IS THE ABILITY OF MARINE ISOLATES TO GROW ON M-TOLUIC ACID PLASMID-ENCODED ?

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ABSTRACT

Two marine isolates capable of growing on m-toluic acid were evaluated to determine if the ability was plasmid-encoded. Plasmid DNA of one marine isolate, XF15, was detected and successfully purified. The marine isolates were not "cured" of the ability to grow on m-toluic acid by growth in benzoate, although *Pseudomonas putida* (*arvilla*) mt-2 was cured of the TOL phenotype by growth in benzoate. Repeated attempts to recover and purify mt-2 plasmid DNA for hybridization experiments were unsuccessful. It has not been conclusively demonstrated that the TOL phenotype of the marine isolates is plasmid- or chromosomally-encoded.

INTRODUCTION

The ability of terrestrial strains of Pseudomonas to degrade toluene and related aromatics by the meta fission pathway (Figure 1) appears always to be mediated by a plasmid (3, 12, 13, 14, 15). In Pseudomonas putida (arvilla) mt-2, the strain originally studied, it was found that the enzymes of the meta pathway were coded for on a transmissible plasmid, the TOL plasmid (12, 14). Subsequently, a number of terrestrial strains able to grow on m-toluate were isolated which contained TOL plasmids (3, 13). Although the plasmids from several strains differed in a number of minor respects, restriction analysis showed that most were markedly similiar to the original TOL plasmid (3, 13).

Recently, Santoro and Hamlett (1983) isolated marine bacteria capable of degrading m-toluate. It was not determined whether this ability was plasmid-mediated. The purpose of the present study was to determine for two of these isolates whether the ability to grow on m-toluic acid is encoded by TOL plasmids. To this end it was desired to determine 1) whether the marine isolates could be "cured" of the ability to use m-toluate, and 2) whether the TOL plasmid DNA of the well-studied terrestrial strain Pseudomonas putida (arvilla) mt-2 would hybridize with chromosomal or plasmid DNA of the marine isolates.

After repeated attempts we were unable to recover and sufficiently purify the mt-2 TOL plasmid for hybridization experiments. However, a small plasmid in one marine isolate, XF15, was detected by gel electrophoresis and successfully purified. Attempts to "cure" the marine isolates of the ability to grow on m-toluic acid were unsuccessful, although the curing procedure used did cause mt-2 to lose the TOL plasmid-related phenotype. This change in phenotype was reflected

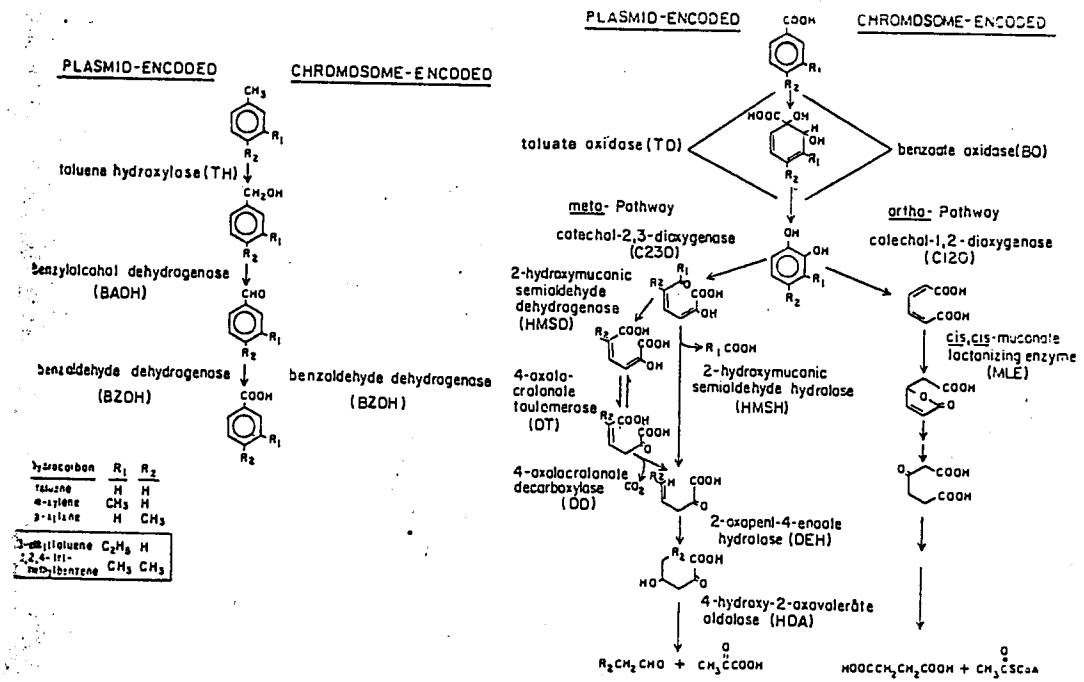

Figure 1. Enzymes of aromatic hydrocarbon and aromatic acid metabolism encoded by TOL plasmids. Toluene or benzoate may be metabolized via either the plasmid-encoded meta pathway or the chromosomally-encoded ortho pathway. Alkyl-substituted toluenes and benzoates are metabolized exclusively via the plasmid-encoded pathway. (from Kunz and Chapman 1981 (9)).

MATERIALS AND METHODS

Bacterial Strains

The two TOL plasmid-containing bacteria used in this study were Pseudomonas putida PAW and Pseudomonas putida (arvilla) mt-2. The marine isolates XF8 and XF15 were isolated from the Great Sippewissett Salt Marsh, Woods Hole, Massachusetts (11). The plots from which the organisms were isolated were experimental plots which had received applications of sewage sludge from 1974 to the present.

Media

Media were prepared from a modified Baumann and Baumann (1981) medium as described by Santoro and Hamlett (1983) (11). Medium for growth and maintenance of strains with the TOL phenotype contained 5 mM m-toluic acid. Medium for the curing experiments contained 5 mM benzoic acid. The aromatic acids went into solution easily when they were first dissolved in 20 - 40 ml distilled water with approximately 1 ml of concentrated NaOH added. Santoro and Hamlett (1983) used two percent agar for solid media (11). This was difficult to handle and boiled over in the autoclave since, prior to a 1:1 dilution with artificial salt water, the agar concentration was four percent. Reducing the final agar concentration to 1.5 percent was satisfactory and eliminated these problems. Plates used for the isolation of "cured" colonies contained 0.05 percent m-toluic acid and 0.005 percent succinic acid.

Screening of Isolates for Plasmid DNA

A rapid screen method as described by Kado and Liu (1981) was used for the extraction and isolation of plasmid DNA (7). The method employs an alkalinesodium dodecyl sulfate lysis at elevated temperatures, followed by extraction of proteins and cell debris with phenol/chloroform. Several modifications of

night to an optical density of 0.35 - 0.45 in 5 ml shake tubes of m-toluic acid medium (wild types) or benzoic acid medium (curing experiments). Approximately 10 to 20 ml of culture was pelleted and resuspended in 1.5 ml of electrophoresis buffer (E buffer) (7) and transferred to microfuge tubes for lysis and extraction. Samples were handled gently and an Adams suction device and serological pipets were used to transfer the plasmid preparations so that damage of the large mt-2 TOL plasmid did not occur.

A cold sucrose/SDS lysis procedure (6) was also evaluated as a method for plasmid extraction.

Isolation of Plasmid DNA

Plasmid DNA intended for hybridization experiments was extracted and purified according to the method of Kado and Liu (1981) (7). Several modifications of the extraction procedure were evaluated. After repeated attempts we were unable to recover the mt-2 TOL plasmid DNA from ethidium bromide/cesium chloride (EtBr/CsCl) density gradients. Recovery of the purified mt-2 plasmid DNA from preparative agarose gels was also attempted. An XF15 plasmid preparation, which was lysed at pH12.6 at 60°C for 70 minutes, was successfully purified by EtBr/CsCl density centrifugation followed by ethanol precipitation (10).

Cells for the isolation of plasmid DNA were grown in 200 ml of liquid medium in 1 liter shake flasks to an optical density of 0.35 - 0.45. Approximately 400 ml of culture was pelleted and the cells were resuspended in 20 - 30 ml of E bufferand pelleted again before continuing with the extraction procedure. In one attempt to recover the mt-2 plasmid DNA from a preparative gel, 800 ml of culture was used for the extraction procedure.

Gel Electrophoresis

Agarose gel electrophoresis was performed on horizontal apparatus (IBI). For the rapid screen of plasmids &

The preparative gels (100 ml) of 0.5 percent agarose were electrophoresed approximately 12 hr at 30 V. One or two ml samples of mt-2 plasmid preparation were loaded with 1/10th volume of bromphenol blue/xylene cylenol sample loading buffer (10). The gels were stained with 0.5 ug/ml ethidium bromide and viewed with a long wave U.V. light source.

Curing Experiments

Cells unable to utilize m-toluic acid were obtained by growth on benzoate in a manner similiar to that described by Santoro and Hamlett (1983) citing Kunz and Chapman (1981) (9,11). Shake tubes of liquid benzoate medium were initially inoculated with loopful suspensions of m-toluic acid medium-grown cultures. Loopfuls of the benzoate cultures were streaked onto agar plates containing m-toluic acid and succinic acid medium. Suspected cured colonies, ones of extremely small size relative to wild-type colonies, were picked and streaked onto both benzoate and m-toluic acid medium agar plates. Strains were considered cured when isolates were no longer able to grow on m-toluic acid.

RESULTS AND DISCUSSION

The marine isolates XF8 and XF15 were able to grow on m-toluic acid and therefore must have the enzyme capability of at least part of the meta pathway. To what extent the isolates have the entire TOL function is undetermined. It is likely that XF8 and XF15 lack parts of the meta pathway. Santoro and Hamlett (1983) demonstrated that the isolates lacked part of the upper pathway. Both isolates, for example, could grow on m-tolualdehyde but not m-xylene. Isolate XF8 could grow on methylbenzyl alcohol whereas XF15 could not (11).

Perhaps the isolates are also lacking part of the "lower

These peaks were absent in the scans of the mt-2 and PAW culture supernatants. The pigmentation could be some secondary metabolite, or could result from the build-up of an intermediate in the metabolic pathway. It could be 2-hydroxy-6-oxo-2,4-heptaienoate, a product of 3-methylcatechol conversion, which is assayed at 388 (8). The organisms could lack the ability to synthesize the 2-hydroxymuconic semialdehyde dehydrogenase and/or the 2-hydroxymuconic semialdehyde hydrolase (Figure 1).

Several systems have been described in which the chromosomally encoded ortho pathway (Figure 1) is induced by the product of its first enzymes, cis,cis-muconate(2,4,12). Perhaps a pathway, different from that of terrestrial Pseudomonas, is present in the marine isolates which relies on the induction of enzymes by an intermediate metabolite, and results in a substantial build-up of the intermediate metabolite before the enzyme is induced. When cultures of marine isolates were maintained at 30°C for a week or more, the intensity of the yellow color decreased as the turbidity of the cultures increased.

Detection of Plasmids and Curing Experiments

The rapid screening of isolates for plasmid DNA indicates that XF15 has a plasmid which is much smaller than the TOL plasmid of mt-2 and PAW (Figure 2). It is possible that this plasmid encodes for some of the enzymes of the meta pathway. The evidence in all cases studied indicates that the presence of the TOL function is correlated with the presence of the TOL plasmid (3). However, the XF15 plasmid cannot be assigned the TOL function until 1) the isolate is cured of the TOL phenotype with a corresponding loss of the plasmid, 2) the XF15 plasmid DNA is hybridized with parts of the mt-2 TOL plasmid, or 3) comparisons of restriction endonuclease digestion products are made.

If the plasmid present in XF15 does code for the TOL

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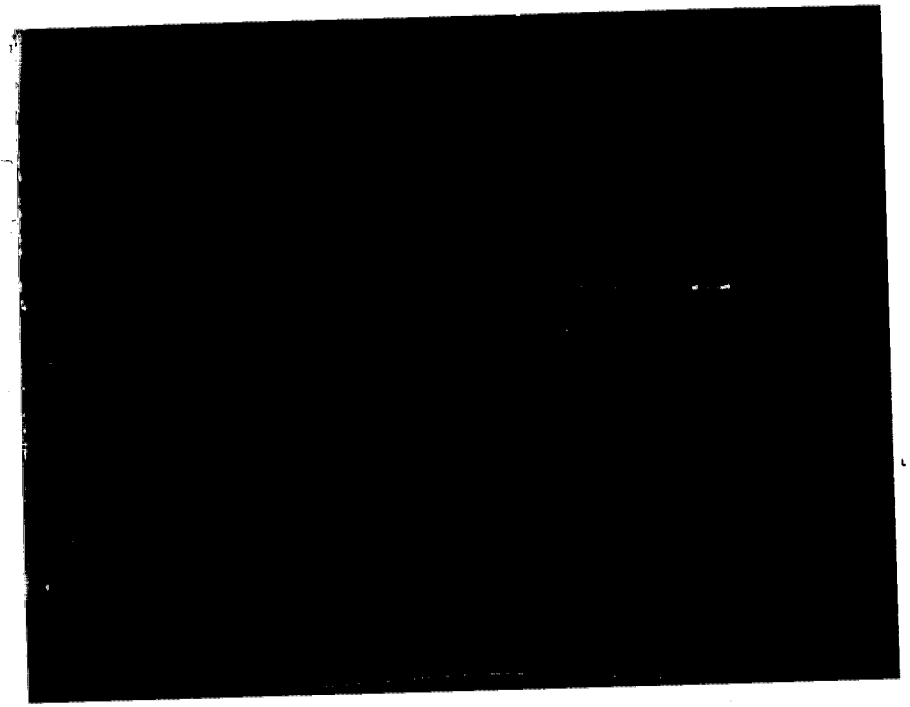

Figure 2. Agarose gel electrophoresis of plasmid DNA isolated by the procedure of Kado and Liu (1981) (7). For A - E, approximately 35 ul of sample loaded into each well. Gels electrophoresed at 80 V for 1 hr. All samples prepared according to the rapid screen method (Materials and Methods: Screening of Isolates for Plasmid DNA) except where noted. (A) mt-2; (B) "cured" mt-2; (C) PAW; (D) XF8; (E) XF15; (F,G,I) Lambda DNA standards 10, 15, and 25 ng respectively; (H) XF15 plasmid DNA after recovery from EtBr/ CsCl gradient but prior to alcohol precipitation (Materials and Methods: Isolation of Plasmid DNA), 5 ul loaded into well.

plasmid may contain only those TOL degradative genes which are like those lost from plasmids of cured strains which have undergone deletion mutations (1, 9).

Marine isolate XF8 does not appear to contain a plasmid on the basis of agarose gel electrophoresis, although the results of the rapid screen procedure cannot be considered conclusive. If there is no plasmid in XF8 coding for the TOL function, then the enzymes of the meta pathway may be chromosomally encoded. This would be a novel finding, since to date a chromosomally-encoded TOL function has not been discovered.

Attempts to cure XF8 and XF15 of the TOL-related phenotype were unsuccessful. This would substantiate the possibility that XF8 does not contain a TOL plasmid and that the TOL genes are chromosomal. Perhaps this is also the case with XF15, and the plasmid revealed by agarose gel electrophoresis has nothing to do with the TOL function.

During the curing experiments we obtained colonies surrounded by a brown halo as described by Kunz and Chapman (1981) (9). However, every time an attempt was made to isolate what appeared to be a cured gram - rod, it was still capable of growth on m-toluic acid. Colonies as described by Santoro and Hamlett (1983) (11) were produced in the curing experiments, but except for the extremely small mt-2 colonies picked from the m-toluic acid/ succinic acid agar plates, all others were able to grow on m-toluic acid.

Pseudomonas putida (arvilla) mt-2 was cured of the TOL phenotype, and this was accompanied by a decrease in the size of the TOL plasmid (Figure 2) suggesting a deletion mutation. Curing experiments with mt-2 have resulted in the loss of the entire TOL plasmid, as wellas in different deletion mutations (1, 9, 12, 13).

Attempts to cure PAW were unsuccessful. This is puzzling, since PAW and mt-2 are the same species

been reported (13).

Isolation of Plasmid DNA

A plasmid preparation of the XF15 isolate was successfully purified using the method of Kado and Liu (1981) (7) (Figure 2). Repeated attempts to obtain a substantially pure preparation of the mt-2 plasmid by the same method were unsuccessful. In large scale preparations a plasmid band was evident in agarose gel electrophoresis of preparations prior to EtBr/CsCl gradient centrifugation (Figure 2). After recovery of the putative plasmid band from the EtBr/CsCl gradient and dialysis against TE, the plasmid band was no longer present in the gels.

The TOL plasmid is very large and could be easily nicked, ending up in the chromosomal DNA plasmid band. A sample of plasmid DNA drawn through a pipetman tip was evidently sheared, as evidenced by agarose gel electrophoresis (Figure 3). However, whether the plasmid band was removed from the cesium chloride gradient with a large bore needle by traditional methods (10) or with a seriological pipet, the plasmid band still disappeared from the preparation.

The possibility that the alkaline lysis procedure was nicking the plasmid was also considered, but gentler methods did not improve the recovery of the plasmid DNA, and were less effective at reducing the amount of chromosomal DNA present in the preparations. The TOL plasmid is present in very low copy numbers. Consequently there is very much chromosomal DNA present in the cell preparations relative to the amount of plasmid DNA present. Lysis at pH 12.3, as well as for shorter time periods, resulted in preparations with far less plasmid DNA and far more chromosomal DNA than samples lysed at pH 12.6 for 70 minutes. The cold sucrose/SDS procedure (6) was even less effective at reducing the amount of chromososcmal DNA and RNA in the preparations. Duggleby and coworkers (1977) report a

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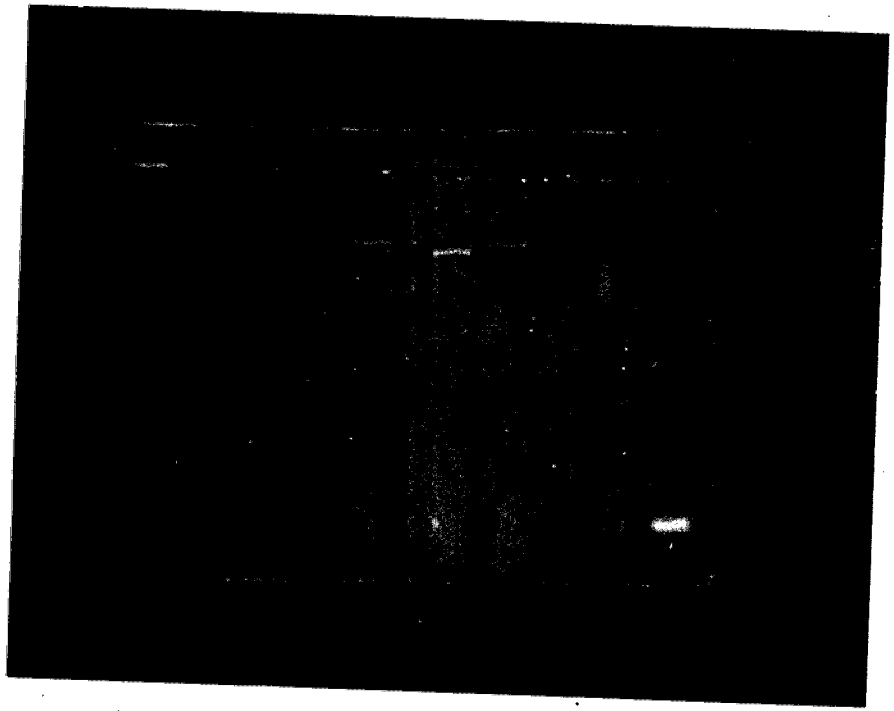

Figure 3. Agarose gel electrophoresis of plasmid DNA, as previously described (Figure 2) except where noted. Approximately 35 ul of each sample loaded into wells. (A-D) Large-scale plasmid preparations (Materials and Methods: Isolation of Plasmid DNA). (A) plasmid preparation before EtBr/CsCl density centrifugation (faint plasmid band present); (B) "sheared" sample of A (plasmid band and a band of smaller MW DNA present); (C) sample A after EtBr/CsCl density centrifugation (no band); (D) 4x concentrated plasmid preparation intended for preparatory gel (a 'v'-shaped "smear" extending down from well). (E-I) rapid screen plasmid preparations: (E) mt-2; (F) "cured" mt-2; (G) PAW; (H) XF8; (I) XF15. (J) XF15 "large-scale" preparation

preparations was not great enough. Even when the amount of culture was doubled and the quantity of reagents was halved, resulting in a 4x concentrated plasmid preparation, there was not enough plasmid DNA in the band to view under long-wave U.V. until after 6 hr of staining with EtBr.

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