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# GENETIC CHARACTERIZATION OF AN ESCHERICHIA COLI MUTANT DEFICIENT IN ORGANOPHOSPHONATE BIODEGRADATION

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An E. coli mutant deficient in organophosphonate biodegradation has been complemented with a cosmid library prepared from a BamHl partial digest of wild-type E. coli W3110. Mutant E. coli SL724, when transformed with cosmid pSL163 and plasmid pSL263, regained the ability to exploit ethylphosphonate as a sole source of phosphorus during growth. In route to complementation, the Tn5 insert of SL724 was subcloned and restriction enzyme mapped. Complementing pSL163 and pSL263 were also characterized via restriction enzyme digests. 

Organophosphonates, such as the herbicide glyphosate 1, possess the advantageous environmental property of being degraded by microbes (1,2) to inorganic phosphate. Although unable to effectively utilize glyphosate as a sole source of phosphorus during growth, Escherichia coli can degrade structurally related aminomethylphosphonates (3,4) such as 2 and alkylphosphonates (5,6) similar to ethylphosphonic acid 3. Analysis of organophosphonate biodegradation by E. coli can take advantage of the enormous reservoir of genetic information available with this organism. Such a feature becomes particularly appealing in view of the widely reported lack of carbon to phosphorus bond degrading activity in cell-free lysate. An E. coli mutant (4) resulting from Tn5 mutagenesis has been isolated which is unable to degrade either aminomethylphosphonate 2 or ethylphosphonate 3. Characterization of this mutant, SL724, is now extended for the first time to the genetic level.

## MATERIALS AND METHODS

### Bacterial Strains

E. coli K12 derivatives W3110 (lac L8Iq), SL724 (4), and DH5 (Bethesda Research Laboratory) were used in this study.

### Medium

LB and M9 medium was described by Miller (7). Ethylphosphonate was prepared according to literature procedure (6). Liquid and solid plate medium containing ethylphosphonic acid as sole phosphorus source was prepared according to literature procedure (6). The concentration of antibiotics used was as follows: a) Tc, 15mg/mL b) Ap, 50mg/mL c) Km, 50mg/mL (in LB or M9 medium) or 15mg/mL (in ethylphosphonate-containing medium) d) streptomycin, 25mg/mL.

### DNA Isolation

Chromosomal DNA was isolated from E. coli W3110 and SL724 as described by Maniatis (8). Plasmid DNA was prepared by using either the alkaline lysis

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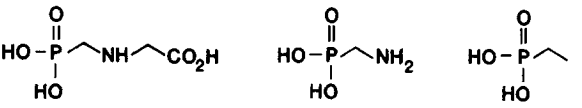

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method of Ish-Horowicz (9) or the boiling preparation method (8). All subcloning from pSL163 was done using DH5 as host. DH5 is recA1 and readily transformed by large plasmids. The endA1 mutation provides a endonuclease free background that improves the quality of plasmid DNA prepared from mini-preps. Cosmid pSL163 was isolated from SL724 and maintained in DH5 to avoid any possible recombinations of DNA and to obtain a better plasmid yield for restriction enzyme mapping.

Construction and Screening of Cosmid Library
The cosmid pLAFR3 was completely digested with BamH1 (37°C, 2hrs) and treated with calf intestinal alkaline phosphatase (Boehringer Mannheim). E. coli W3110 chromosomal DNA was partially digested with BamH1, ligated to vector DNA using T4 DNA ligase (15°C, 15hrs), and packaged into λ packaging system as described by Maniatis (8) using strains BHB2688 and BHB2690 (10). The SL724 Tc transformants were selected for the ability to utilize ethylphosphonic acid as sole phosphorus source by replica plating onto M9/Km/Tc master plates and ethylphosphonate/Km/Tc selective plates. Of the 1300 transformants, 11 colonies were found to be able to utilize ethylphosphonate as sole phosphorus source. Further attention was focused on one of the complementing cosmids, pSL163. Various subclones (see Table 1) from pSL163 were prepared by methods that have been previously described (8). Each strain derived from pSL163 was then introduced into SL724 to test for complementation.

Restriction Enzyme Mapping of the Tn5 insert of SL724
Chromosomal DNA from SL724 was completely digested with EcoR1 (37°C, 2hrs) and ligated to pUC18 vector DNA that was similarly cut with EcoR1 and treated with phosphatase. The plasmid library was transformed into competent DH5 cells and plated onto selective LB/Ap/Km plates. Restriction digests of one of the Ap/Km resistant clones revealed that it contained 2 EcoR1 fragments (4 and 14 kb) cloned into pUC18 vector. The larger EcoR1 fragment, containing the 5.8 kb Tn5 insert, was subsequently subcloned into pUC118.

Gas Analysis
Single colonies of cells able to grow on ethylphosphonate plates were inoculated into 5 ml of ethylphosphonate/Km liquid media. Each test tube was sealed with a rubber septum and shaken at 37°C for 48hr. The headspace was analyzed for ethane and ethylene by gas chromatography using a flame ionization detector and an alumina F-1 column (Alltech Associates).

## RESULTS AND DISCUSSION

As a first step, the genomic DNA surrounding the Tn5 insert of SL724 was restriction enzyme mapped (Figure 1). This afforded clues relative to the types of restriction enzymes to be used during cosmid library construction and subsequent subcloning. E. coli SL724 was digested with EcoRI due to the lack of such a cutting site in Tn5. The resulting fragments were ligated into EcoRI cut pUC118. Digestion of this plasmid with various restriction enzymes indicated an absence of BamHI and HindIII in the genomic DNA. A partial BamHI digest of wild-type E. coli W3110 was subsequently utilized for construction of a cosmid library in pLAFR3 (Figure 2). Cosmid pLAFR3 was chosen as the cloning vehicle since large segments of DNA could be introduced into the cosmid and, in turn, efficiently introduced into SL724. BamHI fragments were ligated into the BamHI site of pLAFR3 and the resulting concatamers treated with phage λ packaging extract. Infection of mutant SL724 was followed by

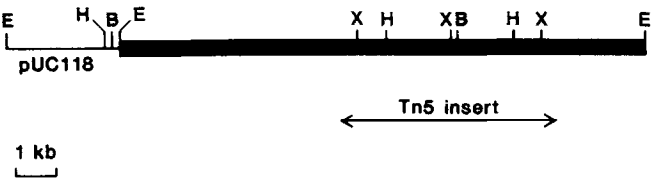


FIGURE 1. Restriction enzyme map of the Tn5 insert and surrounding genomic DNA of E. coli SL724. Enzymes included: EcoRI (E), BamHI (B), HindIII (H), and XhoI (X).

appraisal for complementation on plates where ethylphosphonate was the only source of phosphorus. Cosmid purification from one of the colonies which grew resulted in isolation of pSL163. Obtaining quantities of purified pSL163 suitable for restriction mapping was difficult. As a result, the BamHI fragment was introduced into pUC118 which made subsequent manipulations more convenient. Digestion with restriction enzymes which recognize six base pair sequences led to the restriction map of Figure 3. Curiously, the pUC118

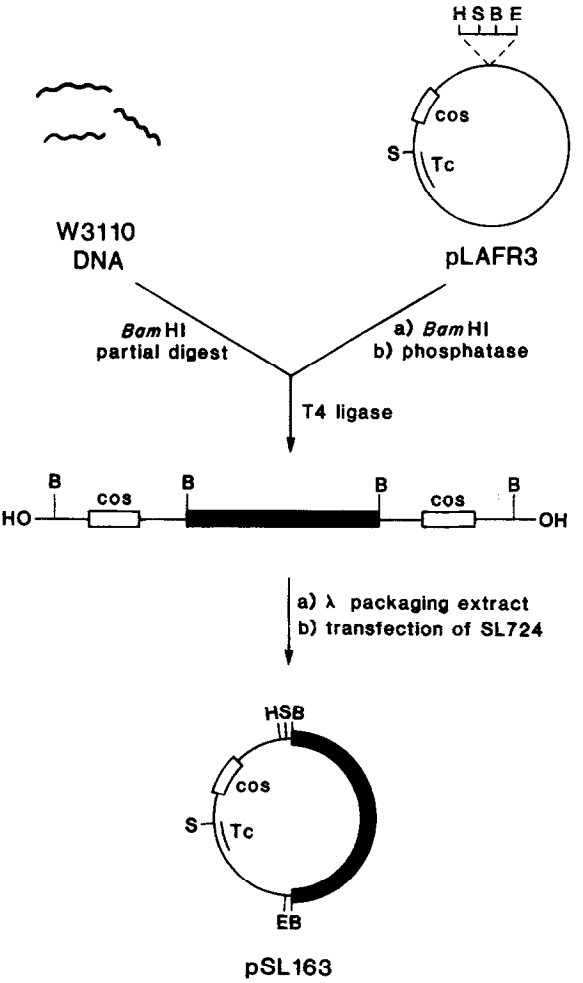


FIGURE 2. Schematic of cosmid library construction.

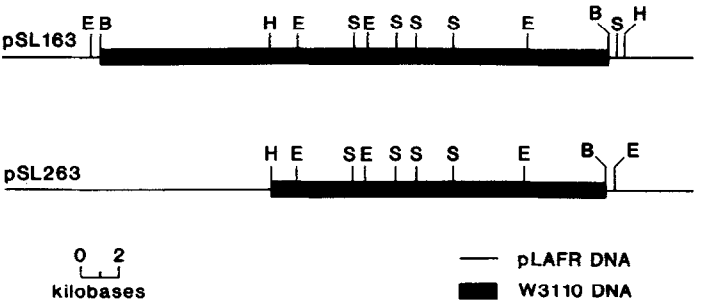


FIGURE 3. Restriction enzyme map of the B fragment of pSL163 and the HB fragment of pSL263. Enzymes included EcoRI (E), BamHI (B), HindIII (H), and SalI.

containing the BamHI fragment isolated from pSL163 was unable to complement the mutation of SL724 (Table 1).

Restriction enzymes used to map pSL163 were subsequently used to generate smaller DNA fragments suitable for subcloning. Because pLAFR3 contains a SalI site, pBR322 had to be used as the cloning vehicle for the double BamHI, SalI digests necessary for construction of pBR263 and pBR363 (Table 1). Fragments derived from pSL163 by cutting with EcoRI and BamHI, HindIII double digests were introduced into pLAFR3 to generate cosmids pSL263, pLA163, and pLA263 (Table 1). Competent E. coli SL724 cells were transformed with the various cosmids and plasmids of Table 1 and complementation determined on selective medium. Only pSL263 which contained the 18 kilobase BamHI, HindIII fragment was capable of utilizing ethylphosphonate 3 as an exclusive source of phosphorus during growth. The headspace of SL724(pSL163) and SL724(pSL263) cultured in ethylphosphonate-containing medium was also examined for degradation products. As observed with wild-type E. coli W3110 (5), both SL724(pSL163) and SL724(pSL263) produced ethane and trace quantities of ethylene.

Other E. coli mutants have been discovered which are unable to use organophosphonates as a sole source of phosphorus. Early work (3) identified an E. coli strain lacking alkaline phosphatase which could not degrade

TABLE 1. Plasmids and Cosmids Constructed During Subcloning of the BH Fragment of pSL163

| plasmid | pSL163 fragment | vector | complementation[a] |
|---|---|---|---|
| pSL263 | BH fragment (18 kb) | pLAFR3 | + |
| pUC163 | BH fragment (27 kb) | pUC118 | - |
| pBR263 | BS fragment (14 kb) | pBR322 | - |
| pLA163 | BH fragment ( 9 kb) | pLAFR3 | - |
| pLA263 | E fragment (11 kb) | pLAFR3 | - |

[a] Growth of transformed SL724 on medium where ethylphosphonic acid is the only source of phosphorus (+). Lack of detectable growth of transformed SL724 on aforementioned medium (-).

organophosphonates. In contrast, E. coli SL724 has been reported to possess active, inducible alkaline phosphatase. Uptake of radiolabeled ethylphosphonate by SL724 is indistinguishable from wild-type W3110. More recent work (11) has focused on E. coli deficient in proteins normally produced under conditions of phosphate starvation. This has lead to the discovery of mutants unable to degrade methylphosphonic acid. Such mutants may be related to SL724, although the random transposon mutagenesis used to derive SL724 could also lead to insertion of Tn5 into a gene involved in C-P bond cleavage which is not part of the phosphate regulon. Viewed in this light, E. coli SL724 constitutes a unique focus for continued genetic appraisal. The insights so derived should prove invaluable to unraveling the cellular machinery used by microbes to degrade organophosphonates.

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