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Identification of pKM101-Encoded Loci Specifying Potentially Lethal Gene Products

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Two pKM101-encoded loci (designated *kilA* and *kilB*) have been identified which elaborate products that are potentially lethal to the bacterial cell. The lethal effects of each of these gene products is inhibited by two other plasmid-encoded loci, designated *korA* and *korB* (for *kil* override). Both *korA* and *korB* are required to control the lethality of either *kil* gene. In the presence of *korA* and *korB* both *kil* genes have other phenotypes: *kilB* is necessary for conjugal transfer, whereas *kilA* is responsible for the small-colony morphology on defined media that is characteristic of pKM101-containing strains (the Slo phenotype).

A number of plasmids have been found to contain genes whose products are potentially lethal to the bacterial host. The IncP plasmid RK2 has three such genes, designated *kilA*, *kilB*, and *kilC* (11). The lethality of these genes is normally inhibited by three other plasmid-encoded genes, designated *korA*, *korB*, and *korC* (for *kil* override). *korA* alone is sufficient to control the lethality of *kilA* (30). In contrast, *korA* and *korB* together are required to control the lethality of *kilB* (D. H. Bechhofer and D. H. Figurski, manuscript in preparation), and similarly, *korA* and *korC* must act together to prevent the lethal phenotype of *kilC* (30). In a strain containing a *rho* mutation, *korC* alone is sufficient to control *kilC*. It has been speculated that *korA* positively effects transcription of *korC*, possibly by antitermination. The DNA sequence of the *korA* gene has been determined (1).

The F episome has also been reported to contain two genes, one having the phenotype of a *kil* gene and the other having the phenotype of a *kor* gene (20). These genes have been implicated in enhancing plasmid stability by coupling host cell division to plasmid replication.

pKM101 is a 35.4-kilobase (kb) self-transmissible IncN plasmid which was derived from the clinically isolated plasmid R46 (6, 13, 18). This plasmid enhances the mutagenic effects of UV and a variety of chemical mutagens (8, 25), and this property has led to its introduction into the Ames *Salmonella* tester strains (15). This enhancement of mutagenesis is due to the *mucAB* genes, which code for analogs of the *E. coli* genes *umuDC* (10, 21). We have also studied the plasmid's system of conjugal transfer (28), entry exclusion (29), and a plasmid-encoded endonuclease (27).

Bacteria which contain an IncN plasmid, including pKM101 (and to a lesser extent, IncP plasmids such as RK2), have the ability to kill strains of *Klebsiella pneumoniae* used as conjugal recipients (23). Cell-to-cell contact is required for this lethality and only transfer-proficient plasmids are capable of mediating this effect (23; S. C. Winans and G. C. Walker, unpublished data).

In the course of trying to subclone various fragments of pKM101, we discovered that it, like RK2 and F, contains genes that are potentially lethal to the host. In the present study we describe two such *kil* genes and two corresponding *kor* genes which are localized near or among the plasmid's transfer genes. At least one and possibly two additional *kil* genes have been identified lying close to *mucAB* (K. Perry and S. Elledge, unpublished data).

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MATERIALS AND METHODS

Strains and plasmids. JC2926 is a *recA13* derivative of AB1157 (27). pKB444 (provided by K. Backman) is a derivative of pBR322 containing, in place of that plasmid's *Pvu*II site, a P_{lac} promoter oriented such that transcription proceeds counterclockwise and an adjacent *Kpn*I site. pPM103 (provided by D. Taylor) is a derivation of pSC101 containing a temperature-sensitive origin of replication (16). pACYC184 (7) was provided by A. Chang.

DNA isolation and manipulation. The method used to isolate analytical quantities of plasmid DNA was a modified version of that described by Rambach and Hogness (22). Restriction endonucleases and T4 DNA ligase were used according to procedures recommended by Maniatis et al. (14). Transformation of bacteria with plasmid DNA was performed by using the $CaCl_2$ method described by Maniatis et al. (14).

Mutagenesis of plasmids with Tn*5*. Transposition of Tn*5* from λ::Tn*5* to a plasmid-containing host was performed as described previously (19), except that cells were plated on LB plates (17) containing 20 μg of kanamycin per ml and 2.5 mM sodium pyrophosphate. Plasmid DNA was then purified from 10^6 pooled transductants and used to transform a recipient strain to Km^r. Tn*5* insertions were also introduced into pKM101 from other plasmids containing cloned pKM101 fragments by homologous recombination. To do this, 2 μg of the donor plasmid containing Tn*5* was digested with *Eco*RI and used to transform to Km^r a *recB21 recC22 sbcB15* host containing pKM101 (Winans et al., J. Bacteriol., in press). At least half of the colonies obtained contained a derivative of pKM101 containing a Tn*5* at the same locus as in the donor plasmid.

RESULTS

Localization of the *kilA* gene. A pKM101-encoded gene having a potentially lethal phenotype was discovered during efforts to subclone the pKM101 DNA between the *Sal*I-1 site and the *Hpa*I-1 site (Fig. 1). To do this we used pGW1582, which is a derivative of pACYC184 containing pKM101 sequences between the *Sal*I-1 and the proximal *Hin*dIII site of the Tn*5* of pKM101 *traD1141*::Tn*5* (Fig. 1). pGW1582 was digested with *Hpa*I and ligated in a sufficiently large volume of buffer that intramolecular ligation would occur preferen-

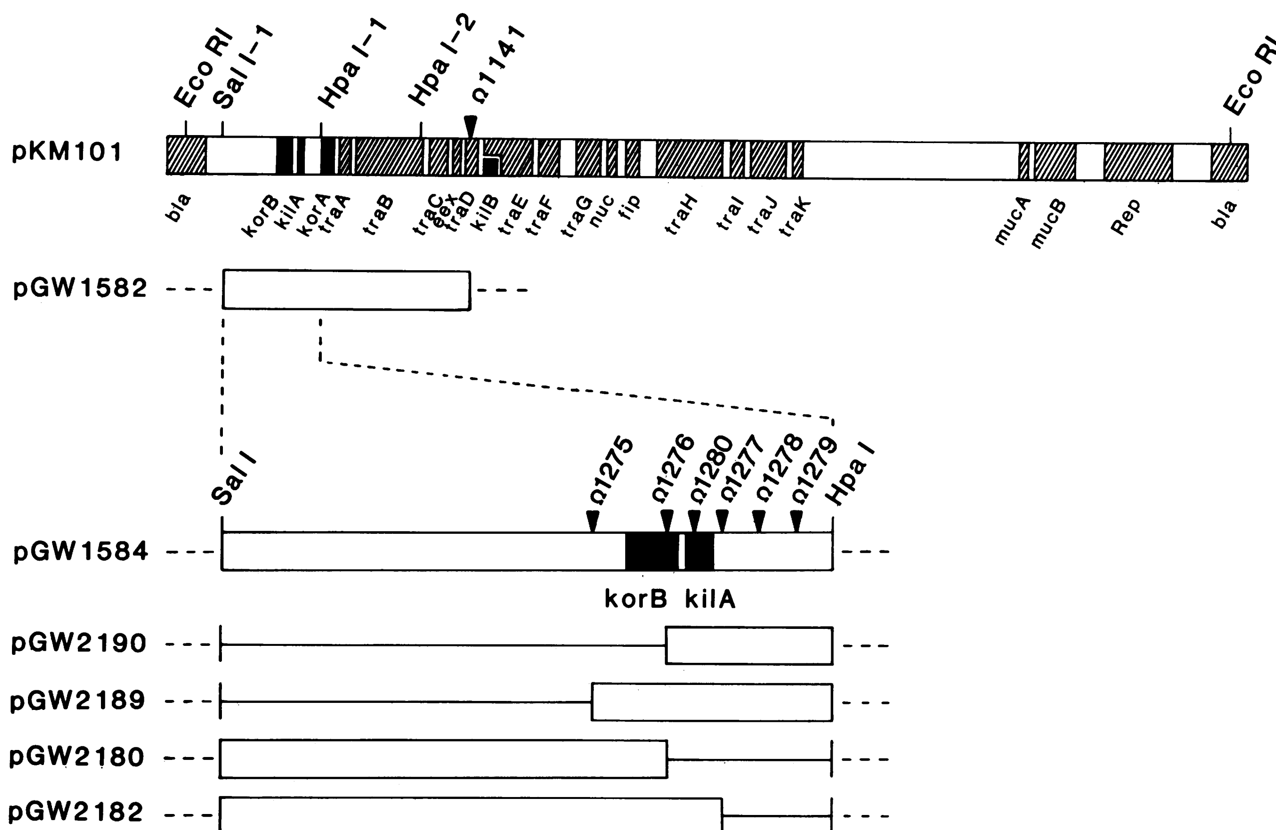

FIG. 1. Localization of *kilA*, using insertion and deletion derivatives of pGW1584. (Top) Construction of pGW1584. Plasmid pGW1582 is a pACYC184 derivative containing pKM101 DNA from the *Sal*I-1 site to the proximal *Hin*dIII site of insertion *traD1141*::Tn*5*, cloned into the gap in the vector created by digestion with the same two enzymes. pGW1582 was digested with *Hpa*I (which creates three fragments) and ligated under conditions favoring intramolecular circularization. Transformation of ligated DNA into JC2926(pKM101) yielded pGW1584. (Bottom) Insertion and deletion derivatives of pGW1584. pGW2189 and pGW2190 were made by deletion of the small *Sal*I fragments of pGW1584Ω1275::Tn*5* and pGW1584Ω1276::Tn*5*, respectively. pGW2180 and pGW2182 were made by deleting the small *Hin*dIII fragments of pGW1584Ω1276::Tn*5* and pGW1584Ω1277::Tn*5*, respectively. Insertion Ω1280::Tn*5* and deletion derivatives pGW2180 and pGW2181 are *kilA*$^-$, whereas insertions Ω1275::Tn*5* and Ω1276::Tn*5* and deletion derivatives pGW2182, pGW2189, and pGW2190 are *kilA*$^+$. Insertions Ω1277::Tn*5*, Ω1278::Tn*5*, and Ω1279::Tn*5* are partially *kilA* deficient. Open bars represent pKM101 DNA retained in each derivative, solid lines represent pKM101 DNA removed in various deletion derivatives, and dashed lines indicate vector or Tn*5* DNA.

tially. Digestion of pGW1582 with *Hpa*I yields three fragments, the largest of which contains the chloramphenicol acetyltransferase determinant and the replication region. Therefore, we expected that the ligation would yield plasmids containing only the largest of the three *Hpa*I fragments of pGW1582. The ligated DNA was used to transform strains JC2926 and JC2926(pKM101) to Cm^r. About 60-fold fewer transformants were recovered with JC2926 than with JC2926(pKM101). We analyzed 12 representatives of each group and found that all of the JC2926(pKM101) transformants contained a second plasmid (designated pGW1584) composed of the largest of the *Hpa*I fragments of pGW1582 (Fig. 1). In contrast, none of the 12 JC2926 transformants had such a plasmid, although several contained plasmids containing the two largest *Hpa*I fragments of pGW1582, arranged in their native configuration (plasmid pGW1585).

DNA prepared from JC2926(pGW1584)(pKM101) was used to transform both JC2926 and JC2926(pKM101) to Cm^r. Approximately 300-fold fewer transformants were obtained with the former host than with the latter. Each of the JC2926 transformants was Ap^r resistant as well as Cm^r, indicating that they had been transformed by pKM101 as well as pGW1584. Since no transformants of JC2926 were obtained which contained only pGW1584, we concluded that this plasmid contains a locus which is lethal to either the bacterium or the plasmid itself and that this lethality can be inhibited by either supplying additional sequences in cis or supplying pKM101 in trans. (Experiments described below indicate that this gene is lethal to the bacterium). This gene has been designated *kilA*.

To localize *kilA* more closely, we constructed a set of Tn*5* derivatives of pGW1584. λ::Tn*5* was used to introduce Tn*5* into JC2926(pGW1584)(pKM101) and about 10^6 Km^r derivatives were obtained. Plasmid DNA purified from pooled colonies was used to transform JC2926 to Km^r and Cm^r. Transformation using this host constituted a direct selection for derivatives of pGW1584 which are *kilA*$^-$, due presumably to insertional inactivation of the gene by Tn*5*. Of the four transformants obtained, three grew extremely poorly and were not characterized further. (We describe below other Tn*5* insertion derivatives of pGW1584 having similar properties). The fourth derivative, pGW1584Ω1280::Tn*5*, was determined to contain an insertion of Tn*5* at the locus indicated in Fig. 1. This plasmid was observed to transform JC2926 and JC2926(pKM101) with equal efficiency and to form colonies of equal size in both hosts. The amount of DNA recovered from cultures containing this plasmid was the same as that obtained from pACYC184, indicating that

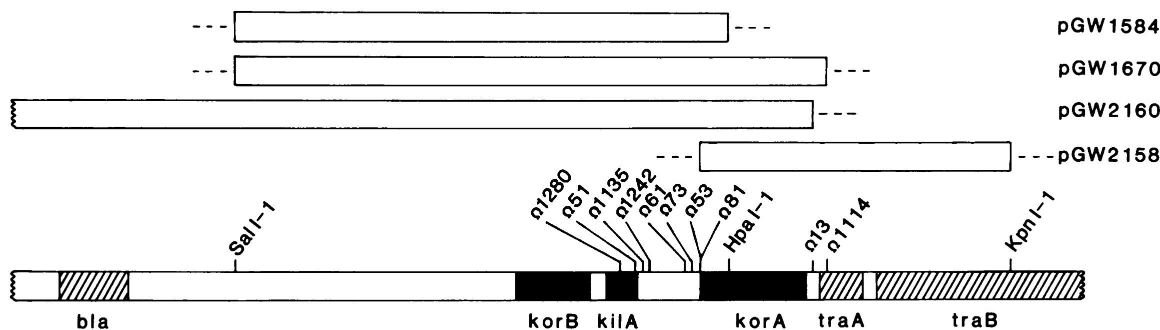

FIG. 2. Localization of *korA*, using insertion derivatives and cloned fragments of pKM101. pGW2158 and pGW2160 are derivatives of pKB444 made by digesting pKM101Ω53::Tn*5* (for pGW2158) or pKM101Ω13::Tn*5* (for pGW2160) with *Hin*dIII and *Kpn*I and cloning the indicated fragments into the gap in the vector created by digestion with the same two enzymes. The left endpoint of the pKM101 DNA cloned in pGW2160 is the *Kpn*I-3 site (which lies in *traH*). pGW1670 is a derivative of pPM103 containing pKM101 DNA from the *Sal*I-1 site to the proximal *Hin*dIII site of pKM101 *traA1114*::Tn*5*. These three plasmids are all *korA*$^+$, indicating that *korA* must lie in the interval bounded by insertions Ω53::Tn*5* and Ω13::Tn*5*. All insertion derivatives of pKM101 are *korA*$^+$ except pKM101Ω81::Tn*5*, which is partially *korA*$^-$. Open bars represent pKM101 DNA present on these clones; dashed lines indicate vector or Tn*5* DNA.

the copy number of the plasmid was normal. pGW1584Ω 1280::Tn*5* therefore has the phenotype expected of a *kilA*$^-$ mutant.

We also used the plasmid DNA that had been purified from Km^r transductants of JC2926(pGW1584)(pKM101) to transform JC2926(pKM101) to Km^r and Cm^r. Transformation using this host allowed isolation of derivatives of pGW1584 bearing insertions of Tn*5* at loci other than *kilA*. The map locations of five of these are shown in Fig. 1. Each of these derivatives was tested to determine whether it was *kilA*$^-$ by using it to transform both JC2926 and JC2926 (pKM101) to Cm^r and Km^r. After overnight incubation we observed that, for all five plasmids, about 300-fold fewer colonies arose with JC2926 than with JC2926(pKM101). All transformants of JC2926 so tested were Ap^r, indicating that they had been transformed by pKM101 as well as the Tn*5* derivative of pGW1584. These results indicate that all of these derivatives are *kilA*$^+$.

After further incubation, however, pGW1584Ω1277::Tn*5*, pGW1584Ω1278::Tn*5*, and pGW1584Ω1279::Tn*5* (Fig. 1) gave rise to additional slow-growing colonies when transformed into JC2926. All such colonies so tested were Ap^s, indicating that they did not contain pKM101. Plasmid DNA prepared from these colonies was recovered in extremely poor yield. These properties are referred to as a "Sic" phenotype, and we speculate that they may be due to low, nonlethal expression of *kilA*. The region in which these three insertions are located is not required for *kilA* function (see below). Since these three insertions all lie to the right of *kilA1280*::Tn*5*, it is possible that their Sic phenotype is due to reduction in *kilA* expression caused by transcriptional polarity. If this is true, then *kilA* must be transcribed from right to left on the map shown in Fig. 1.

A number of these insertion derivatives of pGW1584 were used to create deletion derivatives, four of which are illustrated in Fig. 1. We constructed most of these deletions in JC2926(pKM101) so that those derivatives that express *kilA* could still be recovered. We then transformed the DNA of each of them into JC2926 and JC2926(pKM101) and determined that pGW2182, pGW2189, and pGW2190 are *kilA*$^+$ whereas pGW2180 is *kilA*$^-$. These data confirm that the only DNA necessary for *kilA* function lies within the 0.3-kb interval flanked by Ω1276::Tn*5* and Ω1277::Tn*5*.

Localization of genes necessary to inhibit the lethal phenotype of *kilA*. In an effort to localize the gene or genes required to control the lethality of *kilA*, we transformed DNA prepared from JC2926(pGW1584)(pKM101) into strains containing, instead of pKM101, various insertion or cloned derivatives of pKM101. We constructed two derivatives of the vector pKB444 (pGW2158 and pGW2160) containing segments of pKM101 DNA (Fig. 2). The Kor phenotype of these plasmids was assayed by using pGW1584 to transform strains containing each of these plasmids to Cm^r. Both of them were Kor$^+$, indicating that the *kor* gene lies within the 0.8-kb region flanked by insertions Ω53::Tn*5* and Ω13::Tn*5*. This locus is designated *korA*.

We found that although JC2926(pGW2158) could be transformed to Cm^r, using pGW1584 and most of its derivatives, it could not be so transformed with pGW1584Ω1276::Tn*5* or pGW2190 (Fig. 1). Therefore, the *kilA* gene of these plasmids causes lethality even when *korA* is supplied in trans. Since these plasmids do not kill cells that contain pKM101, the simplest interpretation is that inhibition of *kilA*-mediated lethality requires a second gene in addition to *korA*. This gene, designated *korB* (Fig. 1), was supplied in cis by pGW1584, such that only *korA* had to be supplied in trans. In contrast, survival of strains containing pGW1584Ω1276::Tn*5* or pGW2190 required that *korA* and *korB* both be supplied in trans.

We found that pGW2189 and pGW2182 (Fig. 1) (both of which are *kilA*$^+$) could be transformed into JC2926 (pGW2158), indicating that they are *korB*$^+$. *korB* must therefore lie in the 0.7-kb interval between Ω1275::Tn*5* and Ω1277::Tn*5* (Fig. 1). Two insertions have been obtained within this region: Ω1276::Tn*5* and *kilA1280*::Tn*5*. As stated above, Ω1276::Tn*5* is *korB*$^-$, whereas *kilA1280*::Tn*5* could not be characterized because only *kilA*$^+$ plasmids could be tested by this assay. Since *kilA* is probably distinct from *korB*, we believe that *korB* lies in the 0.5-kb interval flanked by Ω1275::Tn*5* and *kilA1280*::Tn*5*.

Identification and localization of a second *kil* gene. A second *kil* gene was discovered during efforts to delete the region containing *korB*, *kilA*, and *korA*. For example, a deletion of the region between the *Pst*I site of pKM101 and the insertion of Ω13::Tn*5* (plasmid pGW1668, Fig. 3) yielded a plasmid mediating a lethal phenotype. Lethality was prevented by supplying a plasmid such as pGW1670 (Fig. 2) which contained the region that had been deleted.

This second *kil* gene (denoted *kilB*) was localized by using deletion derivatives similar to pGW1668 but removing progressively greater amounts of pKM101 DNA. Two of these deletion derivatives were constructed from pKM101

traD1216::Tn*5* and pKM101 *traE1228*::Tn*5* (see Fig. 3). After these *Pst*I fragments were deleted in vitro, these plasmids were used to transform JC2926(pGW1670) to Ap^r. pGW1670 was chosen to inhibit the lethality caused by *kilB* because it is a derivative of pPM103, a plasmid whose replication is temperature sensitive (16). A strain containing a *kilB*$^+$ plasmid and pGW1670 ought therefore to be temperature sensitive. We found that JC2926(pGW1670)(pGW2156) was temperature sensitive, whereas JC2926(pGW1670)(pGW2155) was temperature resistant, on LB plates supplemented with ampicillin. These data suggest that *kilB* lies at least partly within the DNA flanked by *traD1216*::Tn*5* and *traE1228*::Tn*5*. Data are presented below which indicate that the latter insertion actually disrupted *kilB*.

A 3.8-kb fragment containing this region was cloned into pKB444 (a derivative of pBR322). This plasmid, pGW1672 (Fig. 3), contains pKM101 DNA from the proximal *Hin*dIII site of *traC1134*::Tn*5* to the *Kpn*I-2 site of pKM101 (Fig. 1). We found that pGW1672 did not cause cell lethality. However, it did cause its host to form small, irregularly shaped colonies on media containing antibiotics. This plasmid contains the *bla* gene of pBR322, yet failed to confer resistance to high levels of ampicillin (the 50% lethal dose was about 800 μg/ml compared to 7,000 μg/ml for pBR322 itself). It underwent spontaneous curing at a rate of almost 30% per generation (as measured by the rate of loss of Ap^r during logarithmic growth). Finally, the recovery of plasmid DNA from a strain containing this plasmid was extremely poor, indicating that its average copy number in the population was at least 10-fold lower than that of pBR322. pGW1672 therefore expressed a Sic phenotype similar to certain derivatives of pGW1584 described above. All of these phenotypes were fully suppressed by the presence of pGW1670. It is possible that this Sic phenotype was due to poor expression of *kilB*.

By mutagenizing pGW1672 with Tn*5*, we have been able to show that the locus causing the Sic phenotype is coincident with *kilB*. That pGW1672, in the absence of a *kor*-expressing plasmid, does not confer resistance to high levels of ampicillin provided a direct selection for Sic$^-$ derivatives of pGW1672, since an insertion inactivating this gene should allow growth in high levels of this drug.

Km^r derivatives of JC2926(pGW1672)(pGW1585) were made by using λ::Tn*5*, and plasmid DNA purified from these pooled derivatives was used to transform JC2926 to Km^r and high Ap^r. (pGW1585 is the derivative of pACYC184 containing pKM101 DNA between the *Sal*I-1 site and the *Hpa*I-2 site that was described earlier). We found that 400 μg of ampicillin per ml was sufficient to counterselect Sic$^+$ transformants, suggesting that Sic may lower the 50% lethal dose of ampicillin even more strongly for newly transformed cells than for cells which have contained the plasmid for several generations. In this way, pGW1672Ω1228::Tn*5* (see Fig. 3) was isolated. In addition to allowing growth in high levels of ampicillin, the DNA of this plasmid was recoverable in good yield. It therefore is Sic$^-$. To isolate Tn*5* insertions which flank this gene, we also transformed DNA from the same library of Tn*5* derivatives into JC2926(pGW1585). The site of Tn*5* insertion in two of these *kilB*$^+$ derivatives (Ω1220::Tn*5* and Ω1221::Tn*5*) is shown in Fig. 3, and these help to limit the boundaries of the gene responsible for the Sic phenotype. We then crossed Ω1228::Tn*5* into pKM101 and used this insertion to make the deletion derivative pGW2155 (see Fig. 3). This plasmid is *kilB*$^-$, whereas pGW2156, which contains only 1 kb of additional pKM101 DNA, is *kilB*$^+$.

***kilB* is part of the *traE* complementation group.** Insertions Ω1228::Tn*5* and Ω1221::Tn*5* (Fig. 3), which had both been crossed into pKM101 by homologous recombination, were determined to cause a deficiency in conjugal transfer of at least six orders of magnitude (28). Both insertions were found to be part of the same complementation group (*traE*), suggesting that these insertions are probably in the same transcriptional unit. That only one of these insertions disrupted the *kilB* gene of pGW1672 suggests that the *traE* transcriptional unit may be polygenic. If this were true, since only the former insertion disrupted *kilB*, the latter insertion would lie downstream in this operon from *kilB*. It is also possible that *traE* contains only one gene, but that the

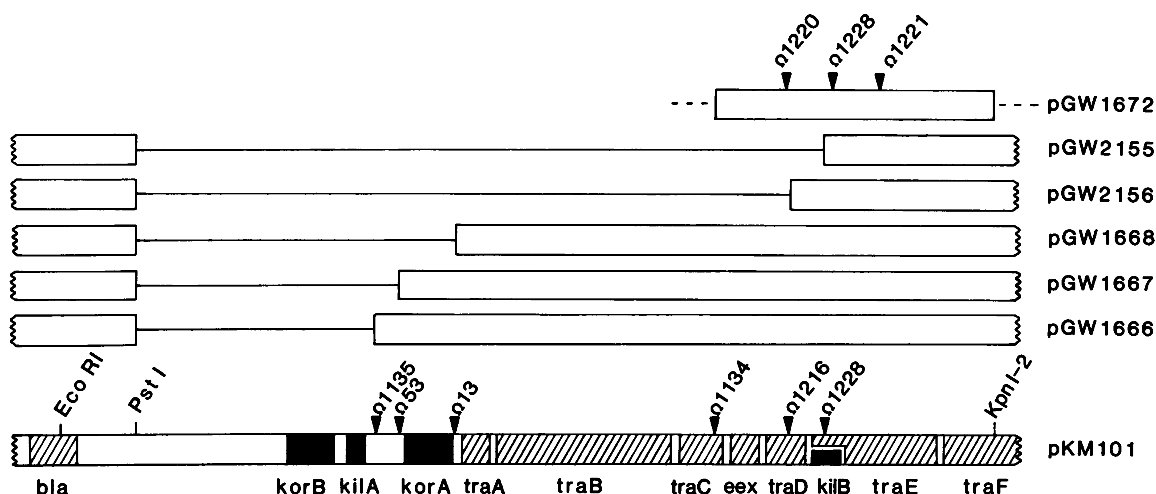

FIG. 3. Localization of *kilB*, using deletion derivatives of pKM101. pGW1666, pGW1667, pGW1668, pGW2156, and pGW2155 were made by deletion of the small *Pst*I fragments of derivatives of pKM101 bearing insertions Ω1135::Tn*5*, Ω53::Tn*5*, Ω13::Tn*5*, Ω1216::Tn*5*, and Ω1228::Tn*5*, respectively. All of these plasmids are *kilB*$^+$ except pGW2155. pGW1672 contains a fragment between the *Hin*dIII site of *traC1138*::Tn*5* and the *Kpn*I-2 site of pKM101 cloned into the *Hin*dIII-*Kpn*I gap of pKB444. Open bars represent pKM101 DNA present on these clones, solid lines indicate deleted pKM101 DNA, and dashed lines indicate vector or Tn*5* DNA present on pGW1672.

TABLE 1. Localization of genes coded by pGW1584 sufficient to suppress the *kilB* gene of pGW1666

Plasmid[a]	Kor phenotype[b]
Deletion derivatives of pGW1584	
pGW2182	+
pGW2180	−
pGW2189	+
pGW2190	−
Insertion derivatives of pGW1584	
pGW1584Ω1275::Tn*5*	+
pGW1584 *korB1276*::Tn*5*	−
pGW1584 *kilA1280*::Tn*5*	+
pGW1584Ω1277::Tn*5*	+
pGW1584Ω1278::Tn*5*	+
pGW1584Ω1279::Tn*5*	+

[a] Map positions of these insertion and deletion derivatives of pGW1584 are shown in Fig. 2.

[b] Each of the plasmids indicated was transformed into JC2926 (pGW1666)(pGW1670). Half of the transformed cells were plated at 30°C, whereas the other half was plated at 43°C, selection being made in both cases for Cm^r. All plasmids transformed this strain with high efficiency when cells were plated at 30°C. "+" indicates that the plasmid yielded transformants with equal efficiency at 30 and at 43°C.

protein coded by this gene is multifunctional, with the amino-terminal fragment being sufficient to mediate the Kil phenotype.

Inhibition of *kilB*-mediated lethality requires *korB*. To localize the genes responsible for the inhibition of *kilB*, we utilized the derivatives of pGW1584 that were described above (Fig. 1) in the localization of *kilA* and *korB*. We used pGW1584 and its derivative to transform JC2926(pGW1666)(pGW1670) to Cm^r and Ap^r, plating cells on rich media and incubating them at 30 and 43°C. At the permissive temperature pGW1670 (Fig. 2) continued to replicate, supplying whatever *kor* gene (or genes) was required by the *kilB*$^+$ *korA*$^+$ *korB*$^-$ plasmid pGW1666 (Fig. 3). At 43°C pGW1670 was lost and transformed cells could form colonies only if the pGW1584 derivative expressed the required *kor* function. Lethality due to introduction of the *kilA* gene of the *kilA*$^+$ *korB*$^+$ *korA*$^-$ plasmid pGW1584 and its derivatives was prevented by using pGW1666, since that plasmid supplied *korA* in trans.

Table 1 shows the results of this experiment. pGW1584 and many of its derivatives (Fig. 1) do inhibit *kilB* lethality. Since cells transformed with either pGW2182 or pGW2189 were viable, the region required for *kilB* inhibition must be contained within the 0.7-kb region flanked by insertions Ω1277::Tn*5* and Ω1275::Tn*5*. Two insertions have been isolated within this region: *kilA1280*::Tn*5* and *korB1276*::Tn*5*. The former is Kor$^+$ for *kilB*-mediated lethality, whereas the latter cannot be tested since it is *kilA*$^+$ *korB*$^-$ and the cell would be expected to undergo *kilA*-mediated lethality. Since it seems unlikely that there is enough space within this 0.7-kb region to the right of *kilA1280*::Tn*5* to code for a gene (0.2 kb), the gene probably lies to the left of *kilA1280*::Tn*5*. Since this same region contains *korB*, it seems likely that inhibition of *kilB*-mediated lethality requires *korB*.

Inhibition of *kilB*-mediated lethality requires *korA*. We sought to determine whether inhibition of *kilB*-mediated lethality might require *korA* in addition to *korB*. To do this, we asked whether three different strains, JC2926(pGW1666)(pGW1670), JC2926(pGW1667)(pGW1670), and JC2926(pGW1668)(pGW1670) (see Fig. 3), when transformed with a plasmid which expresses *korB*, could form colonies at 43°C (Table 2). pGW1666, pGW1667, and pGW1668 all express *kilB*, and the first two, but not the third, also contain *korA*. pGW1670 supplied *korA* and *korB* at low temperature but was lost at high temperature. pGW1584 *kilA1280*::Tn*5* was chosen to supply *korB* because it does not express *kilA*, and therefore the need for *korA* to preveht *kilA*-mediated lethality is abolished. It also lacks the *korA* gene. These three strains were also transformed with two other control plasmids: (i) pACYC184, which is *korA*$^-$ *korB*$^-$; and (ii) pGW2130, which is *korA*$^+$ *korB*$^+$. (pGW2130 is a pACYC184 derivative containing the same pKM101 DNA as pGW1670.)

At 43°C, cells transformed with pGW2130 still formed colonies, since this plasmid contains the same pKM101 DNA as pGW1670. At 43°C, pGW1584 *kilA1280*::Tn*5* also was able to prevent *kilB*-mediated lethality in strains containing pGW1666 and pGW1667. However, it was unable to override the *kilB* gene of pGW1668. pGW1667 and pGW1668 differ by only a 0.8-kb sequence (Fig. 3). This result indicates that this 0.8-kb sequence was needed to prevent *kilB*-mediated lethality. It was exactly this 0.8-kb region which was shown above to contain *korA*. Due to the limited coding capacity of this region, it is proposed that *korA* itself, rather than a second gene, is required for control of *kilB*. Thus control of *kilB*, like that of *kilA*, seems to require the action of both *korA* and *korB*.

***kilA* and *kilB* cause host lethality.** The experiments described above do not distinguish between the possibilities that *kilA* and *kilB* cause cell lethality or that they merely cause the plasmid on which they reside to be unable to replicate. Either phenomenon would result in an inability to recover viable transformant colonies. To distinguish between these possibilities, we made use of strains JC2926(pGW1670)(pGW1584) and JC2926(pGW1670)(pGW1668). When cultured at 43°C, such strains segregate daughter cells lacking pGW1670, thus leaving the *kil* gene of the second plasmid free to express its effect. If this gene causes its replicon to be nonviable, then this plasmid too would be lost from the bacteria, and the cells would not form colonies on selective media; they would, however, still form colonies on nonselective media. If, on the other hand, the *kil* gene causes cell lethality, then these cells will not form colonies at 43°C even on nonselective media.

JC2926(pGW1584)(pGW1670) was cultured at 30°C to late-log phase in LB broth supplemented with chloramphenicol and kanamycin, streaked out onto LB plates, and incubated

TABLE 2. Ability of pGW1584 *kilA1280*::Tn*5* to suppress the *kilB* gene of pGW1666, pGW1667, and pGW1668

Plasmids in recipient strain	Transformation efficiency[a]		
	pACYC184	pGW2130[b]	pGW1584 *kilA1280*::Tn*5*[c]
pGW1670	+	+	+
(pGW1670)(pKM101)	+	+	+
(pGW1670)(pGW1666)	−	+	+
(pGW1670)(pGW1667)	−	+	+
(pGW1670)(pGW1668)	−	+	−

[a] Plasmids indicated were transformed into the strains indicated. Half of the transformed cells were plated at 30°C and the other half were plated at 43°C. Selection was made for Cm^r. At the permissive temperature all plasmids yielded about 1,000 transformants per μg of DNA. At the nonpermissive temperature the strains bearing a "+" formed colonies at the same efficiency as at 30°C, whereas strains bearing a "−" formed fewer than three colonies per microgram of DNA transformed.

[b] pGW2130 is *korA*$^+$ *korB*$^+$.

[c] pGW1584 *kilA1280*::Tn*5* is *korA*$^-$ *korB*+.

at 30 and 43°C. After overnight incubation at 43°C this strain formed barely visible colonies. These colonies did not grow further even after prolonged incubation. This observation indicates that *kilA* does indeed lead to host cell lethality. The same strategy was applied to *kilB*, using JC2926(pGW1670) (pGW1668), and the same result was obtained, indicating that *kilB*, like *kilA*, leads to host cell lethality.

To characterize the kinetics of this loss of viability caused by *kilA*, stationary-phase cultures of strain JC2926(pGW1584) (pGW1670) and the control strain JC2926(pACYC184) (pGW1670) were diluted 1:1,000 into 25 ml of LB broth supplemented with chloramphenicol and cultured with vigorous aeration at 43°C. The turbidity of the cultures was assayed with a Klett colorimeter, and aliquots were periodically drawn off, plated on LB-chloramphenicol plates, and incubated at 30°C. Cells were also examined under a phase-contrast microscope. Figure 4 shows both the change in turbidity of the cultures and the number of CFU per milliliter as a function of time. As can be seen, the CFU per milliliter increases only about fivefold over the entire growth interval. This can be interpreted as being due to a complete cessation of replication of pGW1670 at the high temperature. Since the origin of replication of pGW1670 was derived ultimately from pSC101 (16), this plasmid might be expected to be present at a copy number of five per cell under permissive conditions. After replication ceased, this plasmid would be partitioned to progeny cells until each contained only one. Further cell division would give rise to daughter cells lacking pGW1670, which would not form colonies.

In contrast to the fivefold increase in CFU per milliliter, the turbidity of JC2926(pGW1584)(pGW1670) increased by a factor of >300. It is apparent, therefore, that the pGW1670-free daughters of pGW1670-containing cells are capable of extensive cell division. The most probable explanation is that the *korA* gene product can undergo extensive dilution before it loses the ability to inhibit *kilA*-mediated lethality. JC2926(pGW1668)(pGW1670) was tested in the same way and similar results were obtained, indicating that extensive dilution of the *kor* gene products is required before the cell undergoes *kilB*-mediated lethality.

Examination under the microscope of cells whose growth had ceased due to incubation at 43°C showed no signs of lysis or filamentation. Cells of strain JC2926(pGW1584) (pGW1670) did appear to have a somewhat wider diameter than those of the control strain, although cells of strain JC2926(pGW1668)(pGW1670) did not. We also harvested cultures of JC2926(pGW1584)(pGW1670) and JC2926 (pACYC184)(pGW1670) after 6 h of growth at 43°C and purified their plasmid DNAs to determine if they were in any way distinguishable. As expected, the DNA of the temperature-sensitive plasmid pGW1670 was not detectable by agarose gel electrophoresis. The DNAs of pGW1584 and pACYC184 were readily observable and present in good yield. In fact, the only difference between them was that there was a greater yield of pGW1584 DNA than that of pACYC184. This difference was about 10-fold and was observed in two different trials. Since pACYC184 and its derivatives are amplifiable by agents that inhibit translation (7), one interpretation of this result is that *kilA*-mediated lethality may involve, or result in, an inhibition in translation.

***kilA* and *kilB* are not required for killing of *K. pneumoniae*.** It seemed possible that the *kil* genes of pKM101 might play some role in the ability of the plasmid to kill *K. pneumoniae* (23). We tested pKM101 *kilA1280*::Tn*5* and pKM101 *traE1228*::Tn*5* (which is *kilB*$^-$) for their ability to kill *K. pneumoniae* M5a1. (Since the latter plasmid is transfer deficient, we complemented its defect in trans.) Both of these plasmids killed *K. pneumoniae*. We also constructed a *kilA kilB* double mutant of pKM101, complemented its transfer deficiency, and determined that it too is capable of killing. It is possible, therefore, that these *kil* genes play no role in killing *K. pneumoniae*. However, pKM101 encodes one or more *kil* genes in addition to *kilA* and *kilB* (K. Perry and S. Elledge, unpublished data), and it is possible that all of them play a role in this phenomenon.

***kilA* is required for expression of the Slo phenotype.** Strains containing pKM101 form extremely small colonies on minimal media relative to plasmid-free controls (12, 24). This phenotype (designated Slo) is not seen on media supplemented with 10 μg of adenine or other purines per ml (24). A number of derivatives of pKM101 bearing Tn*5*'s near *korA*

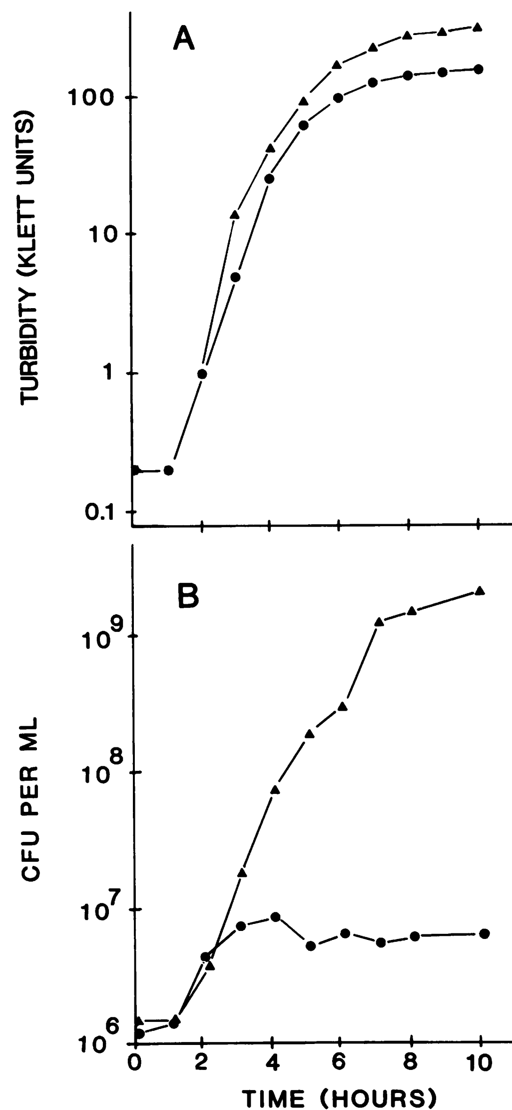

FIG. 4. Growth kinetics of strains JC2926(pGW1584)(pGW1670) (●) and JC2926(pACYC184)(pGW1670) (▲) grown in LB-chloramphenicol broth at 43°C. (A) Turbidity of the culture as measured with a Klett colorimeter. (B) CFU per milliliter as a function of time. Cells were aliquoted from broth cultures, diluted, plated on LB-chloramphenicol plates, and incubated at 30°C.

TABLE 3. Localization of genes sufficient for expression of the Slo phenotype

Plasmid[a]	Slo phenotype[b]
pKM101	++
pGW2158	−
(pGW2158)(pGW1584)	++
(pGW2158)(pGW1584 *kilA1280*::Tn*5*	−
(pGW2158)(pGW1584Ω1277::Tn*5*)	++
(pGW2158)(pGW1584Ω1278::Tn*5*)	++
(pGW2158)(pGW1584Ω1279::Tn*5*)	++
(pGW2158)(pGW2182)	++
(pGW2158)(pGW2189)	++
(pGW2158)(pGW2180)	−
pGW2160	−
(pGW2160)(pGW1584)	++
pKM101Ω13::Tn*5*	−
pKM101Ω81::Tn*5*	+
pKM101Ω53::Tn*5*	++
pKM101Ω73::Tn*5*	−
pKM101Ω61::Tn*5*	−
pKM101Ω1242::Tn*5*	+++++
pKM101Ω1135::Tn*5*	+++
pKM101Ω51::Tn*5*	−
pKM101Ω1280::Tn*5*	−

[a] Plasmids pGW2158 and pGW2160 are shown in Fig. 2. pGW1584, its insertion derivatives (lines 4 through 7), and its deletion derivatives (lines 8 through 10) are described in the legend to Fig. 1. Insertion derivatives of pKM101 (lines 13 through 21) are shown in Fig. 2.

[b] Strains containing the plasmids indicated were streaked out onto minimal medium supplemented with antibiotics and either containing or lacking 10 μg of adenine per ml. After overnight growth, colony size was scored. "+", "++," etc., indicate small-colony morphology. All strains formed large colonies when grown in the presence of adenine.

and *kilA* also do not express this phenotype (12). We provide evidence which suggests strongly that *kilA* is necessary for expression of the Slo phenotype and that *kilA* in combination with *korA* and *korB* is sufficient.

To do this, we made use of various insertion and deletion derivatives of pGW1584 (Fig. 1) in combination with other plasmids necessary to supply *korA*. Both JC2926(pGW2189)(pGW2158) and JC2926(pGW2182)(pGW2158) expressed the Slo phenotype (Table 3), indicating that the 0.7-kb region flanked by insertions Ω1275::Tn*5* and Ω1277::Tn*5* contains all necessary genes. Two insertions, *kilA1280*::Tn*5* and *korB1276*::Tn*5*, have been isolated in this region. The first of these is Slo$^-$, whereas the second cannot be tested because of *kilA*-mediated lethality. These findings strongly suggest that *kilA* is required for expression of the Slo phenotype.

We also replaced pGW2158 with pGW2160 (see Fig. 2) to test the involvement of genes surrounding *korA*. We determined first that pGW2160 alone is insufficient for the expression of Slo even though it contains the *kilA* gene. This result was not unexpected since the pKM101 derivative used to make this clone, pKM101Ω13::Tn*5*, is also Slo$^-$. Evidence is provided below that this may be due to polar effects of this insertion upon *kilA*. pGW2160 in combination with pGW1584 is Slo$^+$, indicating that there is no DNA to the right of Ω13::Tn*5* required for this phenomenon. These data strongly suggest that pGW2158 and pGW2160 play no role in the Slo phenotype other than to supply *korA*.

Table 3 also shows the Slo phenotype of a number of derivatives of pKM101 containing Tn*5* insertions in the 1.4-kb interval between *kilA* and *traA* (see Fig. 2). Some of these derivatives were Slo$^-$, despite the fact that most of these insertions fall into loci which were shown by the analysis above to be dispensible for the phenotype. It is possible that their Slo$^-$ phenotype is due to polar effects on *kilA*. This would indicate that *kilA* is transcribed from right to left and that this transcript originates from a promoter to the right of Ω13::Tn*5*, close to *traA*. This is consistent with the observation described above that insertions in pGW1584 that lie to the right of *kilA* were somewhat deficient in the Kil phenotype. We also observed that two pKM101 derivatives having Tn*5*'s directly upstream from *kilA* express Slo more strongly than pKM101 itself. In fact, strains containing one of these pKM101Ω1242::Tn*5* elements do not form colonies at all in the absence of adenine, yet normal colony morphology is suppressed by addition of 10 μg of adenine per ml. It is possible that these two insertions disrupted some regulatory element or that an operon fusion has been created between *kilA* and a Tn*5* promoter (2).

DISCUSSION

In the present study, two genes mediating potentially lethal functions (*kilA* and *kilB*) have been identified, and two genes that inhibit this lethality (*korA* and *korB*) were also described. It was shown that the expression of both *kor* genes is required to control the lethality of each *kil* gene. A Tn*5*-derived mutation of *kilB* was obtained and crossed into pKM101; this derivative of pKM101 was found to be transfer deficient, indicating either that *kilB* is required for transfer or that this gene lies upstream in the same transcriptional unit as one or more transfer genes. The *kilB*::Tn*5* insertion mutation is part of the *traE* complementation group. A *kilA*::Tn*5* mutant was also isolated and crossed into pKM101. This pKM101 derivative is transfer proficient, but is deficient in the Slo phenotype of pKM101 (12, 24). Evidence was presented that *kilA* itself is required for this phenotype and that *kilA*, *korA*, and *korB* are sufficient to mediate this effect. A set of Slo$^-$ derivatives of pKM101 having Tn*5* insertions lying between *kilA* and *traA* have been described elsewhere (12). We believe that these insertions may be polar on *kilA*.

That both *kor* genes must act together to control either *kil* gene indicates that these four genes are in some way interrelated. The *kil* and *kor* genes of the IncP plasmid RK2 are interrelated in similar ways (see above). This suggests that the two sets of genes may be evolutionarily related. IncP plasmids seem in other ways to be similar to IncN plasmids (3–5). On the other hand, the fact that plasmid N3 (which is related to pKM101) could not inhibit the lethality of the RK2 *kilA* or *kilB* genes suggests that, if these sets of genes do have a common ancestor, considerable divergence must have occurred. Figurski et al. (11) postulated that the RK2-encoded genes may play a role in the extraordinarily broad host range of the plasmid (9). If the functional role of these two sets of genes is related, then they ought not to be sufficient for the broad host range of the latter plasmid, since the host range of pKM101 is narrower than that of RK2.

Many questions about these genes remain unanswered. Both the modes of action of the *kil* genes and their physiological role remain largely unexplored, although the Slo phenotype of *kilA* may provide some clue as to the mechanism of *kilA*-mediated lethality. Likewise, the modes of action of the products of the *kor* genes are not understood. There are many possibilities: they could repress the *kil* genes, they could degrade the products of the *kil* genes, or interact with them in some other way, or they could in some way reverse the damage that the cell suffers from the *kil*

genes. That *kilB* is required for conjugal transfer suggests that the *kor* genes do not prevent synthesis of *kilB*, since the transfer operon of pKM101 is expressed constitutively (26, 28).

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