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Lethality and Survival of *Klebsiella oxytoca* Evoked by Conjugative IncN Group Plasmids

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The transmission of plasmid pCU1 (or other IncN group plasmid) into a population of *Klebsiella oxytoca* cells reduces the viability of the population. A 2,400-bp region adjacent to *traA* is responsible for this phenotype and includes two regions, called *kikA* and *kikC*. *Klebsiella* cells which received this region and survived were found to acquire a chromosomal mutation which renders them immune to killing even after the plasmid is cured from the cells. To obtain insight into the mode of this apparent lethality, an appropriate pCU1*lacZ* derivative was constructed. It could be introduced with high efficiency into *Klebsiella* cells. Analyses of the resultant colonies indicate that the loss of viability is not a consequence of the death of plasmid-free segregants. On the contrary and unlike postsegregational killing by plasmids, cells survived by losing the plasmid or by acquiring, secondarily, a chromosomal mutation which confers immunity to killing.

All conjugative antibiotic resistance plasmids of the incompatibility group N of gram-negative bacteria confer on their hosts a phenotype called Kik$^+$. The phenotype consists of a marked reduction of the viability of *Klebsiella oxytoca* (previously *Klebsiella pneumoniae* [30, 31, 42]) recipients but not of *Escherichia coli* K-12 recipients following matings with the donor hosts on solid surfaces, a condition that is necessary for the efficient conjugative transfer of the plasmid. The phenotype was shown to have an intracellular basis and was initially called Kil (30, 31), but following the use of the designation *kil* for plasmid loci conditionally lethal in *E. coli* (6, 41) and to indicate its host specificity, it was renamed Kik (38). Further studies were undertaken with a plasmid of this group called pCU1 that was the subject of detailed studies in our laboratory. It was shown by transposon Tn*5* mutagenesis that a locus called *kikA* had an important role in this phenotype. The locus mapped close to one end of the conjugative transfer (*tra*) region of the plasmid, but the Tn*5* mutants did not alter the efficiency of conjugative transfer between *E. coli* organisms (38). Hengen et al. (12) cloned and sequenced *kikA* and constructed a plasmid derivative in which *kikA* is controlled by the *tac* promoter. This cloned plasmid could be transformed efficiently into and maintained in *Klebsiella* cells. Upon induction with isopropyl-β-D-thiogalactopyranoside (IPTG), the *Klebsiella* cells lost their viability. This showed that mating was not essential to bring about *Klebsiella* cell lethality and that loss of viability of *Klebsiella* cells could be mediated by events that occurred in them. While these observations showed that *kikA* has an important intracellular role in determining *Klebsiella* lethality, previous observations (11, 12, 18) have shown that there are loci on pCU1 and the closely related plasmid pKM101 that are lethal to *E. coli* (*kil* loci) in the absence or dysfunction of other, cognate loci (*kor* loci). Conceivably, such loci could also contribute to lethality of pCU1 in *Klebsiella* cells.

There are other interesting aspects to *Klebsiella* lethality that were evident from our earlier observations. As stated, after surface matings with auxotrophic *E. coli* carrying pCU1, if prototrophic *Klebsiella* cells were plated on minimal agar without antibiotic, only 1 to 10% of the recipients survived to form colonies. One group of these colonies did not contain any of the plasmid-determined antibiotic markers and could have arisen either by having escaped matings or (as will be shown and discussed in this report) by having acquired and then lost the plasmid during colony development. A second group had all of the plasmid-determined antibiotic markers, contained plasmid DNA with an unaltered restriction pattern, were conjugation proficient, and in a secondary mating could kill another genetically marked group of *Klebsiella* cells with the same efficiency as they did in the *E. coli-Klebsiella* mating, evidence indicating that the plasmid was structurally and functionally unchanged in the surviving *Klebsiella* cells (30, 31). In independent experiments, Gill (10) and Rotheim et al. (32) isolated spontaneous derivatives of these surviving *Klebsiella* cells that had lost all of the plasmid markers and found invariably that such plasmid-cured derivatives were no longer susceptible to the Kik$^+$ phenotype, implying that they were chromosomal mutants.

In this study, we first confirm these earlier observations and identify a region on the plasmid that is both necessary and sufficient for lethality in *K. oxytoca* but not in *E. coli*. Plasmid clones containing this region were unable to transform *K. oxytoca* while transforming *E. coli* efficiently. When an origin of conjugative transfer ($oriT_{RK2}$) was inserted into the DNA of such clones, they could be mobilized into *K. oxytoca* at a low frequency. It is shown here that this occurs because these rare *Klebsiella* cells now have a chromosomal mutation that make them immune to the lethality of the plasmid region. We have identified and sequenced the region on pCU1 that causes this *Klebsiella* lethality and the chromosomal mutation to arise. We compare this sequence with a similar sequence in the closely related plasmid pKM101 (28). It is shown that the region of about 2.4 kbp contains sequences, in addition to those of *kikA*, that are also lethal in *K. oxytoca*. The features that may cause the lethality of this region in *K. oxytoca* but lack of lethality in

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype and/or phenotype	Source or reference
E. coli		
HB101	F$^-$ *hsd20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1* λ^-	3
C600 Nal	F$^-$ *thi-1 thr-1 leuB6 lacY1 tonA21 supE44* λ^- and spontaneous mutation to Nal^r	2
DH5α	*endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* λ^-	9
S17-1	*pro* chr::RP4	36
S17-1λ*pir*	*pro* chr::RP4 λ*pir*	36
CC118	*sup*^{0} (*ara leu*)7697 *lacx74 phoA20 galE galK thi rpsE rpoB argE*(Am) *recA1*	40
K. oxytoca		
M5a1		23
UN2979	*hisD4226 lacZ4001*	23
UN2979::400	Same as that for UN2979 but with pLOF/Km containing 400 bp of pUC18*Sfi*; Km^r	This work
Plasmids		
pACYC184	Cm Tc; cloning vector	4
pUC128	Ap; cloning vector	19
pJF118HE	Ap; cloning vector	7
pNHKan/*oriT*	Ap Km; cloning vector containing $oriT_{RK2}$	13
pUC18*Sfi*	Ap; cloning vector	14
pLOF/Km	Km; cloning vector	14
pMRK400	Km; 400 bp from pUC18*Sfi* cloned into pLOF/Km	This work
pCU1	Ap Sm Sp; naturally occurring plasmid	20
pCU88	Ap Sm Sp Km; Tn*5* insertion in pCU1 at coordinate 10.2	37
pCU109	Cm; pCU1 *tra* region, coordinate 27.7 to 10.2, cloned into pACYC184	38
pCU56	Cm; coordinates 5.6 to 11.4 of pCU1 including its *oriT* region cloned into pACYC184	37
pCU66	Ap Sm Sp Km; Tn*5* insertion in pCU1 at its coordinate 30.2 inactivating *kikA* function	38
pCU403	Km; *Bam*HI deletion derivative of pCU66	38
pCU403-1	Km; *Kpn*I deletion derivative of pCU403	This work
pCU1107 (pCU1107$oriT_{RK2}$)[a]	Cm; *kikA* region from pCU171 cloned in pACYC184	12; this work[b]
pAAG8 (pAAG8$oriT_{RK2}$)[a]	Ap; *Bal* 31 deletion derivative of pCU1107 cloned in pUC128	This work
pAAG7 (pAAG7$oriT_{RK2}$)[a]	Ap; *Bal* 31 deletion derivative of pAAG8	This work
pCU12	Ap Sm Sp; deletion derivative of pCU1	20
pCU1*lacZ*	Ap Sm Sp Km; Tn*5phoA′-1* inserted into a silent site of pCU1	This work; 40
pVT149	Cm Km; Tn*5* insertion in pCU109 at coordinate 6.5	37
pVT149-1	Cm; *Xho*I deletion of pVT149; $oriT_{pCU1}$ deleted	This work
pPH4	Ap; 500-bp *kikA* region cloned in pJF118HE under the control of the *tac* promoter	12

[a] The plasmids in parentheses have the same genotype and/or phenotype as the plasmid listed with it but with Kan/$oriT_{RK2}$ cloned into it.
[b] Second reference applies to plasmid in parentheses.

E. coli are discussed. To obtain insight into the origin of the *Klebsiella* chromosomal mutants, we constructed and used a pCU1*lacZ* derivative which allows *K. oxytoca* transconjugant colonies to develop and be screened under conditions when they are not constrained to maintain the acquired plasmid. From the analyses of the screened or selected colonies, we propose that the chromosomal *Klebsiella* mutants arise late during colony development. These observations are compared with those of other plasmid-determined host lethal systems, and it is suggested that the mode of lethality in *K. oxytoca* imposed by the presence of pCU1 is unlikely to be postsegregational.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* K-12 and *K. oxytoca* strains used are listed with their relevant features in Table 1. Like *E. coli* K-12, the *K. oxytoca* strains (previously *K. pneumoniae*) are being used extensively in genetic and physiological studies (for examples, see references 23 and 24). Plasmids are also listed in Table 1. In the text, strains carrying a plasmid are shown with the plasmid designation in parentheses. None of the several *K. oxytoca* strains derived by spontaneous curing of particular plasmids is listed in this table. When relevant in the text, their origin is indicated. Plasmids were transferred into desired strains by DNA transformation (4) or by mating on the surface of filters placed on L agar (37). When transconjugants were identified by screening for the Lac$^+$ phenotype, dilutions of the mating mixture were spread on the minimal agar (5) supplemented with histidine (requirement for the *K. oxytoca*) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). When transconjugants were selected, this was done on minimal agar with streptomycin or kanamycin. After a two-step purification of the transconjugants or transformants on the selective media, they were analyzed for their plasmid-associated phenotypes, and the restriction pattern of plasmid DNA extracted from them was compared with that of the original plasmid DNA.

Chemicals, media, and growth conditions. Strains were grown routinely in L broth or L agar (33) supplemented when necessary with antibiotics. Antibiotic concentrations for *E. coli* and *K. oxytoca*, respectively, were 50 and 90 μg of ampicillin per ml, 50 and 100 μg of chloramphenicol per ml, 50 and 50 μg of kanamycin per ml, 50 and 100 μg of spectinomycin or streptomycin per ml, and 50 and 100 μg of rifampin per ml. Restriction enzymes and linkers were from New England BioLabs Ltd. (Mississauga, Ontario, Canada), T4 DNA ligase, Klenow enzyme, and 1-kb molecular mass DNA fragment standards were from GIBCO-BRL Life Technologies Ltd. (Burlington, Ontario, Canada), and X-Gal was from Boehringer-Mannheim Canada (Montreal, Quebec). dATP, tetra(triethyl ammonium)salt, [α-^{32}P]dATP, and α-^{35}S-dATP were from Amersham Canada Ltd. (Oakville, Ontario). Other chemicals were from Sigma Chemicals Co., St. Louis, Mo.

DNA extraction and manipulation. For rapid analyses of small amounts of recombinant plasmid DNA, this DNA was extracted as described previously (16). Larger amounts were extracted as described previously (17), as was total bacterial DNA (19). Digestion of DNA with restriction endonucleases and their analysis by agarose electrophoresis were done by standard methods (33), fragment sizes being determined with a program described before (35). DNA fragments of >10 kb were recovered from gels by electroelution, and smaller fragments were recovered with the GeneClean Kit of Bio 101, Inc. (La Jolla, Calif.). Radioactive DNA hybridization probes were labelled, prepared, and used as described by Sambrook et al. (33). Dideoxy sequencing of the DNA of both strands was performed with Sequenase version 2.0 (U.S. Biochemical Corp., Cleveland, Ohio) and the method of Sanger et al. (34). To eliminate ambiguities of sequence in some regions, the regions were resequenced by substituting the dGTP labelling and termination mixtures with dITP mixtures. Sequence analysis was done with Microgenie (Beckman Instruments Inc., Palo Alto, Calif.), MacDNASIS (Hitachi Software Engineering Co., Tokyo, Japan), NCBI Blast (1) and FASTA (27).

Construction of a *K. oxytoca* derivative to serve as a positive control in DNA hybridization experiments. A conclusion that *K. oxytoca* mutants immune to the

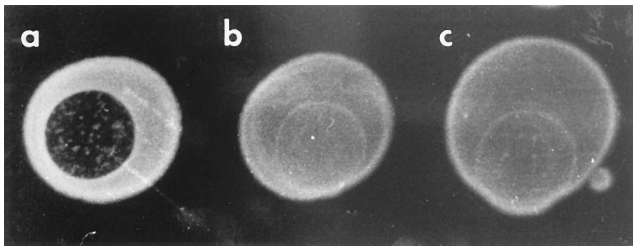

FIG. 1. Illustration of the test used to screen colonies of *Klebsiella* derivatives for their susceptibility to the Kik^+ phenotype of C600(pCU1). Two multipoint probes with different diameters and allowing 25 derivatives to be screened per agar surface on standard petri dishes were used. The device with the larger probes was used to deliver large drops of *Klebsiella* cells in a grid pattern. After the drops dried, a second device with a smaller probe than and the same geometry as that of the first device was used to deliver a smaller drop of *E. coli* C600 (pCU1) on the areas of the *Klebsiella* drops. Only 3 of the 25 areas on a plate are shown. Drops: a, derivative susceptible to Kik^+; b, a control where the same culture that was used in drop a was spotted with C600 that did not carry pCU1; c, immune derivative that was not susceptible.

Kik^+ phenotype contain no part of the plasmid DNA required us to construct a *Klebsiella* derivative that could serve as a positive control. For this purpose, a 400-bp fragment from pUC18*Sfi* (14) was inserted into the suicidal transposon delivery vector pLOF/Km which has the replicon of R6K and the conjugative transfer origin (*oriT*) of RP4 (14). The construct carrying the 400-bp DNA in pLOF/Km is called pMRK400 (Table 1). The function of the R6K replicon in pMRK400 requires the R6K replication gene called *pir* which can be provided in *trans*. pMRK400 was maintained in *E. coli* S17-1λ*pir*, which has both the conjugative transfer system of RP4 and λ*pir* inserted into its chromosome. When S17-1 λ*pir*(pMRK400) is mated with *K. oxytoca* and kanamycin-resistant transconjugants are selected, they arise by transposition of pLOF/Km. Since in pMRK400 the 400-bp DNA from pUC18*Sfi* had been inserted into pLOF/Km, this resulted in transposition of the 400-bp pUC18*Sfi* DNA into the *K. oxytoca* chromosome. A *K. oxytoca* derivative containing pLOF/Km (without the 400-bp insert) served as a control.

Tests of *K. oxytoca* colonies for immunity to the Kik^+ phenotype of pCU1. In some experiments, following the transfer of a plasmid into *K. oxytoca* by conjugation or conjugative mobilization, about 200 of such colonies were tested for immunity to the Kik^+ phenotype of pCU1. To facilitate such tests, the qualitative test that has been described previously (30) was adapted. Cells from single colonies on the selection plates were regrown on L agar for 24 h, and cells from each regrown colony were transferred into separate compartments in a 25-compartment sterile container (Elisa Co., Milan, Italy) with 0.5 ml of sterile 0.85% saline in each compartment. A multipoint sterile inoculator was then used to transfer samples from each compartment simultaneously to the surface of minimal agar to form large drops in an ordered manner. The drops were allowed to dry, and their positions were respotted with a smaller drop of an exponentially growing culture of *E. coli* C600(pCU1). The sterile probes on the inoculator used for this second step had a smaller diameter than those used in the first step. Figure 1 illustrates typical results.

Construction of the plasmid pCU1*lacZ*. An overnight culture of CC118(pCU1) grown in L broth was infected with the λPam phage (25) carrying the Tn*phoA′-1* transposon element essentially as described by Wilmes-Reisenberg and Wanner (40). After overnight incubation at 37°C, the cells were spread on LB plates supplemented with kanamycin, streptomycin, spectinomycin, and ampicillin and incubated overnight. Colonies which appeared on the plates the next day were pooled and mated with *E. coli* C600Nal by standard filter mating. Transconjugants carrying the putative pCU1-Tn*5phoA′-1* construct were then screened for the Kik^+ phenotype, the DNA from one Kik^+ clone was extracted, and the position of the Tn*5phoA′-1* was mapped by restriction analysis.

RESULTS

Identification of a region on pCU1 which when introduced into *K. oxytoca* leads to host-specific lethality. Figure 2 displays a simplified map of pCU1 and of some of its deletion derivatives and clones (Table 1). In matings between *E. coli*, transconjugants of the Tra^+ plasmids shown in this figure (pCU1, pCU109, and pCU403) could be selected at a frequency of approximately 10^{-1}, while in similar matings with *K. oxytoca* as the recipient, the transconjugants arose only at a frequency in the range of 10^{-3} to 10^{-5}. Plasmid derivatives that are not conjugatively self-transmissible can usually be transferred either by conjugative mobilization with a cognate helper plasmid if they contain a functional origin of transfer (*oriT*) as in pCU56 or by plasmid DNA transformation. Among the derivatives displayed in Fig. 2a, pCU12 DNA and pCU56 DNA were each transformed into *E. coli* and *K. oxytoca* with equal and high efficiencies. pCU56 could also be mobilized into both species with equal and high efficiencies.

In contrast to this, the DNA of all other derivatives shown in Fig. 2 did not yield any *Klebsiella* transformants. The smaller members of this group (pAAG7 and pAAG8) transformed *E. coli* efficiently. Of the larger plasmids, only pCU403-1 is not conjugatively self-transmissible. With pVT149-1 (Table 1) (see Materials and Methods) as the helper, this plasmid could be mobilized into *E. coli* at a frequency of 10^{-1} and into *K. oxytoca* at only a frequency of 10^{-5}. These observations indicate that all derivatives shown darkly shaded in Fig. 2 contained a region that was probably host lethal in *K. oxytoca* but not in *E. coli*. The smallest of these derivatives was pAAG7 (Fig. 2b). This plasmid and plasmids pCU403 and pCU403-1 have deletions of the *kikA* locus, indicating that sequences between *kikA* and *traA* constitute a second *Klebsiella*-lethal region that can function independently of *kikA*. This region is called *kikC*.

Nucleotide sequence of the host-lethal region present in pAAG8. The region between the Tn*5* insertion pCU171 in *traA* and pCU66 in *kikA* (37, 38) was sequenced in both strands. The primers used, the sequencing strategy, and the resulting sequence are described in Fig. 3, which indicates all detected open reading frames. The sequence features will be considered further in Discussion.

Conjugative mobilization of pAAG7-*oriT*$_{RK2}$ into *K. oxytoca* and its ability to give rise to mutants that are immune to pCU1 lethality. Since pAAG7 DNA did not yield *K. oxytoca* transformants and since conjugation can be a relatively more efficient process than transformation, we used the Kan/*oriT*$_{RK2}$ cassette that has been described previously (13) to convert pAAG7 to the mobilizable pAAG7-*oriT*$_{RK2}$ and transformed it into the helper strain S17-1 (Table 1). The resulting strain was then mated with *K. oxytoca*. The mobilization frequency was low (10^{-7}). When 200 transconjugants were tested for their susceptibility to the lethality caused by subsequent mating with C600(pCU1), 88 to 90% of the colonies were immune in different experiments. Plasmid DNA isolated from them was unchanged in their restriction patterns (with *Hin*dIII and *Ssp*I) and again gave rise to immune *Klebsiella* colonies at the same frequency (after transforming S17-1 and using the resulting derivative as the donor). The remaining 10 to 12% of the *Klebsiella* colonies were not immune, but plasmid DNA isolated from them was found to have undergone major structural rearrangements. These latter derivatives have not been examined further. Results similar to those obtained with pAAG7-*oriT*$_{RK2}$ were obtained with pAAG8-*oriT*$_{RK2}$ and pCU1107-*oriT*$_{RK2}$ (results not shown).

No detectable sequence of pCU1 is present in the mutant immune *K. oxytoca* derivatives after plasmids have been cured from them. The mutant *K. oxytoca* strains carrying any of the plasmids with the *Klebsiella*-lethal region (shown darkly shaded in Fig. 2) could be cured spontaneously of their respective plasmid markers by growing each strain for about 80 generations in L broth without any antibiotics, plating out for single colonies, and testing 100 single colonies for loss of streptomycin and spectinomycin resistance. Such derivatives were no longer susceptible to the lethality caused by C600(pCU1) when tested by the procedure described in Materials and Methods. When used as recipients, transconjugants of pCU1 now arose with the same high frequency as they did with *E. coli*.

Although no plasmid markers or plasmid DNA was found in

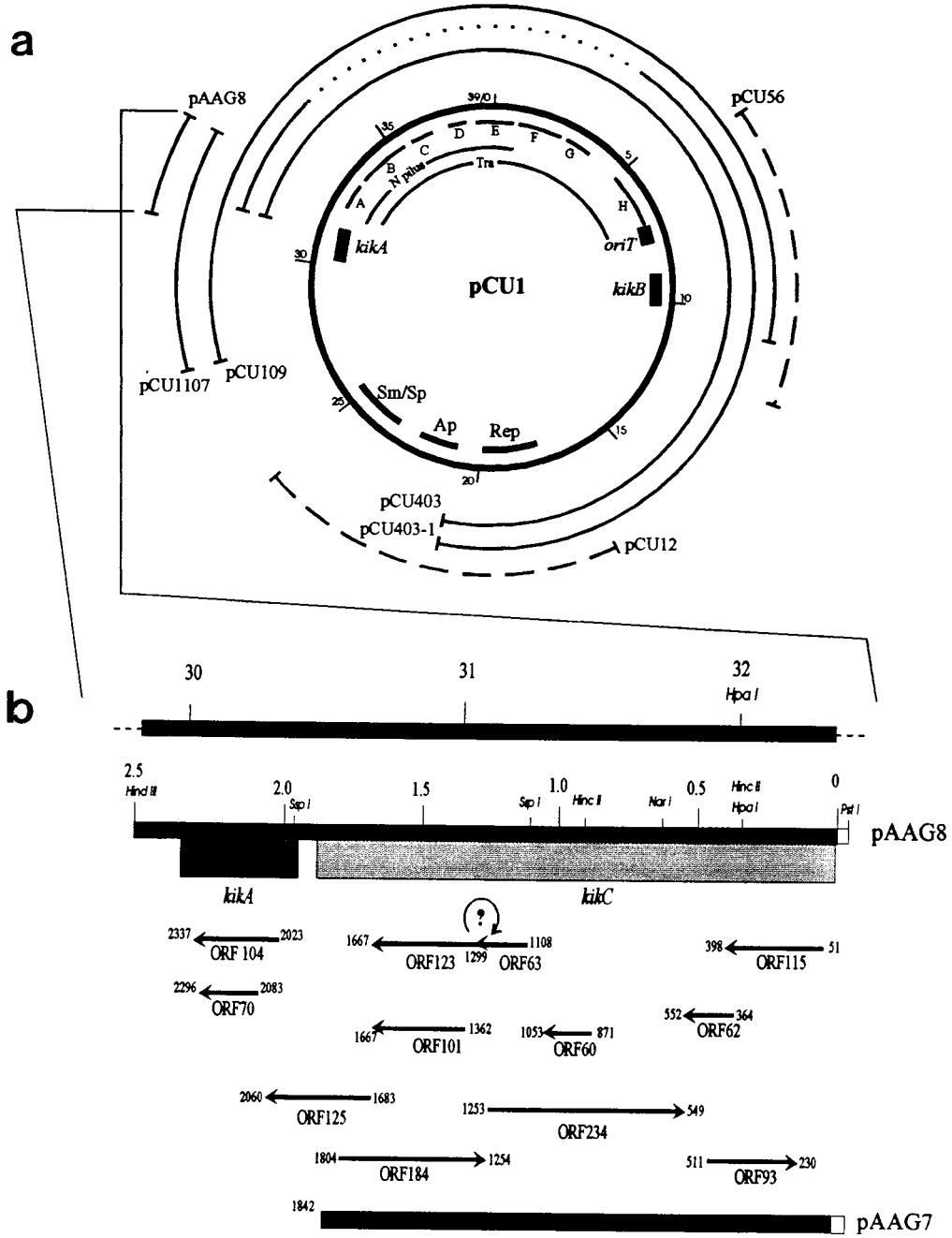

FIG. 2. (a) Derivatives of pCU1 that were used to localize the region that was sufficient to be lethal to *K. oxytoca* (but not to *E. coli*) and to give rise to the mutant immune *Klebsiella* cells. Derivatives that show these phenotypes are shaded dark. The smallest such derivative is pAAG7 (see panel b). The *kikA* locus is deleted in this derivative and in pCU403 and pCU403-1. Derivatives that are not lethal to *Klebsiella* cells (i.e., transform *Klebsiella* cells efficiently) and do not give rise to immune mutants are shown as broken lines of arcs around the circle. (b) Sequence features of the *kikA* and *kikC* regions. Arrows indicate directions of translation. (?) indicates a possible −1 frameshift to bring *orf123* in frame. The small unshaded areas of one end of pAAG8 and pAAG7 are to indicate the presence of a short IS*50* sequence from the terminal portion of the transposon Tn*5* initially present at that location.

these cured derivatives, we wished to determine whether there were any sequences from pCU1 retained by them, perhaps in a form in which they were inserted into the chromosome. Total DNA was isolated from several such cured derivatives of M5a1(pCU1) and UN2979(pCU109), digested with *Hin*dIII, and used to prepare blots for Southern hybridization. These blots were then hybridized with radioactively labelled denatured pCU1 DNA fragments that had been obtained by digesting the plasmid DNA with *Hpa*I. None of the blotted DNA samples hybridized detectably to the probes. To serve as a positive control in such experiments, a 400-bp fragment from pCU18*Sfi* had been inserted into the chromosome of a *Klebsiella* derivative (see Materials and Methods). Chromosomal DNA from this derivative was digested and hybridized to the same probe. Hybridization was detected. The radioactivity was stripped from the membrane, and the stripped membrane was

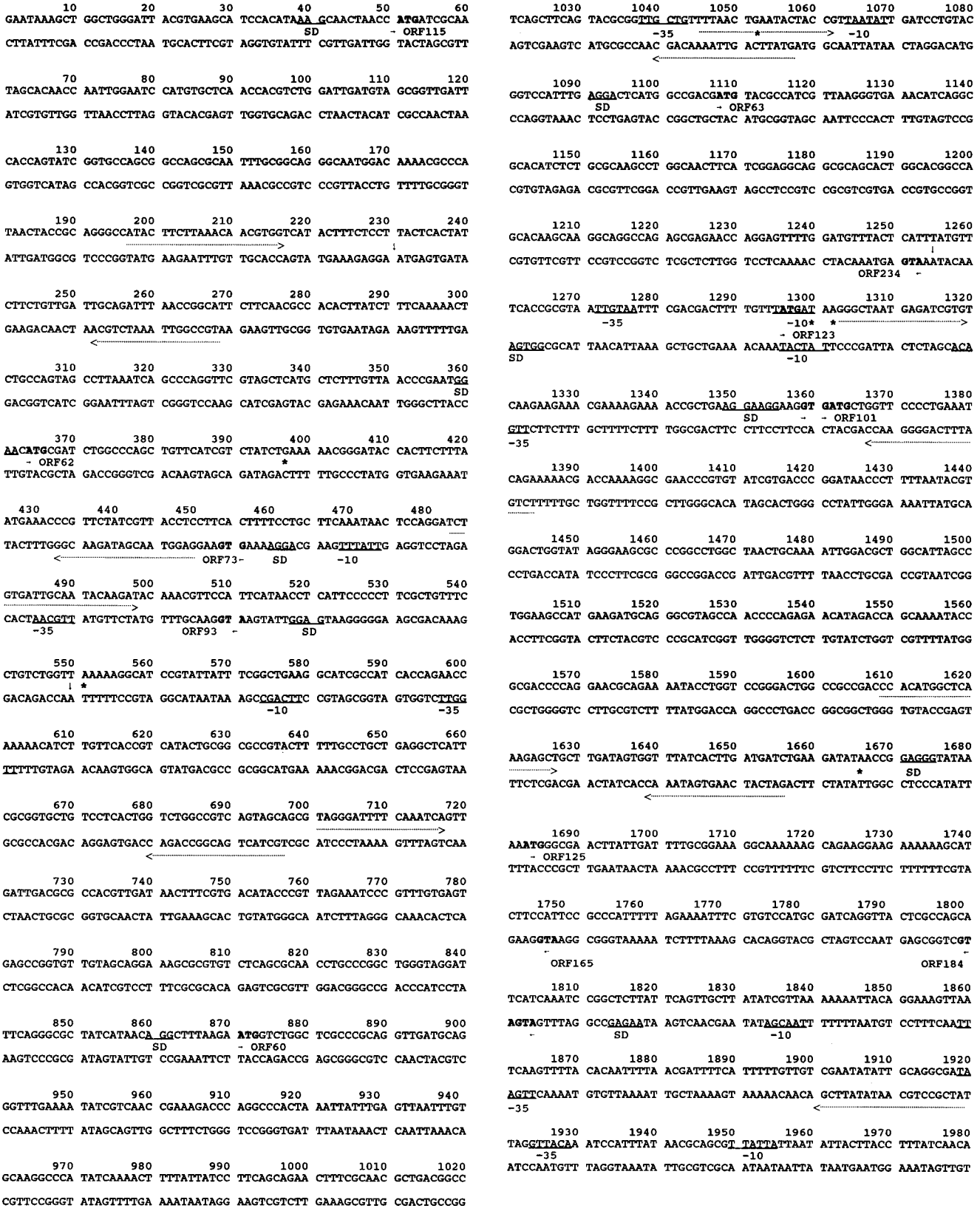

FIG. 3. The sequence of the pCU1 region present in pAAG8 (GenBank accession number U26172). The numbers indicate the nucleotide positions anticlockwise from the pCU1 coordinate 32.35. Putative promoters and Shine-Dalgarno sequences are underlined. Arrows of potential open reading frames (ORFs) pointing right are in the direction from *traA* towards *kikA* (upper strand), and those pointing left are in the direction from *kikA* towards *traA*. Initiation codons are shown in bold type. Potential stop codons on the upper strand are indicated by an asterisk, and those on the lower strand are indicated by a downward arrow. The sequences of the oligonucleotide primers used are indicated with a dotted line. The sequencing strategy is apparent from the positions of these primers.

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      1990        2000        2010        2020        2030        2040
TTCTGGCGAG TGTATATCTG AACAAACTGT TAAGGACAAA AAATGAAGAA ACTCTTAATA
                                    SD          → ORF104
AAGACCGCTC ACATATAGAC TTGTTTGACA ATTCCTGTTT TTTACTTCTT TGAGAATTAT

      2050        2060        2070        2080        2090        2100
CCTCTGATAG CAGCTGGTAG TCTGCTTTAT CTTCCTGCCA GCCATGCTGA AGATCCCTGC
             *           ↓                       → ORF70
GGAGACTATC GTCGACCATC AGACGAAATA GAAGGACGGT CGGTACGACT TCTAGGGACG

      2110        2120        2130        2140        2150        2160
AAAGTTATTA TGTGCATGGC GGGCAAGCTC ACCGGCGATA GCGGCGGAAG CGAGTGTAAC

TTTCAATAAT ACACGTACCG CCCGTTCGAG TGGCCGCTAT CGCCGCCTTC GCTCACATTG

      2170        2180        2190        2200        2210        2220
AGTGCTGAAG CTGCTTTCTT CAATATCGTT AAAAAGAACA AGCACGGCTT TTTACCCAAC

TCACGACTTC GACGAAAGAA GTTATAGCAA TTTTTCTTGT TCGTGCCGAA AAATGGGTTG

      2230        2240        2250        2260        2270        2280
CACACGAAGG ATGCTAGGAA GGCTTTTCTT AATGAATGCC CGGATAATGG CGAAGGTGGA

GTGTGCTTCC TACGATCCTT CCGAAAAGAA TTACTTACGG GCCTATTACC GCTTCCACCT

      2290        2300        2310        2320        2330        2340
AGTAACCAGT CGATGATAAG CCAGATCATA AGTAAATACG GGAAAGTTCG CTTATAGGCC
             *                                                   *
TCATTGGTCA GCTACTATTC GGTCTAGTAT TCATTTATGC CCTTTCAAGC GAATATCCGG
                    ORF80 ←
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FIG. 3—*Continued.*

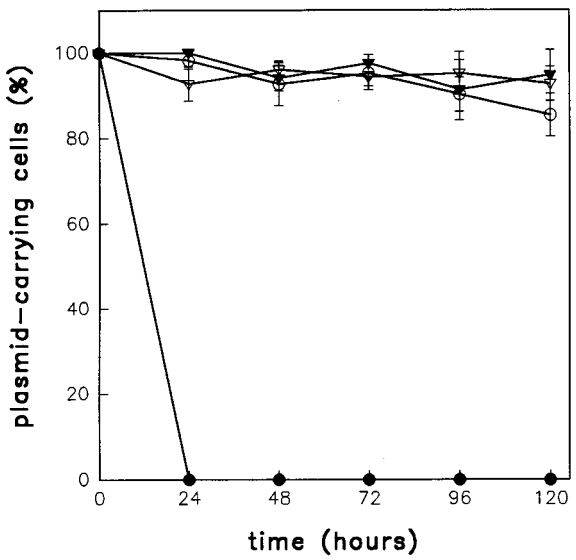

FIG. 4. Stability of the plasmids pPH4 (*tac-kikA*) and pJF118HE (vector) in *Klebsiella* cells in the presence and absence of IPTG. Overnight cultures grown in the presence of ampicillin (900 μg/ml) were diluted 1:100 in fresh L broth without ampicillin and grown in the presence of 0.5 mM IPTG (or distilled water [control]) at 37°C. The cultures were diluted 1:1,000 every 24 h, at which time the samples were withdrawn and plated on L-agar plates. After overnight incubation, 100 colonies of each sample were replica-plated on L-agar plates supplemented with ampicillin. The plates were scored for bacterial growth after overnight incubation. Symbols: ○, −pPH4; ●, −pPH4, +IPTG; ▽, −pJF118HE; ▼, −pJF118HE, +IPTG.

used in a second hybridization with the pCU1 radioactive probe. No hybridization was detected. It was concluded that within the limits of 400 bp, no part of pCU1 DNA exists in such immune *Klebsiella* cells. Furthermore, since *K. oxytoca* cells selected to carry the *kikA* deletion derivative pAAG7, pCU403, or pCU403-1 were also found to be immune following the loss of their respective plasmids, it could be concluded that the *Klebsiella*-lethal locus *kikA* was not essential for the immune mutants to arise. This conclusion was supported by observations that the pCU1 derivatives with Tn*5* insertions in *kikA* also give rise to immune mutant *Klebsiella* cells (result not shown).

Consequences of overexpression of *kikA*. The *kikA* locus has been cloned under the control of the *tac* promoter, and it was shown previously that the induction of *K. oxytoca* carrying this clone by the inducer IPTG results in loss of viability of this host. We have shown elsewhere (15) that the overexpression of *kikA* leads to reversible inhibition of growth, presumably by dissipating the membrane potential. To investigate the effect of *kikA* on the stability of a plasmid derivative carrying it, *Klebsiella* cells were transformed with plasmid pPH4 (*kikA* under the control of *tac* promoter [12]), and the stability of the plasmid in the absence of antibiotic selection and in the presence or absence of inducer IPTG was investigated. As shown in Fig. 4, plasmid pPH4 can be stably maintained in *Klebsiella* cells even without the selection for antibiotic resistance. However, upon induction with IPTG, the plasmid was completely lost from the population within 24 h. We were unable to detect any survivors ($<10^{-7}$).

Events in *K. oxytoca* after receiving the wild-type pCU1 and during colony development. The experiments that have been described indicate that there are two separable but adjacent regions on pCU1 called *kikA* and *kikC*, each of which can independently determine lethality in *K. oxytoca* (see Fig. 2). In addition, there is a region called *kikB* (38) which has not yet been studied in detail but in the absence of which the plasmid undergoes structural rearrangements in *Klebsiella* cells (29a). We wished to monitor the fate of the plasmid in the population of *Klebsiella* recipients by screening rather than selecting transconjugants. To do this, we constructed a derivative of pCU1 called pCU1-*lacZ* carrying the Tn*5*-based transposable element Tn*phoA'-1* described by Wilmes-Reisenberg and Wanner (40). In this element, *phoA* of Tn*phoA* is replaced by *lacZ*. The element also carries a kanamycin resistance (Km^r) marker. The plasmid pCU1*lacZ* was introduced into *E. coli* C600 by mating and maintained in this strain.

C600(pCU1*lacZ*) was mated with the *Klebsiella* histidine auxotroph UN2979 and, separately, with either UN2979 cured of pCU109 (UNC109) or with *E. coli* DH5α. Dilutions of each mating mixture were spread on minimal agar supplemented with the nutritional requirements of each recipient and X-Gal. The results are summarized in Table 2. Transconjugant colonies of UNC109 and of DH5α were uniformly blue, as would be expected for a population that receives and maintains the plasmid in a stable manner. Furthermore, their numbers were

TABLE 2. Frequency and type of transconjugants in matings with an *E. coli* donor of pCU1*lacZ* and *E. coli* and *Klebsiella* recipient UN2979 and UN2979 cured of pCU109 (UNC109)[a]

Recipient	% Survivors on minimal agar[b]		
	Total	On X-Gal[b,c]	On X-Gal plus kanamycin
DH5α	88.37	23.25	24.65
UNC109	91.10	26.30	27.10
UN2979	2.59	0.31	0.0015

[a] Matings were for 1 h on the surface of cellulose nitrate membrane filters placed on L agar as described previously (37). The donor-to-recipient ratio was 1:10. Following the matings, the cells from the membranes were resuspended in saline, and dilutions were spread on the media indicated.

[b] The percentages indicated are relative to a control population of the recipient treated in a manner similar to the mating experiment but without the donor.

[c] On X-Gal, survivors were considered to be colonies showing any evidence of Lac expression, i.e., completely blue colonies in the case of UNC109 or DH5α recipients or predominantly white colonies with blue areas as shown in Fig. 5.

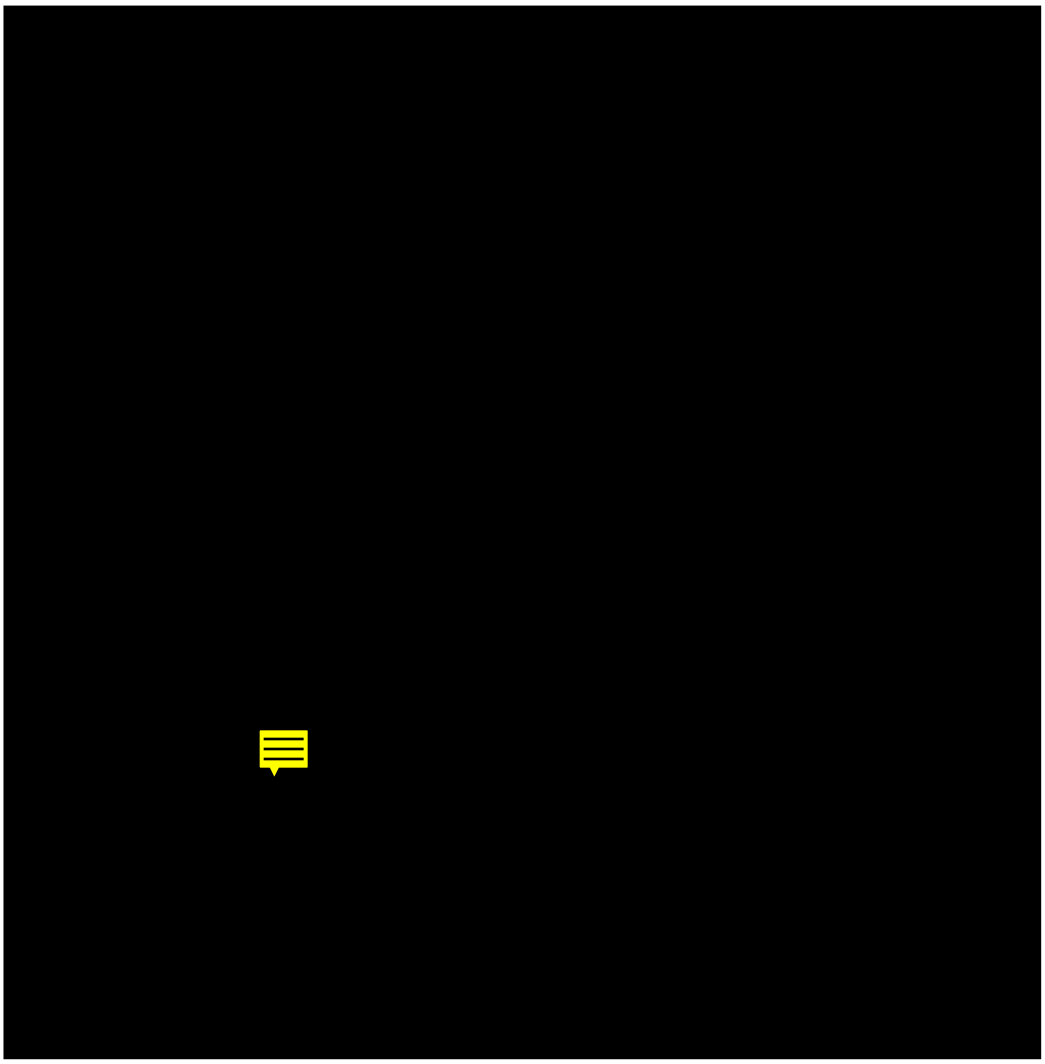

FIG. 5. Appearance of colonies of *K. oxytoca* UN2979 that have received pCU1*lacZ* and lost the plasmid (white area) or retained it in the few cells in the colony that have acquired a chromosomal mutation (blue area). The blue area is at the surface of the colony (not evident in the photograph).

approximately the same whether they were screened as Lac^+ colonies or selected as Km^r colonies (Table 2). These colonies, when tested, were found to have retained all the plasmid-borne and the transposon-borne markers. There was a striking difference when UN2979 was used as the recipient. When transconjugants were screened with X-Gal, their numbers (as determined by colonies showing any evidence of Lac expression) were much greater than when transconjugants were selected as Km^r colonies (Table 2). Furthermore, while the colonies arising at a low frequency on the medium with kanamycin were uniformly blue, those arising on the medium without kanamycin were, for the most part, white or pale blue but with a small and irregular deep blue region (Fig. 5). This region was not a colony sector. It was generally found close to the surface or at the edges, suggesting that the cells giving rise to them arose late in colony development. Cells from the white and blue areas of these colonies were purified to homogeneity and then tested for the presence of the antibiotic markers. None (of 100 tested) of the clones from the white region carried any of the plasmid antibiotic markers (Sm^r, Sp^r, or Ap^r), including the transposon resistance (Km^r) marker. They were inferred to be plasmid-free. Clones purified from the blue regions were now uniformly blue, and they carried all of the antibiotic markers, including the transposon marker. When used as donors with DH5α as the recipient, they also transferred their plasmid efficiently, and when *K. oxytoca* UN2979 genetically marked by a rifampin resistance marker was used as the recipient, all tested derivatives conferred the Kik^+ phenotype.

DISCUSSION

We have defined the location of two regions on plasmid pCU1 which, when present intracellularly either together or separately, cause inhibition of colony formation by *K. oxytoca* but not by *E. coli*. These two regions are called *kikA* and *kikC*. They are adjacent to one another, with *kikC* abutting the *tra* region (Fig. 2B). The evidence for *kikA* being *Klebsiella* lethal has been presented previously (12) and confirmed here (Fig. 5). The evidence that *kikC* is *Klebsiella* lethal and does not simply affect plasmid stability is based on the observation that neither pAAG8 (that has both *kikA* and *kikC*) nor pAAG7 (that has *kikC* alone) (Fig. 2) will transform *K. oxytoca* while they will transform both *E. coli* and the *Klebsiella* mutants efficiently. The *kikA* and *kikC* regions have been shown by

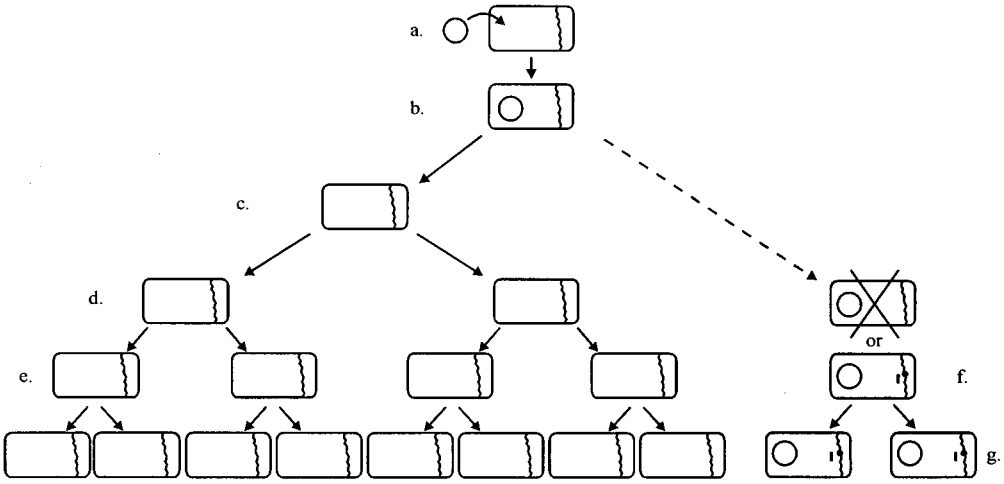

FIG. 6. Diagrammatic interpretation of the consequences of introducing a Kik^+ plasmid into *Klebsiella* cells and the emergence of subpopulations of plasmid-free but nonimmune (majority) and plasmid-carrying but immune (minority) cells within a colony. Following the introduction of a Kik^+ plasmid such as pCU1 into a *Klebsiella* cell (a and b), expression of the Kik^+ phenotype can have one of three outcomes. There can be a segregational loss of the plasmid (c), allowing the cell to survive and multiply (d and e) to give rise to a large number of plasmid-free cells in the colony. A second possibility is that the presence of the plasmid inhibits the multiplication of the cell so that the plasmid is only unilinearly inherited (broken arrow). During such inheritance, the cell can either irreversibly lose colony-forming ability (shown as X) or survive by acquiring, secondarily, a chromosomal mutation (f) that confers immunity to Kik^+ (shown as |). This secondary mutation arises only late in the development of the clone so that immune *Klebsiella* cells constitute a minority of the colony (g). Under conditions where only plasmid-carrying *Klebsiella* cells are selected (e.g., by selecting for an antibiotic resistance marker associated with the plasmid), all selected colonies will be immune even if the plasmid is subsequently cured from them.

DNA hybridization to be conserved among all conjugative IncN group plasmids (12, 28, 21), and in the plasmid pKM101, their relative locations are also similar (28). In addition, the nucleotide sequence of *kikA* in pKM101 is identical and that of *kikC* is nearly identical to that of pCU1 (28), so that it is useful to discuss observations of these regions of both plasmids. With clones carrying mutated *orf104* or *orf70*, it was shown recently that the proteins determined by both of these reading frames in the *kikA* region (Fig. 2) are necessary for the growth inhibitory phenotype of *Klebsiella* cells (15). The *kikC* region is both larger and more complex than the *kikA* region. Two genes that are conditionally lethal in *E. coli* have been recognized to be present in this region of pKM101 (41). One of these was previously called *kilA* (41) and recently renamed *traL* (28). It is similar to *orf234* (Fig. 2b) in most but not all of its sequence but is larger (244 amino acids). To prevent lethality by this gene, it has been proposed that two genes similar to *orf93* and *orf101* (Fig. 2) and called *korA* and *korB*, respectively, are involved (28, 41). The studies on pKM101 also indicated the presence of a second *kil* gene in this region to the left of *traL*, and it was speculated that this might be *kikA* (28). However, it has been reported (12) and confirmed here that overexpression of the *kikA* genes of pCU1 in *E. coli* does not result in growth inhibition of this host. If there is a second *kil* gene to the left of *traL* (*kilA*), as indicated by earlier observations (41), the candidates are *orf184* in one direction and *orf125*, *orf63*, *orf123*, or a protein composed of the 186 amino acids of *orf63* and *orf123* (63 plus 123) that can arise in two ways, either by read-through of stop codons of *orf63* or (since there are two stop codons at the end of *orf63*) by a −1 frame shift (Fig. 2b) that will restore the sequence of *orf123*. Since pAAG8 and pAAG7 transform *E. coli* efficiently, this still-hypothetical *kil* gene must be controlled by sequences present in these plasmids. Further genetic studies will be needed to identify this *kil* gene and the gene(s) (*kor*) that controls it in *E. coli*. Even so, this would not by itself explain why pAAG7 does not transform *K. oxytoca*. Several possibilities can be considered; of these, the most attractive is that the expression of a lethal gene in the *kikC* region or the stability of its product is controlled differentially in *E. coli* and in *K. oxytoca*. The interesting chromosomal mutation(s) in *K. oxytoca* cells which converts them to strains that can be transformed can then be seen as mutations that affect their expression or stability directly or indirectly by affecting the cognate *kor* gene(s). Attempts to identify and characterize the chromosomal mutation(s) in the *K. oxytoca* derivatives are being made.

Our observation of the difference in inherited phenotype between the parent and the mutant *Klebsiella* strains has an important cautionary implication. It is not uncommon to be able to introduce a plasmid at a low frequency into a bacterial host. Barring insertion sequences, it is then assumed that the chromosomal genotype of the recipient is unchanged. This assumption would not be supported by the observations described in this paper.

Although only two regions have been identified in pCU1 that are lethal in *K. oxytoca* without being lethal in *E. coli*, it is possible that there are others. The mating experiments using C600(pCU1*lacZ*) as the donor and *K. oxytoca* as the recipient were intended to examine whether the lethality caused by this entire wild-type plasmid in *K. oxytoca* was mediated by a post-segregational mode, as has been observed recently in *E. coli* with a number of plasmids (8, 22, 26, 39). Because pCU1, upon efficient conjugative transfer to *K. oxytoca*, has been shown here to give rise to *Klebsiella* mutants that maintain the plasmid stably, we chose to examine the mode of lethality indirectly by following the fate of *lacZ* expression from pCU1*lacZ* in developing colonies of *K. oxytoca*. The results are consistent with the interpretation shown in Fig. 6. After the plasmid is received by a *Klebsiella* cell (Fig. 6a), the expression of *Klebsiella*-lethal genes arrests the growth of the recipient (Fig. 6b). However, if the cell is capable of division and loses the plasmid, it will resume growth, resulting in the majority of the colony being white (Fig. 6c to e). The blue areas are neither sectors nor overgrowths but are discrete areas close to the surface or edge of colonies (Fig. 5). We believe that they arise when the plasmid persists in the mother cell for a period that is long enough for a rare secondary chromosomal mutation to occur (Fig. 6f

and g). This mutation confers immunity to the Kik^+ phenotype and occurs late during the development of a colony. An alternative possibility that the chromosomal mutation occurs in a cell that constitutes the plasmid-free part of the colony (white area) and that such a cell subsequently accepts pCU1*lacZ* from the rare mother cell that has not yet succumbed is unlikely because in reconstruction experiments, we have not found evidence for plasmid transfer within a colony. If this chromosomal mutation does not occur (and the cell does not lose the plasmid), it fails to form a colony, which results in the reduction of the observed viability of *Klebsiella* recipients in matings with donors carrying pCU1. Interestingly, the number of colonies showing any evidence of Lac^+ phenotype is much higher when transconjugants are screened rather than selected (Table 2). This could be due to the fact that when transconjugants are selected (as on kanamycin plates), the cells which keep the plasmid are arrested in their growth and synthesis of macromolecules and therefore may be susceptible to the bactericidal effect of kanamycin. This, however, does not occur in the absence of kanamycin, and the likelihood of a chromosomal mutation occurring is therefore higher. Similar results were observed when a *Klebsiella* cell carrying the *kikA* plasmid pPH4 was examined for stability of this plasmid without and with selection for plasmid maintenance. Once the expression of *kikA* was induced by IPTG, the plasmid was lost from the population within several cell divisions, indicating that it is the presence of the plasmid which is detrimental to the cell. However, if the cells were forced to keep the plasmid by selecting for the plasmid-borne antibiotic marker, no colony formation was observed (see also reference 12). It is not clear why selection for the maintenance in *Klebsiella* cells of plasmids carrying *kikA* alone does not give rise to chromosomal mutants while selection for plasmids carrying both *kikA* and *kikC* or *kikC* alone does so. Physiological studies monitoring the expression of the two *kikA* proteins indicate that their effect is bacteriostatic and reversible. Similar studies on the relevant gene(s) in the *kikC* region will be possible once this gene has been identified.

The patterns of inheritance of the plasmid in *Klebsiella* populations thus indicate that the plasmid is deleterious to the host when present in the cell and that loss of the plasmid is not deleterious. The observed lethality is therefore not a consequence of segregational plasmid loss followed by the killing of plasmid-free cells.

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