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Cloning and expression of a pathway for benzene and toluene from *Bacillus stearothermophilus*

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Abstract

Bacillus stearothermophilus strain BR325 demonstrating broad aromatic substrate capability was isolated from petroleum-contaminated soil. The chromosomally-located aromatic pathway from this isolate was cloned into *Escherichia coli* as a 32 kb insert in cosmid pHC79, conferring growth on benzene, phenol, and toluene as sole carbon sources.

Introduction

The broad metabolic capability of the aerobic thermophile *Bacillus stearothermophilus* is well recognized from its important roles in elevated temperature waste treatment (Zinder 1986) and composting (Strom 1985). In view of this capability, it is of interest to examine whether this thermophile is also capable of degradation of environmental contaminants, where rapid growth rates and thermostable enzymes might prove advantageous. Although catabolism of phenol and catechol have been demonstrated by *B. stearothermophilus* isolates in our laboratory (Gurujeyalakshmi & Oriel 1989) and by others (Buswell 1975; Adams & Ribbons 1988; Dong et al. 1992), little is known regarding degradation capabilities for less-oxidized aromatics. In this report, we describe the isolation of a *B. stearothermophilus* strain with a chromosomal pathway for benzene, toluene and other aromatics and the cloning and expression of this pathway in *Escherichia coli*.

Materials and methods

Isolation

BR325 was isolated from contaminated soil near a leaking petroleum pipeline near Mount Pleasant, Michigan. Soil samples were enriched in 5mM toluate in DP medium. DP minimal medium contains per liter: 0.5 g K_2HPO_4, 1.0 g NH_4Cl, 20 mg $MgSO_4 \cdot 7\ H_2O$, 0.2 g yeast extract, 0.1 g casamino acids, and 1 ml trace elements at pH 7.2 (Buswell 1975). Following 55 °C incubation of 1 g soil in 50 ml enrichment medium, samples were periodically spread on DP-toluate plates and incubated at 55 °C. One rapidly-growing colony which showed yellow coloration upon flooding with 0.1% aqueous catechol was designated BR325 and retained for further study.

Growth and degradation experiments

Growth and degradation of nonvolatile aromatics were examined by growing the parental BR325 or its *E. coli* transformant in 250 ml flasks containing 50 ml medium. Growth and degradation studies for volatile aromatics utilized 40 ml serum bottles containing 20 ml medium sealed with Teflon-faced butyl closures. Solution concentrations of aromatics were corrected for evaporation to the headspace using the method of Huang et al. (1992). Cultures were initiated using cells previously grown on the medium under examination which were washed and resuspended in fresh medium. Turbidity was measured using a Gilford spectrophotometer. Growth on plates utilized either soluble aromatics or vapor from aromatics in a glass tube attached

Table 1. Growth of *Bacillus stearothermophilus* BR325 on various aromatic compounds.

Compound	Growth (DP + aromatic)
Benzene (vapor)	***
Toluene (vapor)	***
Phenol (5 mM)	***
Toluate (5 mM)	***
Napthalene (vapor)	*
Salicylic acid	**
m-cresol	**
p-cresol	**
o-cresol	*
no aromatic	*

*** Good growth
** Moderate growth
* Slight growth

to the plate cover. Growth was scored on the basis of colony size.

Phenol degradation was measured using the colorimetric method of Martin (1949). Catechol 2,3 dioxygenase (C23O) activity was determined visually by flooding colonies or resuspending cell pellets with 0.1% catechol and observing the yellow color formation of 2-hydroxymuconic semialdehyde. For quantitation of C23O activity, late-exponential phase culture samples of 10 ml were centrifuged, resuspended in 0.5 M K_2HPO_4 buffer, pH 7.5, sonicated, and clarified by centrifugation. Aliquots of 100 μl were assayed at 37 °C using the assay of Kojima et al. (1961) and converted to specific activity using culture turbidity. Volatile aromatics were measured using a Varian 3700 gas chromatography equipped with a DB624 microcapilliary column and flame ionization detector.

Construction and analysis of Escherichia coli recombinants

Plasmid DNA preparations from *B. stearothermophilus* and *E. coli* were prepared using the procedure of Birnboim & Doly (1979). DNA fragments were isolated after digestion with restriction enzymes followed by agarose gel electrophoresis and 'V' chamber electroelution. Genomic DNA of BR325 was isolated by the method of Saito & Miura (1963) with modifications (Natarajan & Oriel 1991). Following partial restriction digestion of BR325 genomic DNA with BamHI, the fragmented DNA was then ligated to BamHI-restricted, alkaline phosphatase-treated cosmid vector pHC79 according to the recommendations of the manufacturer (Bethesda Research Laboratories, Inc.). Following packaging with commercial extracts, *E. coli* DH5α was infected with the ligation mixture and spread on M9 plates containing 50 μg/ml ampicillin and benzene vapor supplied from a small glass tube attached to the plate covers. Southern hybridization experiments utilized the procedure of Southern (1975). The digested DNA was fragmented by 0.8% agarose gel electrophoresis, transferred onto nitrocellulose membranes (Scheicher and Schuell, Inc., Keene, NH) as described in Maniatis et al. (1982) and baked at 80 °C for 2 h in a vacuum oven. Probe DNAs were nick-translated (BRL-nick translation system) with α-^{32}P dCTP and used to probe the blots. Hybridization was performed for 18 h at 68 °C with hybridization solution (200 mg Ficoll, 200 mg BSA, 200 mg polyvinylpyrolidone, 1 ml of 10% SDS, 15 ml of 20 × SSC, 1 ml of 10 mg/ml denatured calf thymus DNA, 1.2 g Hepes buffer/100 ml) containing ^{32}P-radiolabeled probe. The blots were washed twice in 2 × SSC with 0.1% SDS at 37 °C for 20 min and twice in the same washing solution at 68 °C for 20 min. Hybridizing bands were observed after 5 to 12 h exposure at -70 °C using diagnostic film X-OMAT with an intensifying screen. The 1.6 kb *xylX* fragment used as a hybridization probe was the cloned XbaI/BglII fragment of plasmid pWW53 (Keil et al. 1985) obtained through the courtesy of H. Keil.

Results

Characteristics of the thermophile parent BR325

Following toluate enrichment, one thermophile isolate which demonstrated strong C23O expression on DP/toluate plates was repeatedly streaked on these plates and designated BR325. The rod-shaped isolate exhibited aerobic growth to 70 °C, positive Gram-stain, and sporulation. We therefore tentatively ascribe the isolate to the broadly defined thermophile species *Bacillus stearothermophilus*. The capability of BR325 for aromatic degradation was determined by comparing growth and C23O expression on DP plates in the presence and absence of the aromatic. DP medium supplies low levels of unidentified nutrients frequently required for *B. stearothermophilus* aromatic utilization (Buswell 1975), but supports only low lev-

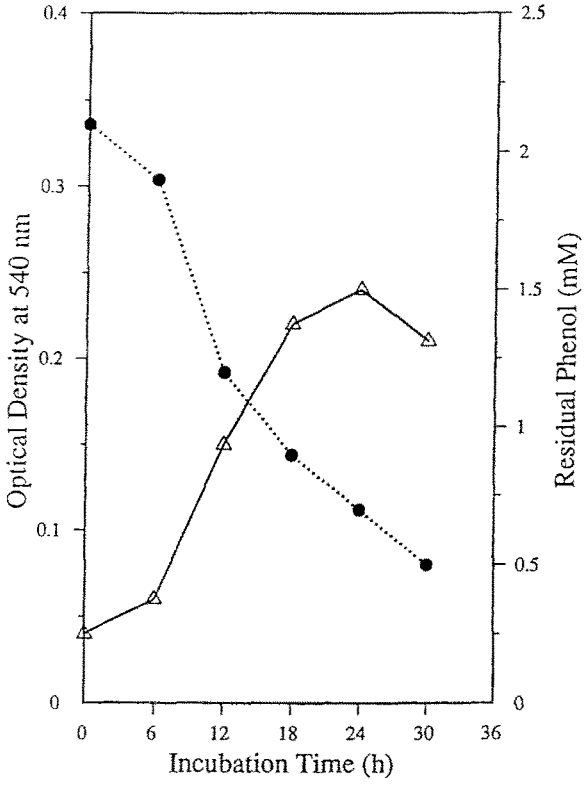

Fig. 1. Growth of *B. stearothermophilus* BR325 on M9 medium with 2mM phenol. Solid circles, residual phenol; open triangles, culture turbidity.

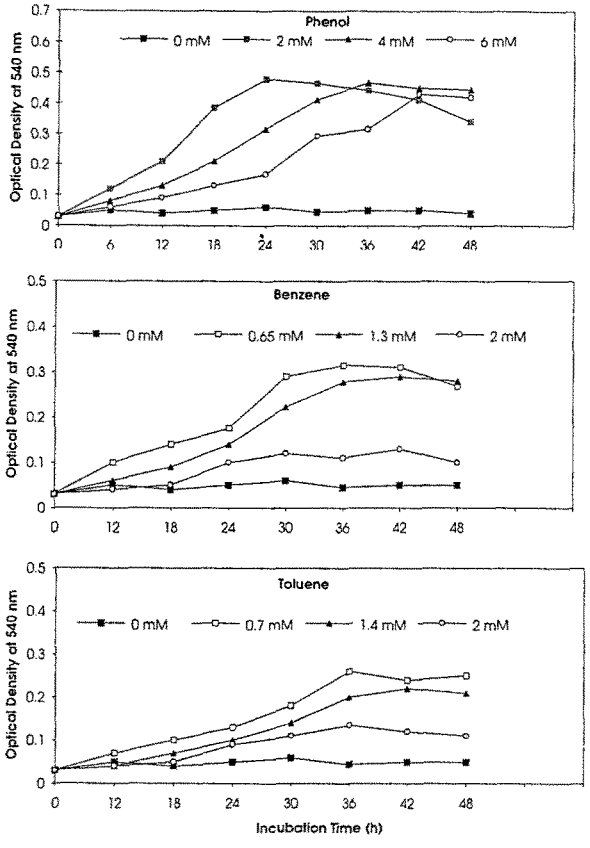

Fig. 2. Growth kinetics of *E. coli* EC365 on M9 medium supplemented with various concentrations of aromatics.

els of growth. As seen in Table 1, BR325 demonstrated enhanced growth with a variety of aromatics, with strongest growth observed for toluene and benzene vapor and soluble toluate and phenol. Expression of catechol 2,3 dioxygenase as an indicator of a *meta* pathway was observed qualitatively for BR325 during growth on these aromatics. While BR325 proved capable of growth on phenol as a sole carbon source (Fig. 1), attempts to demonstrate utilization of benzene and toluene without yeast extract supplementation were unsuccessful.

Characteristics of the E. coli Recombinant EC365

In order to examine the thermophile aromatic pathway and observe utilization of benzene and toluene without the complexities of volatility at elevated temperatures, attempts were made to clone and express the BR325 aromatic pathway in *E. coli*. Following transformation of *E. coli* DH5α with chromosomal fragments of BR325 ligated into the cosmid pHC79, a transformant capable of growth on benzene as sole carbon source was identified and designated EC365.

Transformant EC365 proved capable of growth on phenol, benzene and toluene as sole carbon sources at concentrations up to 6 mM for phenol and 2 mM for toluene and benzene, with more rapid growth at lower concentrations (Fig. 2). In M9 medium with 0.6 mM benzene or toluene, EC365 demonstrated 99% reduction of aromatic in the bottle headspace within 24 h (data not shown). Phenol utilization by EC365 during growth is shown in Fig. 3. Catechol 2,3 dioxygenase expression of EC365 was measured during growth on aromatic and nonaromatic substrates (Table 2). Although C23O activity was observed during growth on pyruvate, higher levels were observed during growth on aromatics, with 10-fold increases observed with benzene and toluene.

Examination of the pHC79 cosmid indicated an insert of approximately 32 kb with a partial restriction map shown in Fig. 4. It should be noted that the large insert size is imposed by pHC79 packaging require-

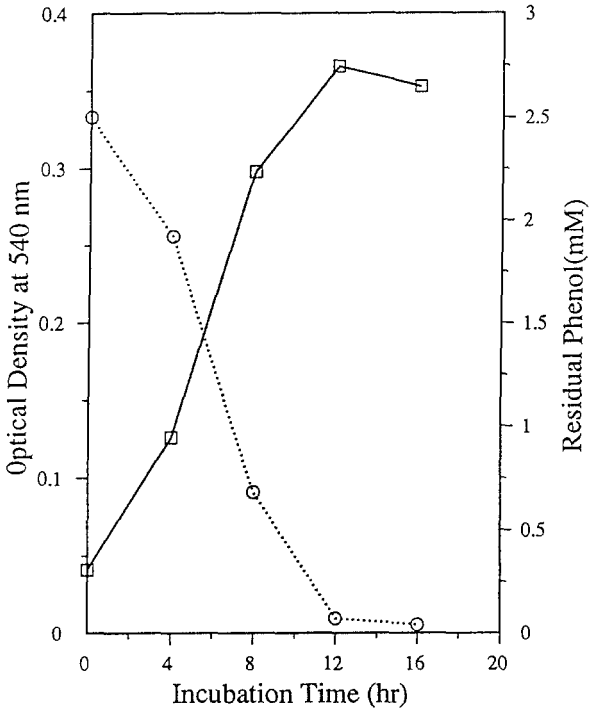

Fig. 3. Growth kinetics of *E. coli* EC365 on M9 medium with 2.5 mM phenol. Open circles, residual phenol; open squares, culture turbidity.

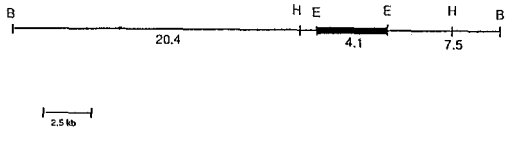

Fig. 4. Partial restriction map of EC365 cosmid insert. Marked area designates fragment hybridizing with 1.6 kb *xylX* gene.

ments, and may not reflect the minimal size of the aromatic pathway. BR325 contains a low copy number plasmid of approximately 70 kb. In order to examine the location of the aromatic pathway, hybridization of the cosmid insert as probe with chromosomal and plasmid DNA of the BR325 parent was carried out. As seen in Fig. 5, hybridization was observed with chromosomal and not plasmid DNA, indicating a chromosomal location. Preliminary hybridization studies using the *xylX* gene from the TOL plasmid indicated homology with a 4 kb EcoRI fragment of the 32 kb insert DNA of EC365 (Fig. 6).

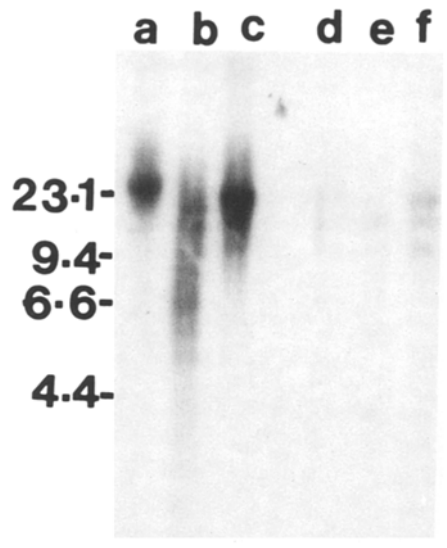

Fig. 5. Hybridization of BR325 genomic and plasmid DNA probed with the EC365 cosmid insert. Lanes a, b and c; genomic DNA restricted with BamHI, EcoRI, and SalI, respectively. Lanes d, e, and f; BR325 plasmid DNA restricted with BamHI, EcoRI, and SalI, respectively.

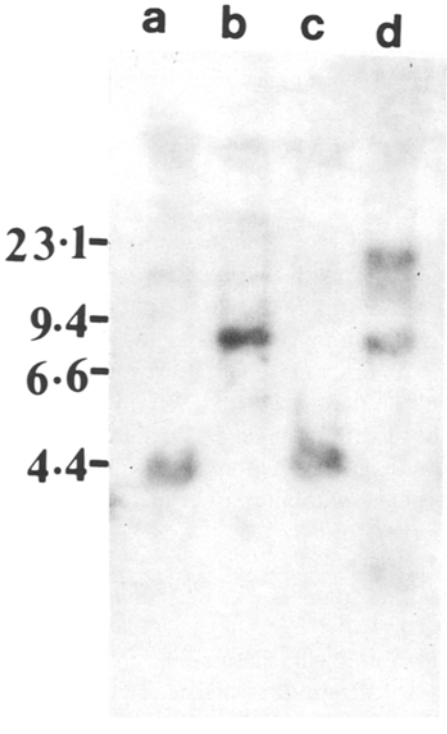

Fig. 6. Hybridization of EC365 cosmid insert restricted, fragments separated by electrophoresis, and probed with the *xylX* gene of pWW53 (lane a, EcoRI; lane b, HindIII, lane c, EcoRI and HindIII; lane d, Pst I.).

Table 2. Catechol 2,3 dioxygenase (C23O) activity of *E. coli* EC365 grown on various substrates.

Growth medium	Cell density (A 540)	C230 culture activity* (A 380)	Specific activity A 380 / A 540
M9 broth no nutrient	No growth	0	0
M9 broth + 5mM Pyruvate	0.421	0.115	0.273
M9 broth + 20 mM Pyruvate	0.922	0.276	0.299
M9 broth + 4 mM Phenol	0.396	0.892	2.25
M9 broth + 1.3 mM Benzene	0.276	0.822	2.98
M9 broth + 1 mM Toluene	0.247	0.787	3.18

* Average of triplicate samples

Discussion

The results of this study demonstrate that *B. stearothermophilus* is capable of utilizing a broad range of aromatics, similar to that documented for Gram-negative mesophiles such as *Pseudomonas putida* F1 (see Zylstra & Gibson 1991) and *Pseudomonas pickettii* PKO1 (Kukor & Olsen 1991). The preliminary indication of homology with *xylX* in this work suggests some similarities between aromatic pathway enzymes from these divergent eubacteria. Comparisons of the sequences will therefore be of interest for clues regarding gene evolution.

Although expression of *Pseudomonas* aromatic pathways has frequently proven difficult in *E. coli* (see Zylstra & Gibson 1991), good expression of *B. stearothermophilus* aromatic pathway is indicated by the rapid growth and degradation of aromatics at concentrations toxic to many mesophiles and at a temperature below the parental growth optimum. To our knowledge, this is the first cloning of a functioning toluene/benzene pathway into *E. coli*. The elevated levels of C23O in the EC365 transformant when grown on aromatics suggests that regulatory functions from the parental thermophile are encoded within the cosmid insert.

The ability of EC365 to effectively degrade aromatic pollutants suggests that utilization of thermophile aromatic pathways for bioremediation and biotransformation need not necessarily be limited to elevated temperatures or use in the thermophile parent.

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