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Recombinant expression of Toluene *o*-Xylene Monooxygenase (ToMO) from *Pseudomonas stutzeri* OX1 in the marine Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125

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Abstract

The psychrophilic bacterium *Pseudoalteromonas haloplanktis* TAC125, isolated from Antarctic seawater, was used as recipient for a biodegradative gene of the mesophilic *Pseudomonas stutzeri* OX1. *tou* cluster, coding for Toluene *o*-Xylene Monooxygenase (ToMO), was successfully cloned and expressed into a "cold expression" vector. Apparent catalytic parameters of the recombinant microorganisms on three different substrates were determined and compared with those exhibited by *Escherichia coli* recombinant cells expressing ToMO. Production of a catalytically efficient TAC/*tou* microorganism supports the possibility of developing specific degradative capabilities for the bioremediation of chemically contaminated marine environments and of industrial effluents characterised by low temperatures.

Keywords: Monooxygenases; Antarctic psychrophilic bacterium; *Pseudoalteromonas haloplanktis* TAC125; *Pseudomonas stutzeri* OX1

1. Introduction

Microbial degradation of aromatic hydrocarbons has been extensively studied with the aim of developing applications for the removal of toxic compounds from contaminated environments. Degradation of aromatic compounds by aerobic bacteria is generally divided into an upper pathway, which produces dihydroxylated aromatic intermediates by the action of monooxygenases, and a lower pathway that processes these intermediates down to molecules that enter in the citric acid cycle. Characterization of the catabolic properties of microorganisms able to grow on toluene as the sole carbon and energy sources showed that different biochemical routes for toluene mineralization evolved among *Pseudomonas* (Bertoni et al., 1996, 1998; Arenghi et

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al., 2001; Harayama and Rekik, 1989). Although many pollution problems occur in seawaters and in effluents of industrial processes which are characterised by low temperatures, considerable efforts have been directed toward the genetic manipulation of mesophilic bacteria to create or improve their ability to degrade various pollutants.

Pseudomonas stutzeri OX1, a mesophilic bacterium isolated from activated sludge (Bertoni et al., 1996), is able to grow on a wide range of aromatics, including phenols, cresols and dimethylphenols, but also on non-hydroxylated molecules such as toluene, *o*-xylene, and benzene (Bertoni et al., 1996, 1998). The pathway for the degradation of these compounds in *P. stutzeri* OX1 is chromosomally encoded. Degradation of aromatic molecules is initiated by mono- and dioxygenases which produce dihydroxylated intermediates (*upper pathway*), which are subsequently catabolized through a *meta*-cleavage pathway (Bertoni et al., 1996). Toluene *o*-Xylene Monooxygenase (ToMO) from *P. stutzeri* OX1 is endowed with a broad spectrum of substrate specificity and with the ability to hydroxylate different positions of the aromatic ring in two consecutive monooxygenation reactions (Cafaro et al., 2004). It is also able to oxidize *o*-, *m*-, and *p*-xylene, 2,3- and 3,4-dimethylphenol, toluene, cresols, benzene, naphthalene, and styrene (Bertoni et al., 1996; Cafaro et al., 2004).

The Antarctic psychrophilic bacterium *Pseudoalteromonas haloplanktis* TAC125 (*Ph*TAC125) (Birolo et al., 2000), originally isolated from a natural marine environment, is able to grow up to a very high cell density in a wide range of temperature (4–28 °C). *Ph*TAC125 is the cold-adapted bacterium so far characterized with the highest specific growth rate at temperature as low as 4 °C although its lowest observed doubling time was detected at 20 °C (31 min) (Medigue et al., 2005). Due to the above properties, *Ph*TAC125 is considered a promising novel host system for recombinant protein production at low temperatures. In fact, the development of a shuttle genetic system for the transformation of the cold-adapted Gram-negative bacterium *Ph*TAC125 (Tutino et al., 2001) made possible the isolation of psychrophilic promoters and the construction of cold expression systems (Duilio et al., 2004). In order to construct a recombinant *Ph*TAC125 able to metabolize aromatic compounds in a broad range of temperatures, *tou* cluster from *P. stutzeri* OX1, coding for ToMO, was cloned into an expression vector under the control of a constitutive psychrophilic promoter (Duilio et al., 2004) and *Ph*TAC125 cells were transformed.

The production of recombinant TAC/*tou* cells endowed with the ability of degrading phenol, *o*- and *p*-cresol at low temperatures indicates that such a strategy can be used to develop engineered psychrophilic bacteria endowed with the ability of degrading aromatic pollutants.

2. Materials and methods

2.1. Bacterial strains and microbiological techniques

The Gram-negative bacterium *P. haloplanktis* TAC125 (Birolo et al., 2000) was collected in 1992 from seawater near the French Antarctic Station Dumont d'Urville (60°40′; 40°01′E); it was grown in aerobic conditions at 4, 15 and 25 °C in minimal medium (1 g/l KH_2PO_4, 1 g/l NH_4NO_3, 10 g/l NaCl, 0.2 g/l $MgSO_4 \times 7H_2O$, 10 mg/l $FeSO_4$, 10 mg/l $CaCl_2 \times 2H_2O$) with 0.4% L-malate at pH 7, supplemented with 100 μg/ml ampicillin when transformed. *E. coli* strain S17-1(λ*pir*) was used as donor in intergeneric conjugation experiments (Tascon et al., 1993). *E. coli* cells were routinely grown in LB broth (Sambrook and Russell, 2001) containing 100 μg/ml of ampicillin if transformed. Antarctic bacterium transformation was achieved by intergeneric conjugation between the transformed *E. coli* S17-1(λ*pir*) cells (donor strain) and the Antarctic host cells (recipient strain) following the procedure previously described (Duilio et al., 2001). Mid-logarithmic-phase cells were used to inoculate flasks to a standard optical density value of 0.05–0.1, and at various times the resultant growth rates were spectrophotometrically measured at 600 nm (Cary UV lamp).

E. coli JM109 cells transformed with plasmid pBZ1260 (Bertoni et al., 1996) were grown in LB (Sambrook and Russell, 2001) containing 50 μg/ml of ampicillin at 37 °C, up to an OD_{600} of 0.5 as reported in Cafaro et al. (2004). Induction of the *lacI*^{q}-regulated *lac* promoter of pGEM-3Z-based plasmid was performed by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentra-