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Genetic Localization and Characterization of a pKM101-Coded Endonuclease

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The genetic and biochemical properties of an endonuclease mediated by the mutagenesis-enhancing plasmid pKM101 have been investigated. Taking advantage of the observation that this endonuclease, unlike host-coded DNases, is active in the presence of EDTA, we have developed an assay with nondenaturing acrylamide gels containing DNA. We have localized the plasmid DNA sufficient for nuclease expression to a 0.8-kilobase sequence that is near regions of DNA necessary for conjugal transfer, and we have determined that this gene is transcribed clockwise on the pKM101 map. The pKM101 gene mediating this activity codes for a 16,000-dalton protein, which is the same molecular mass as the nuclease monomer, leading us to conclude that this gene codes for the nuclease itself rather than for an activator of some host-coded enzyme. Cellular fractionation experiments have shown that the enzyme is localized in the periplasm. We have not been able to demonstrate any physiological role for the enzyme, but we have ruled out a direct involvement of the nuclease in any of the following known plasmid-associated phenotypes: (i) mutagenesis enhancement, (ii) conjugal transfer, (iii) entry exclusion, (iv) fertility inhibition of coresident P-group plasmids, (v) killing of *Klebsiella pneumoniae* used as conjugal recipients, and (vi) plasmid curing induced by treatment of cells with fluorodeoxyuridine. In addition, we have shown that the enzyme does not restrict bacteriophage or affect the ability of the host to utilize DNA as a source of thymine. Finally, we have shown that 11 of the 26 other plasmids tested also elaborated EDTA-resistant DNases.

pKM101 is a 35.4-kilobase (kb) plasmid of the *incN* incompatibility group that was derived from the clinically isolated plasmid R46 by Mortelmans and Stocker (18). pKM101 apparently arose from R46 by the deletion of a single 14-kb region of DNA coding for resistance to three antibiotics and to arsenate (5, 12). It is self-transmissible and renders its host susceptible to the *incN*-specific phage IKe as well as to the phages PR4 and PRD1, which infect cells carrying plasmids of the *incN*, *incP*, and *incW* groups (4, 9). Plasmids belonging to the *incN* group have a broad host range (33) and elaborate pili of the "short brittle" type (3) and inhibit the fertility of P-group plasmids (22). They also have the ability to kill *Klebsiella pneumoniae* strain M5a1 used as conjugal recipients (27). Certain members of this incompatibility group have been shown to be cured by treatment of cell cultures with fluorodeoxyuridine or trimethoprim or by thymine starvation (24, 25). On the basis of indirect evidence, a plasmid-coded nuclease has been postulated to be involved in this process (34).

pKM101 has been studied fairly intensively because of its *recA*$^+$ *lexA*$^+$-dependent ability to increase the susceptibility of cells to mutagenesis by UV light and various chemicals (16, 30). Recent work from this laboratory has indicated that pKM101 exerts its effects on mutagenesis by coding for analogs of the chromosomal *umuC* and *umuD* genes, whose products are needed for "error-prone repair" (23, 35).

Several years ago, Lackey et al. (10) identified a novel endonuclease present in pKM101-containing cells. The enzyme degraded both single-stranded and double-stranded DNA, lacked any apparent site specificity, was ATP-independent, and had a native molecular weight of 76,000. The analysis of several pKM101 mutants defective in enhancing mutagenesis indicated that the nuclease was not required for that process. In this paper we extend our investigations of the endonuclease to a genetic level and describe our efforts to deduce its physiological role.

MATERIALS AND METHODS

Bacterial strains and bacteriophage. Strain AB1157 (2), its *recA13* derivative JC2926, and its *recB21 recC22 sbcB15* derivative JC7623 were obtained from A. J. Clark. RB901, a Δ*recA spr* strain used in maxi-

cells, was obtained from R. Brent (7a). MO1505 (a *his*$^+$, *trpE9758 endA thyA* derivative of AB1157) was obtained from Robert Irbe. GW4201 is a spontaneous *deo* derivative of MO1505 isolated by plating ca. 10^8 stationary-phase cells on M9-glucose plates containing required amino acids plus 2 μg of thymine per ml. λ cI857 b221 *rex*::Tn*5* (Km^r) Oam29 Pam80 was obtained from M. Fox. Mu cts was made by thermoinduction of MAL103, which was obtained from Malcolm Casadaban. λ *vir* and φ80 *vir* were obtained from R. Sauer. T2 and T6 were obtained from S. Luria, and T4d was obtained from J. King.

Nuclease assays with DNA-containing gels. Vertical polyacrylamide slab gels were used for both native and denaturing nuclease assay gels. For assays with native gels, both the gel and the electrode buffer contained 100 mM Tris-phosphate (pH 8.8)–2 mM EDTA. The gel contained in addition 5% acrylamide, 0.13% bisacrylamide, 0.17% Temed, 0.07% ammonium persulfate (all from Bio-Rad Laboratories), and 15 μg of herring sperm DNA (Sigma Chemical Co.) per ml or occasionally 15 μg of pBR322 DNA (purified by CsCl isopycnic centrifugation) per ml. The gels were usually 10 by 4 by 0.15 cm, although for large-scale screening a 14 by 27 by 0.15-cm gel was used. This allowed up to 40 assays to be conducted per gel.

Extracts of bacterial strains were prepared for electrophoresis by growing cells to the late log phase in Luria broth (17), centrifuging 1.5 ml in an Eppendorf centrifuge for 2 min and suspending the cell pellet in 50 μl of 20% sucrose–50 mM Tris-hydrochloride (pH 8.0)–40 mM EDTA–25 mg of lysozyme per ml. This lysate was added directly to the gel. Electrophoresis was carried out in the anodal direction at 4°C at 80 V for 4 h. In earlier work, the pH of the gel and buffer was set at 7.6, and electrophoresis was carried out overnight.

After electrophoresis, the gel was slowly rocked in buffer containing 50 mM Tris-maleate (pH 6.8)–2 mM EDTA–1 μg of ethidium bromide per ml. Nonfluorescing regions could often be visualized with a hand-held UV light in as little as 1 h and continued to develop for up to 2 days. At various intervals, the gels were photographed by using a shortwave UV light source.

Denaturing nuclease gel assays were carried out as described by Rosenthal and Lacks (28), except that (i) the separating gel contained 14% acrylamide, (ii) the extracts were heated to 100°C for 10 min before electrophoresis, (iii) the extracts used were prepared as described for maxicells (29), and (iv) nuclease bands were developed in buffer containing 50 mM Tris-maleate (pH 6.8)–4 mM $MgCl_2$–1 μg of ethidium bromide per ml.

Cellular fractionation. Cultures grown to the stationary phase in Luria broth containing 10 μg of ampicillin and 10 μg of kanamycin per ml were subcultured into 200 ml of Luria broth containing 1 mM isopropylthiogalactoside, grown to 80 Klett units, centrifuged, suspended in 10 ml of 100 mM Tris-hydrochloride (pH 8.0), and centrifuged again. They were then suspended in 5 ml of cold 20% sucrose–30 mM Tris-hydrochloride (pH 8.0), and then 50 μl of freshly prepared lysozyme (1 mg/ml) and 100 μl of 50 mM EDTA were added. The cell suspension (fraction 1) was incubated on ice for 30 min, at which time spheroplasts and any intact cells were separated from the supernatant (fraction 2) by centrifugation at 13,000 rpm for 10 min. The pellet was suspended in 5 ml of 20% sucrose–30 mM Tris (pH 8.0), and the supernatant (fraction 3) and pellet (fraction 4) were again separated by centrifugation. The pellet was lysed by suspending it in 5 ml of cold water. All four fractions were assayed for EDTA-resistant endonuclease by using nondenaturing gels as described above and for β-galactosidase and β-lactamase activities as described elsewhere (17, 21).

Mutagenesis of plasmids with Tn*5*. Transposition of Tn*5* from λ to a plasmid-containing host was performed as described previously (19), except that cells were plated on Luria broth plates containing 20 μg of kanamycin and 2.5 mM sodium pyrophosphate. We plated a sufficient number of infected cells to give rise to approximately 10^4 Km^r colonies per plate. These plates were flooded with 0.85% saline, and the colonies were pooled. Plasmid DNA was purified from them using a protocol described elsewhere (26), and this DNA was used to transform a recipient strain to Km^r Ap^r. Virtually every colony that appeared after transformation was determined by restriction analysis to contain a derivative of the original plasmid bearing a Tn*5* insertion.

Fluorodeoxyuridine-induced plasmid curing. Plasmid curing by fluorodeoxyuridine was performed as described previously (24), except that 300 μg of fluorodeoxyuridine (Sigma) per ml was used and cells were grown in M9-glucose medium. At intervals, cells were diluted and plated on M9-glucose plates, and the proportion of plasmid-free colonies was determined by colony morphology (the plasmid-coded *slo* locus results in small colonies on minimal media [11]).

Other methods. DNA isolation (26) and agarose gel electrophoresis (31) were carried out as described previously. Restriction endonucleases purchased from New England Biolabs were used in buffers recommended by the manufacturers. [^{35}S]methionine labeling of plasmid-coded proteins was carried out by the maxicell technique of Sancar et al. (29). Cation-exchange chromatography was performed as described previously (10), except that fractions were assayed by using the native gels described above.

RESULTS

Nuclease assay and resistance to EDTA. To carry out a genetic analysis of the pKM101-mediated nuclease activity, we felt it necessary to develop an assay suitable for screening large numbers of plasmid derivatives. The procedure we used to follow the activity of the pKM101 endonuclease in most of this work was adapted from a technique described by Rosenthal and Lacks (28). Their method involved subjecting a crude cell extract to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis through a gel containing herring sperm DNA and then subsequent incubation of the gel in several changes of buffer to remove SDS and to allow nucleases to renature and degrade the DNA immobilized in the gel. Regions in which the DNA had been degraded by nuclease appeared as dark bands when the gel was stained with ethidium bromide and viewed under UV light.

We found that we could improve the sensitiv-

ity and reproducibility of the method for detecting the pKM101-coded endonuclease if we omitted SDS from our gels and incubated them in the presence of EDTA rather than $MgCl_2$. The pKM101-mediated endonuclease is active in the presence of EDTA, whereas chromosomally encoded nucleases are inactive (Fig. 1). The pKM101-mediated endonuclease activity (as well as the chromosomally encoded nucleases detected by incubation in $MgCl_2$) created a broad band near the origin of the gel, possibly because of binding of the nucleases to the DNA in the gel. By noting the length and darkness of the nonstaining band appearing after incubation in the presence of EDTA, we were able to estimate the relative amounts of pKM101-mediated endonuclease activity present in various samples.

Although we generally used herring sperm DNA in these gels, we obtained the same results when covalently closed circular pBR322 DNA was used instead, confirming that the pKM101-mediated enzyme that we detected with this assay does indeed have an endonucleolytic activity. It is also worth noting that, although the pKM101-coded endonuclease was active in the presence of EDTA, its activity was moderately stimulated by the presence of 2 mM $MgCl_2$ in the absence of chelating agents. This is apparent in the gels shown in Fig. 1 and was confirmed by partially purifying the pKM101 endonuclease by anion-exchange chromatography (10), exposing plasmid DNA to the enzyme in various buffers, and observing the relative amount of degradation products after electrophoresis in a 0.8% agarose gel (data not shown).

Genetic mapping of the pKM101-mediated endonuclease. By constructing various deletion derivatives of pKM101 and by subcloning regions of pKM101 into the vector pBR322, we were able to localize the region of pKM101 DNA sufficient for expression of the enzyme to a 0.8-kb region of the plasmid. To construct certain deletion mutants of pKM101, we took advantage of the fact that we had available a large number of derivatives of pKM101 having Tn*5* insertions at known locations (11; Winans and Walker, unpublished results). Deletions were easily constructed in vitro between a restriction endonuclease site in the pKM101 DNA and a restriction endonuclease site in the Tn*5* DNA.

The location of the gene required for endonuclease activity was first localized by using a pair of pKM101 deletion mutants, pGW1597 and pGW278. pGW1597, a deletion derivative of pKM101::Tn*5*Ω630 (Fig. 2) that lacks the DNA between the *Sal*I-1 site of pKM101 and the distal *Xho*I site of the Tn*5*, still conferred endonuclease activity upon its host. In fact, the nuclease activity in the extract was increased approxi-

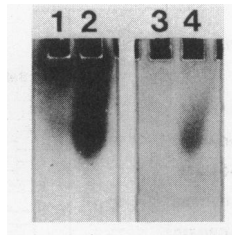

FIG. 1. Assay for EDTA-resistant DNase with nondenaturing polyacrylamide gels containing herring sperm DNA. The gel was prepared and electrophoresis was carried out as described in the text. The DNA degradation shown in lanes 1 and 3 is by extracts of AB1157, whereas that in lanes 2 and 4 is by extracts of AB1157(pKM101). After electrophoresis, the gel was cut lengthwise between lane 2 and 3; the left half was incubated in 2 mM $MgCl_2$, and the right half was incubated in 2 mM EDTA (both buffers also contained 50 mM Tris-maleate, pH 6.8, and 1 μg of ethidium bromide per ml). Photographs were taken after 4 h of incubation.

mately 100-fold over that seen with pKM101, perhaps due to an operon fusion having been created. In contrast, pGW278, a derivative that is deleted from the *Sal*I-1 of pKM101 to the *Sal*I site of the Tn*5* in pKM101::Tn*5* Ω1035, did not confer the endonuclease activity on its host. These observations suggested the gene responsible lay between the points of the Tn*5* insertions in pKM101::Tn*5*Ω630 and pKM101::Tn*5*Ω1035, respectively (Fig. 2).

The position of the gene required for nuclease activity was further localized by using another pair of pKM101 deletion derivatives (Fig. 2). A deletion of the small *Sma*I fragment of pKM101 (pGW1509) did not affect nuclease levels, but a deletion extending from the *Sma*I site of the Tn*5* in pKM101::Tn*5*Ω630 to the *Sma*I-2 site of pKM101 (pGW1510) removed activity. Thus it would seem that the 0.8-kb region between the *Sma*I-1 site of pKM101 and the point of insertion of Tn*5* in pKM101::Tn*5*Ω630 is necessary for expression of the nuclease.

To test whether this region was not only necessary, but also sufficient, for the expression of endonuclease activity, we subcloned the DNA segment between the *Sal*I-2 site and the *Ava*I-1 site of pKM101 into pBR322 cleaved with *Sal*I and *Ava*I to give pGW1503 (Fig. 2). Random Tn*5* insertions were then made into this plasmid, and one of these was mapped to a site within the pKM101 segment about 1 kb away from the *Ava*I site. This derivative (pGW1503::Tn*5*Ω33) was cut with *Hpa*I, diluted, ligated, and transformed by selecting for ampicillin resistance. As expected, the resulting plasmid, pGW1508, contained only the largest of its parent's four *Hpa*I fragments and therefore contained about 1 kb of pKM101 DNA from just

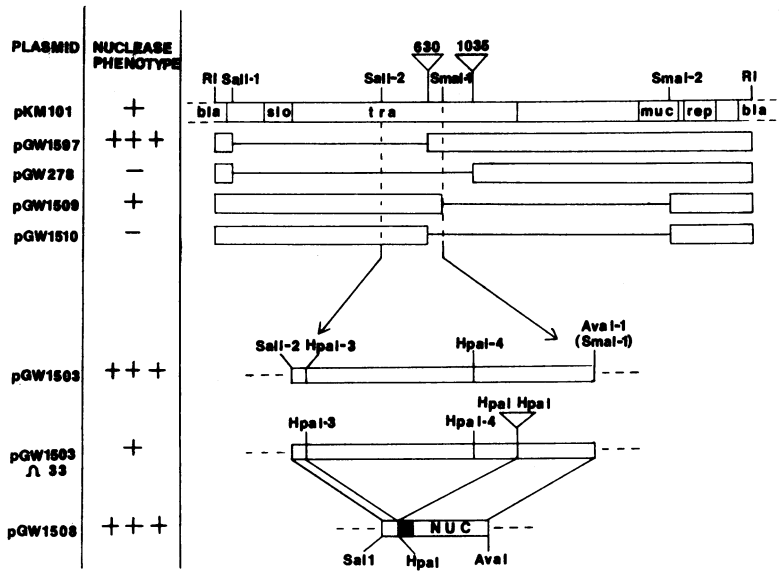

FIG. 2. Genetic localization of the gene sufficient for nuclease expression. Deletion derivatives of pKM101 (pGW1597, pGW278, pGW1509, and pGW1510) and pKM101 DNA cloned into pBR322 (pGW1503, pGW1503::Tn5Ω33, and pGW1508) are depicted. Open bars represent pKM101 DNA present in each plasmid, solid lines indicate deleted pKM101 material, horizontal dashed lines represent pBR322 DNA, and the solid bar in pGW1508 represents Tn5-derived DNA.

to the right of the *Sma*I-1 site (in addition to a very short sequence near the *Sal*I site). A strain containing pGW1508 exhibited the EDTA-resistant endonuclease activity, indicating that the region of pKM101 we had subcloned was sufficient for expression of pKM101-mediated nuclease. It is interesting to note that the locus we had thus identified, denoted as *nuc* in Fig. 2, lies roughly in the middle of the 15-kb region of pKM101 containing genes required for conjugal transfer.

***nuc* is the structural gene for the pKM101 endonuclease.** The above experiments did not distinguish between the possibilities that (i) the pKM101 *nuc* gene codes for the EDTA-resistant endonuclease and (ii) the pKM101 *nuc* gene codes for a positive effector of some chromosomally encoded enzyme. We favor the first possibility, because, as discussed below, we have been able to show that the subunit molecular weight of the EDTA-resistant endonuclease is identical to that of a protein coded for by the 1-kb region of pKM101 that is present in pGW1508.

The proteins coded for by the plasmid pGW1508 and by the control plasmid pBR322 were labeled with [^{35}S]methionine by using the maxicell technique of Sancar et al. (29). Cell extracts were then subjected to SDS-polyacrylamide gel electrophoresis in a gel containing 20 μg of herring sperm DNA per ml. After electrophoresis, the gels were incubated at 37°C for 2 days in several changes of buffer containing 10 mM $MgCl_2$ and then were stained with ethidium bromide. Maxicells containing the plasmid pGW1508 (Fig. 3, lane 1) exhibited a sharp band of nuclease activity that was not present in maxicells containing pBR322 (Fig. 3, lane 2). Since this is an SDS-polyacrylamide gel, the position of the nuclease corresponds to its subunit molecular weight (28).

The gel was then subjected to fluorography; pGW1508 (Fig. 3, lane 3) coded for a 16,000-dalton protein that was not present in pBR322 (Fig. 3, lane 4). The position of this pGW1508-encoded protein on the gel corresponded to the position of the pGW1508-specific nuclease activity. These results are consistent with the *nuc* gene of pKM101 being the structural gene for the EDTA-resistant endonuclease observed in pKM101-containing cells.

Direction of transcription of the nuclease is clockwise. We cloned the DNA fragment between the *Sal*I-2 site of pKM101 and the *Sal*I site of the Tn*5* in pKM101::Tn5Ω1035 (Fig. 2) into the *Sal*I site of pBR322 in both orientations. This fragment cloned in one orientation (pGW1501; Fig. 4, lane 2) leads to far higher levels of nuclease activity than are observed than if it is cloned in the opposite orientation (pGW1500; Fig. 4, lane 1). These results suggested that in pGW1501 the *nuc* gene was being transcribed from the tetracycline resistance promoter of pBR322, and thus that the *nuc* gene

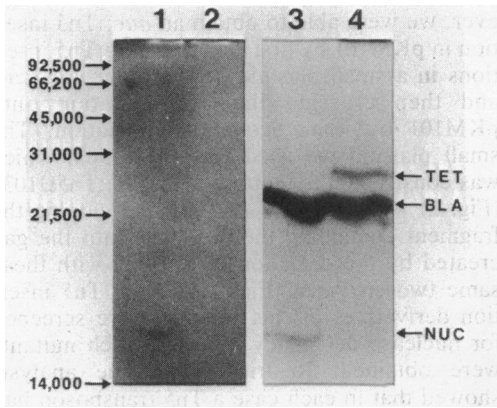

FIG. 3. SDS nuclease assay gel with cell extracts with plasmid-coded proteins labeled with [^{35}S]methionine. Plasmid-coded proteins of strains RB901(pGW1508) (lanes 1 and 3) or RB901(pBR322) (lanes 2 and 4) were labeled by the maxicell procedure and electrophoresed into a denaturing polyacrylamide gel containing 20 μg of herring sperm DNA per ml. Subsequent incubation in buffer containing 50 mM Tris-maleate (pH 6.8)–10 mM $MgCl_2$–1 μg of ethidium bromide per ml revealed a pGW1508-specific band having a molecular weight of 16,000 (lane 1). Fluorography of the same gel revealed that the protein coded by the *nuc* locus also has a molecular weight of 16,000 (lane 3).

must be transcribed in a clockwise fashion on the pKM101 map (11).

To confirm this conclusion, we transposed Tn*5* into various locations in pGW1503 and examined nuclease levels in cells containing these derivatives. pGW1503 mediated the same high nuclease activity that was observed with pGW1501 (Fig. 4, lanes 2 and 3), due we believe to an operon fusion to the tetracycline resistance promoter. All insertions that mapped between the tetracycline resistance promoter and *nuc* (Fig. 4, lane 5, 6, 7, and 8) greatly decreased expression of the nuclease, whereas none of the other insertions obtained (Fig. 4, lanes 4 and 9) had any effects on enzyme levels. The same was true of Tn*5* insertion derivatives of pGW1508 (data not shown).

It is significant that although all Tn*5* insertions lying between the tetracycline resistance promoter and the *nuc* gene decreased the expression of that gene, none abolished it completely. Similarly, a significant level of expression was observed from pGW1500 (Fig. 4, lane 1), even though there was no apparent vector-supplied promoter. These observations suggest that the plasmid sequence cloned into pBR322 may include a promoter adjacent to the *nuc* gene. We have also observed that pKM101 derivatives containing insertions of Tn*5* or Mu *d*(Ap *lac*) less than 0.4 kb counterclockwise from *nuc* are unimpaired in nuclease activity (Winans and Walk-

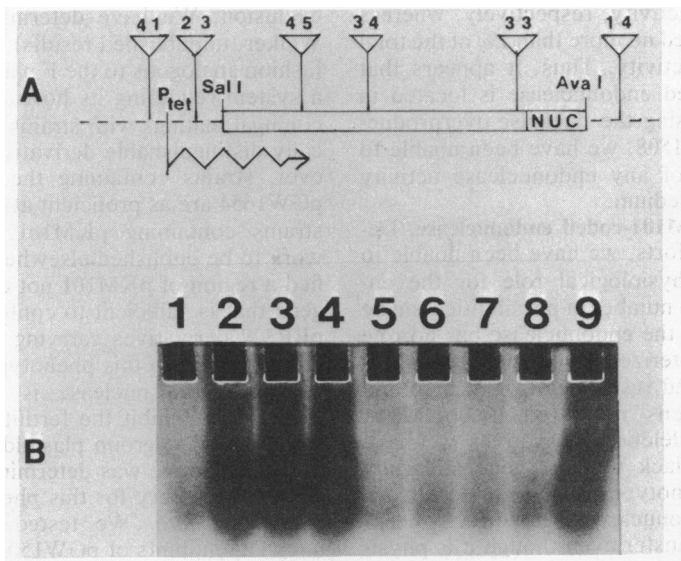

FIG. 4. EDTA-resistant endonuclease activity after electrophoresis on nondenaturing polyacrylamide gels. Extracts of strain JC2926 containing the following plasmids were assayed as described in the text. (B) Lanes: 1, pGW1500 (as described in the text); 2, pGW1501 (the same pKM101 fragment as in pGW1500, but cloned into pBR322 in the opposite orientation); 3, pGW1503 (see Fig. 2). Lanes 4 through 9 represent derivatives of pGW1503 carrying the Tn*5* insertions diagrammed in A as follows: 4, pGW1503::Tn*5*Ω41; 5, pGW1503::Tn*5*Ω23; 6, pGW1503::Tn*5*Ω45; 7, pGW1503::Tn*5*Ω34; 8, pGW1503::Tn*5*Ω33; 9, pGW1503::Tn*5*Ω14.

er, unpublished results). These observations are consistent with the hypothesis that the nuclease is expressed from a nearby promoter on pKM101.

Cellular localization of the endonuclease activity. To determine whether the location of the pKM101-coded endonuclease is cytoplasmic, periplasmic, or membrane bound, we used a modification of the method of Neu and Heppel for the release of periplasmic proteins during the conversion of cells to spheroplasts (20). The strain employed in this experiment was *lacZ*$^+$ and contained both pBR322 and pGW1514, a Km^r Ap^s derivative of pKM101 that carries the *nuc* gene; the cells were grown in the presence of isopropylthiogalactoside so that β-galactosidase was expressed. We were able to monitor the efficiency of our separations by assaying for β-galactosidase and the pBR322-encoded β-lactamase, the former being located in the cytoplasm and the latter being located in the periplasm. We used this particular strain so that we could follow the activity of the well-characterized TEM β-lactamase of pBR322, which has been shown to be located in the periplasm (13) rather than the less well characterized OXA II β-lactamase of pKM101 (15). Most of the EDTA-resistant endonuclease was found in the periplasmic fraction, with all remaining detectable activity being found in a second wash of the spheroplasts with sucrose-Tris buffer (fractions 2 and 3, respectively, as described above). These two fractions contained 93% and 6% of the β-lactamase activity, respectively, whereas they each contained no more than 1% of the total β-galactosidase activity. Thus, it appears that the pKM101-coded endonuclease is located in the periplasm. Using the nuclease-overproducing plasmid pGW1508, we have been unable to detect secretion of any endonuclease activity into the growth medium.

Role of the pKM101-coded endonuclease. Despite extensive efforts, we have been unable to determine any physiological role for the enzyme. However, a number of possibilities can be discounted. First, the endonuclease has no role in the well-characterized mutagenesis enhancement and increased resistance to UV light and chemical mutagens mediated by pKM101 (16,18). Various deletion derivatives we have constructed that lack the *nuc* gene still fully express these phenotypes (11).

Second, the endonuclease is not directly needed for conjugal transfer even though it is physically located on the pKM101 genome between two sets of genes that are required for conjugal transfer. We tried to isolate a plasmid with Tn*5* inserted in the *nuc* gene, but we were still unsuccessful after screening 100 *tra*$^-$ and 160 *tra*$^+$ Tn*5* insertion derivatives of pKM101. However, we were able to obtain an *nuc*::Tn*5* insertion in pKM101 by first isolating *nuc*::Tn*5* insertions in a small plasmid carrying the *nuc* gene and then crossing the Tn*5* mutation onto pKM101 by homologous recombination. The small plasmid we used was pGW1502, which was constructed by cutting pKM101::Tn*5*Ω1035 (Fig. 2) with *Sal*I and *Hin*dIII and cloning the fragment containing the *nuc* gene into the gap created by the digestion of pBR322 with these same two enzymes. Eighty random Tn*5* insertion derivatives of this plasmid were screened for nuclease deficiency, and five such mutants were obtained. Restriction enzyme analysis showed that in each case a Tn*5* transposon had inserted into the nuclease gene. The DNA of one such derivative (pGW1589) was linearized by digestion with *Eco*RI and transformed into a *recB recC sbcB* strain carrying pKM101. Several hundred transformants were obtained from 2 μg of DNA; 40 of these were screened for the ability to donate Ap^r and Km^r in conjugal matings, and 36 were able to do so. Plasmid DNA from four of these was isolated and in each case was shown to have exactly the restriction enzyme pattern expected if the *nuc*::Tn*5* mutation had been recombinationally transferred to pKM101 by a double crossover. One of these plasmids (pGW1654) was chosen for further study and was shown not to mediate EDTA-resistant nuclease activity, but to still conjugate with wild-type efficiency.

Third, the nuclease is not required for entry exclusion. We have determined (Winans and Walker, unpublished results) that pKM101, in a fashion analogous to the F plasmid (1), mediates a system rendering its host a poor recipient in conjugal matings with strains containing genetically distinguishable derivatives of itself. However, strains containing the *nuc*::Tn*5* mutant pGW1654 are as proficient at entry exclusion as strains containing pKM101. Furthermore, in work to be published elsewhere we have identified a region of pKM101 not containing the *nuc* gene that is sufficient to confer this phenotype. pBR322 derivatives carrying just the *nuc* gene do not confer to this phenotype.

Fourth, the nuclease is not required for pKM101 to inhibit the fertility of coresident P incompatibility group plasmids (22). pGW1502, described above was determined to contain the gene(s) necessary for this phenotype as well as expressing *nuc*. We tested two of the five *nuc*::Tn*5* mutants of pGW1502 described above for their ability to inhibit the fertility of RP1; both were able to do so to the same extent as pGW1502.

Fifth, the nuclease is not involved in restricting bacteriophage since pKM101 does not appear to code for a restriction system. The effi-

TABLE 1. Plasmids mediating EDTA-resistant endonucleases

Plasmid[a]	Incompatibility group	Nuclease assay		Source of strain
		pH 7.6	pH 8.8	
TP113	B	−	−	B. Stocker
R805	B	−	−	B. Stocker
R724	B	−	−	B. Stocker
Flac	FI	−	−	B. Bachmann
R124	FIV	−	−	G. Jacoby
RG1521	H	−	++	B. Stocker
R64	Iα	−	++	B. Stocker
R144-3	Iα	+++++	ND[b]	R. Grant
R621-A	Iγ	++	ND	G. Jacoby
COL-IB	Iγ	++	ND	B. Stocker
R391	J	−	−	G. Jacoby
R4466	M	+	ND	R. Grant
N3	N	+++	ND	G. Jacoby
R45	N	++	ND	G. Jacoby
R46	N	++	ND	G. Jacoby
R48	N	++	ND	G. Jacoby
R205	N	++	ND	G. Jacoby
R16	O	−	−	G. Jacoby
RP1	P	−	−	S. Levi
R751	P	−	−	J. Shapiro
R1162	Q	−	−	J. Shapiro
RSF1010	Q	−	−	J. Shapiro
pSa	W	−	−	J. Shapiro
R6K	X	−	−	S. Levi
P1	Y	−	−	A. Clark
P7	Y	−	−	G. Jacoby

[a] Descriptions of these plasmids can be found in reference 8.

[b] ND, Not determined.

ciency of plating and plaque morphology of various phage (P1, λ*vir*, φ80*vir*, Mu, T2, T4, and T6) that had been previously grown on plasmid-free strains was not affected by the presence of pKM101.

Sixth, the nuclease is not involved in the killing of *K. pneumoniae* strain M5a1 that occurs during conjugal matings with pKM101-containing strains (27). *K. pneumoniae* was killed during matings with strains containing pGW1654, the *nuc*::Tn*5* derivative of pKM101.

Seventh, the *nuc* nuclease is not involved in fluorodeoxyuridine-induced plasmid curing (24). Cells containing R46, pKM101, or the pKM101 *nuc*::Tn*5* derivative were treated for 6 h with 300 μg of fluorodeoxyuridine per ml; 1% of the cells survived this treatment, and among the survivors 40% were plasmid free for each of the three strains.

Eighth, the nuclease does not enhance the ability of the host to ingest exogenous DNA. We used a *trp his*$^+$ *endA thyA deo* derivative of AB1157 (GW4201) to determine whether there was a pKM101 endonuclease-dependent ability to utilize high-molecular-weight DNA as a sole source of thymine. Such strains containing either (i) pKM101, (ii) an *slo*::Tn*5* derivative of pKM101 (11), (iii) the nuclease-overproducing plasmid pGW1508, or (iv) pBR322 all grew well in M9-glucose medium containing 4 μg of thymine per ml or 4 μg of thymine plus 3,000 μg of herring sperm DNA per ml, but did not grow at all with 3,000 μg of herring sperm DNA per ml in the absence of extra thymine. Furthermore, none of these plasmids conferred upon a prototrophic *endA* host the ability to utilize 5,000 μg of DNA per ml as a sole source of carbon or nitrogen.

Other plasmids determining an EDTA-resistant endonuclease. In an effort to determine whether plasmid-mediated nucleases were widespread, we screened 26 plasmid-containing strains of *Escherichia coli* K-12 for EDTA-resistant nuclease activity by using native polyacrylamide gel electrophoresis as described above. Since these gels would normally reveal nuclease activity only if the enzyme's isoelectric point were less than pH 7.6, strains containing plasmids that did not appear to determine nuclease activity were reexamined with gels run at a pH of 8.8 so that all but the most basic enzymes would migrate toward the anode. As shown in Table 1, 11 of the 26 plasmid-containing strains tested contained an EDTA-resistant nuclease. In the limited set of strains we examined, we did not observe an instance of an incompatability group containing both nuclease-positive and nuclease-negative members.

DISCUSSION

In the present study we have localized the gene sufficient for expression of a pKM101-encoded endonuclease to an 0.8-kb region lying within a 15-kb region of DNA coding for conjugal transfer functions. We have also determined its direction of transcription, obtained evidence that the product of this gene is the enzyme itself rather than a regulatory protein controlling the expression of some other gene, and shown that the enzyme is confined to the periplasm. We have ruled out as possible physiological functions of the endonuclease roles in (i) mutagenesis enhancement, (ii) conjugal transfer, (iii) entry exclusion, (iv) fertility inhibition of P-group plasmids, (v) phage restriction, (vi) the killing of *K. pneumoniae*, (vii) fluorodeoxyuridine-induced plasmid curing, and (viii) degradation of exogenous DNA. Finally, we have shown that a variety of plasmids mediate EDTA-resistant endonuclease activities.

Except for its resistance to EDTA, the nuclease coded by pKM101 seems not unlike the host-mediated endonuclease I, the product of the *endA* gene. Both are periplasmically localized (6), the activity of both enzymes decays rapidly when cells reach the stationary phase (32; Win-

ans and Walker, unpublished results), and in both cases loss of the enzyme has not been associated with any demonstrable physiological effect (7). The fact that neither enzyme has any effect on the crossing of the periplasm by either entering plasmid or phage DNA indicates that such DNAs must be protected from degradation.

The fact that within any particular incompatibility group all of the plasmids we tested were either nuclease positive or else all were nuclease negative suggests that this property might be useful in classifying plasmids. It is interesting that the *incP* and *incW* plasmids, which have been considered to be closely related to the *incN* plasmids (4), do not elaborate such a nuclease, whereas the *incIα* and *incIγ* groups, which might have been considered to be more distantly related, do have such an enzyme.

The plasmid Rts1 mediates an enzyme that causes a nonstaining halo around colonies grown on DNA-containing agar (14). We have not tested strains containing this plasmid for the production of an EDTA-resistant DNase. However, pKM101-containing strains do not produce such halos, nor do they release endonuclease into liquid media, so that the nucleases of these two plasmids differ in at least this respect.

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