The *kil-kor* Regulon of Broad-Host-Range Plasmid RK2: Nucleotide Sequence, Polypeptide Product, and Expression of Regulatory Gene *korC*

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Broad-host-range plasmid RK2 encodes several *kil* operons (*kilA*, *kilB*, *kilC*, *kilE*) whose expression is potentially lethal to *Escherichia coli* host cells. The *kil* operons and the RK2 replication initiator gene (*trfA*) are coregulated by various combinations of *kor* genes (*korA*, *korB*, *korC*, *korE*). This regulatory network is called the *kil-kor* regulon. Presented here are studies on the structure, product, and expression of *korC*. Genetic mapping revealed the precise location of *korC* in a region near transposon Tn*1*. We determined the nucleotide sequence of this region and identified the *korC* structural gene by analysis of *korC* mutants. Sequence analysis predicts the *korC* product to be a polypeptide of 85 amino acids with a molecular mass of 9,150 daltons. The KorC polypeptide was identified in vivo by expressing wild-type and mutant *korC* alleles from a bacteriophage T7 RNA polymerase-dependent promoter. The predicted structure of KorC polypeptide has a net positive charge and a helix-turn-helix region similar to those of known DNA-binding proteins. These properties are consistent with the repressorlike function of KorC protein, and we discuss the evidence that KorA and KorC proteins act as corepressors in the control of the *kilC* and *kilE* operons. Finally, we show that *korC* is expressed from the *bla* promoters within the upstream transposon Tn*1*, suggesting that insertion of Tn*1* interrupted a plasmid operon that may have originally included *korC* and *kilC*.

Plasmids of incompatibility group P (IncP) can replicate in many different species of gram-negative bacteria (17, 44, 80). The genetic and molecular basis for this extensive host range is not yet understood. However, studies on the IncP plasmid RK2 (30) have revealed an unusual system of genetic interactions involved in the control of plasmid replication and maintenance (22, 80).

Two genetic determinants are required for RK2 replication: *oriV*, the origin of unidirectional replication, and *trfA*, a gene that encodes a polypeptide needed for initiation of replication at *oriV* (20, 33, 41, 46, 47, 53, 54, 59, 64, 66, 74, 78, 81). The *trfA* operon is controlled as part of a complex regulatory network. This network, designated the *kil-kor* regulon, also includes several potentially host-lethal *kil* operons (*kilA*, *kilB*, *kilC*, *kilE*) whose functions are unknown (21, 48, 62; J. Kornacki, C. Chang, and D. Figurski, unpublished data). The *kil* operons and the *trfA* operon are negatively regulated by various *kor* genes (*korA*, *korB*, *korC*, *korE*) (4, 5, 21, 55, 60, 72, 76, 77, 86–88; Kornacki et al., unpublished data). The regulation of *trfA* by *kor* genes directly links the *kil-kor* regulon to control of plasmid replication. Furthermore, coregulation of the *trfA* and *kil* operons hints that the *kil* determinants may be involved in plasmid maintenance or host range.

A distinctive feature of the *kil-kor* regulon is that the operons are regulated by combinations of *kor* genes (Fig. 1). *korA* and *korB* functions inhibit expression of the *trfA* operon (55, 60), the *kilA* operon (4, 21, 87, 88), and the *korA-korB* operon (5, 72, 88). *korA* and *korC* are responsible for the negative control of the *kilC* and *kilE* operons (21, 77, 86; Kornacki et al., unpublished data). In addition, *korE*

contributes to the negative regulation of the *kilA* operon (87) and the *trfA* operon (H. Schreiner, O. Jovanovic, C. Young, and D. Figurski, unpublished data). Only one function, *korB*, is known to be involved in the control of *kilB* (21).

To understand the regulatory interactions of the *kil-kor* regulon, it is necessary to determine the basic properties of the *kor* genes and their products. Studies have shown that *korA* and *korB* are expressed together in an autoregulated operon and encode polypeptides with molecular masses of 11.3 and 39.0 kilodaltons (kDa), respectively (4, 5, 32, 63, 73, 79). Both polypeptides have helix-turn-helix regions that are characteristic of many known DNA-binding proteins (4, 32, 45, 73, 79). Genetic studies indicate that KorA and KorB proteins act as transcriptional repressors at the promoters for the *trfA*, *kilA*, and *korA-korB* operons (5, 55, 60, 72, 87, 88). The nucleotide sequences of these promoters show two operatorlike palindromes whose arrangements are similar in all three promoters (61, 88). One palindrome, which overlaps the −10 region, is believed to be the target for KorA protein (61, 88). The other palindrome, which occurs immediately upstream of the −35 region, is predicted to be the target for KorB protein (61, 88). The nearly identical spacing between the palindromes suggests that KorA and KorB proteins interact to form a corepressor complex.

In this report, we present our studies on *korC*. We mapped the precise location of *korC* and determined the nucleotide sequence of the *korC* region. By constructing specific mutations, we unambiguously identified the *korC* structural gene and its polypeptide product. We found that *korC* expression initiates from the *bla* promoters in the upstream transposon Tn*1* and is not dependent on *korA* function as originally proposed (86). We discuss the possibility that the protein products of *korA* and *korC* function as corepressors in the control of the *kilC* and *kilE* operons.

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korA
korB
korB   korE

korA
korC

korA   korB   korA
korA   korC   korE   korB

→
kilB   trfA   oriV kilC   bla   korC kilE kilA   korA korB

RK2
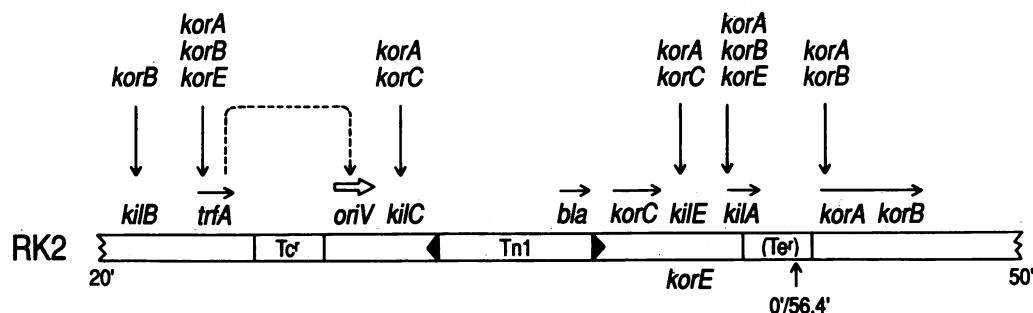20'   Tc^r   Tn1   (Te^r)   50'
korE
0'/56.4'

FIG. 1. Regulatory interactions of plasmid RK2. Only the relevant portion of RK2 is depicted. RK2 map coordinates are based on a total size of 56.4 kilobases (40) and represent the distance in kilobases from the single EcoRI site (0'/56.4'). RK2 genes are described in the text. The positive interaction of trfA with oriV is indicated by a dashed arrow. Negative regulatory interactions of kor gene functions with RK2 operons are shown by vertical arrows. The directions of transcription for the trfA operon (60, 75), kilA operon (88), korA-korB operon (4, 5, 63), korC (this study), and bla (12, 28) are indicated by arrows above the genes. The following determinants were mapped on plasmids RP1 and RP4, which are indistinguishable from RK2 (9). Tc^r shows the region encoding resistance to tetracycline (3, 83); bla, within transposon Tn1, encodes a β-lactamase responsible for ampicillin resistance (3, 27, 28); and (Te^r) indicates the region containing a cryptic tellurite resistance determinant (71).

MATERIALS AND METHODS

Nomenclature. Coordinates of the RK2 physical map are defined as the distance in kilobases from the unique EcoRI site and are designated by a prime (') (e.g., 4.3' to 5.2' region).

Bacteria, bacteriophages, and plasmids. Escherichia coli strains used in this study are described in Table 1. BD2399 is a supE derivative of BW313 (35). MV10 was the host for plasmid constructions except where noted; JM107 was the host for bacteriophage M13 constructions. M13 vectors for cloning and sequencing were M13mp18 (43, 85), M13mp19 (43, 85), M13hc4 (32), M13pz2 (32), and M13pz1, which differs from M13pz2 only in the orientation of the polylinker region. Plasmids used in this study are described in Table 2.

Plasmid cloning vehicles were constructed as follows: pDP8 (pSC101 replicon, tetracycline resistance [Tc^r]), ligation of pSC101 (6, 15) and pHSS6 (56) at their EcoRI sites followed by deletion of the ColE1 replicon-containing NotI fragment; pJAK11 (pSC101 replicon, chloramphenicol resistance [Cm^r], spectinomycin resistance [Sp^r]), insertion of a Sp^r-encoding EcoRI fragment from pHP45Ω (49) and a Cm^r-encoding HindIII fragment from pHP45Ω-Cm (19) into pDP8; pLE3 (P15A replicon, ampicillin resistance [Ap^r], Cm^r), replacement of the kanamycin-resistance (Km^r)-encoding HaeII fragment of pACYC177 (11, 50) with the Cm^r-encoding HaeII fragment of pACYC184 (11, 51); pT7-5B (ColE1 replicon, Ap^r), replacement of the polylinker region of pT7-5 (69) with the polylinker region of M13hc4.

M13 constructs containing a minimal korC region were made as follows: M13jk739, purification of the AluI-TaqI

fragment (RK2 coordinates 4.4' to 4.85') from pRK2086, blunting of the TaqI end with the Klenow fragment of DNA polymerase I, and insertion into the SmaI site of M13mp19; M13jk741, same as M13jk739, but with the AluI-TaqI fragment in the opposite orientation; M13jk752, site-directed mutagenesis of M13jk739 with the oligonucleotide 5'-CGG GAAGTCTAGCACTTGGCC-3'; M13jk761, site-directed mutagenesis of M13jk739 with the oligonucleotide 5'-CGAC CGGCGCTGAAATCCGGG-3'.

Plasmids containing RK2 DNA were constructed as follows: pRK2297, deletion of the NotI fragment (4.3' to 5.2') of pRK2262; pRK2299, insertion of the NotI fragment (4.3' to 5.2') from pRK2262 into the XmaIII site of pBR322 (7, 68); pRK2320, insertion of the PstI-BglII fragment (9.5' to 13.5') of RK2 into pLE3 [RP1770(pRK2086) host]; pRK2454, insertion of a trpE-encoding BssHII fragment from pVH153 (1) into the BssHII site (4.7') of pRK2299; pRK2462, insertion of the NotI fragment (4.3' to 5.2') from pRK2086 into the XmaIII site of pACYC184 (RP1894 host); pRK2464, digestion of pRK2462 with BssHII, blunting of the ends with the Klenow fragment of DNA polymerase I, and ligation with XhoI DNA linkers (the exact number of inserted linkers was not determined); pRK2630, pRK2631, pRK2632, and pRK2633, insertion of the EcoRI-HindIII fragments of M13jk739, M13jk741, M13jk752, and M13jk761, respectively, into pKK223-3; pRK2634, pRK2635, pRK2636, and pRK2637, insertion of the EcoRI-HindIII fragments of M13jk739, M13jk741, M13jk752, and M13jk761, respectively, into pT7-5B; pRK2638, insertion of the NotI-BamHI fragment (4.3' to 6.9') of pRK2103 (21) into pJAK11;

TABLE 1. E. coli strains

Strain[a]	Genotype	Reference or source
BD2399	Hfr PO45 dut-1 ung-1 thi-1 relA1 zbi::Tn10 supE	B. Duncan via H. Klein
BL21(DE3)	F⁻ hsdS gal [λD69 Φ(lacUV5p-T7 gene 1)]	67
JM107	endA1 gyrA96 thi hsdR17 supE44 relA1 λ⁻ Δ(lac-proAB)(F' traD36 proA⁺B⁺ lacI^q ZΔM15)	85
MV10	thr-1 leuB6 thi-1 lacY1 tonA21 supE44 rfbD1 ΔtrpE5 λ⁻	2, 29
RP1770	MV10 ΔtrpE5::[trpE⁺ RK2 (korA⁺ korB⁺)]	86
RP1894	MV10 ΔtrpE5::[trpE⁺ RK2 (korA⁺ korB⁺ korC⁺ korE⁺ kilA⁺ kilE⁺ bla⁺)]	86
S26	Hfr PO2A tonA22 garB10 phoA4(Am) ompF627 relA1 pit-10 spoT1 T2^r	2, 24
S26 Su6⁺	S26 supP51	2, 10

[a] All strains are derivatives of E. coli K-12 except BL21(DE3), which is derived from E. coli B.

TABLE 2. Plasmids

Plasmid	Selective marker(s)	Relevant genotype[a]	Description	Reference or source
pCY2	Tp[rb]		pSM1 replicon	21
pCY5	Km[r]	lacI[q]	P15A replicon with the E. coli lacI[q] allele	55
pKK223-3	Ap[r]	tacp	pBR322 replicon with the tac promoter	8
pRK2086	Km[r]	korC[+] korE[+] kilE[+] kilA[+]	ColE1 replicon with 0' to 6' region of RK2	21
pRK2091	Cm[r] Tp[r]	kilC[+] oriV[+]	P15A replicon with 9.5' to 14.5' region of RK2	21
pRK2161	Tp[r]	kilC[+] oriV[+]	pSM1 replicon with 9.5' to 14.5' region of RK2	21
pRK2262	Km[r]	korC[+]	ColE1 replicon with 3.5' to 6.0' region of RK2 (Fig. 2)	86
pRK2297	Km[r]	ΔkorC	ColE1 replicon with 3.5' to 4.3' and 5.2' to 6.0' regions of RK2 (Fig. 2)	This study
pRK2299	Ap[r]	korC[+]	pBR322 replicon with 4.3' to 5.2' region of RK2 (Fig. 2)	This study
pRK2320	Cm[r]	kilC[+] oriV[+]	P15A replicon with 9.5' to 13.5' region of RK2	This study
pRK2454	Ap[r]	korC::trpE[+]	pRK2299 with trpE-encoding fragment at BssHII site (4.7') (Fig. 2)	This study
pRK2462	Cm[r]	korC[+]	P15A replicon with 4.3' to 5.2' region of RK2 (Fig. 2)	This study
pRK2464	Cm[r]	korC::XhoI linkers	pRK2462 with multiple XhoI DNA linkers at BssHII site (4.7') (Fig. 2)	This study
pRK2630	Ap[r]	Φ[tacp-korC[+](+)]	pBR322 replicon with 4.4' to 4.85' region of RK2 downstream of tac promoter	This study
pRK2631	Ap[r]	Φ[tacp-korC[+](−)]	Same as pRK2630 but with 4.4' to 4.85' region of RK2 in opposite orientation	This study
pRK2632	Ap[r]	Φ[tacp-korC29(Am)]	Same as pRK2630 but with korC29 mutation	This study
pRK2633	Ap[r]	Φ[tacp-korC23]	Same as pRK2630 but with korC23 mutation	This study
pRK2634	Ap[r]	Φ[T7 φ10p-korC[+](+)]	ColE1 replicon with 4.4' to 4.85' region of RK2 downstream of phage T7 φ10 promoter	This study
pRK2635	Ap[r]	Φ[T7 φ10p-korC[+](−)]	Same as pRK2634 but with 4.4' to 4.85' region of RK2 in opposite orientation	This study
pRK2636	Ap[r]	Φ[T7 φ10p-korC29(Am)]	Same as pRK2634 but with korC29 mutation	This study
pRK2637	Ap[r]	Φ[T7 φ10p-korC23]	Same as pRK2634 but with korC23 mutation	This study
pRK2638	Cm[r] Sp[r] Ap[r]	bla[+] korC[+]	pSC101 replicon with 4.3' to 6.9' region of RK2 (Fig. 6)	This study
pRK2639	Cm[r] Sp[r]	Δbla korC[+]	pSC101 replicon with 4.3' to 6.0' region of RK2 (Fig. 6)	This study
pRK2659	Tc[r]	korA[+] korB[+] korC[+] korE[+] kilA[+] kilE[+]	pSC101 replicon with 50.4' to 56.4' and 0' to 6' regions of RK2	This study
pT7-5B	Ap[r]	T7 φ10p	ColE1 replicon with the phage T7 φ10 promoter	This study

[a] Plus and minus signs enclosed by parentheses specify the orientation of the indicated gene relative to the promoter: (+), in the direction of transcription; (−), opposite to the direction of transcription.
[b] Tp[r], Trimethoprim resistance.

pRK2639, insertion of the NotI-PstI fragment (4.3' to 6.0') of pRK2103 into pJAK11; pRK2659, ligation of pDP8 and pRK2102 (21) at their BglII sites followed by deletion of the ColE1 replicon-containing PstI fragment.

Media and reagents. Media for growth of bacteria were LB (37), LB containing 0.1% glucose, M9 (37), and M9 containing 0.5% Casamino Acids (Difco Laboratories, Detroit, Mich.). When required, media were supplemented with L-tryptophan at 50 μg/ml, thiamine hydrochloride at 25 μg/ml, and thymine at 20 μg/ml. Antibiotics were generally used at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 50 μg/ml; kanamycin, 50 μg/ml; penicillin, 150 μg/ml (to select Ap[r]); spectinomycin, 50 μg/ml; tetracycline, 30 μg/ml; trimethoprim, 50 μg/ml. For selection of low-copy-number pSC101 replicons, chloramphenicol and tetracycline were used at 20 and 15 μg/ml, respectively. When necessary, media contained isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) to identify DNA fragment insertions into the lac region of the M13 vectors (39). For induction of the tac promoter, media were supplemented with 1 mM IPTG.

DNA methodologies. Preparation of plasmid DNA and agarose gel electrophoresis have been described previously (31). Restriction endonucleases, synthetic DNA linkers, and T4 DNA ligase were obtained from commercial suppliers and used as recommended. DNA fragments were purified by electroelution from agarose gels with an International Bio-

technologies, Inc. (New Haven, Conn.) electroeluter and the procedure recommended by the manufacturer. DNA fragments containing protruding 5' ends were converted to blunt ends with DNA polymerase I (Klenow fragment) as described previously (37). Transformation and transfection of E. coli was by the method of Cohen et al. (16).

The nucleotide sequence of the 4.3' to 6.0' region of RK2 was determined by the dideoxynucleotide chain termination method (52) with 34 overlapping M13 clones. Both DNA strands were sequenced for the entire region. M13 sequencing primers (New England BioLabs, Inc., Beverly, Mass.) were extended with DNA polymerase I (Klenow fragment) or Sequenase (United States Biochemical Corp., Cleveland, Ohio). Sequencing products were labeled with [α-35S]thiodATP (Dupont, NEN Research Products, Boston, Mass.), separated by gel electrophoresis, and visualized by autoradiography as described previously (32). Band compressions in G+C-rich regions were eliminated by substituting dITP for dGTP in the sequencing reactions (42). The computer programs of BIONET (Intelligenetics, Inc., Mountain View, Calif.) (65) were used for DNA sequence analysis.

Oligonucleotide-directed mutagenesis was by the method of Kunkel et al. (35). Uracil-containing M13 single-strand template was prepared in the dut ung strain BD2399; JM107 was used as the ung[+] host. Synthetic oligonucleotides were obtained from Operon Technologies, Inc. (San Pablo, Calif.). Mutations were identified by nucleotide sequencing.

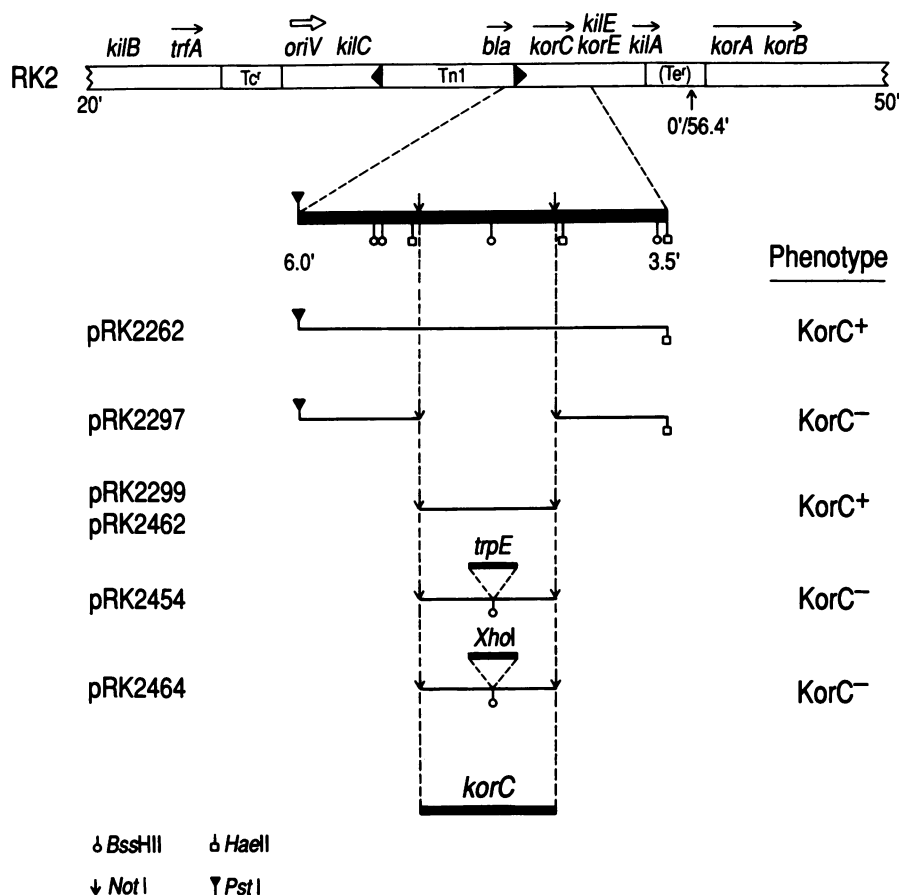**Polypeptide analysis.** The polypeptide product of korC was

FIG. 2. Physical mapping of korC. The genetic and physical map of the relevant portion of RK2 (Fig. 1) is shown on top. The 3.5' to 6.0' region is expanded below; only pertinent restriction endonuclease cleavage sites are depicted. Segments of RK2 DNA present in the various plasmids are indicated. trpE and XhoI represent insertions of a trpE-encoding fragment and multiple XhoI DNA linkers, respectively. KorC⁺ phenotype indicates the ability of the plasmid in E. coli to allow the cell to form a colony after transformation by a kilC⁺ plasmid (pRK2091, pRK2161, or pRK2320) as described previously (21); KorC⁻ phenotype indicates a decrease by at least a factor of 10^3 in the ability to form colonies (relative to a known KorC⁺ strain) after transformation by the kilC⁺ plasmid. Because it had been shown previously that both korA and korC are involved in the control of kilC (86), the initial tests of pRK2262 and pRK2297 included a korA⁺ plasmid. The ability of these plasmids to control kilC in the absence of korA is discussed in the text.

overexpressed and identified with the bacteriophage T7 RNA polymerase-promoter system (67, 70). The host strain was BL21(DE3), which contains the gene for T7 RNA polymerase in the chromosome under control of the inducible lacUV5 promoter. Wild-type and mutant korC alleles were cloned downstream of the T7 φ10 promoter in pT7-5B (pRK2634, pRK2635, pRK2636, pRK2637). To label preferentially the product of the cloned gene, we grew a 10-ml culture of BL21(DE3) containing the plasmid under investigation in LB–0.1% glucose (supplemented with ampicillin) to the mid-logarithmic phase (approximately 2×10^8 cells per ml [50 to 60 Klett units]). The cells were washed twice with M9 medium, resuspended in 10 ml of M9 medium, and incubated at 37°C with shaking. After 60 min, IPTG was added to 1 mM. After an additional 60 min, rifampin was added to 200 μg/ml and incubation was continued for 90 min. Cells from 1 ml of culture were labeled with 5 μCi of ¹⁴C-amino acids (1.71 mCi/mg; ICN Radiochemicals, Irvine, Calif.) for 5 min at 37°C, collected by centrifugation, and suspended in 0.2 ml of protein sample buffer (0.0625 M Tris, 2% sodium dodecyl sulfate, 10% glycerol, 5% β-mercaptoethanol, pH 6.8). Samples of 15 μl were analyzed by electrophoresis through a sodium dodecyl sulfate-20% poly-

acrylamide gel (with a 5% stacking gel) with the discontinuous buffer system of Laemmli (36) as modified by Thomas and Kornberg (82). After electrophoresis, the gel was fixed, stained, and destained as described previously (33). The gel was then dried, placed against Kodak X-OMAT AR film, and exposed at −70°C. ¹⁴C-protein molecular weight markers were obtained from Amersham Corp. (Arlington Heights, Ill.).

RESULTS AND DISCUSSION

Location of korC on RK2. We showed previously that korC is encoded by the 3.5' to 6.0' region, which is present as a cloned HaeII-PstI fragment in pRK2262 (Fig. 2) (86). Deletion of an internal 0.9-kilobase NotI fragment (4.3' to 5.2') from pRK2262 caused loss of korC function (pRK2297; Fig. 2), suggesting that korC is at least partially encoded by this fragment. We cloned the NotI fragment and found that it expresses korC activity (pRK2299, pRK2462; Fig. 2). Thus, korC is located within the 4.3' to 5.2' region of RK2.

When the BssHII site (4.7') within the NotI fragment was interrupted with a trpE-encoding fragment, korC function was destroyed (pRK2454; Fig. 2). Likewise, insertion of

multiple DNA linkers at the *Bss*HII site abolished *korC* activity (pRK2464; Fig. 2). These results suggest that the coding sequence for *korC* overlaps the *Bss*HII site at 4.7'.

Nucleotide sequence of the *korC* region. We determined the nucleotide sequence of the region between the *Not*I site at 4.3' and the *Pst*I site at 6.0' (Fig. 3). The nucleotide sequence was searched for open reading frames that have potential ATG or GTG initiation codons (Fig. 4). Three open reading frames were identified as possible coding sequences for *korC* because they fulfill the following criteria. (i) They are located completely within the *Not*I fragment (4.3' to 5.2'), and (ii) they span the *Bss*HII site at 4.7'. These open reading frames are designated *korC* (nucleotides 1169 to 1426), *orfY* (nucleotides 1195 to 1470), and *orfZ* (nucleotides 1323 to 1153).

We tentatively designated one of these open reading frames as *korC* because it is preceded by a good Shine-Dalgarno sequence for ribosome binding (Fig. 3) (58). This sequence, 5'-TTAGGAGAA-3', is complementary to the 3' end of the 16 S rRNA of both *E. coli* (six bases of complementarity) and *Pseudomonas aeruginosa* (seven bases of complementarity) (58). Also, the spacing between the Shine-Dalgarno sequence and the ATG initiation codon (seven nucleotides) is suitable for efficient initiation of translation (25, 34). The *orfY* and *orfZ* open reading frames are not preceded by any obvious Shine-Dalgarno sequence.

Identification of *korC*. A 454-base-pair (bp) *Taq*I-*Alu*I fragment (nucleotides 1104 to 1557 [4.85' to 4.4']; Fig. 3) containing the three open reading frames (*korC*, *orfY*, *orfZ*) was cloned downstream of the inducible *tac* promoter (pRK2630, pRK2631). In pRK2630, the *Taq*I end (4.85') of the fragment is proximal to the *tac* promoter; pRK2631 carries the fragment in the opposite orientation. These two plasmids were tested for their ability to express *korC* function. pRK2630 was KorC⁺ in the absence or presence of IPTG, whereas pRK2631 was KorC⁻ under both conditions (Table 3). From these results, we conclude that (i) the *Taq*I-*Alu*I fragment (4.85' to 4.4') is sufficient to code for *korC* function and (ii) transcription of *korC* is in the 4.85' to 4.4' direction. Thus, *orfZ* cannot encode *korC* function because it reads opposite to the direction of *korC* transcription.

To determine whether the *korC* open reading frame or *orfY* is responsible for the KorC⁺ phenotype, we constructed nonsense mutations in each of them by oligonucleotide-directed mutagenesis. Codon 29 in the *korC* open reading frame was changed from a TTG (Leu) to an amber stop codon (TAG). This mutation, *korC29*, does not alter the predicted polypeptide product of *orfY* [CTT (Leu) → CTA (Leu)]. Likewise, we changed codon 15 in *orfY* from an AGA (Arg) to a TGA stop codon. This mutation, *korC23*, has no effect on the predicted polypeptide product of the *korC* open reading frame [GCA (Ala) → GCT (Ala)].

Plasmids containing the *korC29* and *korC23* mutant alleles (pRK2632 and pRK2633, respectively) were tested for their ability to confer a KorC⁺ phenotype. To test pRK2632, we used strains S26 and S26 Su6⁺, which are isogenic except for the leucine-inserting amber suppressor (*supP51*) in S26 Su6⁺. Only the nonsuppressing host strain S26 was used to test pRK2633. The results (Table 4) show that in strain S26 the *korC29* mutation (pRK2632) caused loss of *korC* function, while the *korC23* mutation (pRK2633) had no effect on *korC* activity. In addition, pRK2632 was KorC⁺ in the *supP51* strain S26 Su6⁺, indicating suppression of the *korC29* amber mutation. From these results, we conclude that the open reading frame designated *korC* is indeed the *korC* gene.

The *korC* open reading frame consists of 86 codons (Fig. 3). It begins with an ATG codon at nucleotide 1169 and ends with a TGA codon at nucleotide 1426. Analysis of the codon usage in *korC* shows a high percentage (78.8%) of codons having a G or C residue in the third position. This strong preference for codons ending in a G or C residue is also seen in other RK2 genes and reflects the high G+C content of RK2 (32).

Polypeptide product of *korC*. Translation of the nucleotide sequence of *korC* results in a predicted polypeptide of 85 amino acids with a molecular mass of 9,150 Da (Fig. 3). The amino acid composition of KorC polypeptide shows 11 basic residues (Arg, His, Lys) and 9 acidic residues (Asp, Glu) for a net charge of +2.

To overexpress and identify the KorC polypeptide, we used wild-type and mutant *korC* alleles in the bacteriophage T7 RNA polymerase-promoter expression system (Fig. 5). Plasmids pRK2634 and pRK2635 carry the wild-type *korC* region in opposite orientations relative to the T7 promoter in the vector. The plasmid containing *korC* in the direction of transcription showed a single polypeptide with an estimated molecular mass of 7 kDa (pRK2634; Fig. 5, lane 3), a value in reasonable agreement with the expected size of KorC polypeptide. This polypeptide was not observed from the plasmid carrying *korC* in the opposite orientation (pRK2635; Fig. 5, lane 4). Thus, the 7-kDa polypeptide is specific to the *korC* region and expressed in the direction of *korC* transcription.

To determine whether the 7-kDa polypeptide is encoded by *korC*, we examined plasmids containing the *korC23* and *korC29* mutant alleles (pRK2637 and pRK2636, respectively; Fig. 5). The 7-kDa polypeptide was clearly expressed from the plasmid that carries the *korC23* silent mutation (pRK2637; Fig. 5, lane 6). In contrast, the plasmid containing the *korC29* amber mutation failed to express the 7-kDa polypeptide (pRK2636; Fig. 5, lane 5). Because the *korC29* mutation causes loss of both *korC* function and the 7-kDa polypeptide, we conclude that the 7-kDa polypeptide is the product of *korC* and is essential for *korC* function.

FIG. 3. Nucleotide sequence of the *korC* region (GenBank accession no. M32794). The nucleotide sequence of the 6.0' to 4.3' region of RK2 is shown. Numbers refer to nucleotide positions, and landmark restriction sites are indicated. The right end of transposon Tn*1* (28) is denoted by a bracket. The predicted amino acid sequences are shown below the nucleotide sequences of *korC*, *orfX*, the 3' end of *bla* (codons 182 to 287) (28), and the 5' end of *kleA* (codons 1 to 16) (Kornacki et al., unpublished data). Nucleotide changes resulting in the *korC23* and *korC29* mutations are indicated. An asterisk above nucleotide 176 shows a nucleotide difference in the *bla* genes of Tn*1* and Tn*3* (28). Potential Shine-Dalgarno sequences for ribosome binding (58) are underlined and labeled SD. A potential helix-turn-helix region in the KorC polypeptide is boxed; this region (amino acids 28 to 47) is predicted by the Chou-Fasman method (13) to form a helix-turn-helix structure, and it has similarity to the consensus pattern of amino acids in the helix-turn-helix regions of known DNA-binding proteins (45). The t shows a sequence resembling a transcriptional terminator (23): a G+C-rich region of dyad symmetry followed by six thymine residues. The −35 and −10 regions of the *kilE* P1 promoter are boxed (77; Kornacki et al., unpublished data). The pairs of divergent arrows labeled KorA, KorB, and KorC show operatorlike palindromes that are the predicted targets for KorA, KorB, and KorC proteins, respectively (61, 77, 88; Kornacki et al., unpublished data). Dashed arrows indicate the locations of two identical 9-bp direct nucleotide sequence repeats.

*Pst*I

```
CTGCAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGT    100
  A  A  M  A  T  T  L  R  K  L  L  T  G  E  L  L  T  L  A  S  R  Q  Q  L  I  D  W  M  E  A  D  K  V
```

```
                                                                                      *
TGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGATCTCGCGGTATCATTGCAGCACTG    200
  A  G  P  L  L  R  S  A  L  P  A  G  W  F  I  A  D  K  S  G  A  G  E  R  G  S  R  G  I  I  A  A  L
```

```
GGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCT    300
  G  P  D  G  K  P  S  R  I  V  V  I  Y  T  T  G  S  Q  A  T  M  D  E  R  N  R  Q  I  A  E  I  G  A
```

End *bla*
↓

```
CACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGAT    400
  S  L  I  K  H  W  *
```

End Tn*1* | *orfX* →

```
CCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCTTTTATTTCCGAAACGGGCTATCGGTCCCATTA    500
                                                             F  I  S  E  T  G  Y  R  S  H  Y
```

*Bss*HII   *Bss*HII

```
CGACCATGCGCGCGGCGGCATGACGGTGGACCAGGTAGCGGACGGGGTTTTGCGCGCCCTGTTGCGCTCGCATCGCCGGTATCTGGACGCCCGCGACCAG    600
  D  H  A  R  G  G  M  T  V  D  Q  V  A  D  G  V  L  R  A  L  L  R  S  H  R  R  Y  L  D  A  R  D  Q
```

```
GACCGCCTTGCCGACGAGCCACTGCCGGCGTGGCTCGCGGGTATCACGCCACCGCCGCGCCGCGTGCGGGCCGTGGTCGAGGACTGGCGGAAGCCGGACG    700
  D  R  L  A  D  E  P  L  P  A  W  L  A  G  I  T  P  P  P  R  R  V  R  A  V  V  E  D  W  R  K  P  D
```

*Not*I

```
AACTGCCGCCGGGCTTCGCCTGGGTTGATGCCGTGCTGCCGGCGCACCAGGCATTCATCGCCCGCAAATGGGCGGCCAGCGCCAAGGCGAAGCTCGCGGC    800
  E  L  P  P  G  F  A  W  V  D  A  V  L  P  A  H  Q  A  F  I  A  R  K  W  A  A  S  A  K  A  K  L  A  A
```

```
CGCGCGTGCGAAAGCTCAGGAGCCGGCCGGGCAGCGCAGGGAGCCGGTTACACCGGCCAAGCCGGAGCCGGAGCCGGCCAAGGACGAGGACGCGCCGGCG    900
  A  R  A  K  A  Q  E  P  A  G  Q  R  R  E  P  V  T  P  A  K  P  E  P  E  P  A  K  D  E  D  A  P  A
```

```
TGGCCGGCGACGTTCTTTCCTGGCCTGCGGTGCGAGATTGTGAGCGTGCATCACCCGGTTTTTGCCAAGGAAATCGGGAAGCACGTCATCATTACGAAGA    1000
  W  P  A  T  F  F  P  G  L  R  C  E  I  V  S  V  H  H  P  V  F  A  K  E  I  G  K  H  V  I  I  T  K
```

```
TCAGTCCCGAAACGCGACAGGTGTGGGCGCACGACGACAAGCCGCCGCGCTATCGCATCAATCGCAACGGTAGAAAGGTTTGTGAATATGACCCGCGTTG    1100
  I  S  P  E  T  R  Q  V  W  A  H  D  D  K  P  P  R  Y  R  I  N  R  N  G  R  K  V  C  E  Y  D  P  R  C
```

*Taq*I   End *orfX*   SD   Start *korC*
                                        ↓   ↓

```
CATCGAGTCGTGCTACGGCTACGACCAATTGCGGGCCGCCATTTGATAACGATTAGGAGAAAAGCAGCATGAGCGACGTGAATATCCGGCTTGAGTGCCT    1200
  I  E  S  C  Y  G  Y  D  Q  L  R  A  A  I  *            M  S  D  V  N  I  R  L  E  C  L
```

T (*korC23*)   A (*korC29*)   *Bss*HII
↓   ↓

```
GCGCCCGGCGGAACGCTGGGTGCAGCCGACCGGCGCAGAAATCCGGGAAGTCTTGCACTTGGCCGGCCCTCACCGGCGGACAGGCTGCGCGCATCTTGGGC    1300
  R  P  A  E  R  W  V  Q  P  T  G  A  E  I  R  E   V  L  H  L  A  G  L  T  G  G  Q  A  A  R  I  L  G
```

```
TTGGGTGCCAAGGGCGACCGCACGGTGCGGCGTTGGGTTGGCGAGGATTCGCCGATCCCCTATGCCGCCTGGGCGATCCTTTGCGATCTAGCGGGGATTG    1400
  L  G  A  K  G  D  R  T  V  R  R  W  V  G  E  D  S  P  I  P  Y  A  A  W  A  I  L  C  D  L  A  G  I
```

End *korC*   -------→   -------→ KorB
↓

```
GGGCGATCTGGAAAGGCCAGGGCTGACGCCCGCAGGACTTTACGCCAAGGGAGAGGACTTTAGCGGCTAAAACCGCCAACCCTGATCGTTGCGATATGCT    1500
  G  A  I  W  K  G  Q  G  *
```

                    ←   t   →   KorA   -35   KorC   -10
                                            ←   →   ←   →

```
GCCAGCAGCCCGGCCCGCAAGCCGGGTTTTTTCGTGCGCCCGCCGCAAATTGTTTAGCTAAATTTCCTTGACTATCTAGGGCATAATGCCGTAATATAGC    1600
                                                        A/u I
```

              SD   Start *kleA*   *Not*I
                              ↓

```
AATCCAAGGCCGGGCACTTCGCCCAGGTCAGCAACCGGAGGATCAACCCCGATGAAAAGCAAGATCATGTCTTGGCTCGATGAGCTGCCCGGCGCGGCCGC    1701
                                        M  K  S  K  I  M  S  W  L  D  E  L  P  G  A  A
```
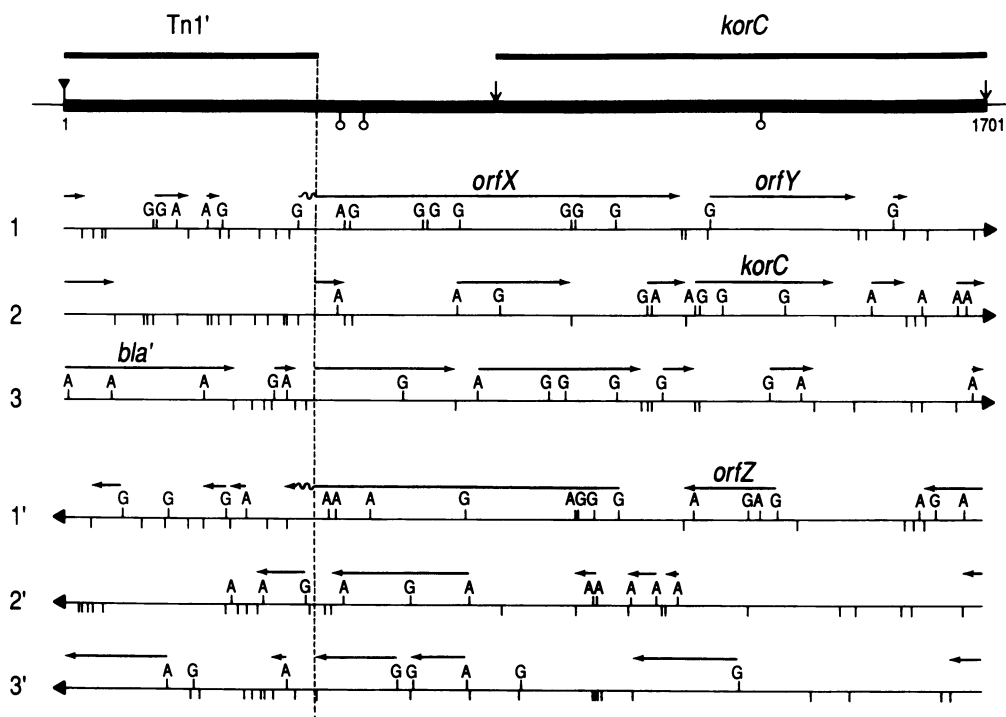
FIG. 4. Analysis of open reading frames in the korC region. The restriction map of the 1,701-bp korC region (Fig. 2 and 3) is shown on top. Tn1' represents a 467-bp segment of the right end of transposon Tn1. The region encoding korC is taken from the mapping experiments shown in Fig. 2. Lines 1, 2, and 3 depict the reading frames phased from nucleotide positions 1, 2, and 3, respectively; lines 1', 2', and 3' represent the reading frames phased from positions 1701, 1700, and 1699, respectively. Arrowheads at the end of each line show the 5' to 3' direction. Potential ATG (A) and GTG (G) initiation codons are represented by marks above the line, and termination codons are denoted by marks below the line. Arrows indicate open reading frames flanked by start and stop codons. Open reading frames labeled orfX, orfY, orfZ, and korC are discussed in the text. bla' shows the 3' end of the open reading frame for bla (28).

Genetic studies indicate that KorA and KorB polypeptides are transcriptional repressors whose predicted structures contain helix-turn-helix regions similar to those in known DNA-binding proteins. We therefore searched for an analogous region in KorC polypeptide. The predicted primary and secondary structures of the polypeptide revealed a single helix-turn-helix region (amino acids 28 to 47) containing the highly conserved amino acids at positions 5 (Ala), 9 (Gly), and 15 (Ile) (Fig. 3). This region may be involved in the regulatory function of KorC polypeptide by recognizing and binding to a specific target in DNA.

korC is expressed from the bla promoters in Tn1. The nucleotide sequence of the region between Tn1 and korC does not show any obvious promoter sequence (Fig. 3). The nearest known promoters upstream of korC are the two overlapping promoters (Pa, Pb) for the β-lactamase gene (bla) of Tn1 (12). Thomas et al. (77) showed that insertions in Tn1 between the bla promoters and korC caused decreased levels of korC activity. Because some transposons can express adjacent genes by transcription from outward-reading promoters (14, 18), it seemed possible that korC is expressed from the bla promoters.

To test this possibility, we constructed two isogenic

TABLE 3. Expression of korC from the tac promoter

Resident plasmid	Relative efficiency of transformation by a kilC⁺ plasmid[a]	
	−IPTG	+IPTG
None	<0.001	<0.001
pRK2659	1.0	0.9
pKK223-3	<0.001	<0.001
pRK2630	1.2	1.1[b]
pRK2631	<0.001	<0.001

[a] JM107 strains with the indicated resident plasmids were transformed with kilC⁺ plasmid pRK2161, and trimethoprim-resistant transformants were selected in the absence or presence of IPTG. The relative competence of each strain was measured by transformation with pCY2 as described previously (21). Values are adjusted for competence differences, which were not more than threefold. Efficiencies of transformation are normalized to that of the pRK2659-containing strain (−IPTG).

[b] These colonies were smaller than those selected in the absence of IPTG. This is a property of pRK2630 because transformation with pCY2 also gave small colonies in the presence of IPTG (data not shown).

TABLE 4. Effect of mutations on korC activity

Resident plasmid	Genotype	Relative transformation efficiencies of recipient strains by a kilC⁺ plasmid[a]	
		S26	S26 Su6⁺
None		<0.001	<0.001
pRK2630	Φ[tacp-korC⁺(+)]	1.0	0.9
pRK2632	Φ[tacp-korC29(Am)]	<0.001	1.2
pRK2633	Φ[tacp-korC23]	1.5	ND[b]

[a] Strains carried the lacI^q-containing plasmid pCY5 and the indicated plasmid. Transformations were done as described in Table 3, footnote a. Efficiencies of transformation are normalized to that of strain S26(pCY5, pRK2630). Values shown are for colonies selected in the absence of IPTG. Plasmids pRK2630, pRK2632, and pRK2633 are extremely deleterious to these strains in the presence of IPTG; for pRK2632, this effect is observed only in strain S26 Su6⁺.
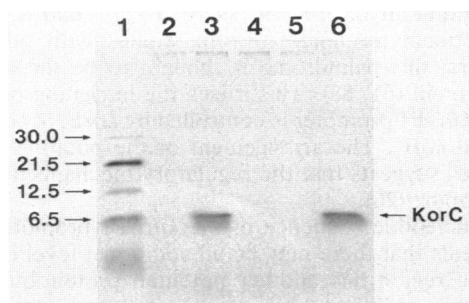
[b] ND, Not determined.

FIG. 5. Identification of KorC polypeptide. Wild-type and mutant korC alleles were expressed in vivo from the bacteriophage T7 φ10 promoter. Polypeptides specified by the cloned genes were selectively labeled with ^{14}C-amino acids, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and visualized by autoradiography as described in Materials and Methods. Lanes: 1, ^{14}C-labeled protein markers (carbonic anhydrase [30 kDa], soybean trypsin inhibitor [21.5 kDa], cytochrome c [12.5 kDa], aprotinin [6.5 kDa], and bovine insulin [5.7 kDa]); 2, pT7-5B; 3, pRK2634; 4, pRK2635; 5, pRK2636; 6, pRK2637. Numbers on left show mass of markers in kilodaltons. The broad band for insulin is due to separation of the A chain (2.3 kDa) and B chain (3.4 kDa).

plasmids that differ in the presence or absence of the bla promoters (pRK2638 and pRK2639, respectively; Fig. 6). Plasmid pRK2638 contains a BamHI-NotI fragment (6.9′ to 4.3′) that carries the bla promoters, the bla gene, and korC; pRK2639 is identical except for a deletion of the BamHI-PstI fragment (6.9′ to 6.0′) that contains the bla promoters and the 5′ end of bla. The cloned fragments are flanked by strong transcriptional terminators to prevent extraneous transcription from expressing korC. The two plasmids were tested for their ability to control a kilC$^+$ plasmid (as described in the legend to Fig. 2). The results showed unequivocally that pRK2638 is KorC$^+$ and pRK2639 is KorC$^-$. We conclude that in wild-type RK2, the bla promoters of Tn1 are responsible for expression of korC.

Expression of korC by the bla promoters suggests that the insertion of Tn1 in RK2 separated korC from its natural promoter. Because kilC is located on the other side of Tn1 (21), it is possible that kilC and korC were once part of the same operon. Such an operon would have been partially autoregulated because kilC is regulated by korA and korC

(86). Following the insertion of Tn1, korC expression by Tn1 promoters would be required to control the lethal effect of kilC. It is noteworthy that the bla promoter regions of Tn1 and the similar transposon Tn3 are identical except for a single-base-pair substitution (12, 28). The nucleotide difference in Tn1 results in two overlapping promoters that express bla at higher levels than the single bla promoter in Tn3 (12, 84). Thus, the need to control kilC may have selected for a mutation in the bla promoter to increase korC expression.

Coregulation of kil operons by korA and korC. In an earlier study, we showed that both korA and korC are involved in the control of kilC (86). We found that the korC$^+$ plasmid pRK2262 (Fig. 2) was insufficient to control kilC unless korA was also present in the cell. This result revealed a role for korA in the regulation of kilC. Because a rho strain obviated the need for korA, we suggested that korA functions as an antiterminator in the expression of korC. However, this explanation is no longer plausible because the results of this study show that some korC$^+$ plasmids are sufficient to control kilC.

Why is pRK2262 uniquely dependent on korA for control of kilC? We suggest an explanation based on our finding that korC is naturally expressed from the bla promoters in Tn1. When the bla promoters are deleted and the korC region is flanked by transcriptional terminators (pRK2639; Fig. 6), korC is not expressed and kilC is not controlled. This is true even when korA is present (data not shown). pRK2262 is identical to pRK2639 with respect to korC and the upstream region, except that korC is not preceded by a transcriptional terminator. Thus, a promoter located on the plasmid vehicle is responsible for some korC expression in pRK2262. We suggest that the level of expression from this heterologous promoter is too low for korC alone to control kilC but sufficient when korA is also present. In the other korC$^+$ plasmids that lack the bla promoters (pRK2299 and pRK2462; Fig. 2), the heterologous promoters express korC at levels sufficient to control kilC. One possible explanation for the rho effect on pRK2262 is that korC expression is increased by allowing transcription from a heterologous promoter to proceed through a rho-dependent termination site located in the plasmid vector.

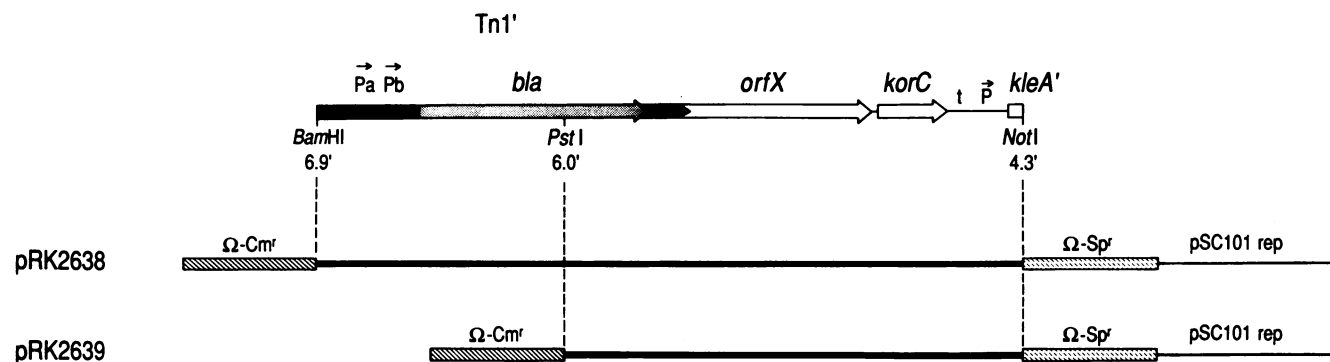This unusual property of pRK2262 was important because it showed that both korA and korC are involved in the

FIG. 6. Structures of plasmids used to study korC expression. The physical and genetic organization of the 4.3′ to 6.9′ region of RK2 (Fig. 3) is shown on top. Tn1′ indicates the bla-encoding right end of transposon Tn1. Pa and Pb show the two overlapping promoters that express bla (12). orfX represents the 3′ end of a putative gene interrupted by Tn1. The location of korC is indicated, and the t represents a potential transcriptional terminator. P shows the location of the kilE P1 promoter; kleA′ indicates the 5′ end of kleA, the promoter-proximal gene of the first kilE operon. Segments of RK2 DNA in pRK2638 and pRK2639 are flanked by vertical dashed lines. Ω-Cm^r and Ω-Sp^r show DNA fragments that contain antibiotic resistance determinants and transcriptional terminators (19, 49).

control of the *kilC* operon (86). The two operons of the recently discovered *kilE* locus are also regulated by *korA* and *korC* (77; Kornacki et al., unpublished data). How do *korA* and *korC* control these operons? Genetic studies on the *kilC* and *kilE* promoters indicate that *korA* and *korC* products act to repress transcription (77; Kornacki et al., unpublished data). The nucleotide sequences of these promoters all show similar operatorlike sequences: a palindrome immediately upstream of the −35 region, believed to be the target for *korA* product, and another palindrome overlapping the −10 region, thought to be the target for *korC* product (77; Kornacki et al., unpublished data). The predicted structures of *korA* and *korC* products show repressorlike characteristics. Both are basic polypeptides with possible helix-turn-helix regions for DNA binding (4, 79; this study). Thus, KorA and KorC proteins may function as corepressors at the *kilC* and *kilE* promoters.

Other features of the *korC* region. The *korC*-encoding region sequenced here also includes the right end of Tn*1* which encodes the 3' end of the *bla* gene (Fig. 3). Tn*1* is a member of a family of nearly identical transposons of which Tn*3* is the prototype (57). Although Tn*1* and Tn*3* are similar, their β-lactamases differ in isoelectric point and Tn*1* confers a higher level of resistance to ampicillin (38, 84). Chen and Clowes (12) have determined the nucleotide sequence of a portion of the *bla* gene from Tn*1* and compared it with the corresponding sequence of Tn*3* (28). They reported four nucleotide differences in the region between the 5' end of the gene and the *Pst*I site (6.0'). Three of these differences are silent changes, whereas the fourth difference results in an amino acid substitution. In this study, we present the remaining nucleotide sequence of the Tn*1 bla* gene from the *Pst*I site to its 3' end (Fig. 3). This sequence identifies another silent change (GGG [Gly] versus GGA [Gly]) at the third position of codon 239 (nucleotide 176 in Fig. 3). The nucleotide sequences of Tn*1* and Tn*3* do not show any differences in the region between the 3' end of *bla* and the right end of the transposon (Fig. 3) (28).

The nucleotide sequence of the region between Tn*1* and *korC* has a large open reading frame designated *orfX* (Fig. 3 and 4). It extends from the end of Tn*1* (nucleotide 468) to a TGA stop codon at nucleotide 1146. Although it contains several potential translational start codons (ATG, GTG), none is preceded by a reasonable Shine-Dalgarno sequence for ribosome binding. However, the codon usage in *orfX* shows striking similarity to the codon usage in RK2 genes (79.5% of *orfX* codons end in a G or C residue) (32). Thus, *orfX* may represent the 3' end of a gene that was interrupted by the insertion of Tn*1*. This gene may have been part of the putative *kilC-korC* operon in an ancestral version of RK2.

Translation of *orfX* yields a predicted polypeptide of 225 amino acid residues (Fig. 3). This polypeptide presumably represents the carboxyl end of a larger polypeptide encoded by the putative gene that was interrupted by Tn*1*. The OrfX polypeptide contains 32.9% charged amino acids consisting of 44 positively charged residues (Arg, His, Lys) and 30 negatively charged residues (Asp, Glu). The amino acid composition of OrfX polypeptide also shows a high percentage of proline residues (10.2%).

The *kilE* P1 promoter and the 5' end of *kleA* (the promoter-proximal gene of the first *kilE* operon) are located in the region between *korC* and the *Not*I site at 4.3' (Fig. 3) (77; Kornacki et al., unpublished data). The nucleotide sequence of the promoter region shows two palindromic sequences that are the predicted targets for KorA and KorC proteins. We were surprised to find another palindrome upstream of

the promoter in the region between *korC* and a potential transcriptional terminator. From studies with other RK2 promoters, this palindrome is thought to be the target for KorB protein (61, 88). This raises the intriguing possibility that the *kilE* P1 promoter is controlled by *korB*, in addition to *korA* and *korC*. The arrangement of the putative operator sequences suggests that the regulatory mechanism involves DNA looping (26).

The nucleotide sequence of the *kilE* P1 promoter region also reveals that there may be an additional level of regulation. The region has another potential protein-binding sequence: two identical 9-bp direct repeats separated by one turn of the DNA helix (11 bp) (Fig. 3). It is interesting that the promoter-proximal end of the second repeat overlaps 4 bp of the putative KorB target.

In summary, this work has added to our understanding of the regulatory interactions that compose the *kil-kor* regulon of RK2. Analysis of *korC* showed that its polypeptide product is a repressorlike molecule. Other studies indicate that the polypeptide products of *korA* and *korB* also act as transcriptional repressors (4, 5, 32, 55, 60, 72, 73, 79, 87, 88). It is fascinating that the operons in the *kil-kor* regulon are controlled by combinations of *kor* gene products. It therefore appears that one strategy for gene regulation in RK2 is the use of multiple repressors to control transcription.

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