Genes Encoding *s*-Triazine Degradation Are Plasmid-Borne in *Klebsiella pneumoniae* Strain 99

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Klebsiella pneumoniae strain 99 degrades the *s*-triazine compound ammelide through cyanuric acid and biuret to yield urea, carbon dioxide, and ammonia. The urea and ammonia formed from the degradation of ammelide or cyanuric acid are utilized as sources of nitrogen for growth of the organism. When plasmids of the IncIα incompatibility group were transferred into *K. pneumoniae* strain 99, the ability to degrade *s*-triazine compounds was lost at high frequency. Analysis of the plasmid profiles of *s*-triazine$^+$ and *s*-triazine$^-$ derivatives of strain 99 indicated that the largest of the at least five plasmids detected in this organism carries the genes encoding the *s*-triazine degradation pathway. Conjugal transfer of this plasmid from wild-type strain 99 into a type strain of *Klebsiella planticola* (ATCC 33531) resulted in exconjugants able to utilize ammelide or cyanuric acid as nitrogen sources. Thus, the genes required for *s*-triazine degradation are present on a large IncIα plasmid in *K. pneumoniae* strain 99.

Keywords: *Pesticide; herbicide; atrazine; biodegradation; heterocyclic ring cleavage*

INTRODUCTION

Herbicides based on the symmetrical triazine ring (*s*-triazine) are important tools in the monoculture of grain crops, particularly corn. Over 83 million pounds of the three major *s*-triazine herbicides (atrazine, cyanazine, and simazine) was used in the cultivation of corn in the United States in 1990 (National Agricultural Statistics Service, 1991). The detection of some of these compounds in ground water samples from agricultural areas has caused concern about the environmental fate of these compounds. A few strains of bacteria or fungi capable of degrading atrazine have been reported [reviewed by Cook (1987)], but little is known about the biochemical and genetic basis of *s*-triazine degradation in microorganisms. Recently, several strains of bacteria that are capable of completely mineralizing atrazine have been reported (Mandelbaum *et al*., 1995; Yanze-Kontou and Gschwind, 1994; Radosevich *et al*., 1995), and genes encoding the enzymes that dechlorinate atrazine or a dealkylated derivative of atrazine have been cloned (DeSouza *et al*., 1996; Shao *et al*., 1995), but little is known about the genetic basis for the degradation of simpler *s*-triazine intermediates. We have studied genetic factors responsible for the metabolism of the simple *s*-triazine compounds melamine (2,4,6-triamino-1,3,5-triazine), ammeline (2,4-diamino-6-hydroxy-1,3,5-triazine), ammelide (2,4-dihydroxy-6-amino-1,3,5-triazine), and cyanuric acid (2,4,6-trihydroxy-1,3,5-triazine) in a group of bacteria isolated by Cook and Hutter (1981) in Switzerland. In these organisms amino-substituted *s*-triazine compounds are sequentially deaminated (Figure 1) to yield cyanuric acid, which is the substrate for the ring cleavage enzyme cyanuric acid amidohydrolase (Cook *et al*., 1985). Cleavage of the *s*-triazine ring yields biuret, which is converted to urea. This collection of *s*-triazine-degrading bacteria included two distinct *Pseudomonas* species and a strain of *Klebsiella pneumoniae.* We have previously shown that these organisms share identical genes encoding the enzymes ammelide aminohydrolase and cyanuric acid amidohydrolase (Eaton and Karns, 1991a); this suggests that gene transfer between species has played an important role in the evolution and spread of *s*-triazine degradative capabilities within the soil microbial community. In this paper we demonstrate that the genes encoding *s*-triazine degrading enzymes are present on a large conjugal plasmid in *K. pneumoniae* strain 99.

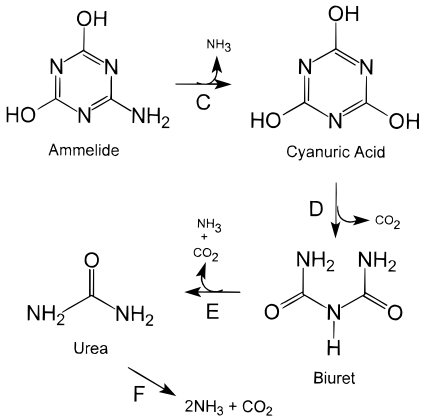

Figure 1. Pathway of *s*-triazine metabolism in *K. pneumoniae* strain 99. Enzymes and genes are as follows: C, *trzC* gene encoding ammelide amidohydrolase; D, *trzD* gene encoding cyanuric acid amidohydrolase; E, *trzE* gene encoding biuret amidohydrolase; F, urease.

MATERIALS AND METHODS

Bacterial Cultures and Media. *K. pneumoniae* strain 99, originally isolated by Cook and Hutter (1981), was obtained from Dr. Homer LeBaron, Ciba-Geigy Corp., Greensboro, NC. The culture was maintained on NFB medium (Tomasek and

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S0021-8561(96)00464-5

Karns, 1989) containing 20 mM citrate or 40 mM glucose as carbon source and 1.5 mM cyanuric acid or 1 mM ammelide as nitrogen source. In some instances BSM medium (Tomasek and Karns, 1989) was used with glucose or citrate as carbon source. For agar plates purified agar (Difco, Detroit, MI; or BBL, Baltimore, MD) was added to 1.2% prior to autoclaving. Citrate, glucose, cyanuric acid, and ammelide were added from filter-sterilized concentrated stocks to autoclaved NFB or BSM. Liquid cultures were incubated at 30 °C with shaking, and growth was monitored with a Klett-Summerson colorimeter equipped with a No. 66 filter.

The wild-type *K. pneumoniae* strain 99 produced a large amount of extracellular slime which interfered with DNA isolation and the preparation of cell-free extracts. A slimeless derivative was isolated by treating a late log phase culture of strain 99 grown in LB (Lennox broth; Gibco, Grand Island, NY) with ethyl methanesulfonate (EMS) according to the procedure of Carlton and Brown (1981). The mutagenized culture was diluted and spread onto NFB−glucose−cyanuric acid plates and grown at 30 °C. The plates were checked after 24, 48, and 72 h to look for any dry (slimeless) colony forms that might be present. Several dry forms were found, of which one was selected and found to be otherwise identical to strain 99 in the ability to degrade *s*-triazines. This dry form was named strain 99d.

Transformation competent *Escherichia coli* strain DH5α was purchased from Bethesda Research Laboratories (BRL, Bethesda, MD) and *Klebsiella planticola* ATCC 33531 was purchased from the American Type Culture Collection (Rockville, MD). Rifampicin resistant derivatives of the *Klebsiella* strains were selected by spreading late exponential phase cells onto L-agar plates containing 50 μg/mL rifampicin and picking colonies that appeared after 24 h of incubation. Antibiotic concentrations used in both minimal and rich media were as follows: rifampicin (Rf), 50 μg/mL; chloramphenicol (Cm), 34 μg/mL; kanamycin (Kn), 50 μg/mL; and tetracycline (Tc), 30 μg/mL.

Plasmids, Molecular Cloning, and Matings. Plasmids R64*drd*11 (Meynell and Datta, 1967), encoding Tc resistance, and R144 (Hedges and Datta, 1973), encoding Tc and Kn resistance, are of the IncIα incompatibility group and were maintained in *E. coli* strains SK1592 and J53, respectively. Plasmid pMMB277 (Morales *et al*., 1991) was the kind gift of Dr. M. Bagdasarian and is a broad host range cloning vector based on the RSF1010 replicon (Bagdasarian *et al*., 1983). It carries a Cm resistance gene, *lacI*^{q}, has a multiple cloning site in *lacZ*α, and is mobilizable by helper plasmids such as pRK2013. Restriction endonucleases and T4 DNA ligase were from BRL or New England Biolabs (Beverly, MA) and used according to the manufacturers' instructions.

A 4.6 kb *Pst*I DNA fragment carrying the *trzC* and *trzD* genes (encoding ammelide aminohydrolase and cyanuric acid amidohydrolase originally from *Pseudomonas* sp. NRRLB12227) was obtained from *Pst*I cut pRE458 (Eaton and Karns, 1991b). The 4.6 kb fragment was separated from the vector on an agarose gel, recovered from the gel by electroelution onto NA45 membrane (Schleicher and Schuell, Keene, NH) according to the manufacturer's instructions, and ligated to *Pst*I cut pMMB277 to form plasmid pJK206. A 2.0 kb *Pst*I−*Hin*dIII fragment containing the *trzD* gene from pRE458 was similarly isolated and ligated to pMMB277 cut with the same enzymes to yield plasmid pJK204. All recombinant plasmids were transformed into competent *E. coli* DH5α according to the method supplied by the manufacturer. Transformed bacteria were plated onto LB agar containing Cm, isopropyl β-D-thiogalactopyranoside (IPTG, 1 mM), and 5-bromo-4-chloroindoyl-β-D-galactopyranoside (X-gal, 50 μg/mL), and transformants having DNA inserts in the *lacZ*α gene of pMMB277 appeared white among blue colonies lacking inserts.

Matings were performed by mixing 0.1 mL each of log phase cultures of donor and recipient cells on L-agar plates and incubating for 16 h at 30 °C. Cells were scraped from the plates and suspended in sterile 50 mM potassium phosphate buffer (pH 7.0) and spread onto selective media. To mobilize pMMB277-based plasmids, *E. coli* HB101 containing plasmid pRK2013 (Figurski and Helinski, 1979) was added to the mating mixture to provide transfer functions for plasmid mobilization in a triparental mating (Tomasek and Karns, 1989).

Derivatives of *K. pneumoniae* strain 99 that were unable to utilize the *s*-triazine compounds ammelide or cyanuric acid as nitrogen sources were isolated by mating Rf resistant strain 99 (or in some cases strain 99d) with an *E. coli* strain containing the IncIα plasmid R144 or R64*drd*11 as described above. *Klebsiella* strains that had received either R144 or R64*drd*11 were selected on BSM−citrate−Rf−Tc plates. Colonies that appeared on these plates were transferred to plates of NFB−citrate−cyanuric acid agar and BSM−citrate agar in a grid pattern using sterile toothpicks. Isolates that grew on the BSM plates but not on the NFB plates were presumed to have lost the ability to utilize *s*-triazines as nitrogen sources. The *s*-triazine$^-$ phenotype was verified by inoculating the isolates into liquid NFB−citrate medium containing either cyanuric acid or ammelide.

Isolation of DNA, Electrophoresis, and Southern Blotting. Plasmid DNA was isolated from *Klebsiella* strains according to the method of Hansen and Olsen (1978). In some instances it was necessary to heat the mixture at 55 °C prior to the alkaline denaturation step to achieve an acceptable degree of cell lysis. For screening purposes the alkaline lysis minipreparation of Birnboim and Doly (1979) was used to view large plasmids in *Klebsiella* strains. Plasmid pMMB277 and derivatives of it containing cloned DNa were isolated from *E. coli* according to the method of Clewell and Helinski (1969). The boiling minipreparation method of Holmes and Quigley (1981) was used to screen recombinant plasmids in *E. coli*. Total DNA was isolated according to the method of Marmur (1961).

Electrophoresis of DNA was accomplished in 0.6 or 0.7% agarose gels in Tris−acetate buffer (Maniatis *et al*., 1982). Gels were stained in a 0.5 μg/mL solution of ethidium bromide and observed under UV light. Southern blotting of DNA from agarose gels onto GeneScreen Plus membranes (NEN/DuPont, Wilmington, DE) was performed as described by Maniatis *et al*. (1982). Dot blots were prepared using a MiniFold II apparatus (Schleicher and Schuell, Keene, NH). DNA was denatured with NaOH, neutralized, and blotted onto a GeneScreen Plus membrane as described by the manufacturer. Radiolabeled DNA probes were prepared by isolating DNA fragments using NA45 membrane as described above and labeling them with [^{32}P]dCTP using the Oligolabelling kit of Pharmacia-LKB (Uppsala, Sweden) according to the instructions supplied with the kit. Prehybridization and hybridization were performed at 65 °C according to the instructions provided by the manufacturer of GeneScreen Plus. Radioactive spots were detected by placing washed filters onto X-ray film for 1−24 h.

Preparation of Cell Extracts and Enzyme Assays. *K. pneumoniae* strain 99 was grown in 1 L of NFB−citrate−cyanuric acid or 1 L of BSM−citrate at 30 °C to late log phase. Cells were pelleted by centrifugation at 6000*g* for 20 min at 4 °C, washed once with 500 mL of ice-cold 25 mM potassium phosphate (pH 7.0), recentrifuged, and suspended in 10 mL of the same buffer. Cells were lysed by passing the suspensions twice through a chilled French pressure cell (4 °C) at 16 000 psi. The lysate was centrifuged at 12000*g* at 4 °C for 15 min, and the resulting supernatant was removed and used as a source of crude extract for enzyme assays. Cell extracts were made in an identical manner from cells of cured strain *K. pneumoniae* 99d-1a and a derivative of this strain carrying the *trzD* gene on pJK204 grown on BSM−citrate containing 0.1 mM IPTG (a gratuitous inducer of the *tac* promoter on pJK204). Protein concentrations in crude extracts were determined according to the method of Bradford (1976) using a kit from Bio-Rad (Richmond, CA).

Cyanuric acid amidohydrolase activity was assayed by adding crude extract to 4 mL of 25 mM potassium phosphate buffer (pH 7.0) containing 1.5 mM cyanuric acid and making repeated injections directly into an HPLC to monitor the loss of cyanuric acid and the buildup of biuret. Samples were chromatographed on a Resolve C_{18} radially compressed column (Waters, Milford, MA) with an isocratic solvent of 5 mM

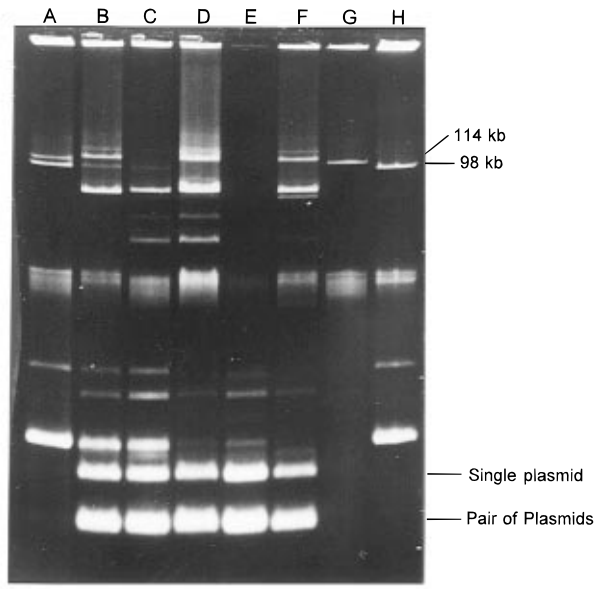

Figure 2. Agarose gel electrophoresis of plasmid DNA from *s*-triazine$^+$ and *s*-triazine$^-$ forms of *K. pneumoniae* strain 99. Lanes: (A) mixture of R144 and R64*drd*11; (B) a derivative of strain 99-5 restored to the *s*-triazine$^+$ phenotype by mating with wild-type strain 99; (C) *s*-triazine$^-$ derivative 99-5; (D) wild-type strain 99; (E) *s*-triazine$^-$ derivative 99-15; (F) 99-15 restored to the *s*-triazine$^+$ phenotype by mating with wild-type strain 99; (G) R64*drd*11 (114 kb in size); and (H) R144 (98 kb in size).

octyltriethylammonium phosphate in 5 mM potassium phosphate (final pH 6.8) at 2 mL/min. Compounds were detected with a Waters 490 multichannel detector monitoring absorbance at 200, 210, and 225 nm.

RESULTS

The introduction of the IncIα antibiotic resistance plasmid R64*drd*11 or R144 into *K. pneumoniae* strain 99 resulted in a high frequency of loss of the ability to utilize the *s*-triazine compounds ammelide or cyanuric acid as nitrogen sources. In each case 32% of the Tc resistant exconjugants tested were unable to utilize *s*-triazine compounds. Strains 99-5 and 99d-1a were *s*-triazine$^-$ derivatives of the slimy and dry forms of strain 99 into which R144 was introdced. Strains 99-15 and 99d-1b were derived through the introduction of R64*drd*11. Mating these *s*-triazine$^-$ with *s*-triazine$^+$ *K. pneumoniae* strain 99 restored the ability to utilize *s*-triazines as nitrogen sources. Wild-type strain 99 contains at least five plasmids (Figure 2, lane D), two plasmids in the range of 3.3−3.5 kb in size, one of about 4.8 kb in size, and a pair of large plasmids. The largest of the two large plasmids is bigger than the R64*drd*11 marker (114 kb) included in lanes A and G, while the other is somewhat smaller than the R144 marker (98 kb) included in lanes A and H. Cured derivative 15 (generated by mating with R64*drd*11) is missing both large plasmids (Figure 2, lane E), while cured derivative 5 (generated by mating with R144) is missing only the largest plasmid (Figure 2, lane C). Both cured derivatives retained the three smallest plasmids. These data suggested that the largest plasmid in *K. pneumoniae* strain 99 carries the genes which allow this strain to utilize ammelide or cyanuric acid as nitrogen source. When Rf resistant derivatives of these cured strains were mated with wild-type strain 99, they regained the ability to utilize ammelide and cyanuric acid as nitrogen sources and they both regained the large (>114 kb) plasmid (Figure 2, lanes B and F), confirming the role of this plasmid in *s*-triazine degradation.

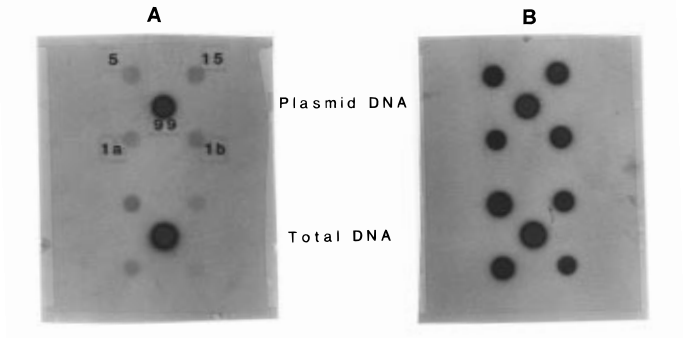

Figure 3. Dot blots of plasmid and total DNA from *K. pneumoniae* strain 99 and cured (*s*-triazine$^-$) derivatives. 5 = 99-5 wild-type strain 99 cured with R144; 1a = 99d-1a slimeless form of strain 99 cured with R144; 15 = 99-15 wild-type strain 99 cured with R64*drd*11; 1b = 99d-1b slimeless form of strain 99 cured with R64*drd*11. The blot in panel A was probed with the 4.6 kb fragment from pJK206. Panel B was probed with a randomly cloned fragment from *K. pneumoniae* strain 99 that is probably part of one of the small plasmids present in this strain. The pattern of dots is the same in each panel.

The complete degradation of cyanuric acid to CO_2 and NH_3 requires three enzymes: (1) cyanuric acid amidohydrolase (*trzD* gene product) converts cyanuric acid to biuret and CO_2; (2) biuret amidohydrolase (*trzE* gene product) converts biuret to urea, CO_2, and NH_3; and (3) urease converts urea to CO_2 and $2NH_3$. Previous studies have shown that the *trzD* gene is located very close to the *trzC* gene (encoding ammelide aminohydrolase) as part of what appears to be an *s*-triazine degradation operon (Eaton and Karns, 1991a), but as yet, the *trzE* gene has not been located. In *Pseudomonas* sp. NRRLB-12227 a high rate of loss of the ability to metabolize biuret while retaining the ability to convert cyanuric acid to biuret suggested that the *trzE* gene was located on a plasmid or DNA element distinct from the DNA element carrying the *trzC* and *trzD* genes (Eaton and Karns, 1991b). In light of this observation it seemed possible that the *trzC,D* gene cluster and the *trzE* gene might also be located on separate DNA elements in *K. pneumoniae* strain 99. To determine which triazine degradation genes were present on the >114 kb plasmid in strain 99, dot blots of plasmid and total DNA from strain 99 and four triazine$^-$ derivatives were probed with the 4.6 kb *Pst*I fragment from pJK206, which carries the *trzC* and *trzD* genes (Figure 3). The probe hybridized strongly to both plasmid and total DNA from wild-type strain 99 but failed to hybridize to DNA from the cured strains (left panel). A randomly cloned fragment from strain 99 plasmid DNA hybridized to all dots (right panel), showing the presence of sufficient DNA in the dots. Thus, the *trzC* and *trzD* genes are definitely located on the large plasmid in strain 99.

To determine whether the *trzE* gene is likely to be located on the same plasmid as *trzC* and *D* or is located elsewhere, plasmid pJK204, containing the *trzD* gene in pMMB277, was transferred into *K. pneumoniae* strain 99d-1a (non-slime-producing *s*-triazine$^-$ derivative cured with R144), with a plasmid profile identical to that seen for cured derivative 5, missing only the >114 kb plasmid. Since the hydrolysis of cyanuric acid by cyanuric acid amidohydrolase does not release any nitrogen for the cell to utilize for growth, both the *trzD* and *trzE* genes are required for release of ammonia from

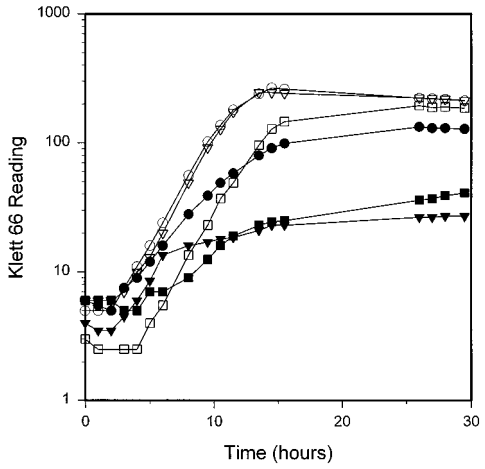

Figure 4. Growth curves of *s*-triazine$^+$ and *s*-triazine$^-$ strains of *K. pneumoniae* strain 99 and *s*-triazine$^-$ strains containing pJK204: (○) strain 99d (*s*-triazine$^+$) on BSM-citrate; (●) strain 99d on NFB−citrate−cyanuric acid; (▽) strain 99d-1a (*s*-triazine$^-$) on BSM−citrate; (▼) strain 99d-1a on NFB−citrate−cyanuric acid; (□) strain 99d-1a containing pJK204 on BSM−citrate; (■) strain 99d-1a containing pJK204 on NFB−citrate−cyanuric acid.

cyanuric acid. If the *trzE* gene is located on one of the other plasmids in this strain or on the chromosome, the presence of the cloned *trzD* gene should yield a strain capable of growth with cyanuric acid as nitrogen source. Enzyme assays showed that crude extracts of the cured strain containing pJK204, grown in BSM−citrate−IPTG medium, had a level of cyanuric acid amidohydrolase activity identical to that seen in NFB−citrate−cyanuric acid grown 99d [65 nmol of cyanuric acid converted to biuret min^{-1} (mg of cell $protein^{-1}$)]. No cyanuric acid amidohydrolase activity was detected in the cured strain when pJK204 was not present. This cured strain containing pJK204 grew no better with cyanuric acid as a nitrogen source than did the cured strain without pJK204 (Figure 4), indicating that no ammonia was released from the biuret produced from cyanuric acid and thus that the *trzE* gene was also absent in this strain.

Conjugal transfer of plasmid DNA from *s*-triazine$^+$ *K. pneumoniae* strain 99d into a Rf resistant derivative of a wild-type strain of *K. planticola* ATCC 33531 resulted in exconjugant *K. planticola* that were able to utilize cyanuric acid and ammelide as nitrogen sources. The original culture of *K. planticola* ATCC 33531 contained no detectable plasmids in our hands (data not shown). Examination of the plasmid profile of *s*-triazine$^+$ *K. planticola* exconjugants revealed that the large (>114 kb) plasmid and the two smallest plasmids of *K. pneumoniae* strain 99 had been transferred (Figure 5). Southern blotting of restriction-digested and whole plasmid DNA from *s*-triazine$^+$ *K. planticola* and hybridization with a ^{32}P-labeled probe made from the 4.6 kb fragment from pJK206 (carrying the *trzC* and *D* genes) confirmed that these genes were present on the >114 kb plasmid (Figure 5). This *s*-triazine degradation plasmid has been named pPDL12.

DISCUSSION

The role of plasmids in the dissemination of biodegradation genes is well documented. Plasmids have been shown to carry genes that encode the enzymes for the degradation of various agricultural chemicals, including organophosphate and ***N***-methylcarbamate insecticides (Serdar *et al.*, 1982; Mulbry *et al.*, 1987; Tomasek and Karns, 1989), as well as the herbicides 2,4-dichlorophenoxyacetate (Amy *et al.*, 1985; Don and Pemberton,

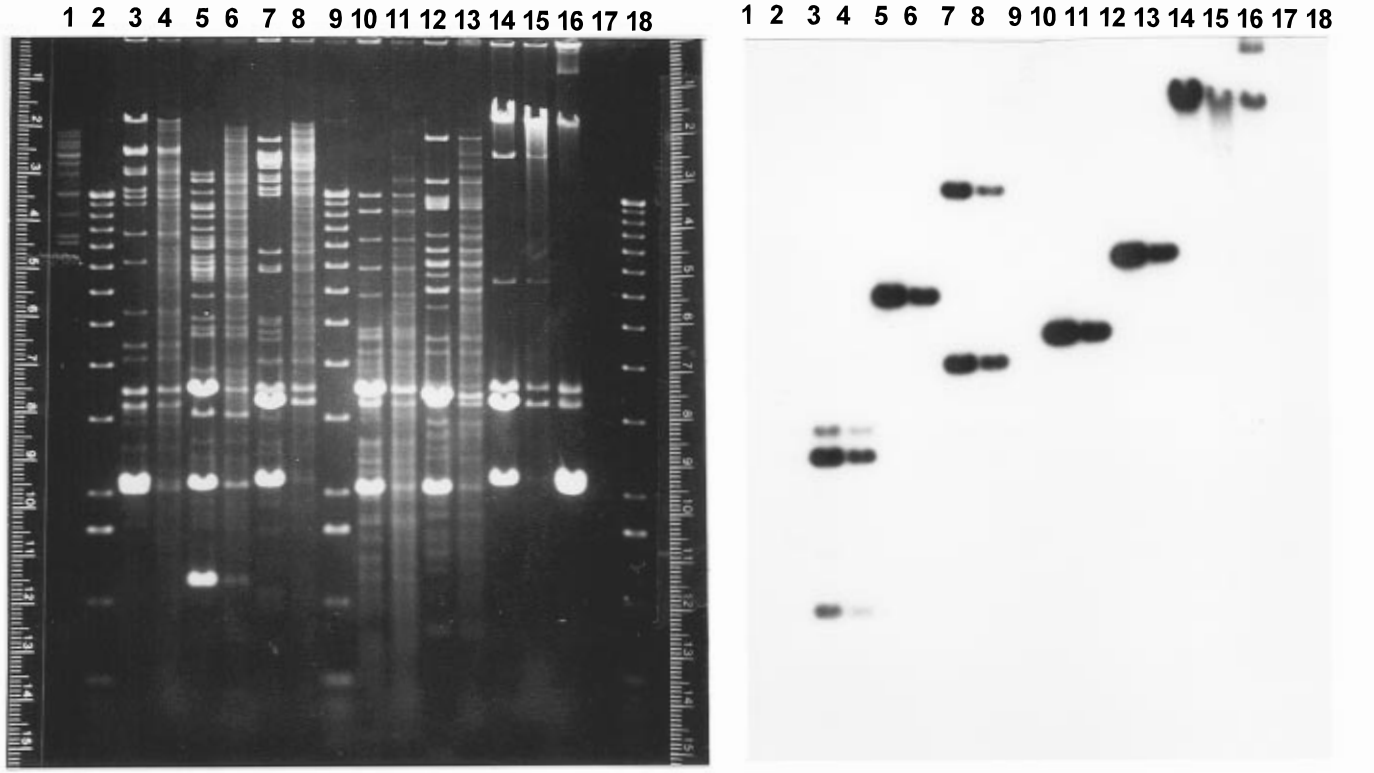

Figure 5. Agarose gel electrophoresis (left) and corresponding Southern blot (right) of plasmid DNA from an *s*-triazine$^+$ exconjugant of *K. planticola* ATCC 33531 after mating with *K. pneumoniae* strain 99: (lane 1) BRL high molecular weight ladder; (lanes 2, 9, and 18) BRL 1 kb ladder; (lane 16) uncut plasmid DNA; all other lanes alternate plasmid or total DNA cut with restriction enzymes [(lanes 3 and 4) *Bam*HI; (lanes 5 and 6) *Eco*RI; (lanes 7 and 8) *Hin*dIII; (lanes 10 and 11) *Pst*I; (lanes 12 and 13) *Sma*I; (lanes 14 and 15) *Xba*I.

1981) and bromoxynil (Stalker and McBride, 1987). This study shows that the genes required for some of the intermediate steps in the metabolism of the *s*-triazine ring structure, a component of several important herbicides (including atrazine), are plasmid-borne in an *s*-triazine degrading strain of *K. pneumoniae*. Direct evidence has shown that the genes encoding ammelide aminohydrolase and cyanuric acid amidohydrolase (*trzC* and *D*, respectively) are present on plasmid pPDL12. Derivatives of *K. pneumoniae* strain 99 that are missing only pPDL12 do not utilize cyanuric acid as a nitrogen source when the recombinant plasmid pJK204 (encoding a functional cyanuric acid amidohydrolase) is present; therefore, the *trzE* gene, encoding biuret amidohydrolase, must also be present on pPDL12. This is confirmed by the fact that transfer of pPDL12 into a type strain of *K. planticola* confers the ability to utilize cyanuric acid as a nitrogen source.

The *s*-triazine degradation plasmid pPDL12 is cured at high frequency when antibiotic resistance plasmids of the IncIα incompatibility group are transferred into *K. pneumoniae* strain 99, strongly suggesting that pPDL12 is a member of the IncIα incompatibility group. In addition, the transfer of this plasmid in mating experiments without the use of helper plasmids suggests that pPDL12, like the IncIα plasmids R64*drd*11 and R144, is self-transmissible, although we cannot rule out the possibility that one of the small plasmids present in strain 99 may provide transfer functions.

In light of the previous observation that identical *trzC* and *D* genes are present in two strains of *Pseudomonas* as well as *K. pneumoniae* strain 99 (Eaton and Karns, 1991a), the finding that pPDL12 is of the IncIα incompatibility group is surprising, since the host range of IncIα plasmids is limited to enteric bacteria (Bukhari *et al*., 1977). Since this plasmid will not replicate in *Pseudomonas*, it seems that it cannot be the agent of propagation of these genes in *Pseudomonas* sp. NRRLB-12227 or *Pseudomonas* sp. NRRLB-12228 unless it has acted as a suicide transposon donor. Indeed, restriction mapping of the DNA surrounding the *trzC,D* gene cluster in all of these strains has shown that the regions of DNA flanking these *trz* genes is markedly different in all three strains (Eaton and Karns, 1991a). Thus, although pPDL12 may be an agent for the dissemination of *s*-triazine degradation genes between bacteria, DNA recombination events must occur for propagation of these genes in other bacterial genera.

ACKNOWLEDGMENT

We thank Ms. Bronda F. Harrison and Mr. Donald Wiggins for technical assistance with portions of this research.

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Received for review June 28, 1996. Accepted December 16, 1996.[⊗]

JF960464+

[⊗] Abstract published in *Advance ACS Abstracts,* January 15, 1997.