In Vivo Cloning of Arylsulfatase-Tyramine Oxidase Genes in *rec* Strains of *Klebsiella aerogenes*

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A general, genetic technique for *in vivo* cloning of bacterial genes is presented. We previously introduced the Mu phage into various genera of bacteria including *Klebsiella aerogenes* with RP4::Mu. Using these strains carrying RP4::Mu *cts* and thermo-inducible Mu prophage in the chromosome, we cloned *in vivo* the arylsulfatase (*ats*) and tyramine oxidase (*tyn*) genes by partial thermo-induction. The donor strains carrying the recombinant plasmids were conjugated with *K. aerogenes rec* strains, which were isolated as UV-sensitive mutants. The resultant recombinant plasmids, pAT1 and pAT2, were purified and used for the transformation of mutant strains deficient in the *ats* and *tyn* genes. The *ats-tyn* genes seemed to be transposed into the RP4::Mu *cts* plasmid together with other chromosomal DNA fragments. This *in vivo* cloning method is applicable to a wide variety of gram-negative bacteria.

Bacteriophage Mu and hybrid plasmid RP4::Mu can mediate the transfer of chromosomal markers by promoting the formation of plasmid primes or by integration of phage DNA or plasmids into the chromosome to form "intermediate Hfr" donor strains.^{1~3)} This property and the development of the transposon Mu dl(Ap^r *lac*) for fusion of genes⁴⁾ indicate that the Mu genomes and RP4::Mu plasmid should be very useful for *in vivo* genetic engineering.

Previously, Dénarié *et al.*⁵⁾ introduced the Mu phage into strains of *Klebsiella* and *Pseudomonas* and we expanded the bacterial host range of coliphage Mu to most gram-negative bacteria.⁶⁾ By using RP4::Mu, we also created intergeneric recombinant strains of enteric bacteria by transfer of some genes.⁶⁾

By combining the procedure for constructing the RP4::Mu primes and transformation of the recombinant plasmids to *rec* strains, we developed a genetic method for *in vivo* cloning of genes of interest into the RP4::Mu vector in gram-negative bacteria. This technique does not require *in vitro* cleavage of the chromo-

some or plasmid by restriction enzymes or *in vitro* ligation of cleaved DNAs.

To illustrate the use of this method, we chose genes involved in the complex system for regulation of sulfate and nitrogen metabolisms in enteric bacteria. In these organisms, arylsulfatase encoded by the *atsA* gene is repressed by inorganic sulfate or cysteine as a corepressor. This repression is released by expression of the tyramine oxidase genes (*tyn*), which is induced by tyramine or catecholamines and subject to catabolite and ammonium repressions.^{7~9)} As a step towards understanding the genetic organization of the *ats-tyn* region, we isolated clones having the *ats-tyn* genes in *Klebsiella aerogenes* by using this method, which will be generally applicable to other bacterial genes and to other RP4::Mu-sensitive bacteria.

MATERIALS AND METHODS

Chemicals and media. The rich media used were Penassay broth (Antibiotic assay medium 3; Difco Labo., Detroit, MI.) and LB medium, consisting of 1% tryptone

TABLE I. LIST OF BACTERIAL STRAINS AND PLASMIDS USED AND THEIR CHARACTERISTICS

Strain	Relevant genotype	Source or reference
<i>K. aerogenes</i>		
W70	Wild-type, P1 ^r	MacPhee <i>et al.</i> ²²⁾
MK9000	P1 ^s	Streicher <i>et al.</i> ²³⁾
K204-1	<i>ats</i> ⁺ <i>tyr</i> ⁺ <i>cys pur</i> (RP4::Mu <i>cts61</i>)	Murooka <i>et al.</i> ⁸⁾
K204-2	<i>ats</i> ⁺ <i>tyr</i> ⁺ <i>cys pur</i> Mu <i>cts61</i> (RP4)	Murooka <i>et al.</i> ⁸⁾
K611	<i>tynA611</i>	Murooka <i>et al.</i> ⁷⁾
K629	<i>tynA611 recA29 srl</i> ⁺	P1 from MKN329 to K642
K642	<i>tynA611 srl-2</i>	Mutagenesis of K611
K646	<i>tynA611 srl-6</i>	Mutagenesis of K611
K648	<i>tynA611 srl-8</i>	Mutagenesis of K611
MKN343-1	<i>tynPA343 tyrR4 atsA343</i>	Oka <i>et al.</i> ²⁴⁾
MKN314	UV ^s <i>recA14</i>	Mutagenesis of MKN343-1
MKN329	UV ^s <i>recA29</i>	Mutagenesis of MKN343-1
KS291	<i>recA29</i> (pAT1)	pAT1 transformed to K629
MT141	<i>recA14</i> (pAT1)	<i>In vivo</i> cloning in MKN314
MT292	<i>recA29</i> (pAT2)	<i>In vivo</i> cloning in MKN329
MT431	<i>tyrR4</i> (pAT1)	pAT1 transformed to MKN343-1
<i>E. coli</i>		
C600-R	<i>hsdR hsdM thr-1 leu-6 thi-1</i> (RP4)	Kiyohara and Yano
BE228	<i>thr-1 leu-6 thi-1</i> (RP4::Mu <i>cts61</i>)	Rao ²⁵⁾
JC5466	<i>trp his recA56 rps</i> (RP4::Mu <i>cts62</i>)	Dénarié ⁵⁾
Plasmid		
RP4	Ap ^r Km ^r Tc ^r	Kiyohara and Yano
RP4::Mu <i>cts61</i>	containing host chromosome	This paper, Rao ²⁵⁾
RP4::Mu <i>cts62</i>	hybrid of RP4 and Mu <i>cts62</i>	This paper, Dénarié <i>et al.</i> ⁵⁾
pAT1	RP4::Mu <i>cts61-ats</i> ⁺ - <i>tyr</i> ⁺	This paper
pAT2	RP4::Mu <i>cts61-ats</i> ⁺ - <i>tyr</i> ⁺	This paper

(Difco), 0.5% yeast extract (Difco), and 0.5% NaCl. The supplemented LB medium used was LBAKT, LB plus ampicillin (Fujisawa Co., Ltd., Osaka, 100 µg/ml), kanamycin (Meiji Seika Co. Ltd., Osaka, 25 µg/ml), and tetracycline (Sigma Chemical Co., St. Louis, MO., 50 µg/ml). The minimal medium used was K medium,⁶⁾ consisting of 0.5% xylose, 0.1% nitrogen source, 0.1 M potassium phosphate buffer (pH 7.2), 0.01% MgCl₂·6H₂O, 1 mM Na₂SO₄, and 0.001% each of NaCl, MnCl₂·4H₂O, and FeCl₃·6H₂O. Unless otherwise mentioned, NH₄Cl was used as a nitrogen source. Media used for isolating clones containing the *ats-tyr* genes were XT(N) which consisting of K medium with tyramine-HCl as the nitrogen source, and XT(N) I which was XT(N) containing indoxylsulfate (Sigma Chemical Co., 0.25 mg/ml) as an indicator of arylsulfatase. *Ats*⁺ colonies become blue on plates containing indoxylsulfate.⁷⁾ For isolating mutants incapable of utilizing sorbitol, sorbitol was used as the carbon source in K medium (KS medium).

Bacterial strains, phage and plasmids. The bacterial strains and plasmids used in this study are described in Table I. The strains of *K. aerogenes* used were derivatives

of *K. aerogenes* W70. Transducing phage Plclr100KM¹⁰⁾ and Mu *cts61* or Mu *cts62*⁶⁾ phage were prepared from our stock cultures of their lysogenes as described previously.

Isolation of mutants. Mutagenesis with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine and chloramphenicol was achieved as described by Sklar.¹¹⁾ The mutagenized cells were treated with penicillin G (3,000 µg/ml) for 3 hr to achieve enrichment of mutants incapable of utilizing sorbitol. For isolation of Rec⁻ mutants, the mutagenized cells at suitable dilutions were spread on LB plates. After overnight growth, each colony was suspended in 0.5 ml of sterilized saline, and then one loopful of the suspension was patched on two LB plates. One plate was irradiated at 20 cm for 30 to 50 sec (about 300~750 erg/mm²) with a National UV lamp, model GL-15 (Matsushita Electronics Co., Ltd., Osaka). The plates were then incubated at 30°C in the dark for 18 hr. Wild-type cells formed a confluent patch of growth, whereas Rec⁻ UV-sensitive strains formed few, if any, colonies. The UV-sensitive (UV^s) colonies were picked up and streaked to obtain single colonies. These purified UV^s colonies were tested for recombination ability by transduction with Plclr100KM phage.

Transduction. Preparation of Plc1r100KM lysates and PI transduction were performed as described previously.^{8,10} PI phages prepared from KM9000 were mixed with UV^s cells (2×10^8) at a multiplicity of infection of 0.1. Scoring of Tyn⁺ transductants was done as described previously.⁷ All strains with *rec* mutations were transduced to *srl* by mixing 0.1 ml of lysate (about 2×10^8 plaque-forming units/ml) with 2×10^8 cells and plating the mixture on KS plates. Each *Srl*⁺ colony was suspended in 0.5 ml of saline, and then one loopful of the suspension was patched on LB plates. After 4 hr of incubation at 30°C, these plates were placed under a UV-light at a distance of 20 cm for 30 sec. As a control, parent strain MKN343-1 and UV^s strains MKN314 and MKN329 were patched on the same plates.

Isolation of clones carrying the recombinant plasmids. For *in vivo* cloning of the *ats* and *tyn* genes, RP4::Mu *cts* or RP4 plasmids were used as a vector. *K. aerogenes* strains K204-1 and K204-2 carrying RP4::Mu *cts61* and both RP4 and Mu *cts61* prophages, respectively, were grown overnight at 30°C in 5 ml of LBAKT medium. The cells were diluted 1:100 with fresh LB medium. Exponentially growing cells of strains K204-1 and K204-2 were heated at 38°C for 16 hr in LB medium without shaking. This partial induction was mutagenic for the host chromosome and killed about 50% of the cells. These cells (about 1×10^9 colony-forming units per ml) were mixed with an equal volume of recipient cells of strains MKN314 or MKN329 in the late exponential phase of growth in LB medium (about 2×10^9 colony-forming units per ml). The mixture was filtered on a Toyo membrane filter (0.45 μ m pore size, 25-mm diameter; Toyoroshi Co., Ltd., Tokyo). Membranes were placed on the surface of a freshly prepared Penassay broth plate and incubated at 30°C for 5 hr. Bacteria were then suspended in 1 ml of saline and samples of 0.1 to 0.2 ml were spread on selective medium XT(N) or XT(N) I containing Ap (100 μ g/ml), Km (25 μ g/ml), and Tc (50 μ g/ml). Donors were counter-selected by omission of an essential nutrient. In control experiments, donor and recipient cultures were filtered on separate membranes and then incubated and plated under the same conditions as those for mating. The purified colonies grown on XT(N) were replicated onto K agar plates in the presence of indoxylsulfate to check arylsulfatase activity, and onto LBAKT and two LB plates, one incubated at 28°C and the other at 41°C. Lysogenes of Mu *cts* grew well at 28°C but very poorly at or above 39°C.

Isolation of plasmid DNA. Cells were grown in 50 ml of LB at 30°C and harvested in the late exponential phase (2×10^8 cells/ml) by centrifugation. The cells were washed with 25 ml of 10 mM sodium phosphate buffer (pH 7.2) and suspended in 1.35 ml of 25% sucrose–0.05 M Tris-HCl (pH 8.0). A cleared lysate was prepared by the alkaline denaturation method of Hansen and Olsen.¹² The plasmid DNA was purified by cesium chloride-ethidium bromide equilib-

rium density gradient centrifugation in a fixed-angle type 50 rotor at 40,000 rpm at 15°C for 40 hr. CsCl was used at a concentration of 1 g/ml. The plasmid band, located with a UV transilluminator, was collected. Ethidium bromide was removed by extraction with isopropanol saturated with 3 M sodium chloride and 0.3 M sodium citrate buffer. Cesium chloride was removed by dialysis against 10 mM Tris-HCl containing 1 mM EDTA, pH 8.0.

Transformation experiments. Transformation was performed as described by Mandel and Higa.¹³ *K. aerogenes recA* strains were grown in LB to about 100 Klett units. The cells were harvested and washed once with sterilized 0.1 M MgCl₂ in 5 mM Tris-HCl, pH 7.4, suspended in cold 0.1 M CaCl₂ and incubated for 20 min on ice. The suspension was centrifuged and the cells were suspended in one-tenth the original volume of cold 0.1 M CaCl₂. A 0.2 ml portion of the purified recombinant plasmid DNA was put into an Eppendorf tube containing 0.2 ml of competent cells. The tubes were incubated on ice for 1 hr, then warmed to 39°C and gently shaken for 2 min. After rechilling, the cells were diluted with fresh LB and incubated for 6 hr at 30°C. The cells were plated on XT(N) I containing Ap (100 μ g/ml), Km (25 μ g/ml), and Tc (50 μ g/ml).

Electron microscopy. The formaldehyde technique of Lang and Mitani¹⁴ was used. The DNA sample (25 μ l) was mixed with 150 μ l of 0.15 M ammonium acetate (pH 7.5), 20 μ l of cytochrome *c* (13 μ g/ml) and 5 μ l of 8% formaldehyde. The layer of cytochrome *c* with plasmid DNA was picked up on grids of polyvinyl formal coated with carbon, and subjected to staining with uranyl acetate and rotary shadowing with platinum palladium at an angle of 10°. The molecular weight of plasmids was calculated by the equation of Lang¹⁵ from measurements on several complete molecules.

Enzyme assay. Arylsulfatase activity was assayed as described previously.⁷ One unit of arylsulfatase activity was defined as the amount causing formation of one nanomol of *p*-nitrophenol per min at 30°C. Tyramine oxidase activity was assayed by a radiometric procedure with [³H]tyramine.⁷ One unit of tyramine oxidase activity was defined as the amount metabolizing one nanomole of tyramine per min at 30°C.

Protein was determined by the method of Lowry *et al.*¹⁶ with bovine serum albumin as a standard.

RESULTS

Rationale

Previously, we created intergeneric recombinant strains by transfer of some genes between *K. aerogenes* and *E. coli* by using Mu phage and RP4 or RP4::Mu plasmids.⁶ At

least part of the transfer was thought to be due to formation of RP4 prime or RP4::Mu prime episomes, which transfer any genes after induction of Mu *cts* prophages on the chromosome, because partial heat induction of Mu results in formation of Mu-bacterial circular DNA which can be integrated at random into a plasmid.^{3,26} Thus, we thought that it would be possible to develop an *in vivo* cloning method for isolating the genes of interest by transposing them into an RP4::Mu vector in gram-negative bacteria. These clones could be selected in a *rec* strain with mutations in appropriate genes and verified by transformation of the *in vivo* constructed plasmid into similar mutant strains.

To demonstrate this method, we isolated plasmids carrying the arylsulfatase and tyramine oxidase genes on RP4::Mu *cts* and transferred them to *K. aerogenes* mutant strains deficient in the *ats*, *tyl* and *rec* genes. The working scheme, which consists of five steps, is shown in Fig. 1: (1) RP4 or RP4::Mu is introduced into auxotrophic cells containing a Mu *cts* prophage or without Mu prophage, respectively. (2) The Mu *cts* prophage on the chromosome is partially induced, leading to *in*

in vivo self-cloning of chromosomal genes. (3) The donor cells are mated with recipient cells carrying mutations of the *ats*, *tyl*, and *rec* genes, and appropriate clones selected. (4) Recombinant plasmid DNA is isolated from the clones. (5) Recombinant plasmid DNA is used to transform secondary recipient cells.

Isolation of *rec* mutant strains

Since *rec* mutations have not been reported in *K. aerogenes*, we tried to isolate mutants of this type. In *E. coli*, at least 6 kinds of *rec* mutations are mapped in different chromosomal loci.¹⁸ Most of these mutations are sensitive to UV irradiation. Therefore, we selected mutants sensitive to UV and tested them for frequency of recombination following transduction by Pl. *K. aerogenes* strain MKN343-1 (*tylPA atsA*) was mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and UV^s colonies were isolated. These puri-

TABLE II. ISOLATION OF Rec⁻ FROM *K. aerogenes* MKN343-1

Strain	UV sensitivity ^a	No. of transductants by Plclr100KM Tyn ⁺ ^b
MKN343-1	R	4380
MKN314	S	0
315	S	>4000
316	S	>4000
323	S	>4000
325	S	880
MKN329	S	0
330	S	4800
331	S	>4000
333	S	1116
338	S	>4000
342	S	>4000
343	S	1624
356	S	>4000

^a R, resistant; S, sensitive.

^b P1 phages prepared from *K. aerogenes* MK9000 were used at a multiplicity of infection of 0.1 for all transductions. Recipient strains used were derived from strain MKN343-1, which is defective in the tyramine oxidase gene, and used at about 1 to 2 × 10⁹ cells for transduction. The Tyn⁺ phenotype was scored on XT(N) plates as described previously.⁸

FIG. 1. Scheme of the Method of *In Vivo* Cloning.

The method consists of five main steps. The details of each step are described in the text. The dotted line shows the second cycle of the process.

TABLE III. GENETIC ANALYSES OF *K. aerogenes* Rec⁻ STRAINS^a

Cross	Donor (genotype)	Recipient (genotype)	No. of Srl ⁺ transductants tested	No. of UV ^s
1	MKN314 (<i>rec-14</i>)	K642 (<i>srl-2</i>)	32	16
2	MKN314 (<i>rec-14</i>)	K646 (<i>srl-6</i>)	50	24
3	MKN314 (<i>rec-14</i>)	K648 (<i>srl-8</i>)	50	28
4	MKN329 (<i>rec-29</i>)	K642 (<i>srl-2</i>)	13	5
5	MKN329 (<i>rec-29</i>)	K646 (<i>srl-6</i>)	50	20
6	MKN329 (<i>rec-29</i>)	K648 (<i>srl-8</i>)	50	21

^a P1 phages prepared from strain MKN314 or MKN329 were used at a multiplicity of infection of 0.1. Scoring of Srl⁺ and UV^s colonies is described under METHODS.

fied mutants were infected with Plc1r100KM phages prepared from wild-type strain MK-9000, and Tyn⁺ transductants were selected. Table II shows that UV^s strains MKN325, 333 and 343 gave low transduction frequencies, and that no transductants of strains MKN314 and MKN329 were obtained. The frequencies of Pl infection in these UV^s mutants were not significantly different from that of the wild-type strain MK9000. These strains also gave no Ats⁺ transductants. The transduction frequencies of the two strains were less than 2×10^{-3} , and both strains were more susceptible to UV irradiation than other UV^s stains.

These phenotypic characters of strains MKN314 and MKN329 are similar to that of the *E. coli* *recA* mutant, which is Pl cotransducible with the gene of sorbitol utilization, *srl*.¹⁸⁾ Thus, we isolated mutant strains that are incapable of utilizing sorbitol from *K. aerogenes* K611 (*tynA*). We chose three mutants strains, K642, K646 and K648, which showed the Srl⁻ phenotype stably, although most Srl⁻ mutations were leaky or back-mutated at frequencies of 10^{-6} to 10^{-8} .

We used Plc1r100KM to determine the linkages of *rec-14* and *rec-29* to the *srl* genes in strains MKN314 and MKN329, respectively. Table III shows the results of transduction

experiments. The Rec⁻ phenotype was scored as UV-sensitivity. The resultant UV^s transductants were chosen at random and shown to have the Rec⁻ phenotype by Pl transduction for the *tynA* gene. When strain MKN314 (*rec-14*) or MKN329 (*rec-29*) was used as a donor and strain K642 (*srl-2*), K646 (*srl-6*) or K648 (*srl-8*) was used as a recipient, the *rec-14* and *rec-29* mutations were shown to be between 50% and 40% cotransducible with the *srl* gene, respectively. These results suggest that the mutations of *rec-14* and *rec-29* are similar to *recA* mutations of *E. coli*, which are located at 58 min on the chromosomal map.¹⁸⁾ Thus, these two *rec* mutations have tentatively been named *recA* of *K. aerogenes*.

Isolation of clones carrying recombinant plasmids containing the *ats* and *tyn* genes

K. aerogenes auxotrophic strains carrying a thermoinducible Mu prophage on the chromosome and RP4 or RP4::Mu *cts61* in the cytoplasm were partially heat induced at 38°C for 16 hr and then mated with *K. aerogenes* recipient strains. The recipient strains had a deletion of the structural gene for tyramine oxidase (*tynPA*), were arylsulfatase deficient (*atsA*), *recA*, and were sensitive to Ap, Km, and Tc, whereas RP4 in the donor confers resistance to Ap, Km, and Tc. The colonies

TABLE IV. ISOLATION OF CLONES CARRYING RP4::Mu-*ats*⁺-*ty*⁺ ^a

Donor strain	Recipient strain	No. of clones				
		Selected maker Tyn ⁺	Unselected maker Ats ⁺	Growth at 28°C 41°C		Drug resistance Ap Km Tc
K204-1	None	0				
K204-1	MKN314	5	4	5	0	5
K204-1	MKN329	4	4	4	0	4
K204-2	None	0				
K204-2	MKN314	1	1	1	0	1
K204-2	MKN329	1	1	1	0	1
None	MKN314	0				
None	MKN329	0				

^a Bacterial strains carrying RP4::Mu *cts61* or RP4 and Mu *cts61* prophage in the chromosome were partially induced at 38°C for 16 hr and mated with *K. aerogenes* strains carrying *ats*, *ty* and *rec*. The resultant Tyn⁺ colonies were replicated onto K plates containing indoxylsulfate and tyramine, LB plates containing Ap, Km and Tc, and onto two plates, one incubated at 28°C, and the other at 41°C. Lysogens of Mu *cts* grew well at 28°C, but very poorly at 41°C.

were purified on XT(N) plates and replicated on K plates containing indoxylsulfate and tyramine, LB plates with Ap, Km, and Tc, and on two LB plates without an antibiotic, one incubated at 28°C, and the other at 41°C. Arylsulfatase positive colonies gave a blue color with indoxylsulfate. Ten of the 11 colonies isolated as the Tyn⁺ phenotype were Ats⁺, and all the Tyn⁺ colonies of *K. aerogenes* strains MKN314 and MKN329 carried RP4::Mu *cts*, even when strain K204-2 (RP4) Mu *cts* was used as a donor (Table IV). Incorporation of the *ats* and *ty* genes into the RP4 plasmid may be mediated by Mu-promoted transposition, resulting in the construction of RP4::Mu-*ats*⁺-*ty*⁺.

Transformation of the recombinant plasmids into *K. aerogenes*

The resulting colonies probably carrying RP4::Mu-*ats*⁺-*ty*⁺ were purified by single colony isolation, and two strains, MT141 (*recA14*, RP4::Mu *cts61-ats*⁺-*ty*⁺) and MT292 (*recA29*, RP4::Mu *cts61-ats*⁺-*ty*⁺), were chosen for isolation of the recombinant plasmids. The plasmids from strains MT141 and MT292 were designated as pAT1 and pAT2, respectively. These plasmids were prepared as described in METHODS. The resultant

plasmid DNA was introduced into Ca²⁺-treated *K. aerogenes* strain MKN343-1 (*ats ty*). Transformants that showed Ats⁺, Tyn⁺ and resistance to Ap, Km, and Tc were selected on XT(N) I plates in the presence of Ap, Km, and Tc. When about 10⁷ to 10⁸ recipient cells and about 1.0 µg of recombinant DNA were used, 5 to 88 colonies of these transformants were obtained per plate. From one of these purified transformants, the recombinant plasmid pAT1 was prepared by the same procedure. The plasmid DNAs were also used to transform Ca²⁺-treated strain K629 (*ty* *recA29*), which was constructed by Pl-mediated transduction of the *recA* allele of strain MKN329. About 4 to 20 colonies of transformants with Ats⁺, Tyn⁺, Ap^r, and Tc^r markers were obtained per plate. These results show that the recombinant plasmids have both the *ats* and *ty* genes.

Observation of hybrid plasmids by electron microscopy

The plasmids prepared from *K. aerogenes* were observed by electron microscopy. Figure 2 shows a molecule of pAT1 in the open circular form. Its average molecular weight was calculated to be 80.3 × 10⁶ on determination of its length by electron microscopy

FIG. 2. Electron Micrograph of an Open Circular Molecule of pAT1 Plasmid DNA.

The bar in the frame is equal to 1 μ m.

with plasmid pBR322 as an internal standard. These results agree well with the size of the fragments formed by digestion with restriction enzymes.²⁰⁾

Expression of the ats and tyn genes in the recombinant plasmid

Expressions of the *ats* and *tyn* genes of RP4::Mu *cts-ats*⁺-*tyn*⁺ plasmids in *K. aerogenes* were investigated. *K. aerogenes* strains KS291, MT141, and MT431, which carry plasmid pAT1 or pAT2, were grown with various sulfur sources and with or without tyramine. The cells were harvested during the exponential phase of growth and assayed for arylsulfatase and tyramine oxidase. As a control, *K. aerogenes* MK9000 without plasmid, and strains MKN314 and MKN329 were cultured under the same conditions. Synthesis of arylsulfatase in strains carrying the pAT1 or pAT2 plasmid was repressed by inorganic sulfate or cysteine as a sulfur source, and this repression was derepressed by addition of tyramine, as

shown previously.⁷⁾ The levels of arylsulfatase in these strains under derepressed conditions were about 4 to 10 units per mg of cells. These levels were similar to or twice that of wild-type strain MK9000. Production of tyramine oxidase in these strains carrying pAT1 or pAT2 was induced by tyramine, and the enzyme levels were 2 to 3 units per mg of cells. These values were about twice of that of *K. aerogenes* MK9000. No arylsulfatase or tyramine oxidase activity was found in strain MKN314 or MKN329.

DISCUSSION

This paper describes an *in vivo* method for construction of recombinant plasmids containing genes from the host chromosome. This method is based on the isolation of RP4 or RP4::Mu primes containing a chromosomal fragment due to insertion of Mu DNA near the gene of interest and transposition of Mu DNAs with a segment of chromosomal DNA of variable size into RP4 or RP4::Mu and on subsequent isolation of transconjugants in a *rec* strain. The recombinant plasmid is isolated and transferred to the cells of interest.

We tested this method by cloning the arylsulfatase and tyramine oxidase genes using a *K. aerogenes* strain carrying RP4::Mu *cts* and *rec* strains, since *in vitro* cloning of intergeneric genes in most bacteria is restricted in Japan, and there is not suitable vector in these bacteria. This method depends on the chance of incorporation of the Mu genome into some neighboring gene and on the frequency of *recA*-dependent recombinational events between homologous regions of Mu DNA¹⁾ and of *recA*-independent transposition of Mu into plasmid.³⁾ Since Mu-promoted mutation occurs at high frequency, [e.g., on average, 2% of randomly isolated lysogens contain auxotrophic mutations caused by insertion of Mu DNA¹⁷⁾], we can expect that formation of Mu-promoted plasmid primes also occurs to some extent. The frequencies of *in vivo* cloning of the *ats* and *tyn* genes were approximately 5×10^{-8} cells per initial donor. These values include the

frequencies of formation of plasmid primes containing the genes and of transconjugation into the *rec*-recipient cells. Since these frequencies are lower than those in our previous report using *Mu* lysogenic recipients,⁶⁾ zygotic induction might occur when recombinant plasmids are transferred into cells without *Mu*-prophage. *K. aerogenes* strain K204-1 carrying RP4::Mu *cts* gave many more clones of genes of *ats* and *tyn* than strain K204-2 carrying RP4 in the cytoplasm and *Mu cts* on the chromosome. We found that the clones obtained from the latter strain also had *Mu cts* in the plasmid. These observations suggest that the transposition of the chromosomal genes into RP4 is also mediated by RP4::Mu, which has been formed by random insertion of *Mu* into RP4.

The molecular weight of arylsulfatase in *K. aerogenes* is about 47,000¹⁹⁾ and that of tyramine oxidase is about 100,000, as determined in preliminary experiments by gel-electrophoresis. Thus, the sum of the two genes is calculated to be about 3 kb. Since the *ats* and *tyn* genes are cotransducible (about 80 to 90%) by phages of both PW52⁷⁾ and Pl,⁸⁾ plasmids pAT1 and pAT2 probably contain these genes in the form of RP4::Mu-*ats*⁺-*tyn*⁺ or RP4::Mu-*ats*⁺-*tyn*⁺::Mu. However, we found that RP4::Mu *cts61* plasmids prepared from *E. coli* BE228 and *K. aerogenes* MK9000 have chromosomal DNA fragments of about 21 kb, whereas RP4::Mu *cts62* prepared from *K. aerogenes* MK9000 does not have any chromosomal DNA and that recombinant plasmids pAT1 and pAT2 also contain the same fragments as those of the 21 kb fragments of RP4::Mu *cts61*.²⁰⁾ These fragments may be derived from the *E. coli* chromosome because this RP4::Mu *cts61* originated from *E. coli*. It is not surprising, however, that we isolated a RP4::Mu prime having a different form of RP4::Mu because the *Mu* genome can be integrated into any site of RP4 or can transpose small DNA fragments to RP4::Mu, and further rearrangements might occur during cultivation of cells carrying RP4::Mu primes. The size of pAT1 was calculated to be about 120 kb by electron

microscopy. The values for pAT1 agree well with the length of DNA molecules estimated from *Eco*RI and *Hind*III digestions.²⁰⁾ More detailed restriction analysis and studies on the molecular basis of recombinant formation are in progress.

The levels of arylsulfatase and tyramine oxidase in *K. aerogenes* strains carrying recombinant plasmids pAT1 and pAT2 were not significantly higher than those of the wild-type strain. These results suggest that the copy number of the recombinant plasmid is the same as that of RP4, *i.e.*, one to three copies per chromosome²¹⁾ if RP4 genes are equally expressed.

Although identification of the Rec⁻ mutant in UV-sensitive strains may be difficult in some bacterial strains without a genetic transfer system, the method described here will be applicable, with some modifications, to a wide variety of genera of bacteria other than *Escherichia* and *Klebsiella*, such as *Enterobacter*, *Citrobacter*, *Salmonella*, *Proteus*, *Erwinia*, *Alcaligenes*, *Serratia*, *Agrobacterium*, *Rhizobium*, *Pseudomonas*, and *Acetobacter*, since RP4::Mu can be introduced and the *Mu* phage is expressed in these bacteria.⁶⁾

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