Deletions in *Klebsiella pneumoniae* R Plasmids Induced by Growth in the Presence of Acridine Orange at High Temperature

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The generation in vivo of plasmids deleted at specific sites in strains of *Klebsiella pneumoniae* containing R plasmids, by treatment with high concentrations of acridine orange (1.2 mg/ml) at 42°C are reported. These deletions seem to be site specific because loss of specific restriction fragments after digestion with restriction enzymes was demonstrated.

From 1979 to 1981, we isolated a number of *Klebsiella pneumoniae* strains resistant to several antibiotics from patients in Children’s Hospital of Buenos Aires (5). The plasmid content of these strains was shown to be heterogeneous, as indicated by their multiplicity (each strain often had many plasmids) and diversity (few strains showed plasmid with the same molecular weight). In spite of their complex picture, the fact that the basic antibiotic resistance pattern was almost identical for all the strains, and the fact that all the strains were isolated at the same hospital, suggested that a relationship could exist among these R plasmids. To understand this relationship, we have begun a detailed study of the plasmid structures of these strains. In our initial experiments, we chose to study two strains, KP11 and KP22, with similar antibiotic resistance patterns (see Table 1). We found that either treatment with acridine orange or growth at high temperature, which are commonly used to cure bacterial cells of plasmids (5), were largely ineffective with these *Klebsiella* strains. More than 6,000 colonies were analyzed by replica plating, after growing the bacteria in the presence of different amounts of acridine orange or at 42°C, and none resulted in antibiotic sensitivity. However, a combined treatment with high concentration of acridine orange (1.2 mg/ml) at 42°C, though not very effective in curing the strains, did result in characteristic alterations of the plasmid structures. These alterations were mainly excision of fragments of plasmid DNA. Analysis of these deletions indicated that excision occurs at specific or quasi-specific regions of plasmid DNA.

The *K. pneumoniae* strains used in this study are listed in Table 1. A previous study of these and other *Klebsiella* strains has been published (5). *K. pneumoniae* KP11 is a spontaneous derivative of a clinical isolate obtained in 1979 that had lost an 85-megadalton (MDa) plasmid without its original resistance pattern being affected. *K. pneumoniae* KP22 is a clinical isolate obtained in 1981 and presumably is a strain representative of several others isolated at that time. Table 1 also shows the basic antibiotic resistance pattern of these strains. Antibiotic sensitivities were tested by disk diffusion (1) and confirmed by measurement of the MICs (9).

The acridine orange-high temperature was performed as follows. Bacteria were grown overnight with shaking in 2 ml of L broth (containing 10 g of tryptose, 5 g of yeast extract, 10 g of NaCl [pH 7.4] per liter). A 0.1-ml portion of this culture was transferred to 3 ml of fresh L broth containing 1.2 mg of acridine orange per ml, and the culture was incubated in a water bath at 42°C. The culture was continuously stirred with a small magnetic bar for 12 to 15 h in the dark. Then, 100-μl culture dilutions were spread on L broth agar plates and incubated overnight at 37°C. Individual colonies were isolated and analyzed for loss of antibiotic resistance by replica plating. Usually the number of bacteria increased about 10 times during incubation in the presence of acridine orange, as indicated by the number of CFU before and after treatment.

Using the described conditions, we derived two strains from strain KP11 which had lost all the resistance markers (KP111 and KP113) (Table 1). Plasmid analysis, performed by the method described by Birnboim and Doly (3) (Fig. 1) showed that the loss of these markers was concomitant with the loss of the 50-MDa plasmid. Another derived strain (KP112) had lost five resistance markers, which included resistance to ampicillin (Ap’), and carbenicillin (Cb’), and three aminoglycosides (resistance to gentamicin [Gm’], tobramycin [Tb’], and sisomicin [Ss’]). It is possible that the concomitant loss of the three aminoglycoside resistances is the result of the loss of a single gene which codes for an enzyme able to inactivate the different aminoglycosides (2). However, the possibility that several genes are involved cannot be discarded. The presence of a kanamycin resistance (Km’) marker in strain KP112 could be indicative of the existence of a second gene coding for an aminoglycoside-modifying enzyme with a narrow substrate range. More information about the resistance mechanisms involved is required to determine the number of genes involved. Analysis of the plasmid from this strain showed that there was a deletion in the 50-MDa plasmid which was now only 36 MDa (Fig. 1). On the other hand, several derived strains obtained through treatment of strain KP22 could be grouped according to the remaining antibiotic resistance traits as follows: (i) strains which remained resistant to chloramphenicol (Cm’) and Ap’ represented in Table 1 by strain KP221; (ii) strains similar to KP223, which remained Ap’ Gm’ Tb’ Km’ Ss’; and (iii) strains similar to KP224, which, in addition to being Ap’ Cm’ Tb’ Km’ Ss’, remained Cb’. The phenotype of group 1 strains (Ap’ Cm’) was the most frequently observed alteration of the antibiotic resistance pattern occurring during acridine orange treatment (approximate frequency, 3 X 10⁻⁴). The plasmid analysis of all the strains within this group demonstrated a deletion in the original 44-MDa plasmid which was now about 33 MDa (Fig. 1 and Table 1). This finding was surprising, since it seems to indicate an excision of a specific (or quasi-specific) portion

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of the plasmid DNA rather than a random deletion. On the other hand, the strains in group 2 (\(\text{Ap}' \text{ Gm}' \text{ Tb}' \text{ Km}' \text{ Ss}' \text{ Cb}'\)) (approximate frequency, \(10^{-2}\)) possessed a plasmid of about 36 MDa (Fig. 1 and Table 1). Loss of \text{Cb}' within this group was not concomitant with the loss of \text{Ap}' as in strain KP112. This suggested that in the original strain KP22 there were two different genes for \text{Ap}', one on the plasmid, which is responsible for \text{Cb}', and a chromosomal gene, which codes for \text{Ap}' but not \text{Cb}'.

Finally, the plasmid analysis of the strains within group 3 (\(\text{Ap}' \text{ Gm}' \text{ Tb}' \text{ Km}' \text{ Ss}' \text{ Cb}'\)) (approximate frequency, \(10^{-1}\)) did not show any detectable change in the 44-MDa plasmid. The high rate of reversion of the strains within this group indicated that the phenotype \text{Cm}' possibly results from a point mutation in the chloramphenicol-plasmid gene.

Since the results of the plasmid analysis were not precise enough to conclude that the deletions were similar for strains within group 1 or 2, a double digestion of plasmid DNA with the enzymes PvuII and SalI (P. L. Biochemicals, Inc.) was performed. Figure 2 shows the restriction patterns for representative strains of each group. The restriction patterns within each group were found to be identical (not shown). Therefore, the deletions within each group were specific within the limits of the restriction analysis. Other information about these patterns can be summarized as follows: (i) the 33-MDa plasmid isolated from strains of group 1 (represented by strain KP221) (\(\text{Ap}' \text{ Cm}'\)) had lost two restriction fragments (8.3 and 1.55 MDa). Also, the 36-MDa plasmid isolated from strain KP112 (\(\text{Km}' \text{ Cm}'\)) which was derived from the 50-MDa plasmid of strain KP11 (Table 1) had lost two similar fragments; (ii) the 50-MDa plasmid from strain KP11 displayed several restriction fragments in common with the 44-MDa plasmid present in strain KP22. This fact, taken together with the observation that both plasmids had lost similar restriction fragments related to carbenicillin and aminoglycoside markers (see above), suggests that some structural relationship, probably in the marker gene region, may exist between these two plasmids that were isolated over a 2-year period at the same hospital; (iii) the 36-MDa plasmids from group 2 strains (represented by strain KP223) (\(\text{Ap}' \text{ Gm}' \text{ Tb}' \text{ Km}' \text{ Ss}'\)) had lost three restriction fragments (3.46, 1.55, and 0.98 MDa); and (iv) the 44-MDa plasmid isolated from group 3 strains (represented by strain KP224) which are \text{Cm}' had lost no detectable restriction fragment. It should be pointed out that the pair of enzymes chosen seems to possess restriction sites very close to the deletion points, since the restriction patterns showed only loss of restriction fragments and no visible fusion segments.

The fact that the observed deletions are specific within the limits of the restriction analysis indicates that at the ends of these deletions there could exist some structural peculiarity.
such as the probable presence of transposition elements on the plasmid DNA.

Further studies are necessary because, to our knowledge, there are few studies of deletions provoked by acridine orange treatment (4), and none of them describe the production of specific (or quasi-specific) deletions like the ones described here.

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LITERATURE CITED