The IncN plasmid pKM101 contains a group of 11 genes thought to be required for the synthesis of its conjugal pilus and mating pore. Within this region are two genes, kilA and kilB, either of which is conditionally lethal to the cell. kilA was previously shown to be allelic with traL, and we now show that kilB is allelic with traE.

In the same region, genetic studies previously defined two loci, korA and korB (kor for kill override), which together prevent lethality mediated by kilA and kilB. We now identify the genes that encode KorA and KorB functions. To determine whether KorA and KorB proteins influence tra gene transcription, we constructed β-galactosidase fusions to three promoters in this region and measured their expression in the presence of KorA, KorB, and both proteins. KorA and KorB together repressed transcription of all three promoters, while neither protein alone affected transcription. We identified all three transcriptional start sites by primer extension analysis. Two putative binding sites for these proteins, designated kor boxes, contain 26 identical nucleotides in a 29-nucleotide region. The electrophoretic mobilities of DNA fragments containing kor boxes were retarded by cell extracts containing both KorA and KorB but were not retarded by extracts containing just KorA or just KorB. DNase I footprinting analysis of one of these promoters demonstrates that KorA and/or KorB binds to a region containing a kor box.

Self-transmissible plasmids that confer resistance to one or more antibiotics play an important role in horizontal transfer of antibiotic resistance genes and therefore are of increasing clinical importance (27). Transfer requires the expression of plasmid-encoded tra genes, which are often controlled by intricate regulatory mechanisms. Although the tra genes of the F plasmid are expressed constitutively, F-like plasmids such as R100 use the FinO protein and the FinP regulatory RNA to repress the traJ gene, whose product activates transcription of other tra genes (6, 33, 34). tra genes of the broad-host-range plasmid RK2 are repressed by the KorB and TrbA proteins, although expression of these promoters remains sufficient for highly efficient conjugation (11, 18, 19, 28). tra genes of the Streptomyces coelicolor plasmid pSN22 are also negatively regulated (14, 21). In contrast, both of the two DNA transfer systems of Agrobacterium tumefaciens Ti plasmids are positively regulated. The vir regulon, whose products transfer T-DNA to plant cells, is activated by the VirA and VirG two-component proteins, while the Ti plasmid tra genes are activated by a quorum-sensing system via the transcriptional activator TraR and the autoinducer synthase TraI (9, 10, 36). We now describe two proteins that negatively regulate tra promoters of the IncN plasmid pKM101.

pKM101 is a deletion derivative of the IncN plasmid R46, which was isolated from Enterobacter cloacae and replicates efficiently in many enteric bacteria (1). Although R46 contains an integron and confers resistance to streptomycin, tetracycline, sulfonamides, arsenate ions, and penicillins, pKM101 mediates resistance only to penicillins (13, 26). pKM101 is used in the Ames Salmonella typhimurium mutagen tester strains, in which it dramatically enhances the sensitivity of these strains to the mutagenic effects of a broad spectrum of mutagenic agents (24). pKM101 has a highly efficient and phenotypically unregulated conjugal transfer system (16, 37). It encodes 14 tra genes, 3 of which are required for conjugal DNA processing and 10 of which are thought to encode a conjugal mating bridge (Fig. 1) (29). These 10 genes plus traL are homologous to the 11 genes of the virB operon of A. tumefaciens, and 9 of them are also homologous to the 9 genes of the ptl operon of Bordetella pertussis, which directs the secretion of pertussis toxin (20, 29, 35).

Two genes in the tra region are able to prevent host cells from forming colonies (38). This so-called Kil phenotype was prevented by providing two additional pKM101 genes, designated korA and korB (kor for kill override) (38). The mechanisms by which kor genes permitted colony formation was not understood. One such Kil gene was shown to be allelic with traL (29), and in this report we show that the other kil gene is allelic with traE. korA and korB were previously localized to a region of pKM101 close to traL (38), but neither gene could be unambiguously assigned to any of the open reading frames (ORFs) recently identified in this region (29). In the present study, we identified the korA and korB genes and used genetic and biochemical techniques to demonstrate that the products of these genes act together to repress at least three tra promoters.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and reagents.** E. coli MC4100 (F− thiAaraD39 dam− lacIqU169 rpsL150 relA1 b5351 dcm1 ptsG25 rbad) was obtained from C. Manoil, University of Washington. Table 1 lists the plasmids used in this study. E. coli RNA polymerase was purchased from Pharmacia Biotech (Piscataway, N.J.). DNase I was purchased from Gibco BRL (Gaithersburg, Md.). Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs (Beverly, Mass.) [α-35S]dATP and [γ-32P]dATP were purchased from Du Pont NEN Research Products (Boston, Mass.). Antibiotics, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), and o-nitrophenyl-β-D-galactopyranoside were purchased from Sigma Chemical Co. (St. Louis, Mo.). Synthetic oligonucleotides were obtained from the Cornell DNA Services. The junction between pKM101 DNA and Tn5 DNA in traE1228::Tn5 was determined using oligonucleotide 5′-GATCAACTCTGCAAGCCCTTTAACGCC-3′, which hybridizes to codons 21 to 29 of traE.

**Subcloning of korA and korB.** PCR amplifications were performed under standard conditions and procedures (15), using Taq DNA polymerase (Promega) and a Hybaid Thermal Reactor thermocycler. korA was amplified by using pKM101 DNA and the oligonucleotides 5′-GGCGGATCCCTTGAAGGGGAAATGGGTTATAGA-3′ and 5′-GGCGGATCCATTCAATATCTTCAGT-3′.
GAT-3' (underlined residues represent restriction endonuclease cleavage sites). The resulting fragment was digested with the restriction endonucleases EcoRI and BamHI and ligated to similarly digested pT718R, creating pMIM291. korB was amplified using pKM101 DNA, using the oligonucleotides 5'-CCGGGTATCTAGATCCGAAGAACTGCGAGACGAGGTTTTGGATGTTTACTCATTTAT-3' and 5'-GGCGAATTCGGAGTTTTGGATGTTTACTCATTTAT-3'. The resulting fragment was digested with the restriction endonucleases BamHI and PstI and cloned into pUC12Cm, creating pMIM292. Representative plasmids carrying korA or korB were sequenced on one strand by using Sequenase (U.S. Biochemicals) and [α-35S]dATP. A plasmid, a subcloning a korA and korB expressed as an operon from the Plac promoter was constructed by digesting pMIM292 with BamHI and PstI and ligating DNA fragment containing korB between the BamHI and PstI sites of pMIM291, creating pMIM293. korA and korB were also introduced by standard recombinant DNA techniques into other plasmid vectors (Table 1).

Subcloning and analysis of three pKM101 promoters. Three putative tra promoters were subcloned by PCR amplification. Pkor was subcloned as a 195-nucleotide fragment by using the oligonucleotides 5'-GGCGAATTCGGAAGTACGGGTTCGCCTTTTGGTCG-3' and 5'-GGCGGATCCCTGACCACCCGCGGATC-3'. Ptra, was subcloned as a 188-bp fragment by using the oligonucleotides 5'-GGCGAATTCGGAAGTACGGGTTCGCCTTTTGGTCG-3' and 5'-GGCGAATTCGGAAGTACGGGTTCGCCTTTTGGTCG-3'. PtraL was subcloned as a 190-bp fragment by using the oligonucleotides 5'-GGCGGATCCCTGACCACCCGCGGATC-3' and 5'-GGCGAATTCGGAAGTACGGGTTCGCCTTTTGGTCG-3'. The fragments containing the Pkor, Ptra, and PtraL promoters were digested with EcoRI and BamHI and cloned into pM1C403 cleaved with the same enzymes (3), which yielded plasmids pMIM277 and pMIM297, respectively. The fragment containing PtraL was digested with EcoRI and cloned into EcoRI-digested pM1C403, yielding plasmid pMIM287.

The DNA sequence of representative clones was checked on one strand, using Sequenase and [α-35S]dATP. β-Galactosidase specific activity was determined as described previously (25) after culturing strains in LB medium at 37°C with vigorous aeration.

Primer extension reactions. Primer extension reactions were performed by using RNA from strain MC1401 containing pMIM277 (PkorB), pMIM297 (PtraL), or pMIM297 (PtraN) in the presence or absence of pMIM299, which expresses KorA and KorB. The oligonucleotide 5'-TGATGGCAATCCCGCGTGGATGTTTACATGCTTTATGCCAGAT-3' was used to detect transcripts originating from PkorB, while the oligonucleotide and 5'-GGTTTCTCAAGTCACCGAAGGTTTTGGATGTTTACTCATTTAT-3' (which hybridizes to vector-derived DNA) was used to detect transcripts originating from both PtraL and PtraN. The oligonucleotide 5'-CAGGAGCGCAAGAACTGCGAGACGAGGTTTTGGATGTTTACTCATTTAT-3' was used to detect transcripts originating from the bla promoter of each plasmid. These oligonucleotides were radioactively labeled by using T4 polynucleotide kinase (New England Biolabs) and [γ-32P]ATP (Amersham). Hybridization reaction mixtures contained 25 ng of primer (approximately 10^6 cpm) and 5 μg of RNA in a buffer containing 40 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPE), pH 6.4, 1 mM EDTA, 0.4 M NaCl, and 80% formamide (30-μl total volume) and were heated to 85°C for 10 min and then incubated overnight at 30°C. Reverse transcription from the annealed primers was carried out by adding 22 μl of an extension mix containing 20 U of avian myeloblastosis virus reverse transcriptase (Promega), 1 U of RNasin (Promega), 1.5 mM each deoxynucleoside triphosphate, and 1 mM dithiothreitol, and incubating the mixture at 37°C for 60 min. Reverse transcripts were size fractionated by denaturing polyacrylamide gel electrophoresis (PAGE) and visualized by autoradiography. The products of DNA sequencing reactions performed with the same primers were size fractionated in adjacent lanes.

Gel mobility shift assays. Plasmids pMIM297 and pMIM277 were digested with EcoRI and BamHI, and the resulting DNA fragments were labeled by using Sequenase and [α-32P]dATP. Protein extracts were prepared by using a French press, centrifuging lysates at 33,000 x g for 20 min, and retaining supernatant fractions. The binding reaction mixtures contained soluble protein extract, 1 ng of labeled DNA, and calf thymus DNA in the amounts indicated in a buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5% glycerol, 1 mM dithiothreitol, and 1 mM EDTA (total of 10 μl). Reaction mixtures were incubated at 25°C for 15 min prior to electrophoresis using 5% native polyacrylamide gels and an electrophoresis buffer containing 3.4 mM sodium acetate, 1 mM EDTA, and 10 mM Tris-HCl (pH 8.0).

DNase I footprinting. The promoter region of traN was subcloned by digesting pMIM297 with EcoRI and BamHI and ligating the fragment containing PtraN between the EcoRI and BamHI sites of pBSK(+). The resulting plasmid, pMIM111, was digested with EcoRI and SacII, and 20 ng of the 218-nucleotide fragment containing PtraN was isolated by preparative electrophoresis using low-melting-point agarose. The EcoRI terminus of this fragment was radiolabeled by using Sequenase, 60 pmol each of dCTP, dGTP, and dTTP, and 5 pmol of [α-32P]dATP in a standard buffer supplemented with 5 mM dithiothreitol (40-μl final volume). A total of 40,000 cpm of labeled DNA was combined with 0.375 μg of competitor DNA and 0.7 μg of a crude extract containing KorA and KorB, incubated for 10 min, and treated with 4 x 10^-4 U of DNase I for 1.5 min. To ensure that only protein DNA complexes were examined in footprinting assay and to obtain a purer preparation of RNA, the protein-DNA complexes were size fractionated on a 5% native polyacrylamide gel and eluted from the gel for 2 h, using a buffer containing 0.6 M ammonium acetate, 0.2% sodium dodecyl...
Slo gene. This Tn conditionally lethal gene was localized in or near traE. The gene or genes conferring KorA function were previously defined with respect to both TraL(KilA)-mediated lethality and TraE(KilB)-mediated lethality. With respect to TraL(KilA)-mediated lethality, korA function was provided by either pGW1670 or pGW2158 but not by pGW1584 (Fig. 1) (38). Assuming that a single genetic locus is responsible, this locus must be fully contained between TraN3::Tn5 and Ω1114::Tn5. With respect to TraE(KilB)-mediated lethality, korA function was provided by pGW1667 but not by pGW1668 (Fig. 1), indicating that it was flanked by insertions TraL3::Tn5 and Ω13::Tn5 (38). Sequence analysis of this region indicates that it contains two ORFs, the one that was provisionally designated korA and a downstream gene designated traM (29) that is essential for conjugation (3a). KorA function could require one or both of these ORFs. However, traM null mutations were isolated in a plasmid that encodes TraL(kilA), suggesting that it was not required for korA function (29). This finding suggested that the upstream ORF was sufficient for KorA function.

kilB is allelic with traE. Previous experiments showed that a conditionally lethal gene was localized in or near traE. First, pKM101 deletion derivative pGW2156 (Fig. 1) is KilB+, while a similar plasmid lacking traN and part of traE (pGW2155) is KilB− (38). Second, a subclone of this region (plasmid pGW1672 [Fig. 1]) conferred a slow-growth phenotype (denoted Slo−), suggesting that it expressed this Kil phenotype at low levels. A derivative of pGW1672 containing Ω1228::Tn5 was Slo−, while all flanking Tn5 insertions in this plasmid were Slo+ (38), suggesting that this Tn5 had disrupted the KilB gene. This Tn5 was also TraE−, but it was not clear whether the Tn5 was located in traE or whether it was located in traN and blocked TraE synthesis by transcriptional polarity. To determine which of these genes was disrupted by this Tn5 insertion, we sequenced the junction between it and flanking pKM101 DNA. This Tn5 insertion is located within the 10th codon of traE, indicating that kilB is allelic with traE (data not shown).

Localization of the korA gene. The gene or genes conferring KorA function were previously defined with respect to both TraL(KilA)-mediated lethality and TraE(KilB)-mediated lethality. With respect to TraL(KilA)-mediated lethality, korA function was provided by either pGW1670 or pGW2158 but not by pGW1584 (Fig. 2) (38). Assuming that a single genetic locus is responsible, this locus must be fully contained between traL53::Tn5 and Ω1114::Tn5. With respect to TraE(KilB)-mediated lethality, korA function was provided by pGW1667 but not by pGW1668 (Fig. 1), indicating that it was flanked by insertions traL53::Tn5 and Ω13::Tn5 (38). Sequence analysis of this region indicates that it contains two ORFs, the one that was provisionally designated korA and a downstream gene designated traM (29) that is essential for conjugation (3a). KorA function could require one or both of these ORFs. However, traM null mutations were isolated in a plasmid that encodes TraL(kilA), suggesting that it was not required for korA function (29). This finding suggested that the upstream ORF was sufficient for KorA function.
To test this hypothesis, we used PCR amplification techniques to subclone korA and to express it from the Plac promoter of pTZ18R. The resulting plasmid (pMIM291) was used to transform strain JC2926(pGW1584, pGW1670) to Ap<sup>+</sup>. pGW1584 encodes traL(KiLA) and korB but not korA, while pGW1670 encodes both kor genes but contains a temperature-sensitive origin of replication (38). Transformants containing pMIM291 formed colonies at 42°C (a restrictive temperature for pGW1670) that were indistinguishable from those formed by using a larger plasmid that expresses korA and several neighboring genes. This finding indicates that pMIM291 provided korA function with respect to TraL(KiLA)-mediated lethality. We demonstrate below that this gene also provides korA function with respect to TraE(KiLB)-mediated lethality.

**Localization of the korB gene.** korB function was previously shown to be provided by either pGW2182 or by pGW2189, indicating that this locus must lie between Ω1275::Tn5 and Ω1277::Tn5 (Fig. 2) (38). This was true both for KiLA(TraL)-mediated lethality and TraE(KiLB)-mediated lethality. This interval contains two ORFs that are likely to encode proteins, the ORF provisionally designated korB and a downstream ORF designated ORF1. Tn5 insertion Ω1276::Tn5 disrupted korB function (38), but it could not be ruled out that it did so by polar effects upon ORF1.

To determine whether the upstream ORF was sufficient for korB function, we PCR amplified this region and cloned the resulting fragment adjacent to the Plac promoter of pUC12Cm. The resulting plasmid, pMIM292, was used to transform JC2926 (pGW1666, pGW1670) to chloramphenicol resistance. pGW1666 expresses TraE(KiLB) and KorA but not korB, while as described above, pGW1670 is a temperature-sensitive replicon expressing both kor genes. Chloramphenicol-resistant transformants formed colonies at 42°C that were indistinguishable from those obtained by using a larger plasmid that expresses korB and several neighboring genes. This finding indicates that pMIM292 expresses korB function. We demonstrate below that this gene also provides korB function with respect to TraL(KiLA)-mediated lethality.

**Construction of a plasmid expressing both kor genes.** To determine whether korA and korB together were sufficient to prevent both traL-mediated and traE-mediated lethality, they were cloned into a single chloramphenicol-resistant plasmid as an operon expressed from the Plac promoter (Plac-korA-korB). The resulting plasmid, pMIM294, was used to transform JC2926 (pGW1666, pGW1670) to chloramphenicol resistance at high temperature. pGW1668 expresses TraE(KiLB) but does not contain korA or korB. The resulting transformants formed colonies at 42°C. In parallel experiments, neither pMIM296 (which expresses korA) nor pMIM292 (which expresses korB) was able to prevent TraE(KiLB)-mediated lethality. We conclude that the korA and korB genes expressed by pMIM294 are necessary and sufficient to restore colony-forming ability to a strain that expresses TraE(KiLB).

To determine whether both kor genes were sufficient to prevent TraL(KiLA)-mediated lethality, korB was introduced into pMIM291, which contains korA. The resulting plasmid, pMIM293, was used transform JC2926(pGW2190, pGW1670) to ampicillin resistance. pGW2190 contains only the traL(kiLA) gene. Ampicillin-resistant transformants formed colonies at 42°C. Neither pMIM291 (which expresses korA) nor pMIM295 (which expresses korB) was able to prevent TraL(KiLA)-mediated lethality in parallel experiments. We conclude that the korA and korB genes expressed by pMIM293 are necessary and sufficient to restore colony-forming ability to strain that expresses TraL(KiLA).

**Sequence similarity between korB and other bacterial DNA-binding proteins.** The predicted amino acid sequences of both korA and korB were used to search the GenBank translated DNA database for homologous proteins. korA was not detectably homologous to any protein in the database. The amino- and carboxyl-terminal halves of korB are similar to each other (Fig. 3a), suggesting that korB arose by duplication of an ancestral gene. Interestingly, both the amino-terminal half and the carboxyl-terminal half of korB showed significant homology to the carboxyl-terminal half of HvrA protein of *Rhodobacter capsulatus* (Fig. 3b and c). HvrA is a positive regulator of light-inducible genes whose products are involved in light-harvesting reactions (2). The amino-terminal and carboxyl-terminal halves of korB appear to resemble each other more closely than either resembles HvrA. In addition, the carboxyl terminal half of korB and to a lesser degree the
TABLE 2. Repression of three pKM101 promoters by KorA and KorB

<table>
<thead>
<tr>
<th>Reporter plasmid</th>
<th>Promoter</th>
<th>β-Galactosidase sp act $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMC1403</td>
<td>None</td>
<td>1</td>
</tr>
<tr>
<td>pMIM277</td>
<td>PkorB</td>
<td>3.234</td>
</tr>
<tr>
<td>pMIM287</td>
<td>PtraL</td>
<td>54</td>
</tr>
<tr>
<td>pMIM297</td>
<td>PtraN</td>
<td>91</td>
</tr>
</tbody>
</table>

$^a$ Miller units (25).
$^b$ Kor plasmid.
$^c$ kor gene.
$^d$ ND, not determined.

amino-terminal half were similar to the carboxyl termini of members of the H-NS family of proteins (Fig. 3b and c). The closest match was found with the E. coli StpA protein and the Haemophilus influenzae H-NS protein. H-NS plays a role in transcriptional repression of a variety of genes and has been implicated in the organization of the procaryotic nucleoid structure (23). The carboxyl terminus of H-NS serves as DNA binding domain (30), suggesting a DNA binding role for the KorB protein, although we show below that KorB is not sufficient for detectable DNA binding.

KorA and KorB repress transcription of at least three pKM101 promoters. Although it was unclear how KorA and KorB functioned, one possibility was that they inhibited the expression of traL and traE and that unregulated expression of these genes is lethal. Precedent for this is found in the IncP plasmid RK2, where conditional lethality of tra genes is prevented by transcriptional repressors (11). To test this hypothesis, it was necessary to fuse tra promoters to a reporter gene in the absence of any lethal genes. Sequence inspection of this region of pKM101 suggested the existence of three promoters, two overlapping divergent promoters between korB and traL, and a third promoter upstream of traN (Fig. 1) (29). All three promoters were subcloned by PCR amplification and placed upstream of a promoterless lacZYA operon. The resulting plasmids, pMIM277, pMIM287, and pMIM297 (Table 1), expressed β-galactosidase activity (Table 2), indicating that each fragment contains a promoter. Strains containing any of these plasmids formed colonies in strains lacking kor genes, indicating that we had succeeded in separating these promoters from genes encoding lethal products.

To test whether KorA and/or KorB repressed the transcription of any of these promoters, kor genes were introduced singly or in combination, using pMIM288, pMIM298, and pMIM299. Although these plasmids contain korA and/or korB fused to the relatively strong Plac promoter, neither protein was detectable by SDS-PAGE among total cellular proteins (data not shown). Since these strains do not contain the lacI gene, isopropylthiolgalactopyranoside was omitted. Neither pMIM288 (which expresses KorA) nor pMIM298 (which expresses KorB) affected transcription of any of these three promoters (Table 2). However, pMIM299, which expresses KorA and KorB, inhibited expression of each promoter about 7- to 10-fold. We conclude that KorA and KorB function together to repress all three promoters. It is possible that expression of the pKM101 promoters could be further repressed by expressing Kor proteins at higher levels.

Mapping the transcriptional start sites of the KorA- and KorB-regulated operons. We performed primer extension reactions to localize the three KorA- and KorB-regulated promoters. RNA was isolated from six derivatives of E. coli MC4100, each containing either pMIM277 (containing PkorB), pMIM287 (PtraL), or pMIM297 (PtraN) and containing either pMIM299, which expresses korA and korB, or a vector control.

Putative transcriptional start sites were identified for all three promoters (Fig. 1 and 4). The korB transcript appears to initiate at adjacent A and T residues that lie downstream from −10 and −35 motifs (Fig. 1), while the traL transcript appears to initiate at two adjacent A residues, also downstream from −10 and −35 motifs. The significance of an additional apparent start site five nucleotides downstream is unknown. The traN promoter initiates at a single A residue (Fig. 1 and 4), once again downstream from −10 and −35 promoter motifs. Expression of KorA and KorB decreased transcription of all three promoters, confirming data described earlier (Fig. 4).

Specific binding of KorA and KorB to DNA. We performed gel mobility shift assays to determine whether KorA and KorB directly bind to the PkorB, PtraL, and PtraN promoters. The assays were performed with crude extracts from E. coli strains that express KorA, KorB, or both proteins. A crude extract from cells containing both KorA and KorB retarded the migration of DNA fragments containing the korB and traL promoters (Fig. 5A, lane 4) or containing the traN promoter.

![FIG. 4. Identification of the 5′ ends of the transcripts initiated from PkorB, PtraL, and PtraN. Primer extension reactions were performed with RNA from strain MC4100 containing pMIM277 (PkorB; A), pMIM287 (PtraL; B), or pMIM297 (PtraN; C) in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of pMIM299, which expresses KorA and KorB. The lengths of the extension products (including primers) are 71 bases for PkorB, 105 bases for PtraL, and 72 bases for PtraN. Transcripts in lanes 1 and 3 were made by using an oligonucleotide specific for each promoter, while lanes A, C, G, and T correspond to dideoxy sequencing reactions performed with the same plasmids and same primers. Transcripts in lanes 2 and 4 were made by using an oligonucleotide specific for the bla gene of each plasmid.)

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and positions of otides over a 29-nucleotide interval (Fig. 1). The similarities sequences, designated kor or PtraL. Binding reactions were performed with 2.5 μg of competitor DNA (25,000-fold excess compared with labeled fragment). Lanes: 1, no protein extract; 2, crude extract containing KorA; 3, crude extract containing KorB; 4, crude extract containing KorA and KorB; 5, crude extract containing KorA combined with crude extract containing KorB. (B) Same as for panel A but using pMIM277, which contains the trnN promoter.

(Fig. 5B, lane 4). In contrast, no gel retardation was observed in extracts from cells containing only KorA (Fig. 5A and B, lanes 2) or only KorB (Fig. 5A and B, lanes 3). However, when the extract containing KorA was added to the extract containing KorB, a shift was observed (Fig. 5A and B, lanes 5). Thus, the formation of stable protein-DNA complexes requires both proteins.

We also performed gel mobility shift reactions with various protein concentrations (Fig. 6). A dilution of extract containing KorA and KorB resulted in partial shifting of both DNA fragments. A second DNA-protein complex, as well as non specific shifting of all DNA fragments, was observed in the presence of high amounts of crude extract (Fig. 6A and B, lanes 6). However, both effects were prevented by adding higher amounts of nonspecific competitor DNA (data not shown), while addition of even a 25,000-fold excess of competitor DNA did not affect the stability of the specific protein-DNA complex (Fig. 5).

**FIG. 6.** Gel mobility shift assays performed with different amounts of crude extracts containing KorA and KorB. Binding reactions were performed with a fragment containing PkorB/PtraL (A) or PtraN (B) with 0.12 μg of competitor DNA (1,200-fold excess compared with labeled fragment). Reaction mixtures contained no crude extract (lane 1), extract diluted 1:1,296 (lane 2), extract diluted 1:216 (lane 3), extract diluted 1:36 (lane 4), extract diluted 1:6 (lane 5), and undiluted extract (lane 6).

**DISCUSSION**

In this study, we first determined that the transposon kilB1228::Tn5 insertion, which defines the kilB gene, lies within traE. Together with previous evidence (38), this finding proves that kilB and traE are the same gene. We previously showed that kilA is allelic with traL (29). Both kil genes are required for efficient conjugation (37) and are homologous to other genes involved in DNA transfer (29). We have therefore abandoned the designations kilA and kilB in favor of traL and traE, respectively, in recognition of the roles of the products of these genes in conjugation. Nevertheless, the conditional lethality of these genes provided a useful phenotype to identify and localize the korA and korB genes. In this study, we have used these lethal phenotypes together with the recently described DNA sequence of this region (29) to identify korA and korB.

KorA and KorB functions are each encoded by a single, rather small gene. korA encodes a 93-residue 10,956-Da protein, while korB encodes a 101-residue, 11,454-Da protein. Both proteins are extremely hydrophilic, each containing 31 charged amino acid residues. KorA has a theoretical net charge of −5 at pH 7.0, while KorB has a net charge of +5. The amino acid sequence similarity between KorB and the positive transcriptional regulator HvrA of R. capsulatus as well as the DNA binding protein H-NS supports its role in tran-
scriptional regulation. It was speculated that HvrA might contain a flavin-containing chromophore so as to regulate gene expression in response to light (2). While we do not know whether KorB binds this or any cofactor, it seems unlikely that tra genes would be light regulated. The similarity of KorB and HvrA to the C-terminal DNA binding domain of H-NS suggests that the corresponding regions in HvrA and KorB also bind DNA, probably by using a similar DNA binding fold (30).

We showed by gel mobility shift assays that the formation of specific protein-DNA complexes required both KorA and KorB. Although the formal possibility remains that KorA and KorB function indirectly by modifying some chromosomally encoded DNA-binding protein, it seems far more plausible that KorA and/or KorB interact directly with these promoters. It is far less clear whether both of these proteins directly bind DNA or whether only one protein (possibly KorB, given its sequence similarity to DNA binding domains) binds DNA, with the other (possibly KorA) somehow controlling this binding activity. KorA and KorB regulate at least three promoters in the pilus/mating pore gene cluster. It is possible that this region contains additional promoters, although sequence inspection suggests that it may not (29). Similarly, it is possible that KorA and/or KorB regulate promoters elsewhere on pKM101, although the remaining tra genes do not contain sequences similar to the kor box motif (unpublished data).

Our DNase I footprinting data confirmed the hypothesis that the kor box near the traN promoter is a binding site for one or both proteins. The extremely strong similarity between this kor box and that found at the korB and trB boxes suggests that KorA and/or KorB may bind this site as well. Although the positions of the two kor boxes with respect to the three regulated promoters differ somewhat from each other, all of these positions are appropriate for repressor binding sites (5). It is interesting that the kor box at traN contains a strong dyad symmetry that extends well beyond the kor box itself (Fig. 1), while the dyad symmetry at the other kor box is weaker and does not extend beyond the kor box. The fact that this region is protected from DNase I suggests that it may be important for protein binding.

The pKM101 KorA/KorB regulatory system bears a superficial resemblance to one found in the IncP plasmid RK2. The conjugal mating bridge of RK2 is encoded by the trB operon, which contains 12 genes (22). This operon is expressed from two linked promoters denoted PtrA and PtrB (22, 31, 32). The PptrB promoter is negatively regulated by the KorB and TrB proteins, which can act independently (17, 32). The KorA, KorB, and TrB proteins of RK2 are a subset of a family of repressors encoded by this plasmid that function at diverse plasmid promoters (28). However, none of these repressors is detectably homologous to the KorA or KorB protein of pKM101. We propose that the pKM101-encoded repressors and those of RK2 have arisen by convergent evolution. It is remarkable that both regulatory systems were originally discovered by virtue of their ability to prevent lethality caused by other plasmid-encoded proteins (8, 38).

It is interesting that so many conjugal transfer systems of enteric bacteria are negatively regulated. One probable consequence of this negative regulation is zygotic induction, that is, transient high-level expression of tra genes in a transconjugant. For conjugation systems that are efficiently repressed at a phenotypic level, this would cause new recipients to be efficient donors only until repression is established. However, conjugation of pKM101 and of RK2 is not repressed at a phenotypic level, since the repressed level of expression of these genes is sufficient for extremely efficient conjugation (11, 37). It is also conceivable that these repressors can in some conditions repress transcription more strongly but that repression is responsive to some uncharacterized environmental signal. If so, such a signal would have to be provided during the laboratory conjugation experiments. The fact that both korA and korB are encoded within operons that are repressed by KorA and KorB proteins should in principle provide negative autogenous regulation of both operons. This could provide a mechanism to ensure that tra genes are expressed at some level appropriate for efficient conjugation but sufficiently low to minimize their burden on host metabolism.

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