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Gene control in broad host range plasmid RK2: Expression, polypeptide product, and multiple regulatory functions of *korB*

(operon/autoregulation/*korA*/co-repressor/replication control)

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ABSTRACT **The *korB* gene of broad host-range plasmid RK2 prevents host-cell lethality by *kilB* and negatively controls RK2 replication. We precisely mapped the limits of *korB* to a region near *korA*, an autoregulated gene involved in control of several RK2 genes. The following results show that *korA* and *korB* are cotranscribed from the *korA* promoter: (*i*) Mutants deleted for the *korA* promoter fail to express *korB*, even with *korA* function supplied in *trans*; (*ii*) the *korA* promoter is nonessential to *korB* if a heterologous promoter is present; and (*iii*) RNA produced *in vivo* has both *korA*- and *korB*-specific sequences. Analysis of polypeptides synthesized from wild-type and mutant *korB* plasmids in maxicells revealed that *korB* encodes a 52-kDa polypeptide, whose activity is extremely sensitive to changes in its carboxyl terminus but relatively unaffected by replacement of its amino terminus. The minimal *korB*-encoding region allowed us to identify two new regulatory functions, both of which duplicate previously known functions of *korA*. First, *korB* alone was found to control the *kilB1* component of *kilB*, thus resolving the paradox of *korA*-independent control of *kilB*. Second, analysis of polypeptides from the *korA–korB* region in the presence and absence of *korB*, and studies with the *korA* promoter fused to the chloramphenicol acetyltransferase structural gene (*cat*) showed that *korB*, like *korA*, autoregulates expression of the *korA–korB* operon. We suggest that *korA* and *korB* gene products act as co-repressors in the control of certain RK2 genes.**

Plasmids of incompatibility group P (IncP) replicate in greatly diverse Gram-negative bacterial hosts (1, 2). The genetic functions that allow this exceptional host range are not yet understood. RK2, a 56.4-kilobase-pair (kbp) self-transmissible IncP plasmid (3, 4), has two genetic determinants essential to its replication (5–14): an origin of replication, *oriV*, and a gene in the *trfA* operon that specifies a polypeptide needed to activate *oriV*. RK2 also encodes several other genes that are both common among and unique to plasmids of the IncP group: *kil* genes (*kilA*, *kilB1*, *kilB2*, and *kilC*), which are potentially lethal to *Escherichia coli* host cells, and *kor* genes (*korA*, *korB*, and *korC*), which prevent the lethal action (15, 16). They form a regulatory network, in which *korA* is the central control element (16). *korA* negatively controls *kilA* (15, 17, 18), *kilB1* (ref. 19; this work), and its own expression (18), and it has a positive effect on expression of *korC* (20). Less is known about *korB*, which is required to control *kilB2* (15, 19), and *korC*, which regulates *kilC* (15, 20). It is now evident that *kil* and *kor* functions are involved in the control of RK2 replication by regulating expression of the essential replication gene *trfA* (16, 21–23).

We report here our studies on *korB*. By mapping the gene precisely and by constructing specific mutants, we unambiguously identified its polypeptide product and showed that *korB* is expressed in an operon with *korA*. In addition to their known functional overlap in the control of *trfA* (16, 21–23), two new examples of redundant control were found. Like *korA*, *korB* can (*i*) prevent the action of *kilB1* and (*ii*) autoregulate expression of the *korA–korB* operon. We argue that *korA* and *korB* gene products sometimes function as co-repressors in RK2 gene control.

MATERIALS AND METHODS

Nomenclature. RK2 coordinates (distance from the *Eco*RI site, in kbp) are indicated with a prime (e.g., 50′–56.4′ region). Superscript "o" shows that a relevant plasmid gene is not present (e.g., *korA*°).

Bacterial Strains. *E. coli* MV10 (24) and the maxicell strain CSR603 (25) were grown in LB, M9, or M9-CAA media supplemented, if necessary, with L-tryptophan at 50 μg/ml (15). Transformation of *E. coli* (26) and concentrations of antibiotics used for selection (15) have been described.

Manipulation and Analysis of Nucleic Acids. Methods for preparation and gel electrophoresis of plasmid DNA are detailed elsewhere (27). Enzymes and DNA linkers were purchased. ^{32}P-labeled DNA fragments were prepared by nick-translation with DNA polymerase I (28). Extraction, fractionation, and hybridization analysis of RNA from *E. coli* were done as described (17, 29, 30).

Analysis of Polypeptides. Plasmid-encoded polypeptides were selectively labeled with a mixture of ^{14}C-labeled amino acids (ICN) in maxicells (25), separated by electrophoresis through 12% $NaDodSO_4$/polyacrylamide gels (with 5% stacking gel), and visualized by autoradiography essentially by a published protocol (11).

RESULTS AND DISCUSSION

Location of *korB* on RK2. Interruption of the *Sst* II site in the 50′–56.4′ region of RK2 causes loss of *korB* function (15). We therefore cloned the 3.1-kbp *Sst* II-containing *Hin*cII fragment and found that this segment expresses *korB* regardless of its orientation (pRK2177, pRK2178; Fig. 1). *korA* maps to one end of the *Hin*cII fragment (Fig. 1) (17). The *Hin*cII site actually occurs within the −35 region of the *korA* promoter, but cloning the fragment into the *Hin*cII site of the vector regenerated a reasonable −35 sequence, and the plasmids express *korA* (17).

Deletion analysis showed that it was possible to remove the internal *Not* I fragment (pRK2289) or the *Bss*HII fragments (pRK2300) without losing *korB* function (Fig. 1). However, it was not possible to construct KorB$^+$ plasmids deleted for one

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Abbreviations: bp, base pair(s); Cm, chloramphenicol; Km, kanamycin; ^{r}, resistant.

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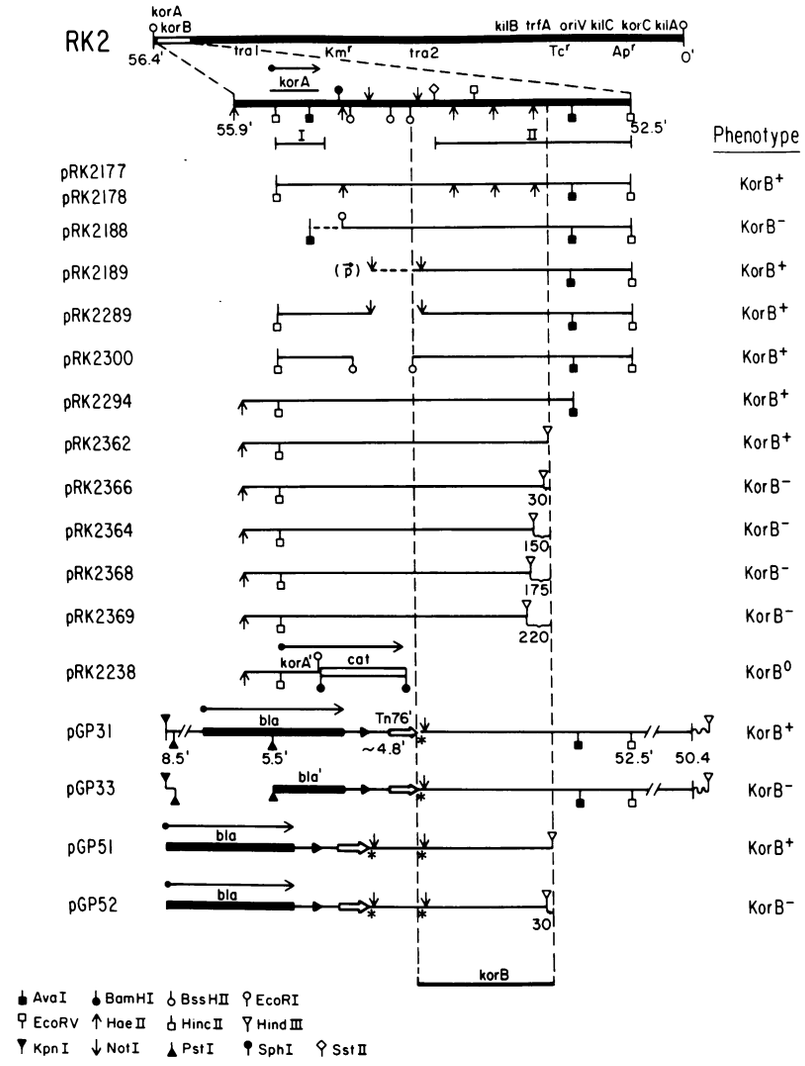

FIG. 1. Physical mapping of *korB*. RK2 map is linearized at the unique *Eco*RI site. Below it is the restriction map of the region encoding *korA* and *korB*. "I" [from pRK2241 (17)] and "II" (*Sst* II/*Hin*cII fragment) show regions used as hybridization probes specific for *korA* and *korB*, respectively (see text). Arrows over genes indicate their direction of transcription. In the text, the leftmost *Hin*cII, *Ava* I, *Bss*HII, and *Not* I sites are designated *Hin*cII^a, *Ava* I^a, *Bss*HII^a, and *Not* I^a, respectively; the rightmost counterparts have superscript b. Plasmids were constructed as follows: pRK2177 (Km^r, P15A replicon), insertion of the 52.5'–55.6' *Hin*cII fragment into the *Hin*cII site of pACYC177 (31) with the nearby *bla* promoter of the vehicle reading opposite to *korA*; pRK2178, as for pRK2177, but in the opposite orientation; pRK2188, BAL-31 deletion of pRK2178 made by insertion of a *trpE*-encoding fragment into the *korA* proximal *Bam*HI site of the vehicle, cleavage at the *Eco*RI site within the inserted fragment, digestion with BAL-31, and recircularization with T4 DNA ligase (dotted line shows region that contains the deletion end point) (32); pRK2189, as pRK2188, but with a different deletion end point and a possible promoter (p); pRK2289 and pRK2300, *Not* I and *Bss*HII deletions, respectively, of pRK2178; pRK2294 [ampicillin resistance (Ap^r), P15A replicon], insertion of the *Ava* I fragment of pRK2178 into the *Ava* I site of the *korA* remnant in pRK2219 (17); pRK2362, pRK2364, pRK2366, pRK2368, pRK2369, BAL-31 deletion mutants of pRK2294 made by cleavage at the *Eco*RI site to the right of *Ava* I^b, BAL-31 digestion, addition of *Hin*dIII linkers, and recircularization with ligase (numbers show approximate sizes of deletions relative to the end point in pRK2362); pRK2238 [Km^r Cm^r, pBR322 replicon (33)], insertion of the *Pst* I Km^r-encoding fragment of pIF11 (27) into the *Pst* I site of pRK2237 (18), which has a promoterless *Bam*HI *cat* cassette from pCM4 (34); pGP31 (Ap^r, *trpE*$^+$, ColE1 replicon), marker rescue of the *korA* deletion of pRP761-6 (35, 36) onto pRK2102 (15); [*bla* is the β-lactamase gene of RK2 present in transposon Tn*1*, one terminus of which is shown by the filled arrow; Tn*76'* is a remnant of transposon Tn*76* (35) with one end at approximately 4.8' on the RK2 physical map and the other end 60 bp from *Not* I^b; wavy line depicts a remnant of phage Mu (15)]; pGP33 (*trpE*$^+$, ColE1 replicon), *Pst* I deletion of pGP31; pGP51 and pGP52 (Ap^r, ColE1 replicon), replacement of the *trpE*-encoding region of pGP31 with the 300-bp *Eco*RI/*Hin*dIII fragment of pMB9 (37) and subsequent replacement of the *Not* I^b to *Hin*dIII region with *Not* I^a/*Hin*dIII fragments of pRK2362 and pRK2366, respectively. (Asterisks indicate the resulting 60-bp duplication.) KorB$^-$ phenotype indicates a decrease by at least a factor of 50 in the ability to form colonies (relative to a known KorB$^+$ strain) after transformation by the *kilB*$^+$ plasmids pRK2133 or pRK2162, as described (15); KorB$^+$, no significant difference. Transformants of pRK2189-containing host give colonies smaller than those of a pRK2178-containing host.

or more of the five *Hae* II fragments that cover this region (unpublished results). These results suggest that at least two separate determinants are required to express the KorB$^+$ phenotype.

We were able to remove the *Ava* I^b/*Hin*cII^b region without destroying *korB* (pRK2294; Fig. 1). BAL-31 exonuclease was then used to construct a set of nested deletions entering the *korB* region from *Ava* I^b. The transition from KorB$^+$ to KorB$^-$ phenotype occurred with pRK2362 and pRK2366, whose deletion end points are separated by ≈30 bp. We suggest that a terminus of one *korB* determinant occurs within this interval.

We also made BAL-31-generated deletions from the *korA* end of the *korB*-encoding *Hin*cII fragment. One derivative,

pRK2189 (Fig. 1), has a large deletion that removes *korA*. It confers a reasonable KorB$^+$ phenotype. Thus the *korA* region is not required, and all of the sequences essential to *korB* function must therefore lie between *Not* I^a and *Ava* I^b (Fig. 1).

Operon Structure of *korA* and *korB*. Another derivative, pRK2188, is typical of the other BAL-31-generated mutants. Its deletion removes at least part of *korA* (Fig. 1); but unlike pRK2189, it confers a KorB$^-$ phenotype, even when a *korA*$^+$ plasmid is maintained in *trans* (unpublished results). Therefore, some determinant in the *korA* region is required in *cis* for expression of the KorB$^+$ phenotype. This is consistent with the earlier results with the *Hae* II deletions.

It seemed possible that *korB* might normally be expressed from the *korA* promoter, but that in the exceptional pRK2189, a new promoter was fused to the structural gene. Evidence in support of this came from analysis of another *korA*$^-$ *korB*$^+$ mutant, pRP761-6. This plasmid arose by an *in vivo* rearrangement of pRP761, a host-range mutant of RP4 (identical to RK2) (35, 36). By marker rescue, we transferred the rearranged region onto a smaller plasmid (pGP31; Fig. 1). Its structure shows that pRP761-6 has undergone a deletion that removed *korA*, *kilA*, and *korC* and placed *korB* just downstream of the β-lactamase gene (*bla*). Deletion of the *Pst* I fragment that carries the *bla* promoter abolished *korB* expression (pGP33; Fig. 1). This clearly shows that *korB* expression is dependent on the heterologous *bla* promoter in pGP31 and that *korB* is transcribed in the same direction as *korA* in RK2. Thus the minimal region essential for *korB* does not have its own promoter, but instead requires a promoter further upstream. This is consistent with the results of Smith and Thomas (38). Using transcriptional fusions to *galK*, they suggested that transcription in the *korB* region occurs primarily from an upstream promoter at or near the *Hin*cII site. This is the *korA* promoter (17).

To test whether *korA* and *korB* form a single transcriptional unit, we did blot hybridization analysis of RNA synthesized *in vivo*. DNA fragments specific for *korA* and *korB* (see Fig. 1) were radioactively labeled and hybridized to total RNA that was isolated from cells carrying RK2 or pRK2108 [a high copy number plasmid with the cloned 50.4′–56.4′ region of RK2 (15)] and separated on agarose/formaldehyde gels. The *korB* probe detected five RNA species, four of which were the same size as those detected by the *korA* probe (Fig. 2). The probes do not cross-hybridize (unpublished results), and the *korA* RNAs are known to be synthesized in the same direction on RK2 (17). Thus, nearly all of the transcription products from the *korA–korB* region have sequences from

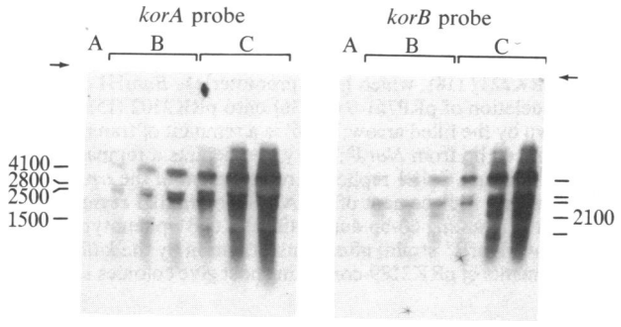

FIG. 2. Hybridization analysis of *korA*- and *korB*-specific RNA synthesized *in vivo*. Blot hybridization of RNA from *E. coli* strains was done with ^{32}P-labeled DNA fragments specific for the *korA* or *korB* region (see Fig. 1). Lanes A contain 10 μg of RNA from MV10; lanes B contain 5, 10, and 15 μg of RNA, respectively, from MV10(RK2); lanes C contain 5, 10, and 15 μg of RNA, respectively, from MV10(pRK2108). Arrows mark the sample origin. Numbers refer to approximate lengths of the RNA species in bases.

both genes. This and the genetic evidence shown above lead us to conclude that *korB* is primarily expressed as part of an operon with *korA*.

Polypeptide Product of *korB*. Polypeptides specified by the various plasmids in maxicells (25) are shown in Fig. 3. The *korA*$^+$ *korB*$^+$ plasmids pRK2177, pRK2178, and pRK2294 showed five polypeptides in common (Fig. 3; lanes 5–7). In addition, RK2 itself shows polypeptides that comigrate with those specified by the cloned region (lane 4). We can tentatively identify the 13-kDa polypeptide as the product of *korA* because it is predicted from the nucleotide sequence (17). Plasmids pRK2177 and pRK2178 carry the *Hin*cII fragment in opposite orientations relative to a nearby *bla* promoter in the vehicle. Because pRK2178 shows higher polypeptide levels (lanes 5 and 6), the direction of transcription of the genes for these polypeptides is the same as that expected for *korB*.

To identify *korB* product, we examined the BAL-31-generated deletion mutants of pRK2294 (Fig. 4). All *korB*$^-$ plasmids showed loss of the 52-kDa polypeptide. In its place are new polypeptide species of different mobilities, as expected for deletion mutations that enter the 3′ end of the structural gene for the 52-kDa polypeptide. Because these deletion mutations cause loss of both *korB* function and the 52-kDa polypeptide, we conclude that the 52-kDa polypeptide is essential for *korB* function.

We also examined mutants in which the region upstream of the *korB* determinant is deleted (Fig. 5). Of the five polypeptides shown to be specific to the *korA–korB* region, only the 13-kDa *korA* product and the 52-kDa polypeptide are expressed from a plasmid (pRK2300) that is missing the *Bss*HII fragments (lane 4). Thus the 52-kDa product appears to be necessary and sufficient for *korB* function.

From a comparison of various *korB*$^+$ and *korB*$^-$ plasmids, Smith and Thomas (38) predicted that a 49-kDa polypeptide is involved in *korB* function. Our results with specific well-defined isogenic mutants, whose fusion products were identifiable, show unequivocally that this is the *korB* product, although our estimate of its mass is 52 kDa.

If the end of the structural gene for the 52-kDa product occurs in the interval between the deletion end points of pRK2362 and pRK2366 (Fig. 1), then its start is estimated to occur between the two *Not* I sites of the *korA–korB* region.

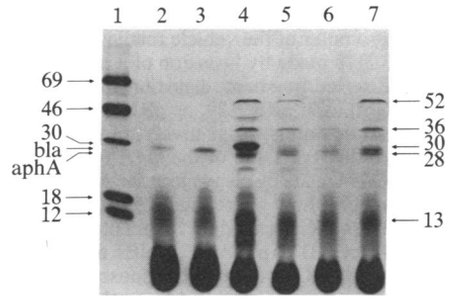

FIG. 3. Polypeptides specified by the *korA–korB* region. Plasmid-encoded polypeptides were specifically labeled with ^{14}C-labeled amino acids in maxicells, separated by NaDodSO$_4$/polyacrylamide gel electrophoresis, and visualized by autoradiography. Lanes: 1, markers [^{14}C-labeled bovine serum albumin, ovalbumin, carbonic anhydrase, lactoglobulin, and cytochrome *c* (69, 46, 30, 18, and 12 kDa, respectively) from New England Nuclear]; 2, pKJ1; 3, pDB6; 4, RK2; 5, pRK2178; 6, pRK2177; 7, pRK2294. For pKJ1, a P15A ampicillin-resistant (Ap^r) plasmid (19), and pDB6, a P15A Km^r plasmid (32), 75-μl samples were used for electrophoresis; all other samples were 50 μl. Numbers on left and right refer to mass in kDa of markers and *korA–korB* region-specific polypeptides, respectively. bla is the β-lactamase responsible for Ap^r of pKJ1, and pRK2294 (39); aphA is the aminoglycoside 3′-phosphotransferase responsible for Km^r of pDB6, pRK2177, and pRK2178 (40).

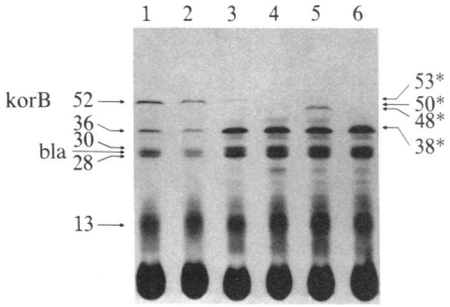

FIG. 4. Identification of the *korB* product. Polypeptides specified by BAL-31-generated deletion mutants of pRK2294 were labeled in maxicells, separated by NaDodSO$_4$/polyacrylamide gel electrophoresis, and visualized by autoradiography, as described in *Materials and Methods* and in Fig. 3. All samples were prepared from the same number of cells. *korB*$^+$: lane 1, pRK2294; lane 2, pRK2362. *korB*$^-$: lane 3, pRK2366; lane 4, pRK2364; lane 5, pRK2368; lane 6, pRK2369. Numbers on left show mass in kDa of *korA–korB* region-specific polypeptides; asterisks denote fusion polypeptides from pRK2366 (53 kDa), pRK2364 (50 kDa, not visible on this exposure), pRK2368 (48 kDa), and pRK2369 (38 kDa).

Indeed, a plasmid deleted for the *Not* I fragment (pRK2289; Fig. 1) does not express a normal 52-kDa polypeptide. In its place is a 71-kDa fusion product (Fig. 5, lane 3). We know the fusion retains the reading frame used for the 52-kDa product because *Not* I deletions of the BAL-31-generated mutants pRK2366, pRK2364, pRK2368, and pRK2369 all give new fusion products whose sizes differ by the same amounts as the original fusion products (unpublished results). Remarkably, the phenotype of pRK2289 is KorB$^+$. Thus the *Not* I deletion of pRK2289 has resulted in an in-frame fusion of an upstream translational start to the *korB* structural gene without destroying *korB* function.

These data show that the 5′ end of *korB* occurs between *Bss*HII^b and *Not* I^b (Fig. 1). Because pRK2289 controls *kilB* efficiently, it appears that the amino terminus of the *korB* product is less important to its regulatory activity than the carboxyl terminus, where an apparently small change had a profound effect (e.g., pRK2362 vs. pRK2366).

Redundant Control of *kilB1*. The *kilB* determinant consists of at least two components: *kilB1* and *kilB2* (19). Although the *korA* region cannot control the complete *kilB* determinant, it does control the *kilB1* component (19). We therefore expected that *korA* would be necessary for control of the complete *kilB* determinant. The results presented above showing that *korA* is not essential suggested that *korB* alone can also regulate *kilB1*. To test this, we used a *kilB1*$^+$ *kilB2*$^-$ plasmid to transform cells carrying the minimal *korB* region (pGP31, pGP51; Fig. 1). The results (Table 1) show clearly that *korB* alone, like *korA*, is able to control *kilB1*.

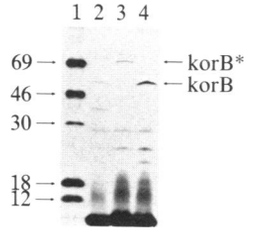

FIG. 5. Effect of deletion of the *Bss*HII and *Not* I fragments on polypeptides from the *korA–korB* region. Labeling, gel electrophoresis, and autoradiography of polypeptides is described in *Materials and Methods*. Lanes: 1, markers; 2, pRK2178; 3, pRK2289 (*Not* I deletion); 4, pRK2300 (*Bss*HII deletion). korB and korB* show the 52-kDa *korB* product and the 71-kDa fusion product, respectively.

Table 1. Control of *kilB1* by *korB*

Resident plasmid*	Genotype	Relative transformation efficiency by a *kilB1*$^+$ plasmid†
None	*korA*° *korB*°	<0.001
pRK2294	*korA*$^+$ *korB*$^+$	1.0
pRK2323	*korA*$^+$ *korB*°	1.0‡
pRK2324	*korA*$^-$ *korB*°	<0.001
pGP31	*korA*° *korB*$^+$	0.8
pGP51	*korA*° *korB*$^+$	1.7‡
pGP52	*korA*° *korB*$^-$	<0.001

*pRK2323 and pRK2324 are identical to pRK2240 and pRK2241 (17), respectively, except for a *trpE*-encoding fragment inserted at the *Hin*dIII site in the tetracycline-resistant (Tc^r) determinant. Other plasmids are shown in Fig. 1.

†The *kilB1*$^+$ plasmid was pRK2172, constructed by cloning the Tc^r *trfA–kilB1*-encoding *Eco*RI fragment of pRK2164 (19) into the pSM1 vehicle pCY2 (15). Cells were transformed with pRK2172 and Tc^r colonies were selected. Efficiency of transformation is normalized to the pRK2294-containing strain. Relative competence of the strains was monitored as described (15).

‡These colonies were noticeably smaller.

***korB* Regulates Expression of the *korA–korB* Operon.** The *korB*$^-$ plasmids showed obvious increases in the levels of the other polypeptide species (Fig. 4). This suggested that *korB* function might exert negative control on expression of genes in the *korA–korB* operon.

We tested the possibility that *korB* function inhibits transcription initiated at the *korA* promoter. In pRK2238, the structural gene for chloramphenicol acetyltransferase (*cat*) is expressed from the *korA* promoter (Fig. 1), such that cells carrying this plasmid are chloramphenicol resistant (Cm^r). It also carries a constitutively expressed gene for kanamycin resistance (Km^r) elsewhere in the vehicle. Transformation of *kor*° cells with pRK2238 yields approximately equal numbers of Km^r and Cm^r colonies (Table 2). However, if a *korA*$^+$ or *korA*$^+$ *korB*$^+$ plasmid is present in the transformed cells, the ratio of the number of Cm^r colonies to that of Km^r colonies is decreased by at least a factor of 100. This is expected because *korA* negatively controls the *korA* promoter (18). We also tested *korA*° *korB*$^+$ plasmids and found that *korB*, like *korA*, negatively regulates the *korA–korB* operon by inhibiting expression from the *korA* promoter.

In summary, this work expands the complex network of *kil* and *kor* interactions to include the following: (*i*) *korB* is expressed as part of an operon with the important regulatory

Table 2. Effect of *korB* on expression of *cat* fused to the *korA* promoter

Resident plasmid*	Genotype	Relative transformation efficiency by a p*korA–cat* plasmid, Cm^r/Km^r†
None	*korA*° *korB*°	0.9
pRK2108	*korA*$^+$ *korB*$^+$	<0.01
pRK2292	*korA*$^+$ *korB*°	<0.01
pGP31	*korA*° *korB*$^+$	<0.01
pGP51	*korA*° *korB*$^+$	<0.01
pGP52	*korA*° *korB*$^-$	0.6

*pRK2108 is a pSM1 plasmid with the 50.4′–56.4′ region of RK2 (15). pRK2292 is a pSM1 plasmid with the *korA* structural gene expressed constitutively as in pRK2240 (17). Other plasmids are shown in Fig. 1.

†Recipient strains were transformed with pRK2238 and plated for Cm^r (100 μg/ml) or the constitutively expressed Km^r marker. Values indicate the ratio of the number of Cm^r transformants to the number of Km^r transformants.

gene *korA*; (*ii*) *korB*, like *korA*, can autoregulate expression of this operon; and (*iii*) either gene is able to control the *kilB1* component of the *kilB* determinant. Other studies have shown that *korA* and *korB* can each regulate expression of the essential RK2 replication gene *trfA* (16, 21–23). Thus, *korA* and *korB* functions display remarkable redundancy in the control of RK2 genes.

Why should *korA* and *korB* show such duplication of their control functions, and why should they be coexpressed in an operon that can be regulated by either component? One possibility is that *korA* and *korB* products act as co-repressors. It would therefore be logical for them to be expressed together to maintain the appropriate stoichiometry. There are two operator-like palindromes (I and II) in the promoter regions of *trfA* and *korA* (41). Palindrome I (near the −35 region) is predicted to be the target for *korB* function (41), and palindrome II (in the −10 region), the target for *korA* function (18, 41). The *kilA* promoter sequence also includes both palindromes (18), suggesting that *korB* may enhance regulation of *kilA* as well, a possibility we are examining. In all three promoters, the spacing between the two palindromes is nearly identical. We suggest that this distance is important to allow specific contacts between korA and korB proteins to produce a stable co-repressor complex that is highly effective in its interaction with the promoters.

It is interesting that *korA* and *korB* are only two genes of an operon that specifies at least three other polypeptides in *E. coli* (refs. 11 and 38; this work), two of which are clearly visible in maxicells carrying parental RK2 (Fig. 3). Several studies implicate other maintenance functions for the *korA–korB* region, including incompatibility (36, 38, 42) and host-range determinants (35, 36, 43, 44), that are distinct from *korA* and *korB*. Thus, it seems that the *korA–korB* operon is of major importance to the survival of RK2 in nature.

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