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Mutagenesis of *Erwinia carotovora* subsp. *carotovora* with Bacteriophage Mu d1(Ap[r] *lac* cts62): Construction of *his-lac* Gene Fusions†

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The bacteriophage Mu d1(Ap[r] *lac* cts62) obtained from an *Escherichia coli* double lysogen carrying the defective Mu d1 phage and a Mu-P1 hybrid phage was utilized as a vector for phage mutagenesis in *Erwinia carotovora* subsp. *carotovora*. Among ampicillin-resistant transductants, 1.4% were auxotrophs. The synthesis of β-galactosidase was derepressed upon starvation for histidine in two different *his-lac* fusion strains.

Gene or operon fusions (2, 6) have become a powerful tool for studying gene regulation. Since β-galactosidase, which is encoded by *lacZ*, can be measured readily, the *lac* genes of *Escherichia coli* have been used extensively to obtain operon fusions. Casadaban and Cohen (5) have constructed a derivative of phage Mu, Mu d1(Ap[r] *lac* cts62), which can facilitate operon fusions in Mu-sensitive bacteria in a one-step procedure. Since this phage carries a gene encoding a β-lactamase, lysogens can be selected on medium containing ampicillin. In addition, this phage carries near one end of its genome the *lac* genes without a functional promoter. When phage DNA is inserted in the proper orientation within a bacterial host gene, the expression of β-galactosidase comes under the control of the promoter of the inactivated gene. Since phage Mu apparently can integrate randomly into the host chromosome, this procedure has been used to study the regulation of various *E. coli* operons (4, 18).

The limited host range of Mu (certain strains of *E. coli*, *Shigella dysenteriae*, *Citrobacter freundii*, and *Klebsiella pneumoniae* [10, 15, 17]) is a hindrance in the application of the one-step procedure in constructing operon fusions in other bacteria. However, a number of techniques have been devised to introduce Mu into bacteria which are not natural hosts for Mu (7, 9, 13, 16). *Erwinia carotovora* subsp. *carotovora*, one of the major agents of microbial spoilage of a wide range of vegetable crops, is normally resistant to infection by Mu (13, 14). However, the insertion of Mu into broad-host-range P-1 incompatibility group plasmids such as RP4 has facilitated the introduction of Mu into *Erwinia carotovora* and many other bacteria (13). Recently Csonka et al. (7) developed a technique for introducing Mu d1 into P1-sensitive strains of *Salmonella typhimurium*. This method is based on the fact that the host range genes of Mu, apparently deleted from Mu d1, are provided by a helper phage. Csonka et al. (7) constructed an *E. coli* double lysogen carrying the defective Mu d1 fused to plasmid R751 and a Mu-P1 hybrid helper phage that confers the P1 host range. Here we describe the introduction of Mu d1(Ap[r] *lac* cts62) into a P1-sensitive strain of *Erwinia carotovora* subsp. *carotovora*, using the Mu d1 lysate obtained from the *E. coli* double lysogen KC89 (7), and its subsequent behavior in this host.

The bacterial strains used are listed in Table 1. Among several wild-type strains of *Erwinia carotovora* subsp. *carotovora* which were tested for their sensitivity to infection by phage P1 Km *clr*100, only Brig-P1A showed sensitivity to P1 infection comparable to that of *E. coli* K-12 CU1 (unpublished data). This strain was used to select spontaneous Str[r] (resistant to 100 μg of streptomycin ml^{-1}) mutants. One of the Str[r] mutants was used to select *Erwinia carotovora* strains which were resistant to 10 μg of nalidixic acid ml^{-1}. Ten Nal[r] isolates were tested three times for pathogenicity, pectolytic activity, and growth in the presence of 100 μg streptomycin ml^{-1} and 10 μg of nalidixic acid ml^{-1}. One of the pathogenic isolates, designated AH2, was used for infection with Mu d1(Ap[r] *lac* cts62).

Lysates of Mu d1(Ap[r] *lac* cts62) with a P1 host range were obtained from *E. coli* KC89 by thermal induction (7). Titers (as determined on *E. coli* K-12 CU1) of ca. 10^9 PFU ml^{-1} were obtained. These lysates were used to infect (multiplicity of ca. 0.3 PFU per cell) *Erwinia carotovora* which had been grown overnight on L broth (LB) containing 5 mM $CaCl_2$. After 20 min at 30°C without shaking, transduction mixtures were diluted twofold with LB and incubated for an additional 30 min on a reciprocating shaker. In preliminary experiments, transductants failed to appear when expression of ampicillin resistance was not allowed to occur in liquid culture (data not shown). The transduction mixtures were centrifuged in a tabletop centrifuge and then washed three times with minimal medium to remove exogenous β-lactamase. The cells were suspended in LB, and 0.1-ml suspensions were spread on LB ampicillin (30 μg ml^{-1})-streptomycin 50 μg ml^{-1}) plates which were incubated at 30°C for ca. 30 h. Ampicillin-streptomycin-resistant colonies were replicated to minimal medium containing ampicillin and streptomycin. Colonies which failed to grow on minimal medium were considered putative auxotrophs and were further characterized as described by Davis et al. (8). In control experiments, plating of either cells or phage lysate alone on LB ampicillin-streptomycin plates did not yield any ampicillin-resistant colonies. Mu lysates from *E. coli* MAL103 (which does not contain Mu-P1) did not produce any ampicillin-resistant colonies, thus indicating that *Erwinia carotovora* is resistant to Mu phage and that transduction required the presence of Mu-P1 helper phage.

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TABLE 1. Bacterial strains

Strain[a]	Genotype or phenotype	Source
E. coli K-12		
CU-1	Wild type	H. Umbarger[b]
MAL103	F⁻ Mu d1(Ap^r *lac* cts62) Mu cts62 Δ(*gpt-lac*)XIII *rpsL*	M. Casadaban (5)
KC89	F⁺ Mu cts62 hP1#1 *araD*/ R751::Mu d1(Ap^r *lac* cts62)	L. Csonka (7)
Erwinia carotovora subsp. *carotovora*		
Brig-P1A	Wild-type pathogenic, pectolytic	M. Harrison[c]
AH2	Str^r Nal^r, pathogenic, pectolytic	See text[d]
RJ39	AH2 *his*39::Mu d1(Ap^r *lac* cts62)	See text
RJ87	AH2 *his*87::Mu d1(Ap^r *lac* cts62)	See text

[a] Bacterial strains were maintained routinely on LB agar medium (12). The minimal medium E of Vogel and Bonner (19) was used for auxotrophic analysis. Ampicillin, streptomycin, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), when needed, were added to medium to a final concentration of 30, 50, and 40 μg/ml, respectively. All liquid cultures and plates were incubated at 30°C unless indicated otherwise. Liquid cultures were grown with shaking on a reciprocating shaker.

[b] Department of Biological Sciences, Purdue University, W. Lafayette, Ind.

[c] Characterized as belonging to the subspecies *carotovora* by M. Harrison, Department of Botany and Plant Pathology, Colorado State University, Fort Collins.

[d] Isolated from Brig-PlA.

Among 5,845 ampicillin-resistant colonies, 84 (1.4%) were unable to grow on minimal medium. The failure to obtain any spontaneous ampicillin-resistant colonies and the high percentage of auxotrophs among the ampicillin-resistant transductants suggest that the mutations result from Mu insertions. The nutritional requirements of the 84 auxotrophs are shown in Table 2. A wide range of phenotypes indicates that the insertion of Mu is random and that Mu can be used as an effective mutagen in *Erwinia carotovora*.

Mu d1(Ap^r *lac* cts62) has been used for constructing *lac* operon fusions within *E. coli* and *S. typhimurium* genes (6, 7, 9, 16). Such gene fusions are likely to be extremely useful to study host-*Erwinia carotovora* interactions during the process of pathogenicity. Although the *Erwinia carotovora* AH2 strain used in this study is Lac⁺, it has no detectable β-galactosidase activity on glucose minimal medium. Any measurable β-galactosidase expression in the ampicillin-resistant transductants grown on glucose minimal medium thus reflects the fusion of Mu d1(Ap^r *lac* cts62) to functional promoters. Of the ampicillin-resistant transductants, ca. 50% displayed blue color of various intensities on minimal glucose X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) medium. The remaining 50% remained white, presumably because they either have Mu d1(Ap^r *lac* cts62) inserted in the opposite orientation or failed to insert the *lacZ* gene near a promoter capable of being expressed on minimal glucose medium.

The *his* operon in *S. typhimurium* and *E. coli* is a cluster of nine genes whose expression increases coordinately in response to histidine starvation (1, 3). The *his* operon can be derepressed in His⁻ auxotrophs by restricting the availabilty of histidine (1). This can be achieved by providing L-histidinol (a poor source of histidine) as the source for histidine (1). To determine whether *lac* genes had been fused to a functional promoter, two histidine auxotrophs (RJ39 and RJ87) which expressed β-galactosidase on X-gal indicator plates in the presence of 1 mM L-histidinol were characterized further. The remaining four His⁻ auxotrophs did not express β-galactosidase in the presence of L-histidinol. These two His⁻ auxotrophs were grown in the presence of 0.1 mM histidine or 1.0 mM histidinol, and histidinol dehydrogenase and β-galactosidase were measured during growth. Both mutants grew rapidly (doubling time of ca. 0.8 h) on medium containing histidine but grew slowly (doubling time of ca. 5.5 h) when L-histidinol was provided. In both His⁻ auxotrophs, histidinol dehydrogenase activity was induced by histidinol to a level 25- to 30-fold higher than that observed when the mutants were grown in the presence of histidine (Fig. 1). The β-galactosidase levels increased five- to sixfold during growth in the presence of L-histidinol. Thus, induction of β-galactosidase was not as great as induction of histidinol dehydrogenase.

In the absence of a genetic system in *Erwinia carotovora* subsp. *carotovora*, we cannot demonstrate that Mu d1(Ap^r *lac* cts62) actually inactivated a gene of the *his* operon. From the nutritional requirements of His⁻ auxotrophs of *S. typhimurium*, it is clear that the histidine pathway has no branch points leading to other essential metabolic routes (11). If the same biosynthetic pathway is operative in *Erwinia carotovora* subsp. *carotovora*, then all His⁻ mutants obtained in this study are likely to have resulted from insertions of Mu d1(Ap^r *lac* cts62) into the structural genes of the *his* operon. Since *lac* expression in the two His⁻ auxotrophs studied is under the control of the *his* promoter, it seems certain that *his-lac* gene fusions have been obtained in *Erwinia caroto-*

TABLE 2. Classes of Mu d1(Ap^r *lac* cts62)-induced auxotrophic mutants of *Erwinia carotovora*

Supplement required for growth	No. of mutants[a]
Threonine	3
Leucine	2
Isoleucine	6
Proline	2
Phenylalanine	1
Tryptophan	12
Tyrosine	1
Histidine	6
Methionine	3
Cysteine or methionine	9
Glycine or serine	4
Arginine plus pyrimidine	5
Isoleucine plus valine	3
Lysine	1
Glutamic acid	1
Adenine	2
Purine	4
Pyrimidine	6
Guanine	1
Pantothenate	3
Thiamine	1
Unknown	8

[a] Ampicillin-resistant transductants were selected as described in the text. Independent isolates (5,845) were scored for their ability to grow on a glucose-minimal salts medium. Mutants unable to grow on minimal medium but able to grow on LB were purified, and their requirements were determined (8).

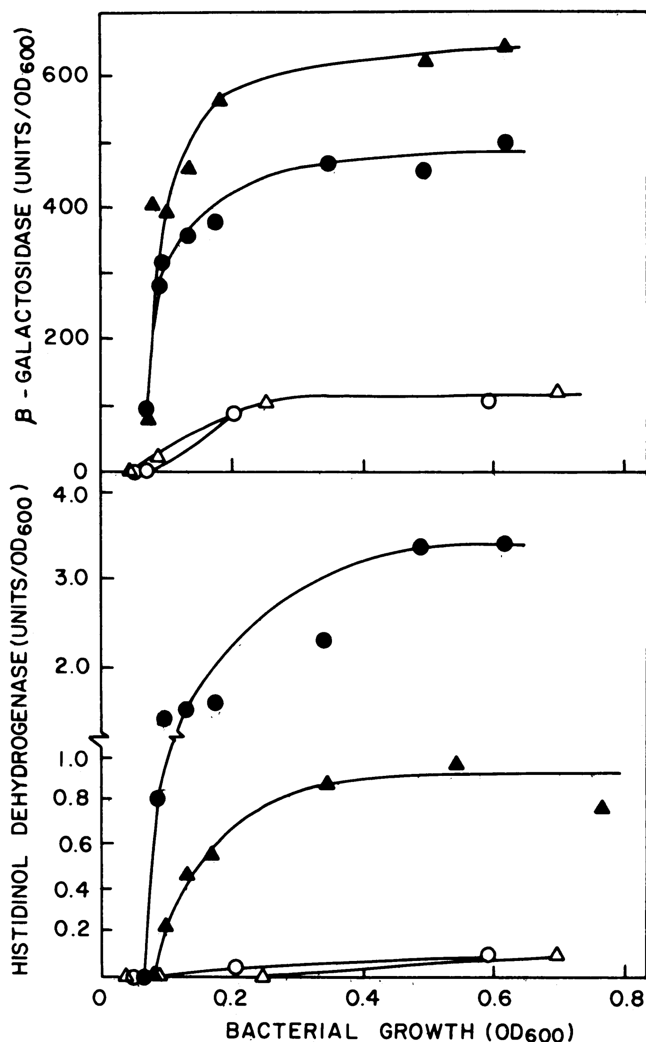

FIG. 1. Kinetics of histidinol dehydrogenase and β-galactosidase expression in *his-lac* fusion strains of *Erwinia carotovora*. Overnight LB cultures of two *his-lac* fusion strains were washed three times with minimal medium. Washed cells were suspended (optical density at 600 nm [OD_{600}], ~0.05) in fresh minimal glucose medium supplemented with 0.1 mM histidine or 1 mM histidinol. At different time intervals, portions of the cell cultures were removed to determine activities of histidinol dehydrogenase and β-galactosidase. The histidinol dehydrogenase was assayed as described by Martin et al. (11). The activity of β-galactosidase was measured as described by Miller (12). *Erwinia carotovora* subsp. *carotovora* RJ39 (△, ▲) and *Erwinia carotovora* subsp. *carotovora* RJ87 (○, ●) were grown in the presence of histidinol (closed symbols) or histidine (open symbols).

vora subsp. *carotovora* by Mu d1(Ap^r *lac* cts62) mutagenesis.

Our results show that Mu d1(Ap^r *lac* cts62) can be used as a generalized mutagen of *Erwinia carotovora* subsp. *carotovora*. In addition, the isolation of *his-lac* fusions illustrates the potential use of this technique for studying the regulation of different operons including any that may be involved in the pathogenicity of this organism.

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