAVID H. FIGURSKI[1]*

Department of Microbiology and Cancer Center, College of Physicians and Surgeons, Columbia University, 701 West 168th Street, New York, New York 10032,[1] and Department of Biology, Colgate University, Hamilton, New York 13346[2]

Received 16 October 1992/Accepted 12 February 1993

The *kil-kor* regulon of the self-transmissible, broad-host-range plasmid RK2 is a unique network with eight coregulated operons. Among the genes encoded by the *kil-kor* regulon are *trfA*, which encodes the replication initiator, and several *kil* loci (*kilA*, *kilB*, *kilC*, and *kilE*), each of which is lethal to the host cell in the absence of appropriate negative regulatory elements encoded by the *korA*, *korB*, *korC*, and *korE* determinants. We have proposed that the functions of the *kil* loci are related to RK2 maintenance or host range. Here, we report the nucleotide sequence of a 2.44-kb region that includes the lethal *kilB* determinant. We identified the first three genes of the *kilB* operon (designated *klbA*, *klbB*, and *klbC*), and we determined by deletion analysis that the host-lethal phenotype requires *klbB*. The predicted amino acid sequence of the 34,995-Da *klbA* product reveals a potential ATP-binding fold. The *klbB* product is predicted to be a membrane protein with a molecular mass of 15,012 Da with homology to the RK2 KlaC membrane protein encoded by the *kilA* operon. The amino acid sequence of the 12,085-Da *klbC* product contains a perfect match to the leucine zipper motif common to eukaryotic regulatory proteins. Primer extension analysis revealed unambiguously that transcription of the *kilB* operon begins 46 nucleotides upstream of *klbA*. No transcription was initiated from the sequence previously presumed by other investigators to be the *kilB* promoter. The abundance of *kilB* transcripts is reduced in the presence of KorB, consistent with the prediction that KorB acts at the level of transcription. A degenerate KorB-binding site that contains a perfect half-palindrome overlaps the *kilB* promoter, but this site is insufficient for regulation by KorB. The region containing a KorB-binding site located 183 bp upstream of the transcriptional start is required for regulation by KorB, indicating that KorB acts at a distance to regulate transcription of *kilB*. Our studies with the mutant plasmid pRP101, a transfer-defective derivative of the RK2-like plasmid RP4, demonstrated that the *kilB* operon includes the conjugal transfer and surface exclusion genes of the Tra2 region. Nucleotide sequence analysis revealed that the transposon Tn*7* insertion in pRP101 is located in the *klbC* gene, and complementation analysis showed that this mutation has a strong polar effect on the expression of genes for conjugal transfer and surface exclusion located several kilobases downstream. A *klbA* mutant was constructed and found to be both transfer defective and complementable, thus demonstrating a requirement for the *klbA* product in plasmid transmissibility. These results have demonstrated a role for the *kilB* operon in conjugal transfer. The *kil-kor* regulon of RK2 is the only known example of plasmid-mediated coregulation of replication and transfer.

The self-transmissible plasmids of incompatibility group P (IncP) are remarkable for autonomous replication in a wide variety of gram-negative bacterial hosts (17, 67, 98). In addition, the range of organisms to which IncP plasmids can transfer by conjugation includes gram-positive (102) and gram-negative bacteria (30) and *Saccharomyces cerevisiae* (33). Efforts to understand the genetic basis for the replicative and transfer promiscuity of the closely related, if not identical, IncP plasmids RK2, RP1, RP4, R18, and R68 (14, 103) have revealed a genetic complexity unmatched by other plasmid replicons. Eight operons specifying at least 20 genes, including the essential replication initiator gene, *trfA*, are transcriptionally coregulated by various combinations of the negative-control determinants *korA*, *korB*, *korC*, *korE*, *korF*, and *trbA* (12, 22, 36–38, 43, 84, 85, 88, 94, 96, 100, 108–110) (Fig. 1). Five of the operons constitute four so-called *kil* loci (*kilA*, *kilB*, *kilC*, and *kilE*), whose expression is toxic to *Escherichia coli* host cells in the absence of the *kor* regulatory functions (22, 28, 43). This unusual plasmid regulatory network, known collectively as the *kil-kor* regulon (23), also includes the *kfrA* operon, which specifies a DNA-binding protein with an unknown function (38), and an autoregulated operon containing several regulatory genes (*korA*, *korB*, *korFI*, and *korFII*) (12, 36) and *incC*, which is thought to be involved in plasmid maintenance (59, 63).

None of the four *kil* loci is essential for replication of RK2. Genetic studies have shown that only two plasmid determinants are absolutely required for the replication of RK2 (6, 82, 83): *trfA*, which encodes two replication initiator proteins (21, 44, 87, 91), and *oriV*, the origin of replication and target for the TrfA proteins (21, 60, 92, 99). The genetic requirements for conjugal transfer are considerably more complex than those for replication. Two large regions, designated Tra1 and Tra2 (10, 71) (see Fig. 1), include genes for

* Corresponding author.
† Present address: Progenics Pharmaceuticals, Inc., 777 Old Saw Mill River Road, Tarrytown, NY 10591.

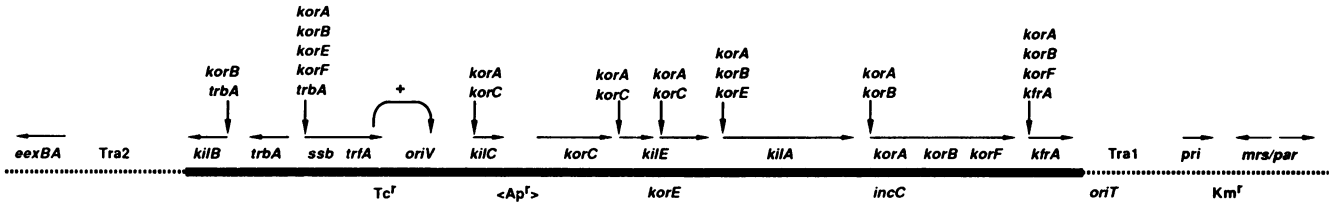

FIG. 1. The *kil-kor* regulon of RK2. Shown here is the relative arrangement of genes and operons on the RK2 genetic map and their regulation. The region containing *kor*-regulated genes is indicated by the solid line. Horizontal arrows indicate known transcriptional units. Genes above the vertical arrows are involved in negative regulation. The curved arrow indicates the requirement of *trfA* for initiation of replication at *oriV*. Tra1 and Tra2 are two large regions (not drawn to scale) involved in conjugal transfer. All genes are indicated in the text except *ssb*, which encodes a single-stranded DNA-binding protein (39), *pri*, which encodes a DNA primase (47), and *mrs/par*, a multimer-resolution and plasmid stability locus (25, 29, 75, 76). Ap^r, Tc^r, and Km^r show determinants for resistance to ampicillin, tetracycline, and kanamycin. The brackets around Ap^r indicate transposon Tn*1*.

assembly of the sex pilus, site-specific nicking and mobilization of RK2 DNA to recipient cells, and surface exclusion (30). In considering possible functions for the *kil* loci, we originally noted that only *kilB* was not excluded from having a role in the conjugal transfer of RK2 (22). Plasmids deleted for *kilA*, *kilC*, and *kilE* are clearly conjugation proficient (21), but a transfer-defective mutant (pRP101) of the IncP plasmid RP4 was found to have a transposon Tn*7* insertion in the *kilB* region (9). In addition to the transfer defect, the mutation affected two other phenotypes. (i) It eliminated the surface exclusion phenotype conferred by two genes (*eexA* and *eexB*) located several kilobases outside the *kilB* region (50, 52). (ii) It abolished sensitivity to bacteriophage PRD1 (73), which is thought to use the sex pilus of IncP plasmids as a receptor (68, 93). One interpretation of the pleiotropy of the mutation in pRP101 is that the *kilB* region is within a large operon involved in transfer functions, including surface exclusion and pilus assembly, and that the Tn*7* insertion exerts a polar effect on expression of these genes.

In this paper, we report our molecular and genetic studies of the *kilB* locus, its regulation, and its relationship to the conjugal transfer system of RK2. Our results revealed that the *kilB* locus is within a multicistronic operon, in which the first gene (*klbA*) is required for conjugal transfer and the second gene (*klbB*) is required for the host-lethal phenotype. A third downstream gene (*klbC*) in the *kilB* operon was also sequenced and found to be the site of the Tn*7* insertion in the transfer-defective mutant pRP101. The nucleotide sequence of the first gene of the *kilB* operon and the involvement of this gene in RK2 transmissibility was reported recently by Motallebi-Veshareh et al. (62). Our results differ significantly from theirs in the identification of the genes responsible for the *kilB* host-lethal phenotype, the location of the *kilB* promoter, and the regulation of *kilB* expression.

MATERIALS AND METHODS

Nomenclature. Coordinates of the RK2 map are defined by the distance in kilobases from the single *Eco*RI site. Superscript o indicates the absence of a plasmid gene (e.g., kor^o). The uniform nomenclature for genes of the *kil* loci has been previously defined (5, 28).

Bacterial strains, plasmids, and phages. The *E. coli* strains used in this study were C600 (*thr-1 leuB6 thi-1 lacY1 tonA21 supE44 rfbD1*) (7); DH5α [*supE44 ΔlacU169 hsdR17 recA1 endA1 gyrA96 thi-1 relA1* (φ80 *lacZΔM15*)] (31); EKA0076, a spontaneous rifampin-resistant isolate of JA221 (4); JA221 (*hsdR recA1 lacY leuB6 ΔtrpE5*) (from C. Yanofsky); JM107 [*endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*) λ^- (F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZΔM15*)] (107); MV10 (C600 Δ*trpE5*) (34); SM10 [C600 Δ*recA* (Muc^+) Ω(RP4-2-Tc::Muc^+)] (89); VT1258, a spontaneous rifampin-resistant mutant of MV10; VT1259, a spontaneous nalidixic acid-resistant mutant of MV10; and VT1284 [SM10(pBK1)].

All plasmids and their relevant properties are listed in Table 1. Previously unpublished plasmids were constructed as follows (RK2 coordinates are in parentheses): pBK1, by insertion of the *Bam*HI ΩCm fragment from pPG25 into pMMB67HE (24); pCH8, by deletion of the *Eco*RI-*Nru*I fragment from pCH1 (28), with cohesive ends filled in with the Klenow fragment of DNA polymerase I and ligation with T4 DNA polymerase; pPG25 (27), by insertion of the *Eco*RI ΩCm fragment from pHP45ΩCm (19) into pIC-20H (55); pRK2709, by insertion of the 9.8-kb *Kpn*I-*Sal*I (kb 14 to 23.8) fragment from RK2 into pUC19 (107) (see Fig. 2); pRK2709Δ36, by partial *Bss*HII digestion and ligation of pRK2709 (see Fig. 2); pRK2819, by insertion of the 1.0-kb *Bss*HII fragment (kb 18.8 to 19.8) from pRK2710 (5) into pHC7 (84) (see Fig. 2); pRK2838, by insertion of the *Sfi*I-*Bss*HII fragment (kb 16.9 to 18.8) from M13ss43 (see below) into *Bss*HII- and *Sph*I-cleaved pVT3 after the *Sfi*I and *Sph*I single-stranded ends were made blunt with T4 DNA polymerase (see Fig. 2); pRK2912, by insertion of the 1.0-kb *Bss*HII fragment (kb 18.8 to 19.8) from pRK2819 into pRK2838 (see Fig. 2); pRK2914, by insertion of the *Kpn*I ΩCm transcription-terminator fragment (19) from pRK2730 (28) into the *Kpn*I site of pRK2709Δ36; pRK21400, by insertion of the 420-bp *Sty*I fragment (kb 18.2 to 18.6) from pRK2827 (5) into the *Sma*I site of pUC18 (107); pRK21407, by insertion of the 290-bp *Eco*RI-*Xmn*I fragment (kb 18.2 to 18.5) from pRK21400 into *Eco*RI- and *Sma*I-cleaved pCH8 (see Fig. 4); pRK21408, by insertion of *korB* on the *Bss*HII-*Hin*dIII fragment of pRK2362 (12) downstream of the *trc* promoter in pSE380 (13) and joining of the *lacI*^{q}-*trcp-korB* region from this plasmid with the *trpE*$^+$ mini-R6K plasmid pRK353 (40); pRK21413, by use of synthetic oligonucleotide primers 5′-GGAATTCCGTCGGCTCGCGCATCATATC-3′ and 5′-TGCAGGTCGACTCTAGAGGATC-3′ to amplify a portion of the *kilB* promoter region on pRK21407 by polymerase chain reaction, digestion of the amplified product with *Eco*RI and *Xba*I, and insertion into pCH8; pRK21416, by insertion of the 3.3-kb *Nde*I fragment (kb 16.3 to 19.6) into pVT10 (see Fig. 2); pRK21417, by insertion of the 132-bp *Sty*I fragment (bp 889 to 1020; see Fig. 3) from pRK2827 (5) into pVEX1212 (6); pRK21418, by insertion

TABLE 1. Plasmids[a]

Plasmid	Selective marker(s)	Relevant properties	Description	Reference
pBK1	Ap Cm	IncQ rep Mob$^+$	Mobilizable by IncP transfer system	This study
pCH8	Ap	Cloning vector	pMB1 rep with polylinker followed by *rrnB* T1 and T2 transcriptional terminators	This study
pRK2108	*trpE*$^+$	*korA*$^+$ *korB*$^+$	pSM1 rep (IncFII) with kb 50.4 to 56.4 of RK2	22
pRK2135	Km *trpE*$^+$	*klbA*$^+$ *klbB*$^+$ *klbC*$^+$	R6K rep with kb 14.0 to 23.8 of RK2	22
pRK2138	Km	*klbA*$^+$ *klbB*$^+$ *klbC*$^+$	pSM1 rep with kb 14.0 to 23.8 of RK2	22
pRK2292	Tp	*korA*^{c}	pSM1 rep with kb 55.1 to 55.6 of RK2; *korA* is expressed constitutively	5
pRK2659	Tc	*korA*$^+$ *korB*$^+$	pSC101 rep with kb 50.4 to 56.4 and kb 0 to 6 of RK2	42
pRK2709	Ap	*klbA*$^+$ *klbB*$^+$ *klbC*$^+$	pUC19 with kb 14.0 to 23.8 of RK2 (Fig. 2)	This study
pRK2709Δ36	Ap	*klbA*$^+$ *klbBΔ104*	pUC19 with kb 14.0 to 19.8 of RK2 (Fig. 2)	This study
pRK2819	Ap	*klbBΔ104*	pHC7 with kb 18.8 to 19.8 of RK2 (Fig. 2)	This study
pRK2837	Ap Cm Sp	*klbA*$^+$ *klbB*$^+$ *klbC*$^+$	pUC19 with kb 14.0 to 23.8 of RK2 between transcriptional terminators	5
pRK2838	Ap	*klbAΔ119*	pMB1 rep with kb 16.9 to 18.8 of RK2 between transcriptional terminators (Fig. 2)	This study
pRK2912	Ap	*klbA*$^+$ *klbBΔ104*	pMB1 rep with kb 16.9 to 19.8 of RK2 between transcriptional terminators (Fig. 2)	This study
pRK2914	Ap	*klbA*$^+$ *klbBΔ104*	pUC19 with kb 14.0 to 19.8 of RK2 followed by a transcriptional terminator	This study
pRK21407	Ap	*kilBp* with upstream KorB-binding site	pMB1 rep with kb 18.2 to 18.5 of RK2	This study
pRK21408	*trpE*$^+$	Φ[*trcp-korB*$^+$]	R6K rep with kb 53.3 to 54.3 of RK2; *korB* is expressed from the *trc* promoter	This study
pRK21413	Ap	*kilBp* lacking the upstream KorB-binding site	pMB1 rep with kb 18.3 to 18.5 of RK2	This study
pRK21416	Cm	*klbA*$^+$ *klbBΔ47*	P15A rep with kb 16.3 to 19.6 of RK2 (Fig. 2)	This study
pRK21417	Sp	*klbA'*	P1 rep with kb 18.6 to 18.7 of RK2	This study
pRK21418	Ap Km Tc Sp	Tra$^-$ Eex$^+$ Dps$^-$	RK2 derivative with an insertion in *klbA*	This study
pRK21430	Km Tp Sp Sm	*klbA*$^+$ *klbB*$^+$ klbC::Tn*7*	pRK2135 carrying the Tn*7* insertion from pRP101	This study
pRP101	Km Tc Ap Tp Sm Sp	Tra$^-$ Eex$^-$ Dps$^-$	RP4 with a Tn*7* insertion in the *kilB* region	9
pVEX1212	Sp	Cloning vector	P1 rep	6

[a] rep, replicon.

of pRK21417 into the *klbA* gene in RK2 by homologous recombination and selection for a spectinomycin-resistant transconjugant; pVT3, by insertion of the *Bam*HI ΩSp transcriptional terminator fragment from pHP45Ω (74) into pCH1 (28); pVT8, by insertion of the *Bam*HI ΩSp fragment from pHP45Ω into pACYC184 (15); and pVT10, by digestion of pVT8 with *Bss*HII, filling in of cohesive ends with Klenow, and ligation with *Nde*I linkers, which also recreated the *Bss*HII sites at both ends of the fragment.

Phage M13 derivatives were constructed as follows: M13ss43 (77), by insertion of the 2.9-kb *Bss*HII fragment (kb 15.9 to 18.8) from pRK2710 (5) into M13hc5, which differs from M13hc4 (41) only in the orientation of the polylinker; and M13vt1, by insertion of the 2.6-kb *Sph*I fragment (kb 19.2 to 21.8) from RK2 into M13mp18 (65, 107). M13vt2 differs from M13vt1 only in the orientation of the polylinker. The phage PRD1 has been described elsewhere (68).

Media and reagents. Media for growth of bacteria were Luria-Bertani (LB) and M9 (53) containing 0.5% Difco Casamino Acids. When required, media were supplemented with L-tryptophan at 50 μg/ml and thiamine hydrochloride at 25 μg/ml. Antibiotics were used at the following concentrations: ampicillin, 50 μg/ml; chloramphenicol, 50 μg/ml; kanamycin, 50 μg/ml; nalidixic acid, 20 μg/ml; rifampin, 100 μg/ml; spectinomycin, 50 μg/ml; and tetracycline, 30 μg/ml. When appropriate, media contained 240 μg of isopropyl-β-D-thiogalactopyranoside (IPTG) per ml and 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml to identify DNA insertions into the *lacZα* region of the M13 and pUC vectors (58). For induction of the *trc* promoter, media were supplemented with 1 mM IPTG.

DNA techniques. DNA was transformed by the method described by Cohen et al. (16). Plasmid DNA preparation (3, 35, 40), polymerase chain reaction (78), and gel electrophoresis (40, 53) have been described previously. Enzymes were purchased from commercial suppliers and used under the recommended conditions.

Nucleotide sequence determination. The complete nucleotide sequence for the *kilB* region was determined for both strands by using double-stranded template from pRK2912, pRK2819, and pRK21422 or single-stranded template from M13vt1, M13vt2, and M13ss43. The sequence was determined with an Applied Biosystems 370A DNA sequencer and by a modification of the dideoxynucleotide chain-termination method (79), in which Sequenase (U.S. Biochemical Corporation) in place of DNA polymerase I Klenow fragment was used. All sequencing was done with dITP or 7-deaza-dGTP in addition to dGTP to eliminate ambiguities caused by band compression in G+C-rich regions (61). The sequencing products were labeled with α-^{35}S-thio-dATP, separated by gel electrophoresis, and visualized by autoradiography as described previously (28, 41). To determine the site of the Tn*7* insertion in pRP101, the fragment derived from cleavage of the *Bgl*II site in Tn*7* and the *Sph*I site in

klbA was subcloned. A 21-mer oligonucleotide primer (5′-CTATTTTGTTCAGTTTAAGAC-3′) specific for one end of Tn*7* (51) was used for nucleotide sequence determination as described above, except with [γ-^{32}P]dATP.

Sequence analysis. The sequenced region was analyzed with the programs of the Genetics Computer Group package (18) and DNA Strider (54). A modified version of the Macintosh MacTargSearch 1.1 program (64) was used to search for *E. coli*- and RK2-specific ribosome-binding sites. Putative polypeptides were analyzed with the MacPattern implementation of the Prosite data base to determine whether they contained known structural motifs. Homologies to known proteins in the GenBank, EMBL, and SwissProt collections were identified by the FASTA and Smith-Waterman algorithms. The Genetics Computer Group and MacTargSearch programs were used to search for prokaryotic factor-independent terminators, RK2-specific consensus sequences, direct repeats, inverted repeats, and *E. coli*- and RK2-specific promoter sequences.

Primer extension analysis. Promoter-containing regions were cloned into pCH8. A synthetic DNA primer (35-mer, 5′-AAGCTTGCATGCCTGCAGGTCGACTCTAGAG GATC-3′), complementary to a region of the transcribed polylinker downstream of the cloned promoters, was used to determine the transcriptional start site. Another synthetic DNA primer (*bla*, 5′-TCTGGGTGAGCAAAAACAGGAAG GCAAAATGCCGC-3′), complementary to the *bla* transcript, was used as an internal control in each of the primer extension reactions. The 5′ end of the *bla* primer is 112 nucleotides from the *bla* transcriptional start site. Extraction of RNA and primer extension with avian myeloblastosis virus reverse transcriptase were done as described previously (28). The reference nucleotide sequence was generated from double-stranded plasmid DNA by the chain termination method and with the end-labeled 35-mer primer.

Conjugation and entry exclusion. Overnight cultures of donor and recipient strains were diluted 1:100 in selective media and grown to mid-log phase. The cultures were centrifuged, washed twice with LB medium, and resuspended in either the original volume or 1/10 volume of LB medium. One milliliter of the donor strain was added to 1 ml of the recipient strain in a 250-ml culture flask and incubated at 37°C, without shaking, for at least 1 h. If the transconjugant selection included a tryptophan requirement, the mating mixtures were centrifuged and resuspended in an equal volume of M9 Casamino Acid broth before plating. To measure the efficiency of mating, the donor-to-recipient ratio was 1:10. The donor hosts were the *recA* strains DH5α and EKA0076; the recipient strains were C600 and VT1259. To measure entry exclusion, the donor-to-recipient ratio was 10:1. The donor was VT1284; the recipient hosts were VT1258 and EKA0076.

PRD1 methods. To prepare PRD1 phage lysates, PRD1 was spotted on a lawn of an RK2-containing strain on an LB plate and incubated overnight at 37°C. A 50-ml LB culture was inoculated with 100 μl of an overnight culture of the sensitive strain and infected with PRD1 phage scraped from the phage spot with a toothpick. The culture was shaken overnight at 37°C. The lysed culture was centrifuged to pellet debris, and the supernatant was retained. The efficiency of plating was determined by comparing PFU on various strains with that on an RK2-containing strain.

Nucleotide sequence accession number. The nucleotide sequence and amino acid sequence data reported here have been submitted to the GenBank nucleotide sequence data bases under accession number LO5600.

RESULTS

Localization and nucleotide sequence of the host-lethal *kilB* determinant. The *kilB* region of RK2 was originally defined by the 9.8-kb *Sal*I-to-*Kpn*I fragment (coordinates, kb 14.0 to 23.8), which is present on plasmid pRK2709 (Fig. 2). *E. coli* cells are able to maintain pRK2709 only if they carry the RK2 *korB* gene to control expression of *kilB*. A convenient assay for the host-lethal (Kil$^+$) phenotype of *kilB* is the inability to obtain viable transformants of *E. coli* cells lacking *korB*. Mutants defective in *kilB* can therefore be readily identified by their ability to produce transformants of *korB*$^+$ and *korB*^{o} strains with equal efficiency.

The 9.8-kb *Sal*I-to-*Kpn*I fragment in pRK2709 also contains the *trfA* operon (Fig. 2). We have shown previously that uncontrolled transcription from the strong *trfA* promoter into a plasmid vehicle can sometimes interfere with the selection of transformants in a *kor*^{o} strain (5). This potential complication was eliminated by providing *korA*, which regulates *trfA* but not *kilB*. Thus, deletion mutants of pRK2709 were constructed in the presence of *korA* and *korB* and subsequently tested for their ability to produce colonies after transformation of *korA*$^+$ *korB*$^+$ and *korA*$^+$ *korB*^{o} strains. Only plasmids defective in *kilB* are able to yield viable transformants of the *korA*$^+$ *korB*^{o} host.

The Kil phenotypes of a series of deletion mutants generated by partial *Bss*HII digestion of pRK2709 showed that *kilB* is located in the region encompassing the 1.0- and 2.9-kb *Bss*HII fragments (Fig. 2). That sequences from both fragments are required for expression of *kilB* was indicated by the Kil$^-$ phenotypes of plasmid pRK2819, which carries the 1.0-kb *Bss*HII fragment alone, and pRK2838, which contains the *kilB*-relevant 1.9-kb *Bss*HII-*Sfi*I region of the 2.9-kb *Bss*HII fragment. Insertion of the 1.0-kb *Bss*HII fragment from pRK2819 into the *Bss*HII site of pRK2838 to regenerate the *kilB* region restored the Kil$^+$ phenotype, but only in the wild-type orientation (pRK2912). The cloned *Nde*I fragment (pRK21416), which includes the same region except for a difference of 165 bp at the end of the *kilB* region, conferred a Kil$^-$ phenotype. Thus, an essential part of the *kilB* determinant lies within the 165-bp region between the *Nde*I and the *Bss*HII sites.

We tested the possibility that plasmids with the newly defined *kilB* region failed to produce transformants of the *kor*^{o} strain because of transcriptional read-through from a strong promoter, similar to the phenomenon we previously observed for the *trfA* (5) and *kilA* (28) promoters. A transcriptional terminator was positioned downstream of the *kilB* region on pRK2709Δ36 (Fig. 2). The resulting plasmid, pRK2914, displays a Kil$^+$ phenotype, thus confirming that a lethal gene product is expressed.

We determined the nucleotide sequence of the region between the beginning of the published *trfA* sequence and the *Bss*HII site at coordinate kb 19.8 in the *kilB* region. Because our analysis suggested that the *Bss*HII site was within an open reading frame (ORF), we continued to sequence an additional 480 nucleotides beyond the *Bss*HII site. The sequence of this region is shown in Fig. 3.

Determination of the *kilB* transcriptional start site. The nucleotide sequence revealed two closely spaced promoter-like sequences (*p2* and *p3* in Fig. 3). Both are related to the consensus sequence for σ^{70} promoters (32) and point in the same direction as that predicted for expression of *kilB* (88), but neither coincides with the sequence previously identified by others as the *kilB* promoter (90) (*p1* in Fig. 3). To determine which, if any, of these sequences is a functional

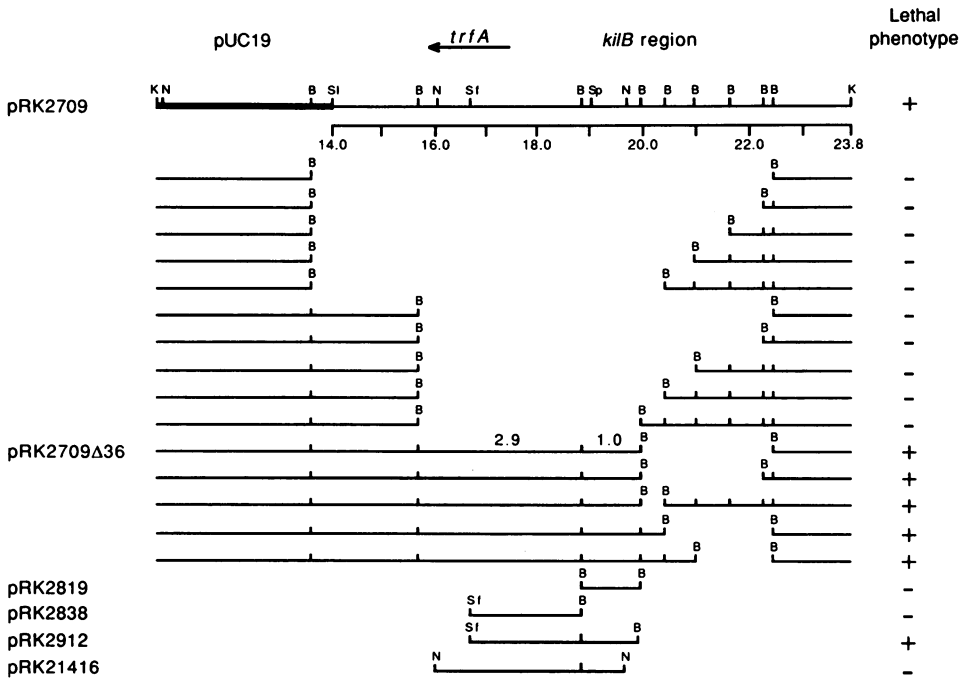

FIG. 2. Physical mapping of the host-lethal *kilB* determinant. Plasmid pRK2709 contains *kilB* on a 9.8-kb *Sal*I-*Kpn*I fragment. RK2 map coordinates are shown below the linear representation of pRK2709. The pUC19 vector is indicated by a thick line. The locations of the *trfA* operon and the *kilB* region are indicated. Lines below pRK2709 show DNA contained in 15 partial *Bss*HII deletions of pRK2709 and several subclones. Plasmid designations refer to specific plasmids discussed in the text. + indicates that the plasmid encodes the *kilB* lethal phenotype; − indicates that the plasmid is not lethal. The assay for the lethal phenotype is described in the text. B, *Bss*HII; K, *Kpn*I; N, *Nde*I; Sf, *Sfi*I; Sl, *Sal*I; Sp, *Sph*I.

promoter in vivo, we mapped the transcriptional start site by primer extension.

The 285-bp *Sty*I-to-*Xmn*I fragment that contains *p1*, *p2*, and *p3* was cloned upstream of a transcriptional terminator in the vector pCH8 to produce pRK21407 (Fig. 4). Two 5′-end-labeled oligonucleotide primers were added to RNA extracted from strains containing pRK21407 or the pCH8 vector. The *bla* primer hybridized to the *bla* mRNA transcript of the pCH8 vector and provided an internal control; the 35-mer primer was designed to hybridize to any RNA transcript expressed from the cloned region. The bound primers were extended by avian myeloblastosis virus reverse transcriptase and separated by polyacrylamide gel electrophoresis (Fig. 5). Analysis of RNA extracted from the pRK21407-containing cells revealed the presence of the *bla* transcript and another RNA initiating from within the cloned region. The 5′ end of the RNA was consistent with initiation of transcription from either *p2* or *p3*. Because *p2* and *p3* overlap, additional analysis will be required to determine which of the two is the actual promoter. We found no evidence of transcription initiating from the previously predicted *kilB* promoter (*p1*), even after prolonged exposure of the gel. We conclude that *p1* is not involved in the expression of *kilB* and that the *kilB* promoter is *p2* or *p3*.

The region containing the upstream KorB target sequence at −183 is required for the regulation of *kilB* by KorB. For studies of KorB regulation of *kilB*, we constructed plasmid pRK21408, which has the *korB* structural gene positioned downstream of the inducible *trc* promoter. After transformation of a pRK21408-containing strain with the *kilB*$^+$ plasmid pRK2138, viable colonies were recovered only in the presence of IPTG, which induces the *trc* promoter and expression of *korB*. A control plasmid lacking *kilB* produced viable transformants both in the presence and in the absence of IPTG (data not shown). This result confirms our earlier analysis (12), which indicated that *korB* is sufficient to control the host-lethal phenotype of the *kilB* region.

To test for KorB regulation of the *kilB* promoter, we used primer extension analysis to assay the relative levels of RNA initiated from the *kilB* promoter in the presence and absence of *korB* in vivo. The abundance of the *bla* transcript was used as an internal control for the relative amounts of RNA in the samples. Figure 6 (lanes 1 to 3) shows that the abundance of *kilB*-initiated RNA is reduced at least fivefold in the presence of *korB*. This result is consistent with transcriptional control by *korB*.

KorB dimers bind to the sequence 5′-TTTAGC(G/C) GCTAAA-3′ (8). The occurrence of this sequence that is closest to the *kilB* promoter is 183 bp upstream of the start site for *kilB* transcription (Fig. 3). To determine whether this upstream KorB box is required for regulation of *kilB*, we compared the effects of *korB* on transcripts initiated from the wild-type *kilB* promoter (pRK21407) and a mutant promoter deleted for the upstream KorB box (pRK21413). The *kilB* promoter deleted for the upstream region gives a lower level of transcription than the wild-type promoter (Fig. 6, lanes 2 and 4), indicating that the upstream region may influence the activity of the promoter. Nevertheless, the level of transcription from the mutant promoter was not significantly altered by the presence of *korB* (Fig. 6, lanes 4 and 5). Thus, the upstream region, which includes the KorB box, is required for KorB-mediated regulation of transcription from the *kilB* promoter.

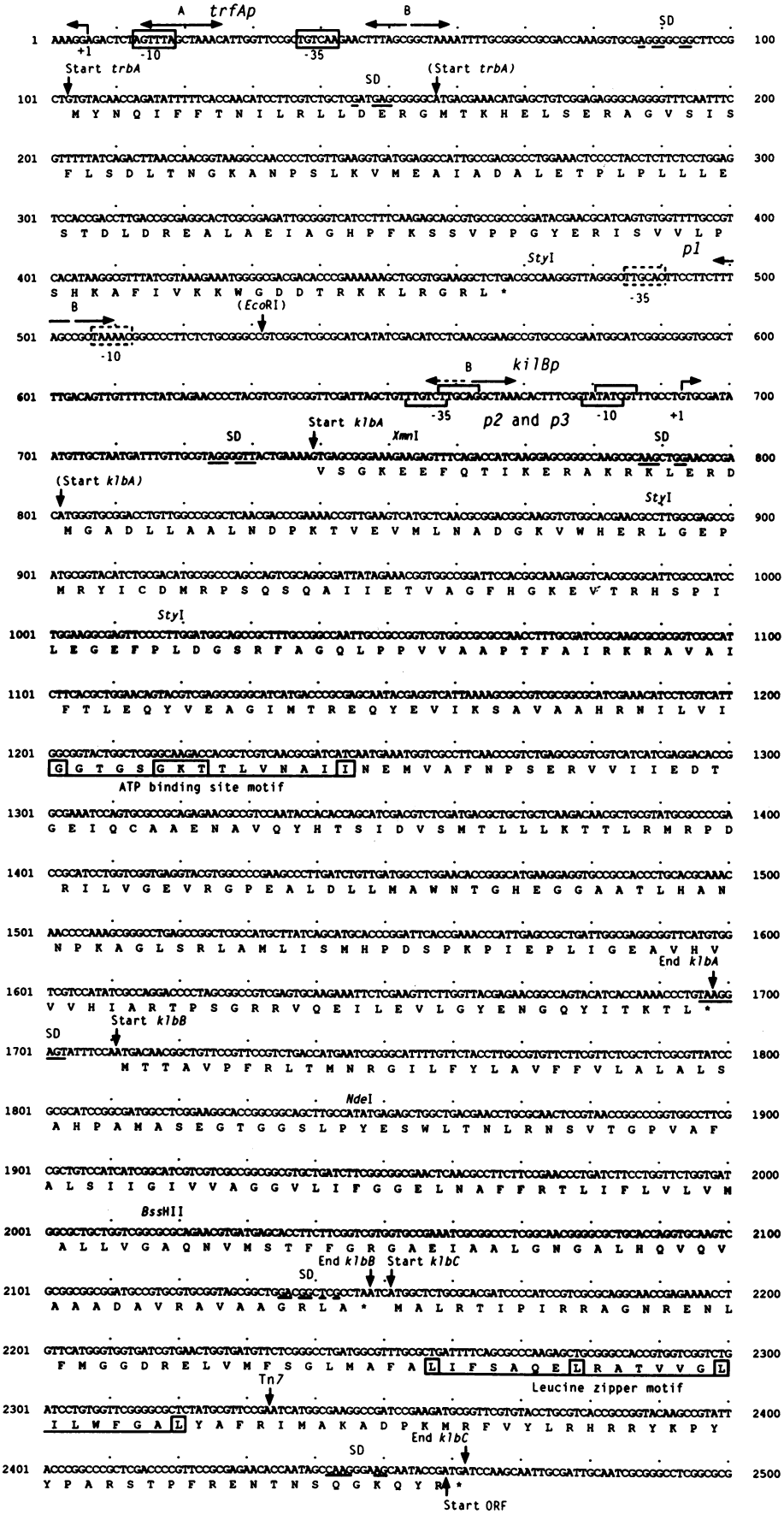
trfAp
A
B
SD
+1
-10
-35
Start trbA
(Start trbA)
SD
p1
StyI
-35
B
(EcoRI)
-10
klBp
B
-35
-10
+1
p2 and p3
Start klbA
XmnI
SD
SD
(Start klbA)
StyI
StyI
ATP binding site motif
End klbA
Start klbB
SD
NdeI
BssHII
End klbB
Start klbC
SD
Tn7
Leucine zipper motif
End klbC
SD
Start ORF

FIG. 3. Nucleotide sequence of the *kilB* region (GenBank accession number LO5600). Shown here is the nucleotide sequence of a 2,500-bp region that encodes the lethal *kilB* determinant. The *trfA* promoter (bp 1 to 65) (90) was determined previously. The transcriptional start sites of the *trfA* (72) and *kilB* (the present work) operons are designated by +1; the arrows indicate the directions of transcription. The −10 and −35 regions of the *trfA* promoter are boxed. The −10 and −35 regions of the previously predicted *kilB* promoter, *p1*, are boxed with dashed lines. The −10 and −35 regions of the overlapping potential promoters *p2* and *p3* are noted with paired half boxes below and above the sequence, respectively. The KorA- and KorB-binding sites are indicated by divergent arrows labeled A and B, respectively. The arrow above the suboptimal half of the partial KorB box in the *p2/p3* promoter is broken. Nucleotides corresponding to an *E. coli* or *P. aeruginosa* Shine-Dalgarno sequence are underlined and designated by SD. The *Sty*I site (bp 471) and the *Xmn*I site (bp 751) were used to clone the promoter region in pRK21407. The *Eco*RI site, 163 bp before the *kilB* transcriptional start site, was introduced on a primer used in polymerase chain reaction amplification of the region from −163 to +60 for construction of pRK21413, as described in Materials and Methods. Shown also are the *Sty*I sites at bp 888 and 1017, used for constructing pRK21417; the *Nde*I site at bp 1844, used for constructing pRK21416; and the *Bss*HII site at bp 2015, used for constructing pRK2912. Tn*7* indicates the site of the Tn*7* insertion in pRP101. The amino acid sequences of the predicted polypeptides of *trbA*, *klbA*, *klbB*, and *klbC* are shown below the nucleotide sequence. Two potential starts are indicated for *trbA* and *klbA*. The ATP-binding motif present in the KlbA sequence and the leucine zipper motif in KlbC are indicated.

Identification of potential structural genes in the *kilB* operon. The nucleotide sequence was examined for ORFs. Three ORFs predicted to be protein-coding regions by the method described by Fickett (20) were located downstream of the *kilB* promoter. They were found to have possible Shine-Dalgarno sequences (86) for *E. coli* and *Pseudomonas aeruginosa* and to exhibit the G+C content and codon structures characteristic of previously identified RK2 genes (Table 2). These putative genes were named *klbA*, *klbB*, and *klbC* (*klb* is pronounced klib [from *kilB*]), in accordance with our previously established nomenclature for genes of the *kilB* locus (5, 28). A fourth ORF was identified in the region between the *kilB* and *trfA* promoters (Fig. 3). Its G+C content and codon structure showed a slight deviation from those of other known RK2 genes (Table 2). This ORF has recently been identified as the *trbA* gene, which is involved in the conjugal transfer of RK2 and negative control of *trfA* and *kilB* (37, 48).

***klbB* is required for the host-lethal phenotype of the *kilB* region.** Plasmid pRK21416 carries the *kilB* promoter, the entire *klbA* gene, and a portion of *klbB* up to the *Nde*I site at position 1844 in the nucleotide sequence. Because its phenotype is Kil$^-$, we conclude that *klbA* is not sufficient for expression of the host-lethal phenotype of the *kilB* region. In contrast, plasmids pRK2709Δ36 and pRK2912, whose cloned *kilB* fragments end at the *Bss*HII site near the end of *klbB* at position 2015, were both Kil$^+$. These results demonstrate that at least some of the *klbB* coding region between the *Nde*I and *Bss*HII sites is required for the lethal phenotype.

The function of the *kilB* operon. The transposon Tn*7* insertion in the *kilB* region of pRP101, a derivative of the RK2-like plasmid RP4, resulted in loss of conjugal transfer and surface exclusion (9). We sequenced the junction of the Tn*7* insertion in pRP101 and found that the transposon disrupts the *klbC* coding sequence (Fig. 3). Therefore, the insertion is located within the *kilB* operon but downstream of the region shown to be responsible for host lethality. To

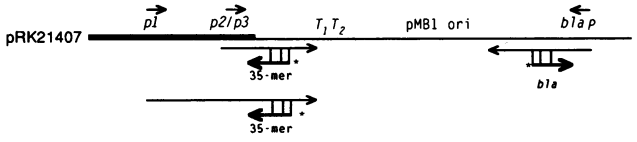

FIG. 4. Strategy for determining the transcriptional start site of the *kilB* operon. The 285-bp *kilB* promoter region (thick line), which contains all three possible promoters (*p1*, *p2*, and *p3*), was cloned upstream of the *rrnB* transcriptional terminators T_1 and T_2 of the vector pCH8 (thin line). The possible RNA transcripts are depicted as thin arrows below the pRK21407 map. The thick arrows represent the ^{32}P-labeled 35-mer primer, which hybridizes to any transcript originating from the *kilB* promoter region. *blap* shows the promoter for the *bla* gene. *bla* indicates the *bla*-specific primer.

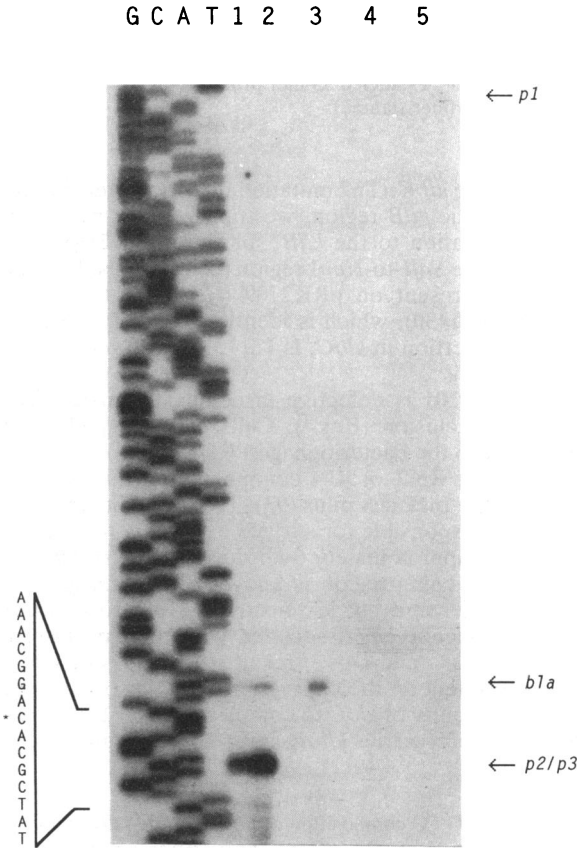

FIG. 5. Transcriptional start site of the *kilB* operon. The ^{32}P-end-labeled 35-mer and *bla* primers were annealed to RNA extracted from cells containing the appropriate plasmid and extended by reverse transcriptase. The labeled DNA products were separated on polyacrylamide sequencing gels and visualized by autoradiography as described in Materials and Methods. The reference sequences were obtained by the dideoxynucleotide chain termination method with the end-labeled 35-mer primer and plasmid pRK21407. RNAs for primer extension were isolated from strains containing the following plasmids: pRK21407 (lanes 1 and 2 [lane 1 has one-fifth the amount shown in lane 2]), pCH8 (lane 3), untreated ^{32}P-labeled 35-mer primer (lane 4), and untreated ^{32}P-labeled *bla* primer (lane 5).

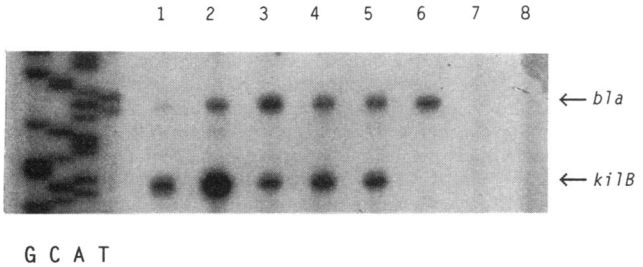

FIG. 6. Effect of *korB* on abundance of transcripts initiated from the *kilB* promoter. The ^{32}P-end-labeled 35-mer and *bla* primers were annealed to RNA extracted from cells containing the appropriate plasmid and extended by reverse transcriptase. Strains carrying the inducible *korB* plasmid pRK21408 were grown in the presence of IPTG. Plasmid pRK21407 contains the *kilB* promoter and the upstream KorB box; pRK21413 carries the *kilB* promoter but is lacking the upstream KorB box (Fig. 3). The labeled DNA products were separated on polyacrylamide sequencing gels and visualized by autoradiography as described in Materials and Methods. The reference sequences were obtained by dideoxynucleotide chain termination method with the end-labeled 35-mer primer and plasmid pRK21407. RNA for primer extension was isolated from strains containing the following plasmids: pRK21407 (lanes 1 and 2 [lane 1 has one-fifth the amount in lane 2]), pRK21407 and pRK21408 (lane 3), pRK21413 (lane 4), pRK21413 and pRK21408 (lane 5), pCH8 (lane 6), untreated ^{32}P-labeled 35-mer primer (lane 7), and untreated ^{32}P-labeled *bla* primer (lane 8).

confirm that the *klbC*::Tn*7* mutation does not alter the lethal phenotype of the *kilB* region, we transferred it by homologous recombination to the *kilB*$^+$ plasmid pRK2135, which carries the same *Sal*I-to-*Kpn*I region (coordinates, kb 14.0 to 23.8) that is present on pRK2709 (Fig. 2). The resulting plasmid (pRK21430), which is identical to pRK2135 except for the Tn*7* insertion in *klbC*, is Kil$^+$, as expected (data not shown).

Plasmid pRP101 is defective in conjugal transfer (Tra$^-$) and surface exclusion (Eex$^-$). Cells carrying pRP101 are also resistant to the bacteriophage PRD1 (Dps$^-$) (73), which normally infects RK2- or RP4-containing cells and is thought to adsorb to the IncP sex pilus (93) (Table 3). The only genes known to be responsible for surface exclusion are *eexA* and *eexB*, located approximately 6 kb downstream of *klbC* (50, 52). This intervening region is known to contain genes for conjugal transfer and phage sensitivity (10). To determine whether the phenotypes associated with the Tn*7* insertion (i.e., Eex$^-$, Tra$^-$, and Dps$^-$) result from a loss of *klbC* or from a polar effect on the expression of downstream genes, we tested the ability of *klbC* to complement pRP101 in *trans*. Plasmid pRK2135 carries *klbA*, *klbB*, and *klbC* and a region of approximately 4 kb downstream of *klbC*, but it does not include *eexA* or *eexB*, which are located 2.6 kb further downstream (49), and is unable to confer a surface exclusion phenotype (data not shown). We tested the ability of pRK2135 to complement the pRP101 transfer defect, PRD1 resistance, and entry exclusion defect in a *recA* host (Table 3). The results show that pRP101 is unable to transfer, even in the presence of pRK2135. Surface exclusion was indicated by the reduced ability of the IncP transfer system to mobilize the compatible IncQ plasmid pBK1 to recipients containing RK2. Table 3 shows that pBK1 was mobilized into an RK2-containing strain 30-fold less efficiently than into the plasmidless strain. In contrast, pBK1 was transferred to strains containing pRP101 alone or pRP101 and pRK2135 with efficiencies similar to that of the plasmidless recipient. We also tested these strains for sensitivity to bacteriophage PRD1. Only the RK2-containing strain allowed the formation of plaques by PRD1.

These results show that none of the defects of pRP101 is complemented by pRK2135 and that the multiple effects of the Tn*7* insertion in pRP101 most likely result from a polar effect on the expression of downstream genes. Thus, the *kilB* operon is predicted to extend for a considerable distance beyond *klbC* and include Tra2 region genes required for conjugal transfer, surface exclusion, and phage sensitivity.

To confirm the role of the *kilB* locus in conjugal transfer, we constructed an RK2 derivative, pRK21418, that contains an insertion within the *klbA* gene and examined its phenotype with respect to conjugal transfer, entry exclusion, and PRD1 sensitivity. We found that pRK21418 is transfer-defective and resistant to PRD1 (Table 3). However, unlike pRP101, pRK21418 expresses surface exclusion. The Eex$^+$ phenotype of pRK21418 suggested that the insertion is nonpolar on the expression of *eexA* and *eexB* and that the Tra$^-$ and Dps$^-$ phenotypes should be complementable by *klbA* in *trans* (pRK21416). The results in Table 3 confirm this prediction and demonstrate that *klbA*, the first gene of the *kilB* operon, is required for conjugal transfer and phage sensitivity of RK2.

TABLE 2. G+C composition of ORFs in the *kilB* region

ORF	% G+C content			
	Base position in codon			Coding region
	1	2	3	
RK2 average[a]	66.3	45.4	70.0	63.9
klbA	63.6	41.8	77.3	60.9
klbB	65.1	46.6	80.1	63.9
klbC	58.6	45.2	76.9	60.3
ORF4	56.6	40.2	65.6	54.1

[a] Values are the averages for genes of RK2 and RK2-like plasmids whose proteins have been identified (11, 25, 36, 41, 42, 50, 76, 95, 97, 100, 111).

TABLE 3. Phenotypes and complementation analysis of pRP101 and pRK21418

Test plasmid(s)	Relative transfer efficiency[a]	Relative entry exclusion index[b]	PRD1 EOP[c]
None		1.0	$<10^{-7}$
RK2	1.0	0.03	1.0
pRP101	$<10^{-5}$	1.4	$<10^{-7}$
pRP101 + pRK2135	$<10^{-5}$	0.6	$<10^{-7}$
pRK21418	$<10^{-5}$	0.05	$<10^{-7}$
pRK21418 + pRK21416	11.0		1.0

[a] The *recA* donor strains contained the test plasmid(s). Transfer efficiency is the ratio of transconjugants per milliliter to donors per milliliter. Relative transfer efficiency is normalized to the transfer efficiency of the RK2-containing strain.

[b] Recipient strains contained the test plasmid(s). Entry exclusion index is the ratio of transconjugants per milliliter to recipients per milliliter. Relative entry exclusion index is normalized to the entry exclusion index of the plasmidless strain.

[c] EOP, efficiency of plating. EOP is the number of PFU of PRD1 on each strain relative to that of the RK2-containing strain.

DISCUSSION

Genetic, molecular, and nucleotide sequence analyses of the *kilB* region were used to identify and characterize the

first three genes of the *kilB* operon, designated *klbA*, *klbB*, and *klbC*. The nucleotide sequence also revealed a fourth ORF between the *kilB* and *trfA* promoters, which was recently identified as the *trbA* gene (37, 48). We determined that (i) transcription initiates 46 nucleotides upstream of *klbA*, (ii) KorB regulation of *kilB* requires a KorB-binding site located 183 bp upstream, (iii) *klbB* is required for the host-lethal phenotype of the *kilB* operon, (iv) *klbA* is required for conjugal transfer, and (v) the *kilB* operon is likely to be a large transcriptional unit encompassing genes of the Tra2 region involved in conjugal transfer and surface exclusion. Both *kilB* and the replication initiator gene, *trfA*, are regulated as part of the *kil-kor* regulon of plasmid RK2. Therefore, the *kil-kor* regulon is a unique example of plasmid-mediated coregulation of conjugal transfer and vegetative replication.

The amino acid sequences of the predicted polypeptide products of *klbA*, *klbB*, and *klbC* are shown beneath the nucleotide sequence in Fig. 3. There are two potential in-frame translational start sites in *klbA*. The GTG at position 739 and the ATG at position 802 are both preceded by potential ribosome-binding sites. Initiation of translation would result in polypeptides with molecular masses of 34,995 and 32,465 Da, respectively. Evidence has been presented recently in favor of the GTG start (62). The predicted amino acid sequence of KlbA shows an ATP-binding site motif (Fig. 3). In addition, the sequence has possibly significant homology (30% identity and 52% similarity) to the product of *virB11*, a gene of *Agrobacterium tumefaciens* Ti plasmids involved in transfer of T-DNA to plant cells (106). These properties have also been noted by Motallebi-Veshareh et al. (62). The product of *klbB* is predicted to be a 145-amino-acid polypeptide with a molecular mass of 15,012 Da. Hydrophobicity analysis of the amino acid sequence suggests that KlbB has three potential membrane-spanning domains (data not shown). The KlbB polypeptide displays 36% identity and 61% similarity to the RK2 membrane protein KlaC, the product of the lethal *klaC* gene of the *kilA* operon (28, 105). The *klbC* product is predicted to be a basic, 103-amino-acid polypeptide with a mass of 12,085 Da. The *klbC* gene ends with a TGA stop codon that overlaps the ATG start codon of another ORF, indicating that the *kilB* operon contains at least one additional gene. This overlapping start-stop motif, which suggests the possibility of translational coupling (1, 69), is common to several adjacent RK2 genes, including *korA* and *incC2* (97), *incC* and *korB* (41), *klaB* and *klaC* (28), *traI* and *traG*, *traG* and *traF*, *traL* and *traM* (111), and *parB* and *parC* (25, 75). The nucleotide sequence (Fig. 3) was compared with the recently completed Tra2 sequence of IncP plasmid RP4 (49); the RK2 sequence presented here is identical to that of the comparable region of RP4.

Our analysis of the deduced amino acid sequence of KlbC revealed a perfect match to the leucine zipper motif, Leu-6X-Leu-6X-Leu-6X-Leu, known to be involved in protein-protein interactions of several eukaryotic proteins (45). Chiral wheel analysis predicted the putative KlbC leucine zipper to have the amphipathic property of known functional eukaryotic leucine zippers. There are few examples of the leucine zipper motif in prokaryotic proteins. The only other reported perfect match to the leucine zipper motif occurs in the MetR activator protein of *E. coli* (57). In this case, the leucines in the heptad repeat regions were shown to be involved in the formation of a DNA-binding homodimer. Leucine heptad repeats are also important in the formation of tetramers of Lac repressor (2) and intramolecular interactions within σ^{54} (80) and have been postulated to be involved in interactions of plasmid replication initiators (26). The putative leucine zipper in KlbC might therefore be involved in the interaction of KlbC monomers with each other or with other proteins. At present, we have no evidence for the biochemical function of KlbC. Given that most known leucine zipper proteins bind DNA (104), there is reason to consider the possibility that KlbC is a DNA-binding protein. Although KlbC displays no obvious DNA-binding motif, such as the zinc finger or helix-turn-helix (70), its leucine zipper region, like that of the MetR regulatory protein (57), is followed by a basic region. Another possibility is that KlbC participates in the formation of protein complexes needed for the assembly of the sex pilus, which is considered a major function of the Tra2 region (10, 48, 71).

Our results do not support the conclusion of Motallebi-Veshareh et al. (62) that the first gene of the *kilB* operon is responsible for the host-lethal (Kil^+) phenotype of the *kilB* region. The deletion analysis presented in the present study shows that (i) *klbA* is not sufficient for killing host cells and (ii) at least some of the *klbB* structural gene is required. Since the polar *klbC*::Tn*7* of pRP101 does not abolish the Kil^+ phenotype, the portion of the *kilB* operon that includes *klbA* and *klbB* constitutes the host-lethal *kilB* determinant.

When we first reported the existence of *kilB*, we noted that it was the only *kil* determinant not excluded from a role in conjugal transfer (22). Lessl et al. (50) were the first to propose that the *kilB* operon is responsible for the expression of transfer genes in Tra2. They identified the *eexA* and *eexB* genes for surface exclusion by plasmid R18 but detected no promoter-like sequences in the immediate upstream region. In addition, they found that a Tn*7* insertion several kilobases upstream abolished the surface exclusion phenotype and suggested that the insertion blocked expression of the *eex* genes from the *kilB* promoter. Recently, Motallebi-Veshareh et al. (62) presented the nucleotide sequence of the first gene of the *kilB* operon and showed evidence that this gene is required for conjugal transfer. The transfer-defective phenotype of the *klbA* mutant constructed in the present study and its ability to be complemented in *trans* is consistent with their results. In addition, we have identified two other genes of the *kilB* operon, *klbB* and *klbC*, located directly downstream of *klbA* and found that a Tn*7*-insertion in *klbC* is responsible for the transfer-defect and absence of surface exclusion exhibited by plasmid pRP101. Additional evidence supporting the possibility that the *kilB* promoter expresses the transfer genes in the Tra2 region was recently provided by Lessl et al. (48). They found by electron microscopy that the only detectable RNA polymerase-binding sites in the Tra2 region were upstream of the first gene of the *kilB* operon. In addition, it has recently been shown that the only occurrences of possible σ^{70} promoter sequences are upstream of the *klbA* counterpart in RP4 (49). All of these results stand in contrast to those of Palombo et al. (71), who deduced the existence of at least six operons in the Tra2 region, on the basis of partial complementation of transposon Tn*5*-induced mutations that were assumed to be polar. The results presented here showing polarity from the Tn*7* insertion of *klbC* suggest that the Tn*5* insertion mutations described by Palombo et al. (71) were not completely polar on the expression of downstream genes. Our results therefore support the conclusion that the *kilB* operon extends through the Tra2 region and includes genes for conjugal transfer and surface exclusion. We do not know whether the *kilB* promoter is the only promoter involved in the expression of Tra2 genes. Although the evidence suggests

that there are no promoters downstream of the *kilB* promoter, it is possible that the upstream *trbA* promoter also participates in the expression of the operon.

The *trbA* gene located between the *kilB* and *trfA* promoters (Fig. 3) may be involved in conjugal transfer (48), and its overexpression leads to reduction in the expression of *trfA* and *kilB* operon fusions (37). Its role in the regulation of transfer and the *kilB* host-lethal determinant is not yet known. Our results demonstrate that *trbA* is insufficient to control the *kilB* determinant because *kilB*$^+$ plasmids that also contain *trbA* are Kil$^+$, unless KorB is present in the cell (Fig. 2). Our studies also show that regulation of the *kilB* promoter does not require *trbA*, since KorB is able to reduce transcription from the *kilB* promoter in the absence of *trbA* (Fig. 6). However, it is possible that *trbA* is important for regulation of the *kilB* operon. This may explain why we (101) and others (46) have not been able to mutate *trbA* in *kilB*$^+$ plasmids, even in the presence of KorB.

The transcriptional start site of the *kilB* operon, determined by primer extension analysis, is located 46 nucleotides upstream of a potential *klbA* start codon. This start site follows two overlapping sequences (*p2* and *p3*) predicted to be reasonable σ^{70} promoters by the matrix analysis of Hawley and McClure (32). Recently, Lessl et al. (48) mapped RNA polymerase-binding sites in the Tra2 region by electron microscopy and predicted that the sequence corresponding to *p3* is the *kilB* promoter. Our results are consistent with this prediction. However, while *p3* has better spacing to the transcriptional start site, *p2* is calculated by the Hawley-McClure analysis to be a stronger promoter. We have not yet determined which of these overlapping sequences is the functional promoter. Nevertheless, the transcriptional start does not correspond to the *p1* sequence, which was predicted to be the *kilB* promoter by Smith et al. (90) and by Motallebi-Veshareh et al. (62). *p1* scores significantly lower than *p2* and *p3* in the Hawley-McClure analysis, and our experiments showed no evidence of transcription initiating from *p1*. We conclude that the *kilB* promoter must be *p2* or *p3*.

Our finding that transcripts initiating from the *kilB* promoter are less abundant in cells expressing KorB is consistent with evidence from operon fusions that KorB controls gene expression by functioning as a transcriptional repressor (12, 22, 36, 85, 88, 94, 109). The 39-kDa KorB protein (12, 41, 95) exists as a dimer in solution and recognizes the palindromic DNA sequence 5′-TTTAGC(G/C)GCTAAA-3′ (8). This sequence occurs at least 12 times in the RK2 genome, but only four occurrences have been clearly implicated in gene regulation. In the *trfA*, *kilA*, and *korA* promoters, the KorB box is located 4 bp upstream of the −35 sequence (90, 110). Presumably, when KorB is bound to this site, RNA polymerase is prevented from interacting with the promoter. In the *kfrA* promoter, the KorB box is positioned 39 nucleotides upstream of the −35 sequence for the primary promoter and overlaps the −10 region of a possible second promoter (36, 100). In this case, KorB regulation of *kfrA* expression depends on the presence of the KorF proteins. It has been proposed that the DNA is wrapped around the histone-like KorF proteins such that KorB is brought into close proximity to the RNA polymerase-binding site (36). Surprisingly, the closest KorB box to the *kilB* promoter is located 183 bp upstream of the transcriptional start site, and deletion of this KorB box abolishes regulation of *kilB* transcription by KorB. In addition, KorB alone is sufficient to reduce transcription from the *kilB* promoter. It seems unlikely that a KorB dimer bound to this site alone would sterically block the binding of RNA polymerase to the *kilB* promoter. We envisage two possible models to explain the regulation of *kilB* transcription by KorB. (i) The upstream region contains an enhancer of transcription, and the activity of the enhancer is blocked by binding of KorB to the region. Indeed, we find that deletion of the region upstream of −163, which includes the KorB box, results in reduced expression from the *kilB* promoter. However, additional studies will be required to test whether the lower activity results from the loss of specific RK2 sequences upstream of the *kilB* promoter. (ii) KorB directly inhibits initiation of transcription at the *kilB* promoter. This could be accomplished by the looping of DNA to allow KorB bound to the upstream target to interact with the RNA polymerase directly or with the promoter. There is considerable precedent for the involvement of DNA loops in transcriptional regulation (56, 81). Efficient regulation of the *E. coli lac* promoter requires DNA looping by tetrameric Lac repressor which is bound simultaneously to an operator within the promoter (O_1) and a second lower-affinity operator located 92 bp upstream (O_3) or 401 bp downstream (O_2) (66). Examination of the *kilB* sequence shows that the *kilB* promoter contains a partial KorB box with a perfect match to one arm of the palindrome and three matches in the remaining seven nucleotides of the binding site (Fig. 3). In addition, Balzer et al. (8) have presented evidence that tetramers of KorB may interact with the KorB box. Binding of a tetramer to the upstream KorB box would dramatically increase the local concentration of KorB. Thus, with DNA looping, even the lower-affinity partial KorB box may be occupied, such that binding of RNA polymerase to the *kilB* promoter is inhibited. We are currently testing the prediction that mutations in the partial KorB box will reduce or eliminate sensitivity to KorB.

The arrangement of KorB boxes in the region that contains the divergent *trfA* and *kilB* promoters suggests other possibilities for their coordinated regulation. The upstream KorB box involved in *kilB* regulation is also 451 bp upstream of another KorB box in the *trfA* promoter (Fig. 3). If a KorB tetramer were to interact with these two targets, then *trfA* expression would be inhibited, while *kilB* expression would be favored. Alternatively, a tetramer of KorB might simultaneously block both *trfA* and *kilB* expression by interacting with the KorB box within the *trfA* promoter and the partial KorB box within the *kilB* promoter. We are currently investigating these possibilities.

ACKNOWLEDGMENTS

We thank Bindu Kamal, Robin Pruett, and Mike Nuveen for their assistance with various experiments and Steve Winans, Soheyla Saadi, and Paul Goncharoff for their interest in the work. We are grateful to Erich Lanka for providing the RP4 Tra2 sequence prior to publication.

This study was supported by NIH grants R01-GM29085 and R01-GM26863 to D.H.F. and Cancer Center support grant CA13696 to Columbia University. V.J.T. was partially supported by NIH training grant T32-AI07161.

REFERENCES

1. **Aksoy, S., C. L. Squires, and C. Squires.** 1984. Translational coupling of the *trpB* and *trpA* genes in the *Escherichia coli* tryptophan operon. J. Bacteriol. **157**:363–367.
2. **Alberti, S., S. Oehler, B. Wilcken-Bergmann, H. Krämer, and B. Müller-Hill.** 1991. Dimer-to-tetramer assembly of the Lac repressor involves a leucine heptad repeat. New Biol. **3**:57–62.
3. **Albrechtsen, B., B. M. Ross, C. Squires, and C. L. Squires.** 1991. Transcriptional termination sequence at the end of the *Escherichia coli* ribosomal RNA *G* operon: complex termina-

tors and antitermination. Nucleic Acids Res. **9**:1845–1852.
4. **Ayres, E. K., and D. H. Figurski.** Unpublished results.
5. **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.
6. **Ayres, E. K., V. J. Thomson, G. Merino, D. Balderes, and D. H. Figurski.** Precise deletions in large prokaryotic 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., in press.
7. **Bachmann, B. J.** 1987. Derivations and genotypes of some mutant derivatives of *Escherichia coli* K-12, p. 1190–1219. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
8. **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.
9. **Barth, P. T.** 1979. RP4 and R300B as wide host-range cloning vehicles, p. 399–410. *In* K. N. Timmis and A. Pühler (ed.), Plasmids of medical, environmental, and commercial importance. Elsevier/North Holland Biomedical Press, Amsterdam.
10. **Barth, P. T., N. J. Grinter, and D. E. Bradley.** 1978. Conjugal transfer system of plasmid RP4: analysis by transposon Tn*7* insertion. J. Bacteriol. **133**:43–52.
11. **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.
12. **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.
13. **Brosius, J.** 1989. Superpolylinkers in cloning and expression vectors. DNA **8**:759–777.
14. **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.
15. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134**:1141–1156.
16. **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.
17. **Datta, N., and R. Hedges.** 1972. Host-ranges of R-factors. J. Gen. Microbiol. **70**:453–460.
18. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387–399.
19. **Fellay, R., J. Frey, and H. Krisch.** 1987. Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for *in vitro* insertional mutagenesis of gram-negative bacteria. Gene **52**:147–154.
20. **Fickett, J. W.** 1982. Recognition of protein coding regions in DNA sequences. Nucleic Acids Res. **10**:5303–5318.
21. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. USA **76**:1648–1652.
22. **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.
23. **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 Corporation, New York.
24. **Fürste, J. P., W. Pansegrau, R. Frank, H. Blöcker, P. Scholz, M. Bagdasarian, and E. Lanka.** 1986. Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tacP* expression vector. Gene **48**:119–131.
25. **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.
26. **Giraldo, R., C. Nieto, M.-E. Fernandez-Tresguerres, and R. Diaz.** 1989. Bacterial zipper. Nature (London) **342**:866.
27. **Goncharoff, P., and D. Figurski.** Unpublished results.
28. **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.
29. **Grinter, N. J., G. Brewster, and P. T. Barth.** 1989. Two mechanisms for the stable inheritance of plasmid RP4. Plasmid **22**:203–214.
30. **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.
31. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557–580.
32. **Hawley, D. K., and W. R. McClure.** 1983. Compilation and analysis of *E. coli* promoter DNA sequences. Nucleic Acids Res. **11**:2237–2255.
33. **Heinemann, J. A., and G. Sprague.** 1989. Bacterial conjugative plasmids mobilize DNA transfer between bacteria and yeast. Nature (London) **340**:205–209.
34. **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.
35. **Holmes, D., and M. Quigley.** 1981. A rapid method for the preparation of bacterial plasmids. Anal. Biochem. **114**:193–197.
36. **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.
37. **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.
38. **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.
39. **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.
40. **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.
41. **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.
42. **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.
43. **Kornacki, J. A., C.-H. Chang, and D. H. Figurski.** Manuscript in preparation.
44. **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.
45. **Landschulz, W. H., P. F. Johnson, and S. L. McKnight.** 1988. The leucine zipper: a hypothetical structure common to a new

class of DNA binding proteins. Science **240:**1759–1764.
46. **Lanka, E.** Personal communication.
47. **Lanka, E., and P. T. Barth.** 1981. Plasmid RP4 specifies a deoxyribonucleic acid primase involved in its conjugal transfer and maintenance. J. Bacteriol. **148:**769–781.
48. **Lessl, M., D. Balzer, R. Lurz, V. L. Waters, D. Guiney, and E. Lanka.** 1992. Dissection of IncP conjugative plasmid transfer: definition of the transfer region Tra2 by mobilization of the Tra1 region in *trans*. J. Bacteriol. **174:**2493–2500.
49. **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.
50. **Lessl, M., V. Krishnapillai, and W. Schilf.** 1991. Identification and characterization of two entry exclusion genes of the promiscuous IncP plasmid R18. Mol. Gen. Genet. **227:**120–126.
51. **Lichtenstein, C., and S. Brenner.** 1982. Unique insertion site of Tn*7* in the *E. coli* chromosome. Nature (London) **297:**601–603.
52. **Lyras, D., E. A. Palombo, and V. A. Stanisich.** 1992. Characterization of a Tra2 function of RP1 that affects growth of *Pseudomonas aeruginosa* PAO and surface exclusion in *Escherichia coli* K12. Plasmid **27:**105–118.
53. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
54. **Marck, C.** 1988. 'DNA Strider': a 'C' program for the fast analysis of DNA and protein sequences on the Apple Macintosh family of computers. Nucleic Acids Res. **16:**1829–1836.
55. **Marsh, J. L., M. Erfle, and E. J. Wykes.** 1984. The pIC plasmid and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation. Gene **32:**481–485.
56. **Matthews, K. S.** 1992. DNA looping. Microbiol. Rev. **56:**123–136.
57. **Maxon, M. E., J. Wigboldus, M. Brot, and H. Weissbach.** 1990. Structure-function studies on *Escherichia coli* MetR protein, a putative prokaryotic leucine zipper protein. Proc. Natl. Acad. Sci. USA **87:**7076–7079.
58. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101:**20–78.
59. **Meyer, R., and M. Hinds.** 1982. Multiple mechanisms for expression of incompatibility by broad-host-range plasmid RK2. J. Bacteriol. **152:**1078–1090.
60. **Meyer, R. J., and D. R. Helinski.** 1977. Unidirectional replication of the P-group plasmid RK2. Biochim. Biophys. Acta **478:**109–113.
61. **Mills, D. R., and F. R. Kramer.** 1979. Structure-independent nucleotide sequence analysis. Proc. Natl. Acad. Sci. USA **76:**2232–2235.
62. **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.
63. **Motallebi-Veshareh, M., D. A. Rouch, and C. M. Thomas.** 1990. A family of ATPases involved in active partitioning of diverse bacterial plasmids. Mol. Microbiol. **4:**1455–1463.
64. **Mulligan, M. E., D. K. Hawley, R. Entriken, and W. R. McClure.** 1984. *Escherichia coli* promoter sequences predict *in vitro* RNA polymerase selectivity. Nucleic Acids Res. **12:**789–800.
65. **Norrander, J., T. Kempe, and J. Messing.** 1983. Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. Gene **26:**101–106.
66. **Oehler, S., E. R. Eismann, H. Krämer, and B. Müller-Hill.** 1990. The three operators of the *lac* operon cooperate in repression. EMBO J. **9:**973–979.
67. **Olsen, R. H., and P. Shipley.** 1973. Host range and properties of the *Pseudomonas aeruginosa* R factor R1822. J. Bacteriol. **113:**772–780.
68. **Olsen, R. H., J.-S. Siak, and R. H. Gray.** 1974. Characteristics of PRD1, a plasmid-dependent broad host range DNA bacteriophage. J. Virol. **14:**681–699.
69. **Oppenheim, D. S., and C. Yanofsky.** 1980. Translational coupling during expression of the tryptophan operon of *Escherichia coli*. Genetics **95:**785–795.
70. **Pabo, C. O., and R. T. Sauer.** 1992. Transcription factors: structural families and principles of DNA recognition. Annu. Rev. Biochem. **61:**1053–1095.
71. **Palombo, E. A., K. Yusoff, V. A. Stanisich, V. Krishnapillai, and N. S. Willetts.** 1989. Cloning and genetic analysis of *tra* cistrons of the Tra2/Tra3 region of plasmid RP1. Plasmid **22:**59–69.
72. **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.
73. **Pohlman, R. F., and D. H. Figurski.** 1983. Conditional lethal mutants of the *kilB* determinant of broad host range plasmid RK2. Plasmid **10:**82–95.
74. **Prentki, P., and H. Krisch.** 1984. *In vitro* insertional mutagenesis with a selectable DNA fragment. Gene **29:**303–313.
75. **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.
76. **Roberts, R. C., and D. R. Helinski.** 1992. Definition of a minimal plasmid stabilization system from the broad-host-range plasmid RK2. J. Bacteriol. **174:**8119–8132.
77. **Saadi, S., and D. H. Figurski.** Unpublished results.
78. **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.
79. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
80. **Sasse-Dwight, S., and J. D. Gralla.** 1990. Role of eukaryotic-type functional domains found in the prokaryotic enhancer receptor factor σ^{54}. Cell **62:**945–954.
81. **Schleif, R.** 1992. DNA looping. Annu. Rev. Biochem. **61:**199–223.
82. **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.
83. **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.
84. **Schreiner, H. C.** 1988. Replication control in broad-host-range plasmid RK2: *kil* and *kor* functions affect expression of the essential replication gene *trfA*. Ph.D. thesis. Columbia University, New York, N.Y.
85. **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.
86. **Shine, J., and L. Dalgarno.** 1974. The 3′ terminal sequence of *E. coli* 16S ribosomal RNA: complementarity to nonsense triplet and ribosome binding sites. Proc. Natl. Acad. Sci. USA **71:**1343–1346.
87. **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.
88. **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.
89. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. BioTechnol. **1:**784–791.
90. **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.
91. **Smith, C. A., and C. M. Thomas.** 1984. Nucleotide sequence of

the *trfA* gene of broad host-range plasmid RK2. J. Mol. Biol. **175**:251–262.
92. **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.
93. **Thatte, V., D. E. Bradley, and V. N. Iyer.** 1985. N conjugative transfer system of plasmid pCU1. J. Bacteriol. **163**:1229–1236.
94. **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.
95. **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.
96. **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.
97. **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.
98. **Thomas, C. M., and C. A. Smith.** 1987. Incompatibility group P plasmids: genetics, evolution, and use in genetic manipulation. Annu. Rev. Microbiol. **41**:77–101.
99. **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.
100. **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.
101. **Thomson, V., and D. H. Figurski.** Unpublished results.
102. **Trieu-Cuot, C., C. Carlier, M. Martin, and P. Courvalin.** 1987. Plasmid transfer by conjugation from *Escherichia coli* to gram-positive bacteria. FEMS Microbiol. Lett. **48**:289–294.
103. **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.
104. **Vinson, C. R., P. B. Sigler, and S. L. McKnight.** 1989. Scissor-grip model for DNA recognition by a family of leucine zipper proteins. Science **246**:911–916.
105. **Walter, E. G., C. M. Thomas, J. P. Ibbotson, and D. E. Taylor.** 1991. Transcriptional analysis, translational analysis, and sequence of the *kilA*-tellurite resistance region of plasmid RK2Te^r. J. Bacteriol. **173**:1111–1119.
106. **Ward, J. E., D. E. Akiyoshi, D. Regier, A. Datta, M. P. Gordon, and E. W. Nester.** 1988. Characterization of the *virB* operon from an *Agrobacterium tumefaciens* Ti plasmid. J. Biol. Chem. **263**:5804–5814.
107. **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.
108. **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.
109. **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.
110. **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.
111. **Ziegelin, G., W. Pansegrau, B. Strack, D. Balzer, M. Kröger, V. Kruft, and E. Lanka.** 1991. Nucleotide sequence and organization of genes flanking the transfer origin of promiscuous plasmid RP4. DNA Sequence **1**:303–327.