Minicircular ColE1-related DNA in Strains of Klebsiella aerogenes
Selected for Fast Growth on Xylitol

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We have previously described a large family of mutants of Klebsiella aerogenes which were selected by continuous culture on xylitol and which superproduce ribitol dehydrogenase. One of these strains was found to harbour a high copy number $2.1 \times 10^6$ dalton plasmid. This plasmid is a deletion derivative of a low copy number $3.5 \times 10^6$ dalton plasmid present in the ancestral strain of K. aerogenes. However, since these plasmids do not contain the genes required for pentitol catabolism and some enzyme-superproducing strains have lost all DNA homologous to the plasmids, they are not implicated in the fast growth on xylitol.

The plasmids contain regions of homology with the Escherichia coli plasmid ColE1.

INTRODUCTION

Strains of Klebsiella aerogenes which express the ribitol catabolic operon (rbt) constitutively are able to grow slowly on xylitol, since this is a poor substrate for ribitol dehydrogenase (Wu et al., 1968). We have previously described directed evolution experiments in which mutants which grow faster on xylitol than the parental strain were selected by xylitol-limited continuous culture (Rigby et al., 1974). The genealogy of some of the 'evolvant' strains is depicted in Fig. 1. Many of these fast-growing mutants synthesize elevated levels of ribitol dehydrogenase and, in some, gene duplication is implicated in the amplification of enzyme synthesis (Rigby et al., 1974). An increase in rbt gene dosage could be achieved by the incorporation of the rbt genes into an independently replicating plasmid present at several copies per cell. Extraction of total DNA from several ribitol dehydrogenase superproducers revealed that one of the mutants growing fast on xylitol (strain A111) contained large quantities of minicircular DNA. We have therefore screened other K. aerogenes evolvants for the presence of this or other plasmids. In addition, we have used $\lambda$ prbt, a specialized transducing phage for the rbt operon (Neuberger & Hartley, 1979), as a hybridization probe to identify DNA fragments containing regions of homology to the rbt genes.

METHODS

Bacterial strains. Klebsiella aerogenes 1033 strain A' is a Gua+ revertant of strain XI (arg gua rbt-constitutive) of Wu et al. (1968) from which a family of mutants was selected which grew faster on xylitol than the parent rbt-constitutive strain (Fig. 1).

Phage. The structure and properties of the specialized transducing phage $\lambda$ prbt were described by Neuberger & Hartley (1979).

Screening for bacteriocin activity. Testing for bacteriocin activity in culture supernatants was performed according to the critical dilution method described by Mayr-Harting et al. (1972).

Extraction of DNA. Total bacterial DNA was isolated by the method of Marmur (1961) from 200 ml cultures grown on M9 medium (containing, per litre, 5.8 g Na$_2$HPO$_4$, 3.0 g KH$_2$PO$_4$, 0.5 g NaCl, 1.0 g...
Fig. 1. Genealogy of *Klebsiella aerogenes* evolvants that grow fast on xylitol (Rigby et al., 1974; Hartley et al., 1977). All evolvants arose spontaneously, unless otherwise indicated (UV, mutagenesis with ultraviolet light; NG, mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine). All the evolvants shown, except those in square brackets, were examined for the presence of plasmids: P, presence of plasmid pKA69; p, presence of high copy number deletion derivative of pKA69; --, absence of detectable plasmid.

NH₄Cl and 0·12 g MgSO₄·7H₂O supplemented to 0·2% (w/v) in xylitol, except that the final propan-2-ol purification step was omitted. Plasmid DNA was isolated by density gradient centrifugation of cleared lysates in CsCl/ethidium bromide as described by Clewell (1972). The chloramphenicol amplification step was omitted for plasmids extracted from *K. aerogenes*. DNA from phages λ c1857S7 and λ prbt was purified as described previously (Neuberger & Hartley, 1979).

**Digestion and electrophoresis of DNA.** DNA was digested in 10 mM-Tris/HCl pH 7·5, 10 mM-MgCl₂, 10 mM-NaCl with restriction endonuclease *Bst*I (a gift from Dr C. M. Clarke) or *Hinfl*. Endonuclease *Hinfl* was purified by the method of Roberts et al. (1975) for *HaeIII* from cell paste obtained from the Microbiological Research Establishment, Porton Down, Wilts. DNA was examined by electrophoresis in 0·4 to 2·0% agarose slab gels containing 2 μg ethidium bromide ml⁻¹ in the Tris/borate buffer system described by Greene et al. (1974). The sizes of DNA fragments were estimated using an *EcoRI*+*Bst*I digest of λ DNA or a *Hinfl* or *HaeIII* digest of pBR322 DNA to generate markers of known length (Haggerty & Schleif, 1976; Sutcliffe, 1978).

**Nick translation of DNA.** DNA was labelled *in vitro* to high specific activity using deoxyribonucleoside [α-³²P]triphosphates (The Radiochemical Centre, Amersham) by a modification of the method of Rigby et al. (1977) as described previously (Neuberger & Hartley, 1979).

**Gel transfer hybridizations.** Hybridization of nick-translated DNA to DNA fragments which had been transferred on to nitrocellulose filter sheets (Schleicher & Schuell, Dassel, Germany) after electrophoretic separation according to the procedure of Southern (1975) was carried out as described previously (Neuberger & Hartley, 1979).

**Colony hybridization.** Hybridization of nick-translated DNA to the DNA of individual bacterial colonies that had been immobilized on nitrocellulose filter sheets (Millipore) was performed as described by Grunstein & Hogness (1975).

**Electron microscopy.** A sample of spreading solution (50 μl) containing 0·5 μg DNA ml⁻¹ and 0·1 mg cytochrome c ml⁻¹ (Calbiochem) in 10 mM-Tris/HCl pH 7·5, 0·1 mM-EDTA was spread on to a hypophase of 0·25 M-ammonium acetate. The film was picked up on parlodion-coated copper grids and the DNA was stained with uranyl acetate and shadowed with platinum/palladium as described by Davis et al. (1971). Grids were viewed in a Philips 301 electron microscope, photographed on 35 mm film and contour lengths were measured with the aid of a Hewlett-Packard 9821 computer-digitizer.

**RESULTS**

**Identification and properties of plasmid pKA23**

Analysis of total DNA extracted from *K. aerogenes* strain A111 by electrophoresis in agarose gels revealed a large quantity of a fast-migrating DNA species. This suggested the presence of a plasmid in strain A111, a prediction that was confirmed by isolating covalently
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Fig. 2. Electron micrograph of plasmid pKA23. Both supercoiled and relaxed forms are present; a molecule of plasmid pBR313, which was used as a size marker (molecular weight $5.8 \times 10^6$), is also visible (arrowed).

closed circular DNA by CsCl/ethidium bromide density gradient centrifugation and visualization in the electron microscope (Fig. 2). Contour length measurements of this plasmid, pKA23, gave an estimate for its molecular weight of $2.2 \times 10^6$, using plasmid pBR313 [molecular weight $5.8 \times 10^6$ (Bolivar et al., 1977)] as a standard. pKA23 contains a single target for restriction endonuclease BstI and the mobility of linear pKA23 in a 1% agarose gel gave an estimate for its molecular weight of $2.1 \times 10^6$. pKA23 contains no sites for endonucleases EcoRI, HindIII, BglII or PstI. This plasmid is clearly present in high copy number in strain A111. The yield of plasmid DNA from a mid-exponential phase culture suggests a copy number in excess of 100.

Plasmids present in other K. aerogenes evolvants

Plasmid pKA23 was not found in strains A11, A1 and A', the ancestors of A111 (Fig. 1). However, the ancestral K. aerogenes rbt-constitutive strain A', as well as most of the evolvants derived from it, harbour a plasmid of molecular weight $3.5 \times 10^6$ at low copy number. This plasmid, designated pKA69, has a single target for endonuclease BstI and no target for EcoRI.

pKA23 is not involved in enzyme superproduction

Strain A111, which was selected by its fast rate of growth on xylitol, synthesizes ribitol dehydrogenase as about 20% of its total soluble protein. This contrasts with strain A' in which ribitol dehydrogenase accounts for only about 1.5% of the total soluble protein. It therefore appeared possible that pKA23 was involved in ribitol dehydrogenase superproduction. To determine whether the ribitol dehydrogenase structural gene (rbtD) was present on pKA23, we tested whether nick-translated pKA23 would hybridize to phage λ prbt DNA in a gel transfer hybridization. No hybridization was detected (Fig. 3). Therefore there is no DNA homologous to the rbt operon on plasmid pKA23. Several other lines of evidence also suggest that plasmid pKA23 is not involved in the fast growth of its host on xylitol. Strain A111 segregates derivatives growing more slowly on xylitol at a frequency of about 0.5%; examination of six such derivatives showed that all retained plasmid pKA23 at high copy number. Furthermore, as described below, several ribitol dehydrogenase superproducing evolvants of K. aerogenes strain A' have lost all DNA homologous to the plasmids pKA23 and pKA69.
Fig. 3. Hybridization of various DNA samples with radioactive (pKA23 + pKA69) DNA. (a) Unrestricted and (b) BstI-digested plasmid DNA from strain A11 after electrophoresis in a 0.7% agarose gel; both pKA23 and pKA69 are visible. (c) to (o) DNA samples were subjected to electrophoresis in a 0.9% agarose gel. After electrophoresis, DNA samples from (d) to (o) were transferred on to nitrocellulose filter paper and hybridized with $5 \times 10^4$ c.p.m. per track of nick-translated (pKA23 + pKA69) DNA (sp. act. $5 \times 10^8$ c.p.m. $\mu$g$^{-1}$) as described in Methods. The autoradiographs (right) have been aligned with the photographs of the DNA in the agarose gels (left). For comparison, (c) contains purified BstI-digested pKA23. (d) Strain A' DNA, unrestricted; (e) strain A11 DNA, unrestricted; (f) strain A22 DNA, unrestricted; (g) strain A3 DNA, unrestricted; (h) strain A5 DNA, unrestricted; (i) strain A12 DNA, unrestricted; (j) strain A2111 DNA, unrestricted; (k) strain A’ DNA, BstI-digested; (m) strain A11 DNA, BstI-digested; (n) phage $\lambda$ prb3 DNA, BstI-digested; (o) pBR313 DNA, BstI-digested. (p) Hybridization of nick-translated (pKA23 + pKA69) DNA to a HindIII digest of ColEl DNA which has been subjected to electrophoresis in a 1.8% agarose gel.
Table 1. Sizes of fragments produced by digestion of pKA69 and pKA23 with HinfI

Fragment sizes were estimated by their electrophoretic mobility in 1.8% (w/v) agarose gels, using pBR322 cut with HinfI and with HaeIII to generate marker fragments of known lengths (Sutcliffe, 1978). Only fragments larger than 140 base pairs could have been detected. Fragment sizes are given to the nearest 10 base pairs.

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<th>Plasmid</th>
<th>730</th>
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<th>590</th>
<th>390</th>
<th>340</th>
<th>310</th>
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**Origin of plasmid pKA23**

Hybridization of nick-translated (pKA23+pKA69) DNA to individual colonies of *K. aerogenes* strain A' and the ribitol dehydrogenase superproducing evolvants of Fig. 1 showed that evolvants A13, A22 and A3 contain no DNA homologous to the pKA plasmids. This was confirmed by the lack of hybridization of nick-translated (pKA23+pKA69) DNA to both unrestricted and to BstI-digested total DNA from these strains in gel transfer hybridizations (Fig. 3). Thus A13, A22 and A3 have lost plasmid pKA69 and, furthermore, there is no DNA on the chromosomes of these evolvants homologous to the pKA plasmids. This implies that, unless strain A111 acquired plasmid pKA23 from a culture contaminant, plasmid pKA23 is most probably a derivative of plasmid pKA69.

We therefore compared the fragmentation patterns obtained on digestion of plasmids pKA23 and pKA69 with restriction endonuclease HinfI. The results (Table 1) entirely support the hypothesis that pKA23 is a deletion derivative of pKA69. As pKA23 and pKA69 co-exist in evolvant A111, the two plasmids must be compatible.

Nick-translated (pKA23+pKA69) DNA hybridized to high molecular weight DNA as well as to the supercoiled monomeric plasmid in unrestricted DNA samples from strains containing pKA69 (Fig. 3). This hybridization was to DNA species of lower mobility than the fragmented chromosomal DNA. Hybridization of (pKA23+pKA69) DNA to BstI-digested total DNA from strain A' revealed only a single radioactive band corresponding to linear pKA69, suggesting that the hybridization observed to high molecular weight species in unrestricted total DNA was not due to integration of pKA69 into the bacterial chromosome. We believe that the hybridization to high molecular weight DNA was due to the presence of plasmid multimers. The existence of plasmid multimers in DNA extracted from rec+ hosts has previously been observed by Bedbrook & Ausubel (1976).

We observed that evolvant A2111, but not its parent A211, also harbours a high copy number plasmid which is intermediate in size between pKA23 and pKA69 and hybridizes with nick-translated (pKA23+pKA69) DNA but contains no target for endonuclease BstI. It therefore appears likely that the generation of high copy number deletion derivatives from pKA69 is not an infrequent event.

**pKA plasmids contain homology with ColE1**

As shown in Fig. 3, the pKA plasmids contain regions of homology with the *E. coli* plasmid pBR313. Plasmid pBR313 is a ColE1-derived plasmid with resistance determinants to ampicillin and tetracycline (Bolivar et al., 1977). *Klebsiella aerogenes* strain A' is sensitive to 10 µg tetracycline ml⁻¹ and, although it is resistant to 100 µg ampicillin ml⁻¹, the strains cured of pKA69 remain ampicillin-resistant. Thus the homology between the pKA plasmids and pBR313 is likely to lie in the ColE1-derived portion of the pBR313 genome. This was confirmed by the demonstration that DNA of the pKA plasmids hybridizes to many of the restriction fragments generated by HinfI cleavage of ColE1 DNA (Fig. 3).

We attempted to detect bacteriocinogenic activity in culture supernatants of *K. aerogenes*...
1033 strains A' and A11 both before and after exposure to u.v. light by testing on plates for inhibition of growth of K. aerogenes 1033 strains A3 and A13, K. aerogenes types NCIB 8267, NCIB 8021 and W70, K. pneumoniae and E. coli K12. No bacteriocinogenic activity was detected. We have not been able to identify any phenotypic trait resulting from the presence of the pKA plasmids.

DISCUSSION

Several workers have previously reported the presence in E. coli strains of small, high copy number plasmids containing homology with ColE1 (Cozzarelli et al., 1968; Goebel & Schrempf, 1972; Hershfield et al., 1976). It is interesting in this context to note that about 5% of the covalently closed circular DNA in a normal ColE1 preparation is in the form of ColE1 deletion derivatives (Goebel & Kreft, 1974). In this work, the two deletion derivatives of pKA69 identified were both harboured by mutants which had been isolated following u.v. mutagenesis and it is possible that u.v. stimulates the formation of plasmid deletions by causing an increase in the frequency of DNA recombination. Out of 14 evolvants screened, three had lost pKA69. This loss of plasmid is more likely to be due to a high rate of spontaneous curing than a strong selective disadvantage conferred by plasmid maintenance, as pKA69 has been retained in several evolvants which have been grown in xylitol-limited continuous culture for more than 600 generations.

Whilst pKA23 presents a suitable plasmid from which to develop a cloning vector for K. aerogenes, we have recently found that the extensively characterized E. coli plasmid pBR322 replicates to high copy number in K. aerogenes and expresses its tetracycline resistance. (The strains of K. aerogenes used in this work are all resistant to ampicillin and therefore expression of the pBR322 Amp' gene would not have been detected.) Thus a satisfactory K. aerogenes cloning vector already exists in pBR322.

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REFERENCES


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