

Engineering of a psychrophilic bacterium for the bioremediation of aromatic compounds

Ermengilda Parrilli,^{1,2} Rosanna Papa,³ Maria Luisa Tutino^{1,2} and Giovanni Sannia^{1,*}

¹Dipartimento di Chimica Organica e Biochimica; Università di Napoli Federico II—Complesso Universitario M.S. Angelo via Cinthia 4; Naples, Italy;

²Facoltà di Scienze Biotechnologiche Università di Napoli Federico II; Naples, Italy; ³Dipartimento di Scienze di Sanità Pubblica “G. Sanarelli” Università

“La Sapienza” Piazzale Aldo Moro; Rome, Italy

Microbial degradation of aromatic hydrocarbons has been studied with the aim of developing applications for the removal of toxic compounds. Efforts have been directed toward the genetic manipulation of mesophilic bacteria to improve their ability to degrade pollutants, even though many pollution problems occur in sea waters and in effluents of industrial processes which are characterized by low temperatures. From these considerations the idea of engineering a psychrophilic microorganism for the oxidation of aromatic compounds was developed.

In a previous paper it was demonstrated that the recombinant Antarctic *Pseudoalteromonas haloplanktis* TAC125 (*PhTAC/tou*) expressing a toluene-*o*-xylene monooxygenase (ToMO) is able to convert several aromatic compounds into corresponding catechols. In our work we improved the metabolic capability of *PhTAC/tou* cells by combining action of recombinant ToMO enzyme with that of the endogenous *P. haloplanktis* TAC125 laccase-like protein. This strategy allowed conferring new and specific degradative capabilities to a bacterium isolated from an unpolluted environment; indeed engineered *PhTAC/tou* cells are able to grow on aromatic compounds as sole carbon and energy sources. Our approach demonstrates the possibility to use the engineered psychrophilic bacterium for the bioremediation of chemically contaminated marine environments and/or cold effluents.

The main objective of the work was to engineer a marine psychrophilic bacterium in order to confer it new and specific degradative capabilities for the bioremediation of chemically contaminated cold environments.

This aim was achieved constructing a recombinant strain of the psychrophilic bacterium *P. haloplanktis* TAC125,¹ with the ability to convert a wide spectrum of aromatics into the corresponding catechols and subsequently combining this recombinant enzymatic activity with the endogenous *P. haloplanktis* TAC125 laccase-like activity, which turned out to be able to attack functionalized aromatic compounds.

P. haloplanktis TAC125 was engineered² for the production of the aromatic oxidative activity encoded by toluene-*o*-xylene monooxygenase from the mesophilic bacterium *Pseudomonas sp.* OX1.³ ToMO enzyme is able to convert a broad range of aromatics, including phenol, cresols and dimethylphenols, but also non-hydroxylated molecules such as toluene and *o*-xylene into the related catechols.^{3,4} *PhTAC/tou* recombinant cells expressing ToMO resulted to be able to actively grow in the presence of phenol but cellular death was observed during the late exponential phase probably due to the increased toxicity of the medium.² Indeed catechol deriving from phenol oxidation produced by ToMO enzyme is toxic for *P. haloplanktis* TAC/*tou* and it hampers cell growth (Fig. 1A). Since, as reported by Grass et al.⁵ laccase-like enzymes are able

Key words: psychrophilic bacterium, aromatic compounds degradation, copper-inducible oxidase, toluene-*o*-xylene monooxygenase, cold environmental bioremediation

Submitted: 12/22/09

Revised: 01/20/10

Accepted: 01/27/10

Previously published online:
www.landesbioscience.com/journals/
biobugs/article/11439

*Correspondence to:
Giovanni Sannia; Email: sannia@unina.it

Addenda to: Papa R, Parrilli E, Sannia G. Engineered marine Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125: a promising microorganism for the bioremediation of aromatic compounds. J Appl Microbiol 2009; 106:49–56.

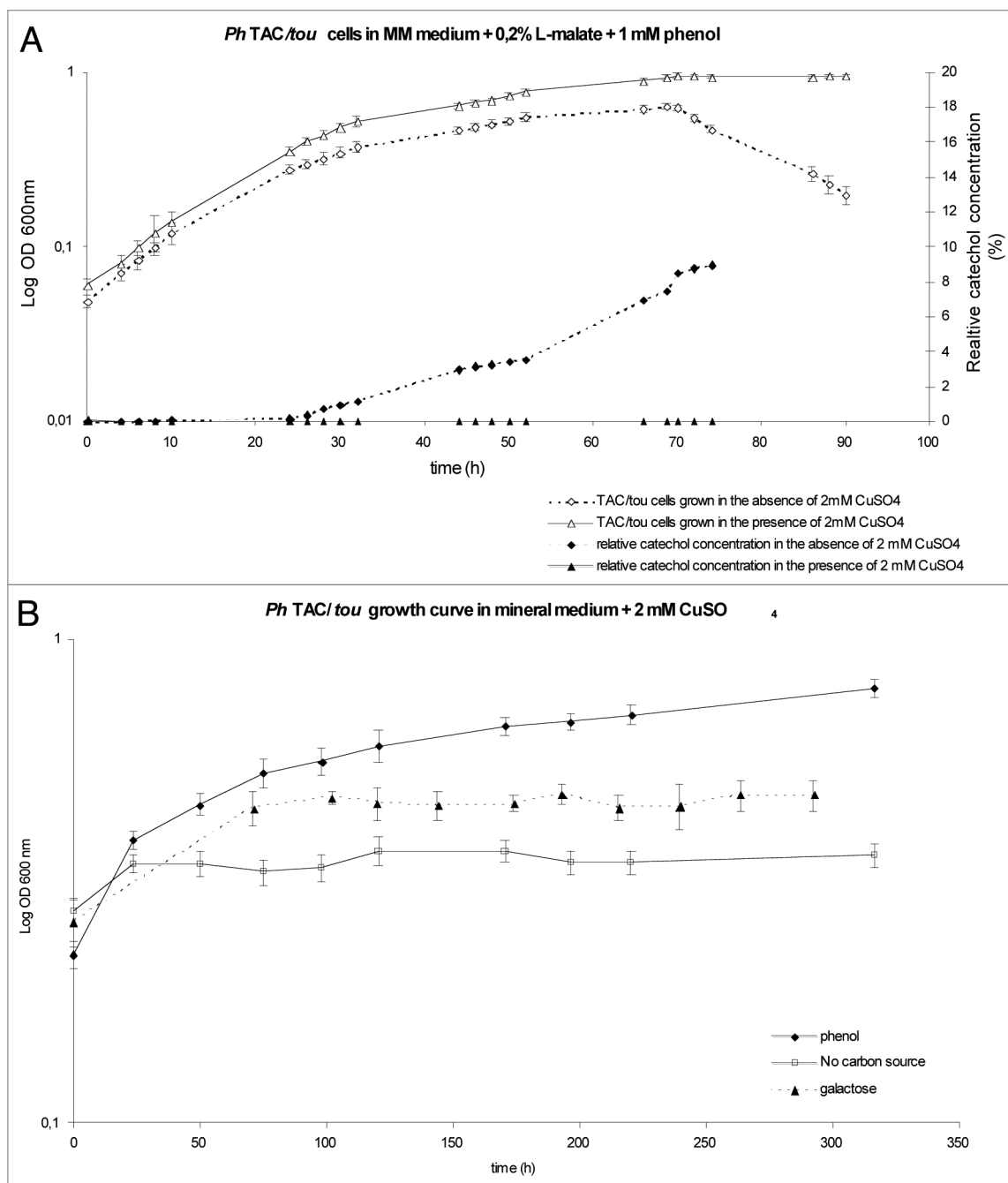


Figure 1. (A) *PhTAC/tou* cells growth curves at 15°C in mineral medium supplemented with 1 mM phenol both in the presence and in the absence of 2 mM CuSO₄. (B) *PhTAC/tou* cells growth curves at 15°C in mineral medium supplemented with 2 mM CuSO₄ in the absence of any carbon source and in the presence of phenol and galactose, respectively.

to oxidize dioxygenated aromatic compounds such as catechols,⁶ *P. haloplanktis* TAC125 genome¹ was searched for the presence of genes potentially encoding laccase. From this analysis, the attention was focused on a gene coding for a periplasmic putative laccase-like protein (*PhcopA*), which belongs to a cluster (*PhcopABCD*) encoding a multicomponent copper

homeostasis system. This system resulted to be under the control of a copper-inducible promoter. The action of the wide substrate range of ToMO was combined with laccase-like enzymatic activity growing *P. haloplanktis* TAC/*tou* cells in the presence of copper as inducer. In this condition there was no catechol accumulation (Fig. 1A) and TAC/*tou* cells were able to

grow on phenol as sole carbon and energy source. Furthermore, in this experimental condition, TAC/*tou* cells showed a faster specific growth rate and reached higher biomass than those detected on a substrate chosen as reference (Fig. 1B). This data suggests that laccase-like enzymatic conversion of catechol occurs efficiently. *PhCOP*A likely converts catechol

Table 1. Substrates tested as sole carbon source for *P. haloplanktis* TAC125 and *PhTAC/tou* liquid culture in marine salt medium in the presence and in the absence of CuSO_4 2 mM at 15°C

Substrates	<i>PhTAC125</i>	<i>PhTAC/tou</i>	<i>PhTAC125</i> + CuSO_4	<i>PhTAC/tou</i> + CuSO_4	<i>P. sp</i> OX1*
Benzene	-	-	-	+	+
Phenol	-	-	-	+	+
Toluene	-	-	-	+	+
<i>p</i> -Cresol	-	-	-	+	+
<i>o</i> -Xylene	-	-	-	+	+
<i>m</i> -Xylene	-	-	-	-	-
<i>p</i> -Xylene	-	-	-	+/-	+/-
Ethylbenzene	-	-	-	+	-
Styrene	-	-	-	-	-
3,4-dimethylphenol	-	-	-	+	+
2,3-dimethylphenol	-	-	-	+	+
Catechol	-	-	-	+	+
Naphthalene	-	-	-	+	-
Chloroform	+	+	+	+	-

*Data were obtained by Baggi et al.;⁴ Bertoni et al.⁵ Data are average results of four independent experiments. +, indicates the conditions in which cells reached final biomass higher (>10%) than that reached by the cells grown in the presence of 1.2 mM galactose after about 300 h. -, indicates the conditions in which cells reached final biomass lower (>10%) than that reached by the cells grown in the presence of 1.2 mM galactose after about 300 h. +/-, indicates the conditions in which cells reached final biomass comparable ($\pm 5\%$) with that reached by the cells grown in the presence of 1.2 mM galactose after about 300 h.

into a benzosemiquinone that may react further either enzymatically or non-enzymatically, therefore the enzymatic activity of psychrophilic laccase allows the mineralization of phenol by the *P. haloplanktis* TAC125 cells. Moreover, the paper demonstrated that the biodegradation mediated by *P. haloplanktis* TAC125 cells grown in presence of copper converts phenol into metabolites not toxic, therefore this strategy permits to overcome the substrate specificity and the formation of toxic products problems generally encountered in complex bacterial biodegradation pathways. So this system comes up to bioremediation expectation that is to transform organic pollutants into harmless metabolites or mineralize the pollutants into carbon dioxide and water.

The ability of *PhTAC/tou* engineered strain was tested and analyzed in the presence of copper, on several aromatic compounds as sole carbon and energy sources (Table 1). In particular a range of aromatics that could be hydroxylated by TOMO (Table 1;^{3,7}) was tested. Growth results were compared with those reported for *Pseudomonas sp.* OX1 (Table 1). The recombinant *PhTAC/tou* cells had acquired the capability to grow on all the tested substrates, with the exception

of *m*-xylene and styrene. These results indicate that *PhTAC/tou* cells are only not able to grow on the same substrates of *Pseudomonas sp.* OX1, but also on ethylbenzene and naphthalene that are not metabolized by *Pseudomonas sp.* OX1. This result is most likely due to the differences in substrate affinity and specificity towards catechols between the putative laccase of *P. haloplanktis* TAC125 and the catechol dioxygenase of the *Pseudomonas sp.* OX1.

Data obtained demonstrates that is feasible to introduce new and specific degradative capabilities into a bacterium isolated from an unpolluted environment (Antarctic seawater) transforming it into a bacterium able to grow on phenol as sole carbon and energy source. Above all, results presented highlight the broad potentiality of *PhTAC/tou* microorganism in bioremediation applications suggesting the use of this engineered psychrophilic bacterium in the decontamination of cold environments. However, in order to apply this strategy to solve pollution problems occurring in cold effluent, it is necessary to stably engineer *P. haloplanktis* TAC125. The first step in this stabilization will be the clean introduction of *tou* cluster expressing TOMO in

P. haloplanktis TAC125 genome using an insertional mutagenesis strategy, avoiding the introduction of any antibiotic resistance encoding gene, such technology has recently become available.⁸ The second step will be the conversion of the inducible gene *Phc* into a constitutive one, by substituting the copper inducible promoter located upstream the *Phc* cluster. This latter will avoid the addition of the copper to the medium, reducing the costs of the entire process.

Acknowledgements

This work was supported by grants of Ministero dell'Università e della Ricerca Scientifica (PRIN 2007).

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