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Structural, Molecular, and Genetic Analysis of the *kilA* Operon of Broad-Host-Range Plasmid RK2

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The *kil* loci (*kilA*, *kilB*, *kilC*, and *kilE*) of incompatibility group P (IncP), broad-host-range plasmid RK2 were originally detected by their potential lethality to *Escherichia coli* host cells. Expression of the *kil* determinants is controlled by different combinations of *kor* functions (*korA*, *korB*, *korC*, and *korE*). This system of regulated genes, known as the *kil-kor* regulon, includes *trfA*, which encodes the RK2 replication initiator. The functions of the *kil* loci are unknown, but their coregulation with an essential replication function suggests that they have a role in the maintenance or host range of RK2. In this study, we have determined the nucleotide sequence of a 3-kb segment of RK2 that encodes the entire *kilA* locus. The region encodes three genes, designated *klaA*, *klaB*, and *klaC*. The phage T7 RNA polymerase-dependent expression system was used to identify three polypeptide products. The estimated masses of *klaA* and *klaB* products were in reasonable agreement with the calculated molecular masses of 28,407 and 42,156 Da, respectively. The *klaC* product is calculated to be 32,380 Da, but the observed polypeptide exhibited an apparent mass of 28 kDa on sodium dodecyl sulfate-polyacrylamide gels. Mutants of *klaC* were used to confirm that initiation of translation of the observed product occurs at the first ATG in the *klaC* open reading frame. Hydrophobicity analysis indicated that the KlaA and KlaB polypeptides are likely to be soluble, whereas the KlaC polypeptide was predicted to have four potential membrane-spanning domains. The only recognizable promoter sequences in the *kilA* region were those of the *kilA* promoter located upstream of *klaA* and the promoter for the *korA-korB* operon located just downstream of a *rho*-independent terminatorlike sequence following *klaC*. The transcriptional start sites for these promoters were determined by primer extension. Using isogenic sets of plasmids with nonpolar mutations, we found that *klaA*, *klaB*, and *klaC* are each able to express a host-lethal (Kil$^+$) phenotype in the absence of *kor* functions. Inactivation of the *kilA* promoter causes loss of the lethal phenotype, demonstrating that all three genes are expressed from the *kilA* promoter as a multicistronic operon. We investigated two other phenotypes that have been mapped to the *kilA* region of RK2 or the closely related IncP plasmids RP1 and RP4: inhibition of conjugal transfer of IncW plasmids (*fiwB*) and resistance to potassium tellurite. The cloned *kilA* operon was found to express both phenotypes, even in the presence of *korA* and *korB*, whose functions are known to regulate the *kilA* promoter. In addition, mutant and complementation analyses showed that the *kilA* promoter and the products of all three *kla* genes are necessary for expression of both phenotypes. Therefore, host lethality, fertility inhibition, and tellurite resistance are all properties of the *kilA* operon. We discuss the possible role of the *kilA* operon for RK2.

Bacterial plasmids of incompatibility group P (IncP) are distinguished by their extraordinary host range (13, 50, 78). Because they transfer to and replicate within a wide variety of gram-negative bacteria, IncP plasmids have become conspicuous as tools for genetic studies of non-*Escherichia coli* hosts (25, 65). In nature, they are likely to be significant agents in the evolution of gram-negative bacteria as a consequence of their demonstrated ability to acquire and disseminate chromosomal genes (25). Of fundamental importance to understanding IncP plasmids is to determine the genetic basis of their remarkable promiscuity. So far, studies on the replication and maintenance of IncP plasmids have revealed an unusual replicon of surprising regulatory complexity (18, 19, 78).

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RK2 is a self-transmissible, IncP plasmid isolated from an antibiotic-resistant *Klebsiella aerogenes* strain (29). It is 56.4 kb in size (45) and closely related, if not identical, to the IncP plasmids RP1, RP4, R18, and R68 from *Pseudomonas aeruginosa* (9, 80). The ability of RK2 to replicate in different hosts is, in part, a property of its two known replication determinants: a single origin of replication, *oriV*, and a gene, *trfA*, that specifies two polypeptide products able to activate *oriV* for initiation of replication (17, 35, 57, 58, 61, 69). Mini-RK2 derivatives relying entirely on these two functions for replication can be maintained in several gram-negative bacteria (57, 58). However, none of these plasmids is as consistently stable in the various hosts as the parental RK2, suggesting that RK2 encodes additional functions for stable maintenance in diverse hosts.

Studies in our laboratory have revealed the existence of four *kil* loci (*kilA*, *kilB*, *kilC*, and *kilE*), originally detected by their deleterious effects on the host (Fig. 1) (18, 34a). While unregulated expression of any one of the *kil* determinants leads to death of *E. coli* host cells, lethal levels of *kil* expression are normally prevented by the RK2 determinants *korA*, *korB*, *korC*, and *korE* (18, 87). All but *korE*, whose

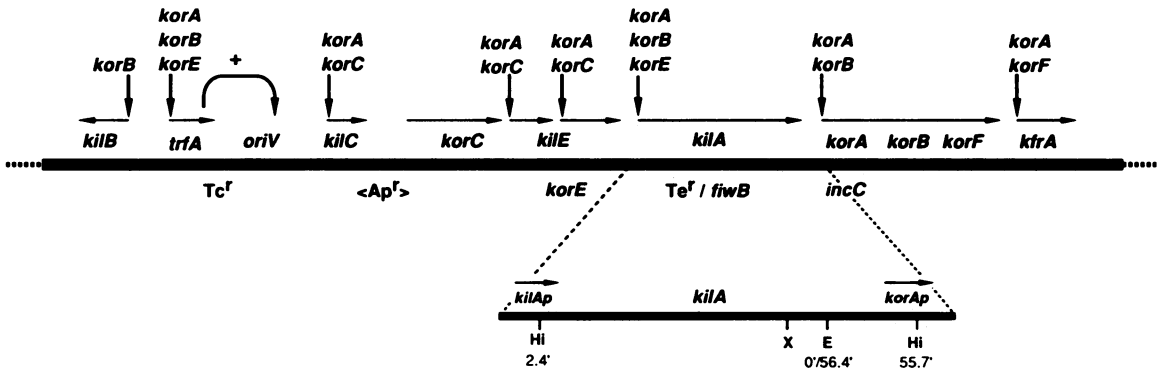

FIG. 1. The *kil-kor* regulon of IncP plasmid RK2. Shown is a genetic and physical map covering approximately 25 kb of RK2. Tc^r indicates the tetracycline resistance determinant (83); Ap^r shows the position of Tn*1*, which encodes ampicillin resistance (3, 27). All other loci are described in the text. Horizontal arrows show directions of transcription. Vertical arrows depict negative regulatory interactions. The curved arrow indicates the positive interaction of the *trfA* replication initiator with *oriV*. The *kilA* region is expanded below the map. *kilAp* and *korAp* indicate the *kilA* and *korA* promoters, respectively. Restriction endonuclease cleavage sites are Hi (*Hin*cII), X (*Xho*I), and E (*Eco*RI). Numbers are map coordinates (primes represent kilobases) based on a total size for RK2 of 56.4 kb (45).

mechanism of action is unknown, appear to encode repressors that interact with specific operator sequences within the various *kil* promoters (4, 5, 33, 34, 66, 74, 75, 87, 88). A remarkable feature of *kor*-mediated regulation is that the target genes are controlled by overlapping combinations of *kor* functions. The *kilA* determinant is regulated by *korA*, *korB*, and *korE* (18, 87, 88). The *kilC* promoter (75, 86) and the two promoters of *kilE* (34a, 75) are each repressed by the *korA* and *korC* products. *kfrA*, a recently discovered gene whose function is unknown, was found to be regulated by *korA* and a new regulatory function *korF* (79). *korA*, *korB*, and *korF* are expressed together in a single operon (5, 67, 79) whose promoter is sensitive to dual autoregulation by *korA* and *korB* products (5, 73, 88). *kilB* is the only determinant that appears to be controlled by a single *kor* function, *korB* (18).

We originally proposed that this unusual regulatory network (the *kil-kor* regulon) may be involved in plasmid maintenance or host range based on the finding that all known IncP plasmids encode *korA*- and *korB*-like functions (18). Subsequent work revealed that expression of *trfA*, the essential replication gene, is also regulated by *korA*, *korB*, and *korE* (58a, 59, 62). Thus, the *kil-kor* regulon includes *trfA* and directly participates in plasmid replication control.

Of the four *kil* determinants, *kilA* is especially interesting because it and *trfA* are regulated similarly. Both are regulated by *korA*, *korB*, and *korE*, with both the *kilA* and *trfA* promoters showing the same arrangement of the putative operators for KorA and KorB proteins (73, 88). This indicates that expression of *trfA* is accompanied by expression of *kilA* and strengthens the suggestion that the *kilA* locus has a role in RK2 maintenance.

The *kilA* region has also been linked to two other phenotypes: tellurite resistance (Te^r) and fertility inhibition. Te^r, normally cryptic or expressed at very low levels in wild-type RP1, RP4, and RK2, was revealed by selecting mutant plasmids that allow the host to grow in the presence of high levels of tellurite (7, 20, 71, 81). The resistance determinant was found to map to a region with considerable overlap to the *kilA* region (71). Fertility inhibition was observed as the ability of IncP plasmid RP1 to inhibit the conjugal transfer of a coresident IncW plasmid (89). RP1 encodes two separate fertility inhibition determinants, and mutations in one of the determinants, *fiwB*, were clustered in a small region that overlaps the *kilA* region (20).

Reported here are studies on the genetic structure of the *kilA* region. We have determined the nucleotide sequence of a 3-kb segment of RK2 that encodes the complete *kilA* locus, including the regions predicted to encode Te^r and *fiwB*. The region encodes a single operon of three genes (*klaA*, *klaB*, and *klaC*) expressed from the *kilA* promoter. We identified the gene products and determined the transcriptional start site. Genetic analysis of the operon demonstrated (i) that each of the three genes expresses a host-lethal phenotype when expressed from the unregulated *kilA* promoter and (ii) that all three genes are required for Te^r and fertility inhibition.

Walter et al. (82) have recently reported the nucleotide sequence of the analogous region from a Te^r derivative of RK2. The mutant was found to encode three genes (*kilA*, *telA*, and *telB*) essentially identical to *klaA*, *klaB*, and *klaC*. Using the sequence reported here, they determined that the basis for the high Te^r conferred by the mutant was a single nucleotide difference in *klaC* (*telB*). We discuss the significance of these studies on the function of the *kilA* region in RK2.

MATERIALS AND METHODS

Nomenclature. Coordinates of the RK2 physical map are defined by the distance in kilobases from the single *Eco*RI site. Superscript "0" indicates the absence of a plasmid gene (e.g. *kor*^{0}). We have adopted a uniform nomenclature for genes of the various *kil* loci: *kla* (e.g., *klaA*, *klaB*, and *klaC*), *klb* (e.g. *klbA* [2]), *klc*, and *kle* designate genes of *kilA*, *kilB*, *kilC*, and *kilE*, respectively.

Bacterial strains, plasmids, and bacteriophages. *E. coli* K-12 JM107 [*endA1 gyrA96 thi hsdR17 supE44 relA1*, Δ(*lac-proA*) (F′ *traD36 proA*$^+$*B*$^+$, *lacI*^{q}*Z* Δ*M15*)] (85) was used for propagating derivatives of bacteriophage M13. The following *E. coli* K-12 strains were used for construction and preparation of plasmids and determination of phenotypes: MV10 (*thr-1 leuB6 thi-1 lacY1 tonA21 supE44 rfbD1* Δ*trpE5* [28]); RP1770 (MV10 with *korA*, *korB*, and *trpE* inserted in the chromosome) (86); JMB9.1 (*leu thi gal1,2 lac xyl ara hsdR* Δ*trpE5*) from C. Yanofsky; DF4015 (JMB9.1 Nal^r) selected

TABLE 1. Plasmids

Plasmid	Selective marker(s)	Genotype or relevant properties[a]	Description[b]	Source or reference
pCH1	Ap^r	Cloning vector	pMB1 rep with polylinker followed by the *rrnB* T1 and T2 transcriptional terminators	This work
pCH4	Ap^r	Cloning vector	Equivalent to pCH1 but with different polylinker	This work
pMK20	Km^r	Cloning vector	ColE1 rep	32
pRK2102	*trpE*$^+$	*korA*$^+$ *korB*$^+$ *korC*$^+$ *korE*$^+$ *kilA*$^+$ *kilE*$^+$	ColE1 rep with kb 50.4 to 9.5 of RK2	18
pRK2108	*trpE*$^+$	*korA*$^+$ *korB*$^+$	pSM1 rep (IncFII group) with kb 50.4 to 56.4 of RK2	18
pRK2294	Ap^r	*korA*$^+$ *korB*$^+$	P15A rep with kb 53.2 to 56.1 of RK2	5
pRK2440	Km^r	*klaA*$^+$ *klaB*$^+$ Δ*klaC194*	ColE1 rep with kb 0 to 2.4 of RK2	88
pRK2443	Km^r	*kilAp*$^+$	*Eco*RI deletion of pRK2440::mini-Tn*3-57*	This work (Fig. 3)
pRK2444	Km^r Cm^r	*kilAp*$^+$	pRK2443 with ΩCm fragment[c]	This work (Fig. 3)
pRK2446	Km^r Cm^r	*klaA*$^+$ *klaB*$^+$ Δ*klaC194*	pRK2440 with ΩCm fragment	This work (Fig. 3)
pRK2484	Ap^r	*klaA*$^+$ *klaB*$^+$ Δ*klaC194*	ColE1 rep with kb 0 to 2.4 of RK2	This work
pRK2487	Ap^r	Δ*klaC15*	ColE1 rep with kb 55.7 to 2.4 of RK2	This work
pRK2489	Km^r Cm^r	*klaA*$^+$ Δ*klaB367*	ColE1 rep with kb 1.5 to 2.4 of RK2 and ΩCm fragment	This work (Fig. 3)
pRK2492	Ap^r	Φ[T7 φ*10p-klaA*$^+$ Δ*klaB367*]	pMB1 rep with kb 1.5 to 2.4 of RK2 downstream of T7 φ*10* promoter	This work (Fig. 3)
pRK2499	Ap^r	Φ[T7 φ*10p-klaA*$^+$ *klaB*$^+$ Δ*klaC194*]	pMB1 rep with kb 0 to 2.4 of RK2 downstream of T7 φ*10* promoter	This work (Fig. 3)
pRK2722	Km^r	*klaA*$^+$ *klaB*$^+$ *klaC*$^+$	ColE1 rep with kb 55.7 to 2.4 of RK2	This work
pRK2727	Km^r Cm^r	Δ*klaA105* Δ*klaB367*	ColE1 rep with kb 1.5 to 2.4 of RK2 with ΩCm fragment and deletions in *klaA* and *klaB*	This work (Fig. 3)
pRK2730	Km^r Cm^r	*klaA*$^+$ *klaB*$^+$ *klaC*$^+$	pRK2722 with ΩCm fragment	This work (Fig. 3)
pRK2734	Km^r	Δ*klaA105* Δ*klaB270* Δ*klaC194*	Equivalent to pRK2446 but with additional deletions in *klaA* and *klaB*	This work
pRK2771	Km^r Cm^r	Δ*klaA105* Δ*klaB270 klaC*$^+$	Equivalent to pRK2730 but with deletions in *klaA* and *klaB*	This work (Fig. 3)
pRK2772	Km^r Cm^r	Δ*klaA105* Δ*klaB270* Δ*klaC303*	Equivalent to pRK2734 but with ΩCm fragment and a larger deletion of *klaC*	This work (Fig. 3)
pRK2774	Km^r Cm^r	Δ*klaA105 klaB*$^+$ *klaC*$^+$	Equivalent to pRK2730 but with a deletion in *klaA*	This work (Fig. 3)
pRK2775	Km^r Cm^r	*klaA*$^+$ Δ*klaB270 klaC*$^+$	Equivalent to pRK2730 but with a deletion in *klaB*	This work (Fig. 3)
pRK2778	Ap^r	Φ[T7 φ*10p-klaA*$^+$ *klaB*$^+$ *klaC*$^+$]	pMB1 rep with kb 55.7 to 2.4 of RK2 downstream of T7 φ*10* promoter	This work (Fig. 3)
pRK2779	Ap^r	Φ[T7 φ*10p*-Δ*klaA105* Δ*klaB270 klaC*$^+$]	Equivalent to pRK2778 but with deletions in *klaA* and *klaB*	This work (Fig. 3)
pRK2781	Km^r Cm^r	*kilAp*::ΩCm *klaA*$^+$ *klaB*$^+$ *klaC*$^+$	pRK2722 with ΩCm fragment inserted within *kilAp*	This work (Fig. 3)
pRK2783	Km^r Cm^r	Δ*klaA105 klaB*$^+$ Δ*klaC303*	Equivalent to pRK2772 but *klaB*$^+$	This work (Fig. 3)
pRK2784	Ap^r Cm^r	Δ*klaA105 klaB*$^+$ *klaC*$^+$	Equivalent to pRK2774 but with P15A rep	This work (Fig. 3)
pRK2785	Ap^r Cm^r	*klaA*$^+$ Δ*klaB270 klaC*$^+$	Equivalent to pRK2775 but with P15A rep	This work (Fig. 3)
pRK2786	Ap^r Cm^r	*klaA*$^+$ *klaB*$^+$ Δ*klaC194*	Equivalent to pRK2446 but with P15A rep	This work (Fig. 3)
pRK2788	Ap^r	Φ[T7 φ*10p*-Δ*klaA105* Δ*klaB270 klaC16*]	Equivalent to pRK2779 but with filled-in *Xho*I site at RK2 kb 0.3	This work (Fig. 3)
pRK2790	Ap^r	Φ[T7 φ*10p*-Δ*klaC15*]	pMB1 rep with kb 55.7 to 0.3 of RK2 downstream of T7 φ*10* promoter	This work (Fig. 3)
pRK2865	Ap^r	*kilAp*	pCH1 with *kilA* promoter	This work
pRK2999	Ap^r	*korAp*	pCH4 with *korA* promoter	This work
pT7-5B	Ap^r	T7 φ*10p*	pMB1 rep with phage T7 φ*10* promoter	34

[a] Δ*klaA105*, 105-codon in-frame deletion of *klaA*; Δ*klaB270*, 270-codon in-frame deletion of *klaB*; Δ*klaB367*, 367-codon deletion of *klaB* (removing all but the first 12 codons); Δ*klaC15*, 15-codon deletion from the 5′ end of *klaC*; Δ*klaC194*, 194-codon deletion from the 3′ end of *klaC* (leaving the first 124 codons); Δ*klaC303*, 303-codon deletion of *klaC* (removing all but the first 15 codons); *klaC16*, filled-in *Xho*I site that creates a frame-shift at *klaC* codon 16.

[b] rep, replicon.

[c] When present, the ΩCm fragment directly follows the *kilA* region of all plasmids except pRK2781, which has ΩCm fragment inserted within *kilAp*.

as a spontaneous nalidixic acid-resistant mutant of JMB9.1; and PG1118 (JMB9.1 *srl*::Tn*10 recA1*) constructed by P1 transduction using strain HS3089 from H. Shuman. The *E. coli* B strain BL21(DE3) {F$^-$ *hsdS gal* [λD69 φ(*lacUV5p*-T7 gene *1*)]}, which carries the gene for T7 RNA polymerase (gene *1*) under the control of the *lacUV5* promoter (70), was used for expression of genes cloned downstream of the phage T7 φ*10* promoter. Bacteriophage vectors used to clone fragments for sequencing were M13mp18 and M13mp19 (48, 85) and M13hc4 (33).

Plasmids are described in Table 1. For construction of plasmids containing portions of the *kilA* region, the host

strains contained *korA* and *korB*. Previously unpublished plasmids were constructed as follows: pCH1, replacement of the *tac* promoter-containing *Sal*I-*Hin*dIII fragment of pKK223-3 (8) with the *Sal*I-*Hin*dIII fragment from the polylinker of pIC-20H (42); pCH4, replacement of the *Eco*RI-*Hin*dIII fragment of pCH1 with that from the polylinker of pUC19 (85); pPG23, insertion of the ΩCm *Hin*dIII fragment from pHP45ΩCm (16) into the *Hin*dIII site of pIC-20R (42); pPG27, insertion of the ΩAp *Eco*RI fragment from pKT254ΩAp (16) into the *Eco*RI site of pACYC184 (10, 53); pRK2443, deletion of the *Eco*RI fragment (containing RK2 kb 0 to 2.3 and most of a mini-Tn*3* transposon) of pRK2440::mini-Tn*3-57* (unpublished; see Fig. 2 for the site of mini-Tn*3* insertion); pRK2444 and pRK2446, insertion of the ΩCm *Eco*RI fragment from pHP45ΩCm into the *Eco*RI sites of pRK2443 and pRK2440 (88), respectively; pRK2484, insertion of the 2.4-kb *Hin*cII-*Eco*RI fragment (Fig. 1) from the *kilA* region of pRK2440 into *Sma*I- and *Eco*RI-cleaved pUC18; pRK2487, cloning of the 1-kb *Xho*I-*Hin*cII fragment (Fig. 1) from pRK2102 into *Sal*I- and *Sma*I-cleaved pUC18; pRK2488, deletion of the *Eco*RI-*Dra*II fragment (RK2 kb 0 to 1.5) of pRK2440, filling in the ends with the Klenow fragment of DNA polymerase I, and religation; pRK2489 and pRK2727, deletion of the *Eco*RI-*Dra*II fragment (RK2 kb 0 to 1.5) of pRK2440 and pRK2724, respectively, filling in the ends with the Klenow fragment of DNA polymerase I, and ligation with a similarly blunt-ended, ΩCm *Eco*RI fragment from pHP45ΩCm; pRK2491, insertion of the *Hin*cII-*Nru*I fragment of pRK2489 (RK2 kb 1.5 to 2.4) into *Nru*I-cleaved pT7-5B (34); pRK2492, deletion of the *Hin*dIII ΩCm fragment from pRK2491; pRK2497, insertion of the *Hin*cII-*Nru*I fragment of pRK2446 (RK2 kb 0 to 2.4) into *Nru*I-cleaved pT7-5B; pRK2499, deletion of the *Hin*dIII ΩCm fragment from pRK2497; pRK2527, digestion of pRK2440 with *Acc*I (RK2 kb 1.9), blunting of the ends with S1 nuclease, and ligation with *Xba*I DNA linkers; pRK2711, deletion of the *Xba*I-*Rsa*I fragment (RK2 kb 1.9 to 2.2) of pRK2527 and insertion of the *Xba*I-*Sma*I fragment from the polylinker region of pUC18 (85); pRK2722 and pRK2736, insertion of the *Eco*RI fragment (containing RK2 kb 55.7 to 56.4) of pRK2487 into the *Eco*RI site of pRK2440 and pRK2734, respectively; pRK2724, digestion of pRK2711 with *Xba*I and transformation of MV10 with the linearized DNA; pRK2730 and pRK2771, insertion of the ΩCm *Sac*I fragment from pPG23 into the *Kpn*I and *Sac*I sites of pRK2722 and pRK2736, respectively; pRK2734, deletion of the *Bal*I-*Dra*II fragment (RK2 kb 0.7 to 1.5) of pRK2724, partial fill-in of the *Dra*II-generated extension by the Klenow fragment of polymerase I with dGTP only, and religation; pRK2738, insertion of the *Sfi*I-*Cla*I fragment (RK2 kb 1.8 to 2.4 and a portion of the ColE1 plasmid vehicle) of pRK2488 into *Sfi*I- and *Cla*I-cleaved pRK2734; pRK2772 and pRK2783, deletion of the *Eco*RI-*Xho*I fragments (RK2 kb 0 to 0.3) of pRK2734 and pRK2724, respectively, and insertion of the ΩCm *Eco*RI-*Sal*I fragment from pPG23; pRK2774 and pRK2775, insertion of the *Eco*RI fragment (containing RK2 kb 55.7 to 56.4 and the ΩCm fragment) from pRK2730 into the *Eco*RI sites of pRK2724 and pRK2738, respectively; pRK2777, replacement of the *Acc*I-*Pst*I fragment of pRK2497 (RK2 kb 0 to 1.9) with that of pRK2730 (RK2 kb 55.7 to 1.9); pRK2778, deletion of the *Hin*dIII ΩCm fragment of pRK2777; pRK2779, insertion of the *Hin*cII-*Hin*dIII fragment of pRK2771 (containing RK2 kb 55.7 to 2.4 with Δ*klaA105* and Δ*klaB270*) into pT7-5B cleaved with *Nru*I and *Hin*dIII; pRK2781, digestion of pPG23 with *Xba*I and *Nru*I, blunting of the *Xba*I-generated end with the Klenow fragment of DNA polymerase I, and insertion of the ΩCm-containing fragment into the *Hin*cII site of pRK2722; pRK2784, pRK2785, and pRK2787, insertion of the *Cla*I-*Sal*I fragment (containing the *kilA* region, ΩCm fragment, and ColE1 remnant) of pRK2774, pRK2775, and pRK2730, respectively, into *Cla*I-and *Sal*I-cleaved pPG27; pRK2786, insertion of the *Nru*I fragment (containing the *kilA* region, ΩCm fragment, and ColE1 remnant) of pRK2446 into the *Nru*I site of pPG27; pRK2788 and pRK2790, cleavage of pRK2779 with *Xho*I and *Xba*I plus *Xho*I, respectively, filling of the ends with the Klenow fragment of DNA polymerase I, and religation; pRK2855, insertion of the 550-bp *Xho*I-*Bam*HI fragment containing the *kilA* promoter from pRK2443 into *Sal*I- and *Bam*HI-cleaved pCH1; pRK2865, replacement of the *Bam*HI-*Hin*dIII polylinker region of pRK2855 with that of pUC19; and pRK2999, insertion of the 275-bp *korA* promoter-encoding *Sau*3A fragment from pRK2219 (4) into the *Bam*HI site of pCH4.

Media. Media used for routine growth of bacteria were LB, LB with 0.1% glucose, M9 minimal salts, and M9 containing 0.5% Casamino Acids (Difco) (40). Solid growth media contained 1.5% agar. Supplements were added to the following concentrations: amino acids, 40 μg/ml; ampicillin, 50 or 100 μg/ml; chloramphenicol, 25 μg/ml; isopropylthiogalactoside (IPTG), 1 mM; kanamycin sulfate, 50 μg/ml; nalidixic acid, 20 μg/ml; penicillin, 150 μg/ml; trimethoprim, 50 μg/ml; and 5-bromo-4-chloro-3-indolylgalactoside (X-Gal), 40 μg/ml.

Enzymes, reagents, and radiochemicals. Restriction endonucleases, S1 nuclease, T4 DNA ligase, *E. coli* DNA polymerase I Klenow fragment, T4 polynucleotide kinase, DNA linkers, T7 DNA polymerase (Sequenase), avian myeloblastosis virus reverse transcriptase, and synthetic oligonucleotides (Operon Technologies) were purchased from commercial suppliers and used with the recommended conditions. [α-^{35}S]dATP (1,400 Ci/mmol), L-[^{35}S]methionine (>1,000 Ci/mmol), and [γ-^{32}P]ATP (6,000 Ci/mmol) were purchased from New England Nuclear. ^{14}C-labeled proteins for molecular weight standards were from Amersham.

Manipulation and analysis of DNA. Cleavage of DNA with restriction endonucleases, DNA ligation, blunting of 5′ and 3′ extensions of DNA with the Klenow fragment of DNA polymerase I and S1 nuclease, respectively, and gel electrophoresis were done according to published procedures (40). Plasmid DNA was prepared by the method of Birnboim and Doly (6) or Kahn et al. (32). Single-stranded M13 bacteriophage DNA was purified as described previously (44). Transformation and transfection of *E. coli* were done essentially as described by Cohen et al. (11). Large DNA fragments (1 to 3 kb) were separated by electrophoresis in low-melting-temperature agarose (40). Small DNA fragments (30 to 300 bp) were eluted from 5% polyacrylamide gels by the crush-and-soak method of Maxam and Gilbert (43).

Nucleotide sequence determination. To subclone small overlapping fragments into M13, pRK2484, which carries kb 0 to 2.4 of RK2 (Fig. 1), was digested with *Hae*III, *Msp*I, *Taq*I, and *Sau*3A individually. Fragments were separated by electrophoresis in 5% polyacrylamide gels, and each RK2-specific fragment was purified and ligated to appropriately cleaved M13mp18 or M13mp19. *Hae*III and *Sau*3A fragments were cloned at the *Hin*cII and *Bam*HI sites, respectively; *Msp*I and *Taq*I fragments were inserted in the *Acc*I sites. The orientations of the cloned *kilA* fragments were determined by the C test (44), using single-stranded DNA from M13 phage derivatives M13ss120 and M13ss121, which carry the *Hin*cII-*Eco*RI fragment of the *kilA* region in

opposite orientations. To generate the sequence of the *Xho*I-*Hin*cII region (Fig. 1) and to obtain the overlap at the *Eco*RI site, pRK2487 was digested with *Nae*I or *Rsa*I and the RK2-specific fragments were inserted into the *Hin*cII site of M13mp19. Fragments, generated by digestion with both *Bss*HII and *Eco*RI, were cloned by ligation to *Bss*HII- and *Eco*RI-cleaved M13hc4.

The complete nucleotide sequence was determined for both strands of the 3-kb *kilA* region from overlapping M13 clones by the chain termination method of Sanger et al. (56). Sequenase was used in place of DNA polymerase I Klenow fragment in the sequencing reactions, and the products were labeled with [α-^{35}S]dATP. To overcome the problem of band compression in some G+C-rich regions, additional sequencing reactions were done with dITP replacing dGTP (47). Gels were run according to Sanger and Coulson (55), and autoradiographs were prepared as described by Sambrook et al. (54). The GCG (14) and DNA Strider (41) computer programs were used for analysis of the sequence.

Identification of *kilA*-encoded polypeptides. Various segments of the *kilA* region were cloned downstream of the phage T7 ϕ*10* promoter in the expression vector pT7-5B. In this system, gene products are overexpressed following induction of T7 RNA polymerase in strain BL21(DE3). Induction and labeling of the polypeptide products were done as described previously (34) except that 1-ml aliquots were removed and labeled with 12 μCi of [^{35}S]methionine for 5 min.

Determination of transcriptional start sites. The *kilA* and *korA* promoters were cloned into pCH1 and pCH4, which carry strong transcriptional terminators downstream of the polylinker. The terminators permit maintenance of the plasmids (pRK2865 and pRK2999) in the absence of *korA* and *korB* regulatory functions, thereby providing an abundance of RNA transcripts. A synthetic DNA primer (5′-AAGCTTG CATGCCTGCAGGTCGACTCTAGAGGATC-3′) that is complementary to a region of the transcribed polylinker downstream of the cloned promoters was used to determine the transcriptional start sites for both promoters. Extraction of RNA, ^{32}P end labeling of the primer, annealing of the primer to RNA, and primer extension with avian myeloblastosis virus reverse transcriptase were done as described previously (1). The DNA products were separated by electrophoresis through a polyacrylamide sequencing gel. The reference nucleotide sequence was generated from double-stranded plasmid DNA by the chain termination method (37), using the 5′-end labeled primer.

Bacterial conjugation. Overnight cultures of donor and recipient strains were diluted 20-fold in LB medium supplemented with the appropriate antibiotic(s) and grown to mid-log phase (approximately 5×10^8 cells per ml). Cells were centrifuged, washed, and resuspended in an equal volume of LB medium and titered. Donors and recipients were mixed at a ratio of approximately 1:9. The mixtures were incubated in broth for 4 h at 37°C without shaking and then vortexed vigorously for 30 s to disrupt mating pairs. Transconjugants were selected on medium supplemented with the appropriate nutrients and antibiotics.

Nucleotide sequence accession number. The sequence data in this study have been given GenBank accession number M62426.

RESULTS

Determination of the nucleotide sequence of the *kilA* region. Our earlier studies showed that the *kilA* determinant encodes at least one host-lethal function in the 2.4-kb region (RK2 kb 0 to 2.4) located 0.7 kb upstream of the *korA-korB* operon (Fig. 1) (18). A single promoter responsible for the lethal expression of *kilA* was previously sequenced and found to overlap the *Hin*cII site at kb 2.4, with the direction of transcription pointing toward the *Eco*RI site at kb 0 (88). To identify the gene(s) of the *kilA* locus, we determined the nucleotide sequence of the 3-kb segment of RK2 that spans the entire region between the *kilA* and *korA* promoters. Seventy-seven unique and overlapping M13 clones were used to resolve the complete nucleotide sequence for both strands. The region between the *Hin*cII sites in the *kilA* and *korA* promoters was found to be 3,026 bp in length. Its sequence and the previously determined sequences of the *kilA* and *korA* promoters are displayed in Fig. 2.

Analysis of ORFs. The nucleotide sequence was examined in both directions for open reading frames (ORFs). Sequences preceding the ATG or GTG codons in each ORF were searched for possible ribosome binding sites (Shine-Dalgarno sequences) for two different RK2 hosts, *E. coli* (5′-TAAGGAGGT-3′) and *P. aeruginosa* (5′-TTAGGAG AG-3′) (60). Four ORFs were found to have reasonable Shine-Dalgarno sequences with acceptable spacing (3 to 7 nucleotides) (24, 36) to the initiation codon.

ORF1, ORF2, and ORF3 read in the same direction as *kilA* transcription. ORF1 is defined by an ATG codon at nucleotide 97 and a TAA stop codon at 868. ORF2 has two possible translation initiation sites: an ATG codon 18 nucleotides downstream of ORF1 at nucleotide 888 and a GTG codon at 987. It ends with a TGA stop codon at position 2024. ORF3 also exhibits two potential translation start sites. The first, an ATG at position 2021, causes ORF2 and ORF3 to overlap by 4 bp in an ATGA motif. A second possible initiation site for ORF3 is the ATG codon at position 2084. ORF3 terminates with a TAA codon at position 2972 and thus is not completely contained within the 2.4-kb region originally shown to encode a host-lethal determinant. ORF4 is the only ORF reading in the opposite direction that is preceded by a sequence with good similarity to the Shine-Dalgarno sequences of *E. coli* and *P. aeruginosa*. It begins with an ATG codon at 1741 and terminates with a TAG codon at 1082.

The percent G+C at each position of the codons from RK2 coding sequences shows a distinctive pattern, with high G/C usage in the first and third positions and low G/C usage in the middle position (Table 2). The *kilA* ORFs all display a similar overall composition of 63.3 to 65.3% G+C, which agrees with the 63.4% overall value for previously sequenced RK2 genes. The use of G or C in the first, second, and third positions of codons from ORF1, ORF2, and ORF3 is remarkably similar to those of known RK2 genes. The pattern for ORF4 is significantly different in G/C frequency in the first two positions. We also compared the four ORFs in their use of synonomous codons for each amino acid. ORF1, ORF2, and ORF3 again show patterns that generally reflect the codon usage of the known RK2 genes, whereas ORF4 displays notable differences in its distribution of codons for Leu, Asn, Glu, and Arg (data not shown). The codon AGG (Arg), which is used sparingly in genes of RK2, *E. coli*, and *P. aeruginosa*, occurs 13 times in ORF4. These results suggest that the most reasonable candidates for protein-encoding genes among the ORFs of the *kilA* region are ORF1, ORF2, and ORF3, all of which read in the same direction as does *kilA* transcription.

Polypeptide products expressed from the *kilA* region. We tested the *kilA* region for its ability to express the polypeptide products predicted for the ORFs. Portions of the *kilA*

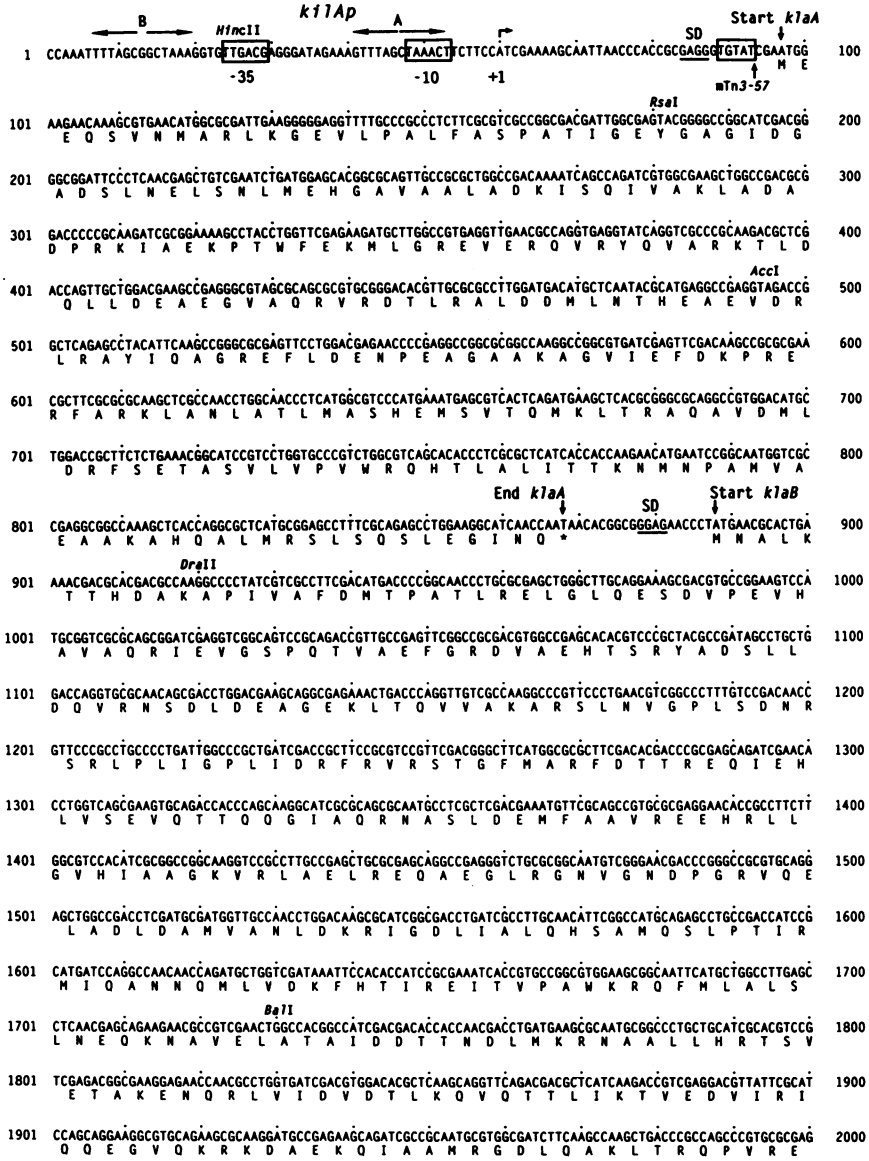

FIG. 2. Nucleotide sequence of the *kilA* operon (GenBank accession number M62426). Numbers refer to nucleotide positions. The predicted amino acid sequences are shown in single-letter code below the nucleotide sequences of *klaA*, *klaB*, and *klaC*. Potential Shine-Dalgarno sequences for ribosome binding are underlined and labeled SD. The boxed ATG codon and the nearby Shine-Dalgarno sequence (SD) in *klaC* indicates a second potential site for initiation of translation (see text). Restriction endonuclease cleavage sites used in the construction of various mutants are indicated above the nucleotide sequence. mTn*3-57* shows the site of insertion of a mini-Tn*3* transposon in plasmid pRK2443; the box indicates the nucleotides duplicated upon insertion. *kilAp* and *korAp* indicate the positions of the promoters for the *kilA* and *korA-korB* operons. The −10 and −35 regions are boxed, and mRNA start sites are indicated by +1 and the angled arrows. The pairs of divergent arrows labeled A and B show the operatorlike palindromes predicted to be the targets for KorA and KorB proteins, respectively. The 3,026-bp *Hin*cII fragment sequenced here includes 38 nucleotides of the *kilA* promoter sequence first reported by Young et al. (88). Two other short nucleotide segments (3033 to 3051 [4] and 2707 to 3051 [77]) overlap previously published sequences. Our data confirm these sequences with the exception that we find an additional A · T base pair at position 2988.

region were cloned downstream of the phage T7 ϕ*10* promoter in expression vector pT7-5B (Fig. 3). Plasmid pRK2778, which carries the entire *kilA* region, expressed three polypeptides of 44, 29, and 28 kDa (Fig. 4A, lane 4). These were specific to the *kilA* region because no labeled polypeptides were visible in the extract for the vector alone (Fig. 4A, lane 1). No polypeptides were observed from a plasmid that has the *kilA* region in the direction opposite that of pRK2778 (data not shown). Thus, ORF4 appears to be expressed poorly or not at all in *E. coli*. This conclusion is consistent with the codon analysis, which indicated that ORF4 is not likely to be an RK2 gene.

To determine which ORFs are responsible for the observed polypeptides, we examined the polypeptides expressed by pRK2492, pRK2499, and pRK2779 (Fig. 3). Plasmid pRK2492, which carries ORF1 and 12 codons of ORF2, expressed the 29-kDa polypeptide only (Fig. 4A, lane 2). The estimated mass of the observed polypeptide is in

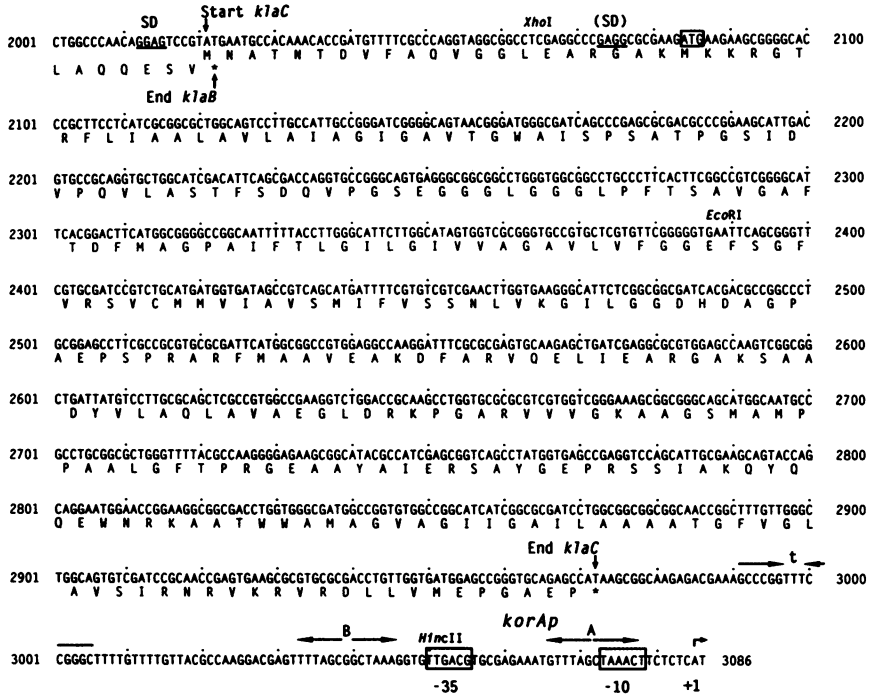

FIG. 2—*Continued*

close agreement with 28,409 Da, the calculated mass of the ORF1 product. Plasmid pRK2499 encodes the complete sequences of ORF1 and ORF2 followed by a truncated version of ORF3 fused to vector sequences. Extracts from induced cells carrying pRK2499 contained not only the 29-kDa polypeptide but also the 44-kDa polypeptide (Fig. 4A, lane 3), indicating that ORF2 encodes the 44-kDa polypeptide. The observed mass of 44 kDa corresponds well with the calculated mass of 42,156 Da for ORF2 product initiated from the first ATG codon. The additional small polypeptide, visible at the bottom of the gel and absent in pRK2778 and pRK2492 extracts, is likely to be the expected truncated ORF3 product. To confirm that the 28-kDa polypeptide visible in extracts of pRK2778 cells is the product of ORF3, we used plasmid pRK2779, which has an intact ORF3 downstream of in-frame deletion mutations in both ORF1 and ORF2. The 28-kDa polypeptide was indeed expressed by pRK2779, while the 29- and 44-kDa polypeptides were not (Fig. 4A, lane 5). In addition, we observed a band that corresponds to the expected size of the shortened ORF2 product. No truncated ORF1 product was detected.

From these results, we conclude that the *kilA* region consists of three protein-encoding genes defined by ORF1, ORF2, and ORF3. We have therefore designated these genes *klaA*, *klaB*, and *klaC*, respectively.

Translational start codon for *klaC*. As discussed above, the *klaC* ORF has two potential sites for initiation of translation. The larger predicted polypeptide would have a calculated mass of 32,380 Da; the mass calculated for the smaller polypeptide is 30,247 Da. The latter value is more consistent with the 28-kDa value for the apparent molecular mass of the observed polypeptide product of *klaC* (Fig. 4). Therefore, it seemed possible that the KlaC polypeptide is initiated at the second ATG.

To determine the translational start site for *klaC*, we constructed two mutant plasmids. Plasmid pRK2788 is a derivative of pRK2779 that has an insertion of 4 bp at the *Xho*I site located between the first ATG and the Shine-Dalgarno sequence of the second ATG (Fig. 2 and 3). This frameshift mutation will cause loss of the 28-kDa polypeptide only if translation initiates at the first ATG. The second plasmid, pRK2790 (Fig. 3), is deleted for the first ATG of *klaC* but retains the second ATG and its corresponding Shine-Dalgarno sequence. Both plasmids failed to express any detectable 28-kDa polypeptide (Fig. 4B, lanes 2 and 4). In addition, we constructed an isogenic set of *klaC-lacZ* gene fusions as a more sensitive assay for the ability of the second ATG to initiate translation. In these derivatives, expression of β-galactosidase was completely dependent on the first ATG codon (data not shown). Taken together, these results indicate that translation of the *klaC* product is initiated at the first ATG codon. We cannot yet distinguish whether the low apparent mass of *klaC* product on sodium dodecyl sulfate (SDS)-polyacrylamide gels relative to its predicted size

TABLE 2. G+C composition of *kilA* genes

Gene or ORF	% G+C content			
	Base position in codon			Coding region
	1	2	3	
RK2 average[a]	66.9	45.0	78.2	63.4
ORF1 (*klaA*)	66.7	40.3	83.0	63.3
ORF2 (*klaB*)	68.9	38.0	85.2	64.0
ORF3 (*klaC*)	66.7	52.8	76.4	65.3
ORF4	38.2	68.6	85.9	64.2

[a] Values represent the average of RK2 genes whose proteins have been identified. These include *korA* (4), *korB* (33, 74), *korC* (34), *kfrA* (79), *incC* (38-kDa polypeptide) (77), *traJ* (90), and *trfA* (13- and 44-kDa polypeptides) (68).

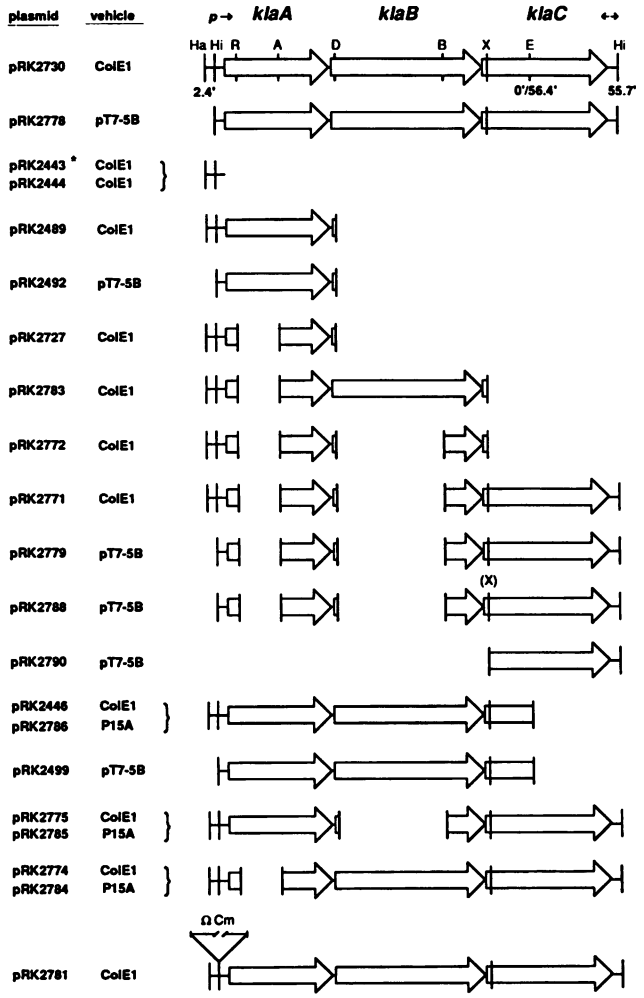

FIG. 3. Plasmids used in the molecular and genetic analysis of the *kilA* operon. Open arrows depict the *klaA*, *klaB*, and *klaC* genes; *p* indicates the *kilA* promoter; and the arrow shows the direction of transcription. Opposing arrows show the location of a potential *rho*-independent transcriptional terminator. Restriction endonuclease cleavage sites are Ha (*Hae*II), Hi (*Hin*cII), R (*Rsa*I), A (*Acc*I), D (*Dra*II), B (*Bal*I), X (*Xho*I), and E (*Eco*RI). (X) indicates a filled *Xho*I site. Numbers refer to map coordinates; primes represent kilobases. All plasmids carry the ΩCm transcriptional terminator fragment immediately downstream of the *kilA* region present in each plasmid except pRK2443 (marked with *), which is lacking ΩCm, and pRK2781, which has the ΩCm fragment inserted in the *kilA* promoter. See Table 2 and the text for details.

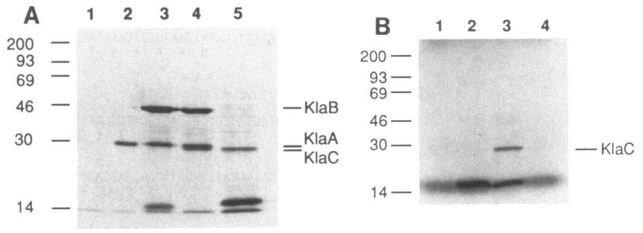

FIG. 4. Analysis of polypeptide products. Regions carrying various wild-type and mutant alleles of *klaA*, *klaB*, and *klaC* were expressed in vivo from the bacteriophage T7 $\phi 10$ promoter. Polypeptides specified by the cloned genes were selectively labeled with [^{35}S]methionine, separated by SDS-polyacrylamide gel electrophoresis, and visualized by autoradiography as described in Materials and Methods. Numbers at the left show the positions and sizes of ^{14}C-labeled molecular weight markers (myosin [200 kDa], phosphorylase *b* [92 kDa], bovine serum albumin [69 kDa], ovalbumin [46 kDa], carbonic anhydrase [30 kDa], and lysosyme [14 kDa]). (A) Identification of the polypeptide products of *klaA*, *klaB*, and *klaC*. Lanes: 1, pT7-5B; 2, pRK2492; 3, pRK2499; 4, pRK2778; 5, pRK 2779. (B) Determination of the start codon for *klaC*. Lanes: 1, pT7-5B; 2, pRK2788; 3, pRK2779; 4, pRK2790.

results from anomalous migration in the gel, possibly due to its hydrophobicity (see below), or from posttranslational processing.

Analysis of the Kla polypeptides. We determined the hydrophobicity profile of each polypeptide by the method of Kyte and Doolittle (38) to assess the possibility of their association with the membrane. The profiles of the KlaA and KlaB polypeptides were consistent with their being soluble proteins (data not shown). However, the analysis for the KlaC polypeptide predicted four potential membrane-spanning domains, suggesting that this polypeptide may be an integral membrane protein (Fig. 5). Three of the membrane-spanning domains reside in the N-terminal half of the polypeptide, and the fourth is near the C terminus, separated from the others by a hydrophilic stretch of 125 amino acid residues. The hydrophobic character of the KlaC polypeptide could be responsible for its anomalous behavior in SDS-polyacrylamide gel electrophoresis.

The nucleotide sequence of the *kilA* region was compared with those of previously identified genes in the GenBank and EMBL nucleic acid data bases, and the deduced amino acid sequences of KlaA, KlaB, and KlaC were compared with sequences of known proteins listed in the PIR data base. No notable similarities were found. In addition, the *kla* genes and products exhibited no marked resemblance to each other or to other sequenced RK2 genes and products.

Structure of the *kilA* operon. Lethal expression of the *kilA* locus occurs from a single promoter at RK2 kb 2.4 immediately preceding *klaA* (88). Because *klaA* is the first gene downstream of the *kilA* promoter, it is clearly part of the regulated *kilA* operon. To provide an indication of whether *klaB* or *klaC* might also be expressed from the *kilA* promoter, we examined the nucleotide sequence for any of the known promoter motifs. The only recognizable promoterlike sequences were the two previously identified σ^{70}-type promoters: the *kilA* promoter located upstream of *klaA* and the promoter for the *korA-korB* operon located downstream of

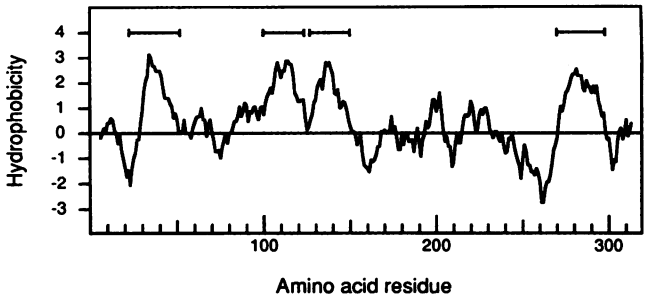

FIG. 5. Hydrophobicity profile of KlaC. Values are calculated by the method of Kyte and Doolittle (38) with a window length of 11 amino acid residues. Hydrophobic and hydrophilic regions appear above and below the line, respectively. Brackets show potential transmembrane segments.

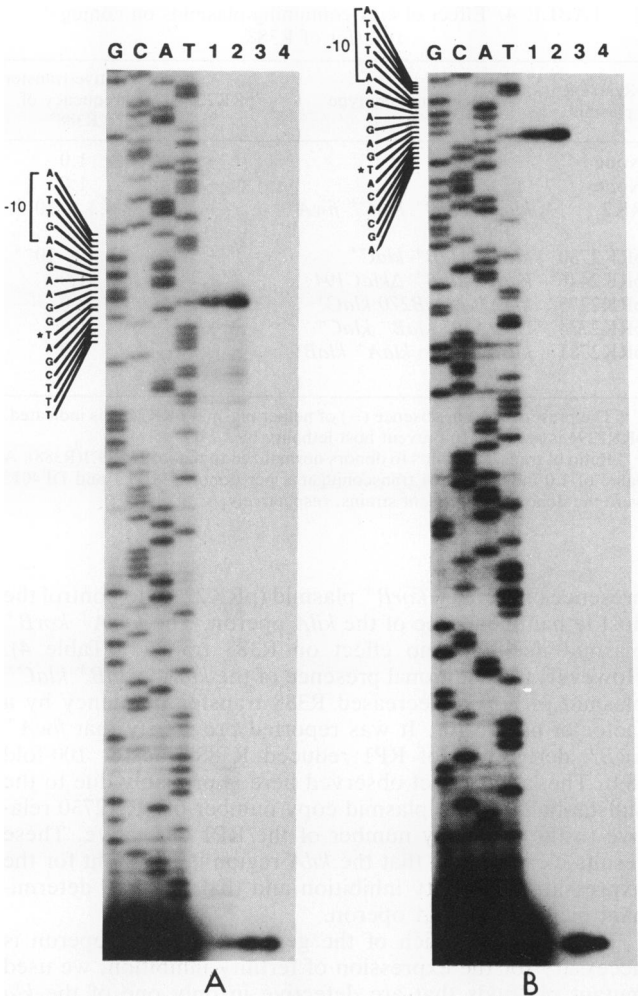

FIG. 6. Mapping the transcription start sites for the *kilA* and *korA* promoters by primer extension. A DNA primer, whose 5′ end was labeled with ^{32}P, was annealed to RNA extracted from cells containing the appropriate plasmid and extended by reverse transcriptase. The labeled DNA products were separated on polyacrylamide sequencing gels and visualized by autoradiography. The reference sequences were obtained by the dideoxynucleotide chain termination method using plasmids pRK2865 and pRK2999 and the same ^{32}P-labeled primer. Asterisks show the positions in the nucleotide sequence that correspond to the start sites for transcription. (A) *kilA* promoter. Lanes: 1, pRK2865, 1:200 dilution of the reaction products; 2, pRK2865, 1:100 dilution; 3, pCH1, 1:100 dilution; 4, untreated ^{32}P-labeled primer. (B) *korA* promoter. Lanes: 1, pRK2999, 1:50 dilution of the reaction products; 2, pRK2999, 1:25 dilution; 3, pCH1, 1:50 dilution; 4, untreated ^{32}P-labeled primer.

klaC (Fig. 2). These promoters were previously identified genetically by showing that disruption of the *Hin*cII sites within the −35 regions abolished expression (4, 88). The promoters were each cloned upstream of strong transcription terminators to allow unregulated transcription while avoiding possible effects of transcription read-through on plasmid maintenance (see below). The first nucleotides of the RNA transcripts were then identified by primer extension with reverse transcriptase (Fig. 6). Transcription for both the *kilA* and *korA* promoters was found to start at the expected +1 positions (Fig. 2).

The nucleotide sequence shows a potential *rho*-independent terminator: a G+C-rich inverted repeat followed by a run of T residues. This sequence, noted previously (77), has the potential to form a stem-loop structure with a 3-base loop and a 6-bp stem or a 12-bp stem with one mismatch. The putative terminator sequence begins 10 bp downstream of the *klaC* termination codon at nucleotide 2985 (Fig. 2).

Thus, the *kilA* region appears to begin with a single promoter, located upstream of *klaA*, and end with a sequence that resembles a *rho*-independent terminator downstream of *klaC* and immediately preceding the start of the *korA-korB* operon. This arrangement suggests that *klaA*, *klaB*, and *klaC* are all members of the *kilA* operon. This conclusion is corroborated by the genetic studies discussed below.

TABLE 3. Relative transformation efficiencies of *kilA*-containing plasmids

Transforming plasmid	Relevant genotype[a]	Relative transformation efficiency[b]	Kil phenotype[c]
pRK2443	*kilAp*	<0.001	NA[d]
pRK2444	*kilAp*-ΩCm	1.9	NA
pRK2730	*klaA*$^+$ *klaB*$^+$ *klaC*$^+$	<0.001	+
pRK2489	*klaA*$^+$ Δ*klaB367*	<0.001	+
pRK2727	Δ*klaA105* Δ*klaB367*	0.5	−
pRK2783	Δ*klaA105* *klaB*$^+$ Δ*klaC303*	<0.001	+
pRK2772	Δ*klaA105* Δ*klaB270*	0.6	−
pRK2771	Δ*klaA105* Δ*klaB270* *klaC*$^+$	<0.001	+
pRK2775	*klaA*$^+$ Δ*klaB270* *klaC*$^+$	<0.001	+
pRK2774	Δ*klaA105* *klaB*$^+$ *klaC*$^+$	<0.001	+
pRK2781	*kilAp*::ΩCm *klaA*$^+$ *klaB*$^+$ *klaC*$^+$	1.0	−

[a] All plasmids except pRK2443 and pRK2781 have the ΩCm fragment after the *kilA* region.

[b] *E. coli* JMB9.1 and JMB9.1(pRK2108) were transformed with approximately 0.2 μg of each plasmid. Relative competence was determined by transformation with pMK20. Differences in competence were corrected for and were never greater than fivefold. Relative transformation efficiency is the ratio of corrected JMB9.1 transformants to JMB9.1(pRK2108) transformants.

[c] Phenotypes were scored after overnight growth at 37°C. The Kil$^+$ phenotype is defined as a 10^2-fold or greater difference in transformation efficiency.

[d] NA, not applicable; see text.

***klaA*, *klaB*, and *klaC* are all potentially host lethal.** The *kilA* locus was first detected by the host-lethal (Kil$^+$) phenotype expressed by the cloned kb 0 to 2.4 region of RK2 in the absence of *korA* and *korB* (18, 86). This region encodes *klaA*, *klaB*, and about a third of *klaC*. To systematically test the individual roles of *klaA*, *klaB*, and *klaC* in the host-lethal phenotype, we constructed an isogenic set of plasmids carrying various portions of the *kilA* operon expressed from the *kilA* promoter (Fig. 3). All plasmid derivatives tested for the host-lethal phenotype contained the ΩCm transcriptional terminator immediately downstream of the *kilA* segment in the plasmid. This was necessary to prevent possible confusion arising from nonlethal interference of plasmid maintenance by unregulated transcription from the *kilA* promoter. This phenomenon is evident in the phenotypes of two plasmids that contain the *kilA* promoter but are missing all the structural genes of the *kilA* operon: pRK2443 (lacking ΩCm) and pRK2444 (containing ΩCm) (Fig. 3). With pRK2443, colonies arise after transformation of the *korA*$^+$ *korB*$^+$ host but not after transformation of the *kor*^{o} host (Table 3). However, pRK2444 can transform *kor*^{o} and *korA*$^+$ *korB*$^+$ cells with equal efficiency. This showed that the failure to transform *kor*^{o} cells by pRK2443 was not due to killing of host cells by the plasmid but rather to transcription

read-through that inhibited plasmid replication or expression of the selective marker. In contrast, the *kor*-sensitive, Kil$^+$ phenotype of a plasmid carrying a host-lethal structural gene is not altered by insertion of the ΩCm transcriptional terminator. Thus, plasmid pRK2730, which contains the complete *kilA* operon (Fig. 3), is unable to produce viable transformants of *kor*^{o} cells despite the presence of the ΩCm terminator because at least one of the gene products is lethal to cells (Table 3).

The ΩCm-containing plasmids were tested to determine the role of each of the *kla* genes in host lethality by comparing their transformation efficiencies in *kor*^{o} and *korA*$^+$ *korB*$^+$ cells (Table 3). Plasmid pRK2489, which contains the *kilA* promoter, the *klaA* gene, and only 12 codons of *klaB* (Fig. 3), was unable to transform *kor*^{o} cells. Thus, its phenotype is Kil$^+$. To prove that the phenotype is caused by expression of the *klaA* structural gene, we made an in-frame deletion of 105 codons in *klaA* (Δ*klaA105*) to produce plasmid pRK2727 (Fig. 3). This derivative displayed a Kil$^-$ phenotype. Therefore, unregulated expression of *klaA* is lethal to host cells. We tested the phenotype of *klaB* alone by using pRK2783 (Fig. 3). This plasmid has the *kilA* promoter and the entire *klaB* coding region downstream of the in-frame Δ*klaA105* deletion of *klaA*. Its phenotype was also found to be Kil$^+$. Inactivation of the *klaB* structural gene by a 270-codon deletion (Δ*klaB270*) (pRK2772; Fig. 3) changed the phenotype to Kil$^-$ (Table 3). Therefore, *klaB* alone is also lethal to *kor*^{o} cells. Finally, combining the in-frame Δ*klaA105* and Δ*klaB270* deletions along with an intact *klaC* coding sequence made it possible to test the effect *klaC* alone on *kor*^{o} cells. The resulting plasmid, pRK2771 (Fig. 3), was also Kil$^+$ (Table 3). These results show clearly that expression of each of the three *kilA* genes (*klaA*, *klaB*, or *klaC*) is lethal to host cells in the absence of *korA* and *korB* regulatory functions.

***klaA*, *klaB*, and *klaC* are expressed in an operon.** Because each of the *kla* genes confers a Kil$^+$ phenotype, it was possible to test whether all three genes are expressed from the *kilA* promoter. If they are, then inactivation of the *kilA* promoter in a plasmid carrying the complete coding sequences for *klaA*, *klaB*, and *klaC* should result in a Kil$^-$ phenotype. If any of the genes is expressed by another promoter, then the plasmid should remain Kil$^+$. In plasmid pRK2781, the *kilA* promoter is inactivated by insertion of the ΩCm fragment at the *Hin*cII site within the −35 region, but *klaA*, *klaB*, and *klaC* are left intact (Fig. 3). The phenotype of this plasmid is Kil$^-$ (Table 3). Therefore, the *kilA* promoter is essential for the expression of *klaA*, *klaB*, and *klaC* in the production of the Kil$^+$ phenotype. This finding confirms that the three genes comprise a single multicistronic operon.

***klaA*, *klaB*, and *klaC* are all necessary for fertility inhibition.** IncP plasmid RP1 encodes two determinants for fertility inhibition, *fiwA* and *fiwB*. Both loci are responsible for reducing the transfer frequency of a coresident IncW plasmid R388 (89). We confirmed that RK2 also expresses the fertility inhibition phenotype by measuring the transfer frequency of IncW plasmid R388 in the presence and absence of RK2 in the donor cells (Table 4). The transfer frequency of R388 is reduced by a factor of 10^5 to 10^6 in the presence of RK2, a value consistent with that observed for RP1 (20, 89).

Because the *fiwB* determinant was mapped to a region that overlaps the *kilA* operon, we investigated the relationship of *fiwB* and the *kilA* operon. First we tested the *klaA*$^+$ *klaB*$^+$ *klaC*$^+$ plasmid pRK2730 for expression of the fertility inhibition phenotype. These experiments were done in the presence of a *korA*$^+$ *korB*$^+$ plasmid (pRK2294) to control the host-lethal phenotype of the *kilA* operon. The *korA*$^+$ *korB*$^+$ plasmid itself has no effect on R388 transfer (Table 4). However, the additional presence of the *klaA*$^+$ *klaB*$^+$ *klaC*$^+$ plasmid pRK2730 decreased R388 transfer frequency by a factor of nearly 10^4. It was reported previously that *fiwA*$^+$ *fiwB*$^+$ derivatives of RP1 reduced R388 transfer 100-fold (89). The larger effect observed here is probably due to the substantially higher plasmid copy number of pRK2730 relative to the low copy number of the RP1 derivative. These results clearly show that the *kilA* region is sufficient for the expression of fertility inhibition and that the *fiwB* determinant maps to the *kilA* operon.

TABLE 4. Effect of *kilA*-containing plasmids on conjugal transfer of R388

Resident plasmid	Relevant genotype	pRK2294[a]	Relative transfer frequency of R388[b]
None		−	1.0
None		+	1.0
RK2	*klaA*$^+$ *klaB*$^+$ *klaC*$^+$ *fiwA*$^+$ *fiwB*$^+$	+	$<6.4 \times 10^{-6}$
pRK2730	*klaA*$^+$ *klaB*$^+$ *klaC*$^+$	+	1.4×10^{-4}
pRK2446	*klaA*$^+$ *klaB*$^+$ Δ*klaC194*	+	1.0
pRK2775	*klaA*$^+$ Δ*klaB270* *klaC*$^+$	+	2.0
pRK2774	Δ*klaA105* *klaB*$^+$ *klaC*$^+$	+	1.0
pRK2781	*kilAp*::ΩCm *klaA*$^+$ *klaB*$^+$ *klaC*$^+$	+	0.9

[a] The presence (+) or absence (−) of helper plasmid pRK2294 is indicated. pRK2294 is required to prevent host lethality by *kilA*.

[b] Ratio of transconjugants to donors normalized to that of JMB9.1(R388). A value of 1.0 indicates 0.04 transconjugants per donor. JMB9.1 and DF4015 were the donor and recipient strains, respectively.

To determine which of the genes of the *kilA* operon is necessary for the expression of fertility inhibition, we used mutant plasmids that are defective in only one of the *kla* genes. Plasmids pRK2774, pRK2775, and pRK2446 contain the Δ*klaA105*, Δ*klaB270*, and Δ*klaC194* mutations, respectively (Fig. 3). Each was tested for the ability to inhibit transfer of R388. The results show that loss of any one of the genes (*klaA*, *klaB*, or *klaC*) is sufficient to abolish the fertility inhibition phenotype and allow normal transfer frequency of R388 (Table 4).

Because the fertility inhibition phenotype is clearly evident in the presence of *korA* and *korB*, we wished to determine whether the *korA*- and *korB*-regulated *kilA* promoter is responsible for its expression. We therefore tested the promoter-defective but otherwise *klaA*$^+$ *klaB*$^+$ *klaC*$^+$ plasmid pRK2781 for its ability to inhibit R388 transfer. This mutant was found to be defective in fertility inhibition (Table 4). Therefore, all three genes of the *kilA* operon are necessary for fertility inhibition, and expression of the phenotype depends on the *kilA* promoter, even in the presence of *korA* and *korB*.

***klaA*, *klaB*, and *klaC* are all necessary for Te^r.** It is possible to select mutants of RP1, RP4, or RK2 that enable the host cells to survive in the presence of high levels of tellurite (7, 20, 71). The Te^r determinant in these mutants has been mapped to a portion of RK2 that overlaps the *kilA* region. To understand the relationship of the *kilA* operon to Te^r, we examined two phenotypes: (i) expression of low-level resistance to tellurite (<5 μg/ml) from the cloned wild-type *kilA* operon and (ii) selection of mutants resistant to high levels of tellurite (>5 μg/ml).

TABLE 5. Tellurite resistance conferred by *kilA*-containing plasmids

Resident plasmid(s)	Relevant genotype(s)	pRK2108[a]	MIC of K_2TeO_3 (μg/ml)[b]	Frequency of high-level Te^r[c]
None		−	0.3	$<1.4 \times 10^{-9}$
None		+	0.2	$<1.0 \times 10^{-9}$
RK2	*klaA*$^+$ *klaB*$^+$ *klaC*$^+$	−	0.3	1.9×10^{-9}
pRK2730	*klaA*$^+$ *klaB*$^+$ *klaC*$^+$	+	2.0	2.4×10^{-5}
pRK2781	*kilAp*::ΩCm *klaA*$^+$ *klaB*$^+$ *klaC*$^+$	+	0.2	$<2.6 \times 10^{-10}$
pRK2784	Δ*klaA105* *klaB*$^+$ *klaC*$^+$	+	0.5	$<1.6 \times 10^{-9}$
pRK2785	*klaA*$^+$ Δ*klaB270* *klaC*$^+$	+	0.5	$<1.4 \times 10^{-9}$
pRK2786	*klaA*$^+$ *klaB*$^+$ Δ*klaC194*	+	0.3	$<9.1 \times 10^{-10}$
pRK2489	*klaA*$^+$ Δ*klaB367*	+	0.5	$<1.3 \times 10^{-9}$
pRK2783	Δ*klaA105* *klaB*$^+$ Δ*klaC303*	+	0.5	$<5.6 \times 10^{-10}$
pRK2771	Δ*klaA105* Δ*klaB270* *klaC*$^+$	+	0.4	$<9.1 \times 10^{-10}$
pRK2489 pRK2784	*klaA*$^+$ Δ*klaB367* Δ*klaA105* *klaB*$^+$ *klaC*$^+$	+	2.0	1.4×10^{-8}
pRK2783 pRK2785	Δ*klaA105* *klaB*$^+$ *klaA*$^+$ Δ*klaB270* *klaC*$^+$	+	2.0	5.6×10^{-8}
pRK2771 pRK2786	Δ*klaA105* Δ*klaB270* *klaC*$^+$ *klaA*$^+$ *klaB*$^+$ Δ*klaC194*	+	2.0	2.7×10^{-8}

[a] The presence (+) or absence (−) of pRK2108 is indicated. Plasmid pRK2108 is required to prevent the host lethality of *kilA*.

[b] Overnight cultures of PG1118 and PG1118 carrying various plasmids were serially diluted and plated on a series of LB plates supplemented with different concentrations of K_2TeO_3. The concentrations of K_2TeO_3 varied from 0.1 to 1.0 μg/ml in 0.1-μg/ml increments and from 1 to 5 μg/ml in 1-μg/ml increments. The MIC is defined as the concentration at which there is a 10^3-fold or greater reduction in plating efficiency after 24 h of incubation at 37°C.

[c] Overnight cultures were plated on LB agar containing 5 μg of K_2TeO_3 per ml. Colonies were scored after 2 days of incubation at 37°C.

We tested the *klaA*$^+$ *klaB*$^+$ *klaC*$^+$ plasmid pRK2730 to determine whether the cloned wild-type *kilA* operon confers Te^r on the host cells. The host used in these studies expresses a low level of resistance to K_2TeO_3, such that the MIC is 0.2 to 0.5 μg/ml (Table 5). As expected, we were unable to observe any difference in Te^r when the cells contained wild-type RK2 or the *korA*$^+$ *korB*$^+$ plasmid pRK2108, which was used to control the host-lethal phenotype of the *kilA* plasmids used in these experiments. However, the high-copy-number *klaA*$^+$ *klaB*$^+$ *klaC*$^+$ plasmid pRK2730 caused a significant and reproducible 4- to 10-fold increase in Te^r. This finding demonstrated that the wild-type *kilA* operon of RK2 is sufficient to express a low-level Te^r phenotype.

The spontaneous mutation frequency to high level Te^r of plasmidless host cells is extremely low ($<10^{-9}$) (Table 5). The presence of RK2 in the cells increases that frequency by about 10-fold. However, in pRK2730-containing cells, the spontaneous mutation frequency increased at least 1,000-fold. Plasmid pRK2730 did not cause an increase in mutation frequency of resistance to other selective agents (nalidixic acid, streptomycin, and rifampin), and the Te^r phenotype was plasmid associated (data not shown).

We used plasmids pRK2784 (Δ*klaA*), pRK2785 (Δ*klaB*), pRK2786 (Δ*klaC*), and pRK2781 (*kilAp*::ΩCm) (Fig. 3) to test the involvement of each of the *kla* genes in the Te^r phenotype. None of these plasmids conferred the low-level Te^r phenotype, and none gave rise to high-level Te^r mutants (Table 5). For each of the structural gene mutants, both of these defects were complemented by providing the missing gene product in *trans* on another replicon, thereby ruling out any *cis*-dominant effects of the mutations (Table 5). From these experiments, we conclude that the *klaA*, *klaB*, and *klaC* gene products and the *kilA* promoter are all required for both (i) the low-level Te^r phenotype exhibited by the wild-type *kilA* region and (ii) the appearance of high-level Te^r mutants.

DISCUSSION

We have established that the *kilA* locus of RK2 consists of an operon of three genes (*klaA*, *klaB*, and *klaC*) expressed from the *korA*- and *korB*-regulated *kilA* promoter. Each of the genes is sufficient to confer a host-lethal phenotype when expressed from the natural *kilA* promoter in the absence of *kor* regulatory functions. In addition, all three genes and the *kilA* promoter are necessary for two other phenotypes that have been mapped to the region: fertility inhibition of IncW plasmids and Te^r, both of which are evident even in the presence of *korA* and *korB*. Therefore, host lethality, fertility inhibition, and Te^r are three manifestations of the *kilA* operon.

The *kilA* operon begins with the *kilA* promoter at kb 2.4 on the physical map and fills the 3-kb gap to the start of the *korA-korB* operon. The region contains no other recognizable promoterlike sequences, and the genetic studies showed that expression of host lethality, fertility inhibition, and Te^r all depended on a functional *kilA* promoter. Therefore, it is likely that the *kilA* promoter is the only promoter used for expression of *klaA*, *klaB*, and *klaC* in *E. coli*. Our studies do not rule out the possibility that the region encodes cryptic promoters that are active in other gram-negative hosts.

Hydrophobicity profiles of the *klaA* and *klaB* products indicated that these polypeptides are likely to be cytoplasmic proteins, whereas the *klaC* product shows the characteristics of a membrane protein. We established that the first of two ATG codons with good Shine-Dalgarno sequences is used to initiate translation of the *klaC* product. However, we noticed that the amino-terminal region of the hypothetical polypeptide initiated from the second site resembles a signal peptide (51). Although we see no evidence for initiation from the second ATG, it remains possible that this potential translational start is used under certain conditions or in specific hosts. Two other RK2 genes, *trfA* (initiator) and *pri* (primase), are known to initiate translation from two different

start sites (39, 61). In the case of *trfA*, this property may have relevance to the host range of RK2 (15, 63).

The first *klaC* start codon overlaps the TGA termination codon for *klaB* in an ATGA motif also observed in the *korA-incC2* and *incC-korB* overlaps in the *korA-korB* operon (33, 77). This arrangement has been observed for coordinately expressed genes and suggests translational coupling (12). The *klaB* polypeptide was more abundant than the product of *klaA* or *klaC* in extracts from cells carrying the T7 ϕ*10 kilA* fusion plasmids (data not shown). One possible explanation is that *klaB* contains significantly fewer codons considered rare for genes of *E. coli* and *P. aeruginosa*. This property of *klaB* may explain the results of Walter and Taylor (81), who expressed the *kilA* region in vitro by using a coupled transcription-translation system. The only visible product was a 40-kDa polypeptide, which is likely to be the *klaB* product.

These studies have clearly established that three apparently unrelated phenotypes are intimately associated with the *kilA* operon. What do these phenotypes tell us about the possible function of *kilA*?

Host cell lethality arising from uncontrolled expression of any one of the *kla* genes indicates that all three gene products interact with crucial host cell components and that lethality probably results from titration of essential host functions or disruption of important structures. The degree of expression of the *kilA* operon determines whether or not it is lethal to its host. *korA* and *korB* eliminate the lethal phenotype of the *kilA* operon by reducing expression of the *kla* genes, although some expression does occur, as evidenced by the Te^r and fertility inhibition phenotypes. Mutations in the promoter can eliminate the lethal phenotype in the absence of *korA* and *korB*. The −35 region of the *kilA* promoter (TTGACG) overlaps the *Hin*cII site (GTTGAC) (Fig. 2). Insertion of the ΩCm fragment at the *Hin*cII site (pRK2781) changes the −35 region to AGGACG, predicted to be marginally functional, if at all (26). Indeed, this mutation abolishes expression of all of the phenotypes studied here. In an earlier study, we found that deletion of the upstream region and modification of the *Hin*cII site to one of its alternative recognition sequences (GTCGAC) leaves a functional −35 region (TCGACG) that allows some expression, but at levels too low to give a lethal phenotype (88). This mutation is also present in the −35 region of the *kilA* plasmid studied by Walter et al. (82) and is most likely responsible for the absence of a host-lethal phenotype in their derivative.

Some plasmids, like F (46, 49) and R1 (21, 22), are known to encode lethal functions that enhance the maintenance of the plasmid in a population of growing cells. The lethal gene products kill plasmidless segregants after cell division, thereby maintaining a population of plasmid-containing cells. In these types of systems, posttranscriptional regulatory mechanisms are required to control the lethal functions. We have not ruled out such a role for the *kilA* locus of RK2, but at present there is no evidence for posttranscriptional control of lethality.

Fertility inhibition is a widespread phenomenon among plasmids (30). A selective advantage of such a function is apparent in cases where the formation of sex pili is inhibited, thereby reducing the sensitivity of the population to pili-specific phages. The only well-characterized mechanism of fertility inhibition is that directed against the F plasmid of *E. coli* (84). Inhibition of conjugal transfer is caused by plasmid gene products that prevent the expression of a positive activator of the F-plasmid transfer operon. Plasmids with F-plasmid-like transfer functions normally regulate expression of their transfer operons by this mechanism. In contrast, the fertility inhibition exhibited by the RK2 *kilA* operon towards IncW plasmids does not appear to be involved in regulation of RK2 transfer. Plasmids missing the *kilA* region transfer at about the same frequency as wild-type RK2 (17). There is a paucity of information about mechanisms of inhibition of plasmid transfer by unrelated or distantly related plasmids. However, conjugal transfer of DNA is a complex process, and one can imagine several potential targets for inhibition. If we consider the possibility that KlaC protein forms a membrane-bound complex, perhaps with KlaA and KlaB, then this complex might interfere with any of a number of membrane-involving steps in conjugation: sex pilin polymerization or depolymerization, formation of the conjugal pore, or assembly of the DNA localization and transfer apparatus.

The RK2 *kilA* operon specifies one of at least three unrelated classes of plasmid-encoded Te^r. The determinant on the *Alcaligenes* IncHI-2 plasmid pMER610 is inducible and appears to encode five genes, one of which is thought to specify a membrane protein (31). The Te^r encoded by *E. coli* IncHII plasmid pHH1508a involves one or two polypeptides and is expressed constitutively (81). The third class of Te^r determinant is found on the α subgroup of IncP plasmids, which includes RK2 (7, 81). On these plasmids, Te^r is normally cryptic and requires a plasmid mutation to be detected. Our results show that the wild-type RK2 *kilA* operon cloned on a high-copy-number plasmid allows the host cells to grow on significantly higher levels of potassium tellurite. This result agrees with the findings of Fong and Stanisich (20), who detected a slight increase in Te^r with wild-type RP1 in a host expressing a very low background level of resistance. Our studies also revealed that expression of Te^r and the ability to select high-level Te^r mutants requires a functional *kilA* promoter and the products of *klaA*, *klaB*, and *klaC*.

The mechanism of RK2-mediated resistance to tellurite is not known. Bacterial colonies show a gray-black color from accumulation of tellurium formed by reduction of tellurite. Electron microscopy has revealed the formation of tellurium crystals on the surface of resistant cells (72). The possible mechanisms might therefore include (i) a membrane-bound reductase, similar to that used in heavy metal resistance (64), (ii) inhibition of tellurite uptake, and (iii) promotion of tellurite efflux. For mechanisms (ii) and (iii), the observed reduction of tellurite (gray-black colonies) might be catalyzed by the low intrinsic activity exhibited by the host cells, which are now able to survive on higher levels of tellurite. The requirement for all three *kla* gene products and the prediction that KlaC is a membrane protein is consistent with the possibility that a KlaABC complex mediates resistance at the membrane. One way an RK2 mutation could lead to high-level Te^r is to increase the expression of the *kilA* operon. Indeed, one class of Te^r mutants of a plasmid carrying the *kilA* operon shows an alteration in the *kilA* promoter predicted to increase expression (24a). Mutations of this class are limited by the deleterious effects on the cells caused by increased expression of the *kilA* operon. Another expected class of Te^r mutants includes those with alterations in the *kla* structural genes. Recently, Walter et al. (82) have reported the nucleotide sequence of the *kilA* region of a high-level Te^r derivative of RK2. The genes analogous to *klaA*, *klaB*, and *klaC* are called *kilA*, *telA*, and *telB*. By comparing the mutant sequence with that of the wild-type *kilA* region reported here, it was possible to pinpoint the

resistance phenotype to a single nucleotide change in *klaC* (*telB*), suggesting a possible interaction of the KlaC protein with tellurite.

The disparate phenotypes have not made it possible to identify the actual function of the *kilA* operon. Choosing one of the known phenotypes as the function requires that the other phenotypes be explained as inadvertent, although all of the phenotypes should ultimately provide valuable information about mechanism. Host cell lethality per se is not informative because many genes can have deleterious effects when overexpressed. If the function of the *kilA* operon is to provide resistance to tellurite, then fertility inhibition must be a side effect. Moreover, it is curious that Te^r should be cryptic in all IncPα plasmids (7). If the function of *kilA* is to inhibit conjugal transfer of coresident IncW plasmids, the potential for Te^r must then be a fortuitous property of the locus. It seems equally plausible that interaction of a hypothetical KlaABC complex with the membrane has pleiotropic effects that include both Te^r and fertility inhibition.

In first reporting the existence of multiple *kil* loci on RK2, Figurski et al. (18) suggested that these determinants encode auxiliary functions involved in the maintenance or broad host range of IncP plasmids. This view was based on the finding that the *korA* and *korB* functions needed for regulation of the *kil* determinants were conserved on all of 14 IncP plasmids tested, including members of both the α and β subgroups. Heteroduplex analysis of IncP plasmids has indicated that the *kilA* region is clearly present in members of the IncPα subgroup (80). Using probes specific for *klaA*, *klaB*, and *klaC*, we have obtained additional evidence that all three genes of the *kilA* operon are indeed highly conserved among IncPα plasmids (24a). This degree of conservation is consistent with the hypothesis that the *kilA* operon has an important function for these broad-host-range plasmids.

Neither Te^r nor fertility inhibition addresses the conservation of the *kilA* operon or the fact that the *kilA* operon is coregulated with the *trfA* replication initiator. Coordinate regulation of a gene known to be essential for replication hints that the functions are related and supports the proposal that *kilA* is involved in some aspect of plasmid maintenance or host range. Studies with mini-RK2 plasmids have shown that *kilA* is clearly not essential for replication (76) or the functioning of a known partition locus (23, 52). Perhaps the proteins encoded by the *kilA* operon assemble at the membrane (via KlaC) and provide a more controlled environment for initiation of replication or partition in several different hosts. Thus, while not essential for promiscuous replication or partition, the *kilA* locus may nevertheless be important for IncP plasmid survival in nature. Now that the nucleotide sequence of *kilA* is known, it is possible to construct the appropriate mutants of RK2 to test this hypothesis.

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