JOURNAL OF BACTERIOLOGY, Mar. 1988, p. 1076–1081
0021-9193/88/031076-06$02.00/0

Vol. 170, No. 3

Unmasking of Bacteriophage Mu Lipopolysaccharide Receptors in *Salmonella enteritidis* Confers Sensitivity to Mu and Permits Mu Mutagenesis

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Received 18 December 1986/Accepted 30 November 1987

The human pathogen *Salmonella enteritidis* 3b was found to be highly resistant to phage P22 and Mu derivatives. The Mu sensitivity (*musA1*) allele from *Salmonella typhimurium* could be transferred to *S. enteritidis* 3b at low frequency by cotransduction with *hisG*::Tn*10*. Sensitivity to Mu resulted in a large reduction in the number of lipopolysaccharide core-region oligosaccharides that were substituted with O-antigen polysaccharide. The residual high-molecular-weight lipopolysaccharide appeared to be a hybrid displaying O antigens which were immunologically related to those of *S. typhimurium* and not to those of *S. enteritidis*. Consequently, Mu d1(Ap *lac*) could then be transduced into Mu^s strains forming stable lysogens. On temperature induction, Mu transposition could easily be used to generate mutations in genes coding for cell surface antigens including fimbriae, lipopolysaccharide, and flagella.

Salmonella species are important enteropathogens of humans, most commonly associated with gastroenteritis (42). The pathogenesis of salmonellosis involves initial colonization of the gastrointestinal tract followed by invasion of the intestinal mucosa (38, 42). In certain strains cytotoxic enterotoxins and other cytotoxic factors appear to be produced (2), and the invading organisms may also penetrate the mucosal blood vessels to reach the bloodstream (29). The composition and structure of surface components such as lipopolysaccharide (LPS), outer membrane proteins, and appendages such as flagella and fimbriae must play important roles in pathogenesis by facilitating colonization and invasion and by allowing the pathogen to escape from or withstand host defenses (32). In the case of *Salmonella typhimurium*, flagella have certainly been implicated in virulence (4), as have the long O-polysaccharide chains of smooth LPS which protect the cell from the bactericidal activity of complement (22, 39). Fimbriae may also contribute to the pathogenesis (19).

Relatively little is known concerning the genetics of *Salmonella* virulence, and the information that is available is largely focussed on *S. typhimurium*. Many other *Salmonella* serotypes produce disease, and one serotype that consistently ranks in the top three serotypes associated with salmonellosis in humans is *Salmonella enteritidis* (11, 42). Yet virtually no information is available concerning the genetics of surface components involved in the virulence and antigenicity of this serotype. To obtain specific information concerning the virulence and antigenicity of *S. enteritidis* we have established a mutagenesis system in this organism which permits the generation of mutants at high frequency, including such useful mutations as operon and protein fusions. The wild-type strain we used was *S. enteritidis* 3b, an enterotoxin-producing strain isolated from human feces in India (11).

The mutagenesis system chosen was based on the bacteriophage Mu, since the Mu derivatives developed by Casadaban et al. (4, 5, 7, 13) have proven to be very useful genetic tools. Like wild-type Mu, they insert randomly into their host genome and induce stable polar mutations at a high frequency. Upon insertion they can create transcriptional (Mu d1 and derivatives) or translational fusions (Mu d2 derivatives) to the *lacZ* gene. Therefore, due to the facile monitoring of conferred β-galactosidase activity (12), these phage are ideal tools for studies of regulation or genetic rearrangements, such as those often seen during the expression of surface antigens (12, 23, 28, 33, 35).

Although *Escherichia coli* is commonly used to grow bacteriophage Mu, mutagenesis by Mu has also been used in other species. For instance gene fusions via Mu d1(Ap *lac*) have been isolated successfully in *S. typhimurium* even though the organism is naturally resistant to Mu (8, 15, 20, 21, 24, 30). Like *S. typhimurium*, *S. enteritidis* is highly resistant to Mu. The Mu sensitivity allele (*musA1*) has been installed in *S. typhimurium* and maps near the *rfb* cluster, but the expressed molecular basis of this mutation has not been described (10). In this paper we describe the transfer of *musA1* to *S. enteritidis* and reveal the cell surface alteration it imposes. This permitted us to use Mu to create mutations in various surface antigens and putative virulence determinants of this important human pathogen.

MATERIALS AND METHODS

Media. LB medium was described by Miller (25), and CFA agar was described by Evans et al. (9). Other media were made according to the instructions of the manufacturers (Difco, GIBCO Laboratories). The following antibiotics were used: ampicillin (50 μg/ml), tetracycline (20 μg/ml), and kanamycin (40 μg/ml). Miniswarm and swarm plates contained 1% tryptone, 0.5% NaCl, and either 0.5 or 0.35% agar. Motility buffer contained 10 mM potassium phosphate buffer (pH 7.0) and 0.1 mM potassium EDTA (27).

Genetic manipulations. The bacterial and phage strains used are described in Table 1. Preparation of phage stocks, generalized transduction with phage P22, and determination of phage titers were as described by Miller (25). Cells in the

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TABLE 1. Bacterial strains and phages

Strain or phage	Description or genotype	Source/reference
S. enteritidis 3b	Previously, strain 27655-3b	T. Wadström
122	Tn*10* derivative of *S. enteriditis*	This laboratory (11)
KX1	*hisG9424*::Tn*10*Δ4Δ11 *musA1* (by P22 transduction from MA766) Lac$^+$ Mu d1(Ap *lac*) lysogen	This paper
KX6	Lac$^-$ derivative of KX1	This paper
KX30	Kan^r (Tn*5* introduced by P22 transduction from TT3406)	This paper
KX8	*hisG9424*::Tn*10*Δ4Δ11 *musA1* (obtained by mating MA766 with KX30)	This paper
S. typhimurium		
MA766	HfrK9 *hisG9424*::Tn*10*Δ4Δ11 *musA1 thrA49*	R. Kelln (10)
KR91	*metA27 trpE2 hisF100*g *rpsL201 xyl-1 ilvA99 pgrF231 malA110 gal-851 musA1* [Mu *c*ts62, Mu d1(Ap *lac*)]	R. Kelln
KR98	*cdd-2 usp-3 udp-4 musA1 hisG9424*::Tn*10*Δ4Δ11	R. Kelln
TT3406	*pyrB655* F'ts114 *lac*$^+$ *zzf-701*::Tn*5* (A)	J. R. Roth
SU453	*hisF100 trpB2 metA22 rpsL201 xyl-1*	K. Sanderson
E. coli LA1031	F$^+$::Mu d1(Ap *lac*)/Δ(*lac pro*) *supF trp pyrF his rpsL thi*	G. Wilcox (21)
Phages		
P22	*int3* HT1214	B. Ames
SP6		K. Sanderson
MB78		K. Sanderson
Mu *c*ts62	Thermoinducible	M. M. Howe (16)
Mu d1(Ap *lac*)	Mu *c*ts62::IS*121* d(Ap *trp'* B$^+$ A' ΔW209-*lac'* ZYA) (defective *lac* transcription fusion phage)	M. Casadaban (6)

early-log phase were infected at a multiplicity of infection of 1 to 10, and phage growth was amplified either on plates (8 to 16 h at 30°C) or in liquid culture (16 to 24 h at 30°C with good aeration). Phage titers were routinely between 10^{10} and 10^{13} PFU/ml. For generalized transduction approximately 2×10^8 to 5×10^8 cells were infected at a multiplicity of infection from 0.1 to 10. After 30 min of phage adsorption at 30°C, cells were harvested by centrifugation, suspended in 1 ml of LB medium, shaken at 37°C for 30 to 90 min for phenotypic expression of the antibiotic resistance markers, and plated. Mixed lysates of Mu *c*ts62 and Mu d1(Ap *lac*) were obtained by thermal induction of *S. typhimurium* KR91. Lysate preparation and phage adsorption were as described by Casadaban and Chou (5). Mu d1(Ap *lac*) lysogens of the sensitive *S. enteritidis* 3b derivatives were induced by thermal inactivation of the temperature-sensitive Mu repressor by incubation on plates in an incubator at 42°C for 20 to 40 min followed by growth at 37°C.

We unsuccessfully attempted to introduce Mu d1(Ap *lac*) into *S. enteritidis* 3b by several methods: conjugation with *E. coli* LA1031, P22 phage transduction from *S. typhimurium* KR91, and P22 phage transduction from KX1.

Analytical methods. LPS gel profiles in whole cell lysates were determined by a modification of the procedure of Hitchcock and Brown (14).

Identification of Fim$^-$ mutants. Potential fimbriae defective (Fim$^-$) clones were transferred to sterile nitrocellulose membrane filters (0.45-μm pore size) and grown at 30°C on CFA agar. The presence or absence of fimbriae was determined by immunodot blot assay (11) with a 1:2,000 to 1:4,000 dilution of anti-*S. enteritidis* 3b fimbriae antiserum (11) and either protein A-horseradish peroxidase conjugate (Bio-Rad Laboratories) or goat anti-rabbit immunoglobulin G antibody-peroxidase conjugate (Tago, Inc. Immunodiagnostic Reagents, Burlingame) at a dilution of 1:3,000. The final staining reaction was carried out in TBS (200 mM Tris hydrochloride, 200 mM NaCl at pH 7.5) containing 200 mM NH_4Cl, 0.03% H_2O_2, 0.03% dianisidine, 0.015% 4-chloro-1-naphthol, and 6% methanol.

RESULTS

Isolation of Mu^s strains. Faelen et al. (10) have reported the isolation of Mu-sensitive (Mu^s *S. typhimurium* mutants. The mutation responsible for sensitivity to Mu (*musA1*) has also been moved to *Salmonella typhi* (37), making this serotype Mu sensitive. Several attempts on our part to isolate Mu-sensitive derivatives of *S. enteritidis* 3b were unsuccessful. However, we were able to use phage transduction to move the *musA1* allele from *S. typhimurium* to *S. enteritidis*. Phage P22 was grown on Mu-sensitive *S. typhimurium* MA766, *S. enteritidis* 3b (10^8 cells) was infected with the P22 lysate (~10^{10} PFU), and transductants were selected for Tc^r (cotransduction of *his*::*Tn*10 and *musA1* is approximately 2%). All 122 Tc^r transductants were pooled and screened for sensitivity to Mu by infection with a Mu *c*ts62-Mu d1(Ap *lac*) lysate derived from KR91. In total, four ampicillin-resistant clones were obtained, all of them Lac$^+$ (KX1 through KX4). Sensitivity to Mu was also transferred more efficiently than by P22 transduction, by conjugation of KX30 with MA766 carrying Mu^s and selection for the early transferred and closely linked *his*G::Tn*10* insertion to give KX8. These clones were confirmed as *S. enteritidis* 3b derivatives by their typical colonial morphology as well as their positive reaction with antiserum to the serospecific fimbriae of *S. enteritidis* 3b (11). The presence of the *musA1* genetic region from the parent MA766 was confirmed by the *his*::Tn*10* marker and by a positive reaction with antiserum specific to *Salmonella* serogroup B O antigen. In control assays, this antiserum agglutinated cells of *S. typhimurium* MA766 but did not agglutinate cells of wild-type *S. enteritidis* 3b, which reacted with serogroup D-specific antiserum. This procedure allowed Mu-sensitive derivatives of *S. enteritidis* 3b to be obtained without lysogeny. The efficiency of Mu infection

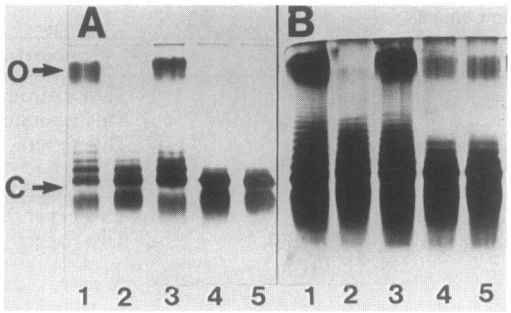

FIG. 1. LPS analysis of Mu-sensitive strains. Cells from one to three midsize colonies were solubilized by boiling for 10 min in 100 μl of 2% sodium dodecyl sulfate sample buffer. After the addition of 100 μl of proteinase K (1 mg/ml in H_2O), the solubilized cells were incubated for 4 h at 60°C; 5 μl (A) or 10 μl (B) of the digest was analyzed on either a 12 or 15% sodium dodecyl sulfate–polyacrylamide gel. After separation LPS was stained with silver (41). The arrows indicate O antigen (*O*), and core region (C). Lanes: 1, *S. typhimurium* SU453 (wild-type LPS); 2, *S. typhimurium* MA766 (Mu sensitive); 3, *S. enteritidis* 3b (wild-type LPS); 4, KX1 (Mu-sensitive derivative of *S. enteritidis* 3b, Lac^+); 5, KX6 (Mu-sensitive derivative of *S. enteritidis* 3b, Lac^-).

was unfortunately extremely low—less than 10^{-8} per PFU—and Mu [Mu dl(Ap *lac*), Mu *c*ts62] itself did not form plaques on these derivatives, presumably due to a restriction barrier or poor phage adsorption.

Characterization of Mu-sensitive salmonellae. Faelen et al. (10) mapped the *musA1* mutation very close to the complex *rfb* region, which codes for LPS O-antigen synthesis. However as their strains still appeared to be phage P22 sensitive, Faelen et al. (10) presumed that the mutation was not in the *rfb* locus. In contrast we found that P22 phage grew poorly on all Mu-sensitive *Salmonella* strains; the plaques were smaller and the titer was ~100-fold lower than in the corresponding wild-type strains. One explanation for this decreased sensitivity to phage P22 was that there was a quantitative change in the availability of P22 phage receptors. Therefore we analyzed the LPS electrophoretic gel profiles of Mu-sensitive strains and compared them with those of their corresponding parent strains (Fig. 1). The LPS gel profiles of Mu-sensitive strains differed significantly from that of wild-type cells. In Mu-sensitive strains, the extent of LPS core substitution with O antigen was markedly reduced (Fig. 1B). However, as seen in overloaded gels, O antigen was not completely missing, which explained why these strains retained sensitivity to phage P22, albeit at a reduced level.

The core oligosaccharide of LPS has previously been identified as the receptor for bacteriophage Mu (18, 31, 32). In wild-type *Salmonella* strains cells are Mu resistant, presumably because of the physical shielding of the Mu receptors by the large number of *O*-polysaccharide chains which substitute the core oligosaccharide stubs. The dramatic decrease in the number of *O*-polysaccharide chains in the mutants apparently serves to unmask the Mu receptors and explains Mu sensitivity.

Mu dl(Ap *lac*) mutagenesis. Since Mu dl(Ap *lac*) could be introduced into Mu-sensitive *S. enteritidis* 3b derivatives only with very low efficiency, another Mu dl(Ap *lac*) mutagenesis system was explored. KX1, one of the four Mu dl(Ap *lac*) transductants, was shifted to 42°C for 30 min to temporarily inactivate the Mu repressor and permit Mu transposition. These bacteria were screened for Lac^- isolates on MacConkey agar containing ampicillin. One Lac^- Ap^r Tet^r isolate was purified and chosen as the progenitor strain for further mutagenesis experiments. By using a Lac^- derivative the frequency of transposition could easily be monitored by plating for Lac^+ on MacConkey agar (Fig. 2). Induction of Mu dl(Ap *lac*) at 42°C on plates or in liquid culture resulted in only a relatively low percentage of Lac^+ clones (≤1%). The viability of the cells dropped after 40 min (liquid culture) or after 60 min (on plates) at 42°C, most probably due to the lethal effect of Mu *kil* gene expression. To obtain a reasonably high percentage of Lac^+ clones, the cells were grown on MacConkey plates at 37°C for 6 to 7 h before induction at 42°C. After that period of growth there were enough cells per microcolony to ensure at least a few survivors even after relatively long induction times (90 min). In this way virtually every colony gave several Lac^+ survivors (Fig. 2), and usually about 5 to 10% of the total cell population was Lac^+ after 30 to 90 min of induction at 42°C. Cells from 2 to 10 plates, equivalent to 5,000 to 25,000 individual Lac^+ spots, were pooled and screened for mutants.

Screening for surface antigen mutants. We chose to demonstrate that induction of Mu dl(Ap *lac*) could be used as an efficient mutagenesis system in *S. enteritidis* 3b by subsequently isolating a variety of mutants involved in the synthesis or expression of LPS, fimbriae, and flagella because these structures are important surface antigens involved in pathogenesis. Flagellar genes are also subject to antigenic phase variation involving genetic rearrangements (35). Fusion of the appropriate gene to *lacZ* is also a useful tool in studying such genetic rearrangements (12). Comparisons in mutational frequencies were made with cells in which Mu had not been induced. By plating *S. enteritidis* cells temperature induced for Mu dl(Ap *lac*) on MacConkey agar many different colony sizes and levels of *lac* expression were found. Presumably Mu had transposed downstream to various promotors, resulting in gene fusions with varied levels of β-galactosidase expression. Uncharacterized auxotrophs, which were not further studied, were obtained at frequencies of 0.5 to 1%.

We first examined the ability of the Mu mutagenesis system to provide mutants in flagellar function, as measured by the loss of motility. Among nonmotile mutants a certain percentage would be nonflagellated. Therefore the motility of cells embedded in miniswarm tryptone agar (about 500 cells suspended in motility buffer per plate) was examined

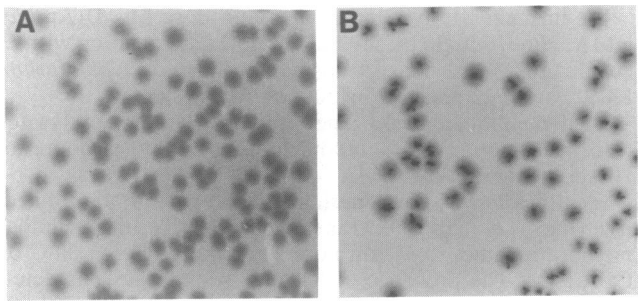

FIG. 2. Induction of Mu dl(Ap *lac*) in KX6. The Lac^- Mu dl(Ap *lac*) lysogen KX6 was plated on lactose MacConkey agar (about 2,000 cells per plate). Cells were grown at 37°C for 6 to 7 h to form microcolonies. Then the plates were transferred to 42°C for different times for phage induction. After induction the cells were incubated at 37°C for 16 to 20 h. After 20 min at 42°C (A) almost no Lac^+ colonies were visible; after 40 min (B) virtually all colonies had several red spots, which arose from individual Lac^+ cells.

after 36 to 48 h of incubation at 30°C. About 1 to 2% of the population were nonswarming or poorly swarming mutants, whereas <0.1% of control cells were motility negative. Fifty randomly picked potential mutants were reexamined on swarm plates; 46 (92%) of these were clearly defective in motility relative to their parent (KX6). Therefore the induction of Mu results in an enhancement of flagellar mutations.

We then examined the ability of the Mu mutagenesis system to provide mutations affecting LPS structure. This was measured by loss of sensitivity to phages SP6 and MB78; since LPS plays an important role in their infectivity, phage-resistant mutants likely have an altered LPS structure. After Mu induction, mutants resistant to both phages were found at a relatively high frequency, 1.5 to 2.5% for SP6 and 3 to 5% for MB78. A high background of phage-resistant mutants was also present with uninduced KX6 as a control, 0.1 to 0.25% for SP6 and 0.5 to 0.75% for MB78. This high background may well have been due to the *musA1* mutation, which affects LPS structure (Fig. 1), and/or the presence of a Mu phage in KX6. SP6-resistant mutants had different colony sizes, so mutations in different genes could contribute resistance to SP6, although it is possible that Mu-induced rearrangements or deletions of adjacent genes could also be a factor. In contrast, MB78-resistant cells all formed uniform colonies. Therefore, Mu induction resulted in an enhancement of LPS mutations.

The ability of the Mu mutagenesis system to provide fimbria production mutants was also tested. Since there is no simple direct selection for nonfimbriated mutants an enrichment technique was required. The medium in a CFA plate was divided into two separate compartments; the two compartments were then connected by a sterile strip of Whatman 3MM filter paper soaked with antiserum against *S. enteritidis* 3b fimbriae. Cells plated on one of the compartments could reach the second compartment only by crossing over the antibody-soaked filter bridge. Fimbriated cells become immobilized by the fimbria-specific antibodies, preventing their transit. Therefore, cells growing on the other side of the filter bridge were enriched for nonfimbriated mutants. After this enrichment procedure was repeated twice, 30 to 50% of the Lac^+ colonies were unable to react with fimbria-specific antibody as detected by colony blotting. Less than 0.1% of control cells enriched in the same way were fimbriae negative. Therefore, Mu induction resulted in an enhancement of fimbrial mutants.

Analysis of restricted chromosomal DNA from the fimbrial mutant clones generated here by Southern hybridization with a *lacZ*-specific mRNA probe indicated that in the various mutants Mu had actually integrated at three to four loci. Two of these loci appeared to be identical in all mutants; in the third locus we found some variation in the different mutants. Specifically, an *Eco*RI-*Pst*I fragment reacting with the probe varied from 6.0 to 6.5 kilobases, indicating that Mu integrated into a fairly narrow region, possibly in the same gene, in different mutants (unpublished results).

DISCUSSION

The *musA1* mutation (10), which makes *S. typhimurium* Mu sensitive, significantly reduced the extent of *O*-polysaccharide substitution of the LPS core oligosaccharide. This was equally true for *S. enteritidis*. Consequently P22 phage grows poorly in these strains, and the efficiency of generalized P22 transduction is reduced in Mu-sensitive strains. This mutation has been located in the *rfb* region on the *S. typhimurium* linkage map (10), and from our data we think it most likely that one of the *rfb* genes is affected by the mutation. In wild-type *S. typhimurium* and *S. enteritidis* the LPS core receptor for Mu is obviously inaccessible. By drastically reducing the extent of core substitution, the Mu receptor became sufficiently exposed and the strain became Mu sensitive. It is possible that the LPS oligosaccharide core structure serves as a receptor for Mu G^+ or G^- phage in other gram-negative bacteria, and these organisms could also be made sensitive by reducing the degree of core substitution either by mutagenesis or nutritional means. Mu phages with an easily selectable marker such as antibiotic resistance could serve as selective agents for the isolation of Mu-sensitive mutants. In specific gram-negative strains with the appropriate LPS core structure, bacteriophage Mu and its derivatives could then be used as genetic tools, allowing genetic manipulations that otherwise might not be possible.

Using Mu sensitivity we have developed a mutagenesis system which works well in a strain of *S. enteritidis* which is pathogenic for humans. Genetic manipulations of this strain, such as Tn*10* mutagenesis with P22Tc10 (11), generalized transduction with P22, or introduction of Mu dl(Ap *lac*), have been found to be hopelessly inefficient. Therefore, to overcome possible phage adsorption and restriction problems, we used a lysogenic Mu phage as mutagen. By using a Lac^- Mu dl(Ap *lac*) lysogen as the parent strain, we were able to easily monitor the efficiency of mutagenesis and enrich for transcriptional fusions to the *lacZ* gene by growing cultures on lactose-containing MacConkey plates. The isolation of Lac^- strains is facile and therefore useful. How this occurs is presumably via a spontaneous deletion or rearrangement either in the Mu phage or adjacent sequences during replicative transposition. There is still a fully functional Mu dl(Ap *lac*) phage in the new Lac^- derivative, since it could be used to generate further Lac^+operon fusions. Therefore, as long as genes of interest (in this case for fimbriation, motility, and LPS) were unaffected, any deletion or rearrangements involved in the strain construction would likely be distant and irrelevant to later transpositions. Mutations and *lacZ* gene fusions were then obtained easily by thermal induction of the Mu dl(Ap *lac*) *c*ts62 lysogenic phage. We were also able to isolate mutants at high frequency in genes potentially involved in the antigenicity and virulence of this organism. Since transcriptional gene fusions can be created by Mu dl(Ap *lac*) mutagenesis, variation in expression of these genes could be readily studied by measuring β-galactosidase activity in appropriate mutants (34).

Normally Mu mutagenesis by zygotic induction of an introduced Mu phage is preferable to induction of a lysogen. However thermoinduction of lysogens seems to be very useful in strains into which Mu can be introduced only at very low efficiency but still form Mu lysogens. The rare lysogens obtained in these cases could be used to generate mutants and gene fusions at high frequency by thermoinduction of the lysogenic Mu phage. The system developed here serves as a useful example for mutagenesis of strains into which Mu can be introduced only at very low efficiency but still allow Mu growth and lysogen formation.

However, there are features of Mu dl lysogens which limit its utility. Since Mu dl phages have a temperature-sensitive repressor, lysogens are temperature sensitive for growth, and secondary Mu-specific transpositions occur at relatively high frequency, making it difficult to study variation in the expression of genes fused to the *lacZ* gene. There are several ways to overcome this problem: for example, by the

isolation of temperature-resistant, transposition-defective mutants (3, 20) and by recombination with transposition-defective derivatives of Mu, which results in temperature-resistant lysogens (1, 17). Mu mutagenesis by induction of a lysogen normally results in mutants with more than one insertion of Mu in the bacterial chromosome. If possible the mutation should be moved to a new genetic background either by generalized transduction or by conjugation. To prevent multiple Mu insertions by zygotic induction the new host has to be Mu immune. Under the partial induction conditions used here Mu may generate DNA rearrangements (40). To our knowledge the frequency of such DNA rearrangements has never been determined accurately under experimental conditions similar to ours. However, according to the models for Mu transposition, rearrangements could occur at high frequency. Therefore it is advisable to examine certain mutants for possible DNA rearrangements. Thus, to facilitate further characterization of mutated genes we have constructed plasmid vectors that readily facilitate the isolation of *lacZ*-specific mRNA hybridization probes, cloning of *lacZ* fusions, and subsequent isolations of mRNA hybridization probes specific for the gene of interest (unpublished results).

ACKNOWLEDGMENTS

This work was supported by a Programme Grant (PG-24) to T.J.T. and W.W.K. from the Medical Research Council of Canada.

We are grateful to Martha Howe and Josiane Feutrier for valuable discussions.

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