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Fluorogenic surrogate substrates for toluene-degrading bacteria—Are they useful for activity analysis?

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Abstract

Cultivated bacterial toluene degraders use one or several of four described pathways for the aerobic degradation of this priority groundwater contaminant. To be able to identify un-cultivated toluene-degrading bacteria within enriched or natural consortia, we attempted to develop a set of staining techniques that invariably label toluene-degrading bacteria while differentiating between the different degradation pathways. In the literature, we found suggestions for pathway-specific labels of individual cells that rely on the conversion of toluene surrogates into specific colored and fluorescent products. These surrogate substrates were phenylacetylene (PA), cinnamonitrile, 3-hydroxyphenylacetylene (3-HPA), and indole. We were able to confirm that the chromogenic reactions reliably verified the pathway-specific reactions of well-characterized toluene-degrading bacterial species. However, it was most surprising to find out that three (PA, 3-HPA and cinnamonitrile) of the four supplied surrogate substrates did not lead to any product fluorescence above the cultures' autofluorescence, neither inside of cells nor in supernatants. More disturbingly, the original surrogate compound 3-HPA was inherently fluorescent and found to stain cells at intensities that depended on their states in the cell cycle. Indoxyl originating from the surrogate substrate indole was the only fluorescent product that was formed. It was detected intracellularly when the cells were sealed with *para*-formaldehyde, but its appearance was unrelated to the presence of expressed toluene degradation pathways. These findings were scrutinized by fluorescence spectroscopy, fluorescence microscopy, and flow cytometry. Activity and growth of the test bacteria were determined by analyzing chromosome numbers and membrane integrity. Our results contradict literature reports that propose the surrogate fluorogenic substrates for the identification of toluene degraders and the identification of specific pathways used by them.

Keywords: Toluene degradation; Fluorescent products; Population dynamics; Flow cytometry; Bacterial activity

1. Introduction

In microscopy, there has been longstanding interest in the development of chromo- and fluorogenic interactions and reactions capable of visualizing gene expression or metabolic activity in living cells. Whereas in living animal cells, the activity of metabolic enzymes has been followed by siRNA or FIVH (fluorescent *in vivo* hybridisation) approaches (Yanagihara et al., 2006; Ho et al., 2006; Chan et al., 2006; Bouzakri et al., 2006; Dirks et al., 2001; Shav-Tal, 2006; Jacobson et al., 1995), serious difficulties limit the application to living microorganisms (Pernthaler and Amann, 2005; Couzin, 2004; Knemeyer et al., 2003; Albertini et al., 2006). Many molecules capable of labelling intracellular targets (e.g. oligonucleotide probes) appear to be too big to pass functional microbial cell walls and membranes. A good alternative to follow metabolic activity is to apply fluorogenic substrates or substrates which become fluorescent after the action of intracellular enzymes and to follow their uptake and fate within the individual cell by using microscopic techniques like laser scanning microscopy or flow cytometry (Achilles et al., 2006; Muthian et al., 2000; Masami et al., 2003).

Since our research concentrates on the analysis of the degradation potential of harmful substances by distinct bacterial species (Kleinsteuber et al., 2006), we were interested to

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establish a quick verification method to observe live and metabolizing single bacteria within enrichment cultures or even in isolates from natural environments. A wide range of fluorescent techniques are available to distinguish living from dead cells, although sometimes these approaches fail for environmental bacteria as we recently noticed applying propidium iodide (PI). This dye is widely used to analyze dead cells, yet was found to enter living, exceptionally fast proliferating microbial cells, too (Shi et al., 2007). Fluorescent viability markers like fluorescein diacetate (FDA), 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and similar probes label unspecifically active bacteria and are therefore not suited to distinguish specific metabolic traits within bacterial communities. It appeared promising to us to use substrates that develop fluorescent products upon transformation by intracellular catabolic enzymes that are indicative of specific pathways.

Toluene-degrading bacteria play a dominant role during both aerobic and anaerobic decontamination of sites polluted with harmful contaminants like BTEX compounds (benzene, toluene, ethylbenzene, and xylene), trichloroethane (TCE) and phenol (Sun and Wood, 1996; Reardon et al., 2000; Vogt et al., 2005). According to Fishman et al. (2004) four different pathways are known for aerobic toluene degradation: the toluene dioxygenase (TDO) pathway, the xylene monooxygenase (TOL) pathway, the toluene 2-monooxygenase (T2MO, with similarity to the ToMO (Cafaro et al., 2004)) pathway and the toluene *para*-monooxygenase (TpMO) pathway (Fig. 1).

To be able to identify un-cultivated toluene-degrading bacteria within enriched or natural consortia, we attempted to develop a set of staining techniques that invariably label toluene-degrading bacteria while differentiating between the different degradation pathways. In the literature, we found suggestions for pathway-specific labels of individual cells that rely on the conversion of toluene surrogates into specific colored and fluorescent products (Keener et al., 1998, 2001; Woo et al., 2000; Kauffman et al., 2003; Clingenpeel et al., 2005). The surrogate substrates were phenylacetylene (PA), cinnamonitrile, 3-hydroxyphenylacetylene (3-HPA), 3-ethynylbenzoate (3-EB) and indole. All reactions are shown in Fig. 2.

In this paper, we provide evidence for the specificity of the chromogenic reactions brought about by distinct toluene-degrading bacterial species, chosen according to the suggestions of the abovementioned authors. However, with exception of indoxyl as the fluorescent product of the oxygenation of indole

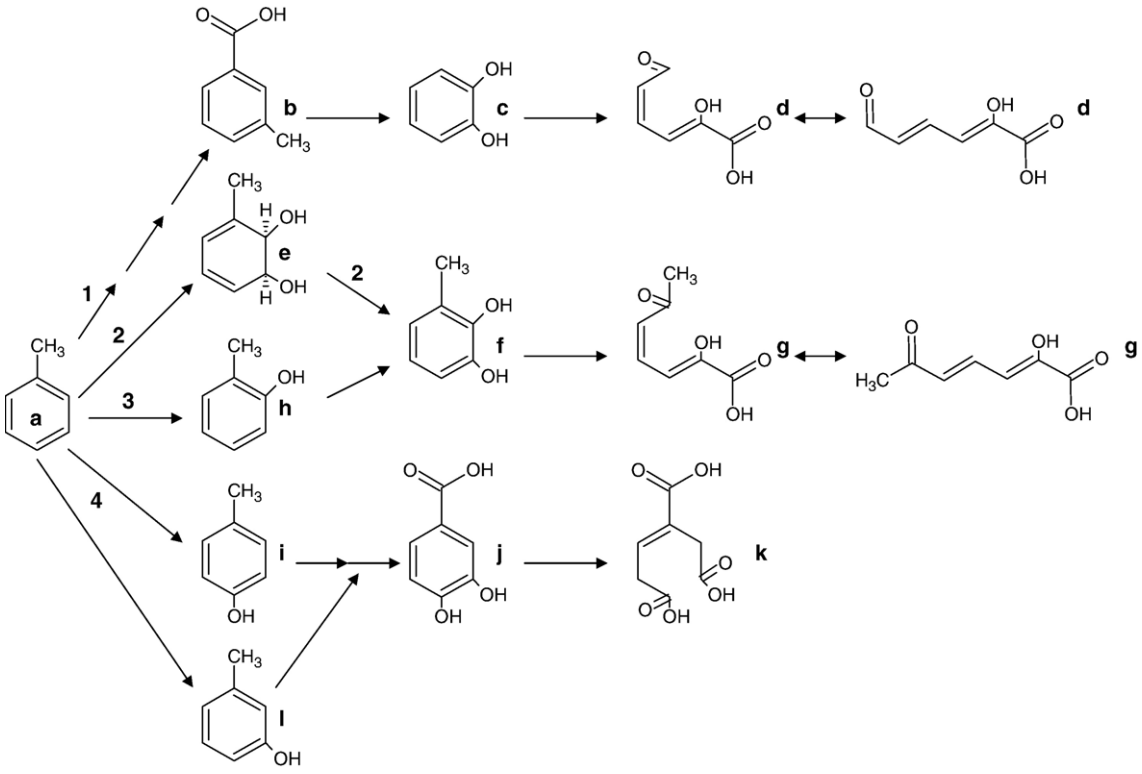

Fig. 1. Pathways of the aerobic degradation of toluene (a) (modified after Clingenpeel et al., 2005; Fishman et al., 2004). 1: Xylene monooxygenase (TOL-pathway; *P. putida* mt-2) initiates the conversion from toluene to 3-methyl-benzoate (b). In further reactions 3-methylbenzoate is transformed via catechol (c) to 2-hydroxy-6-oxohepta-2,4-dienoate (d), which is further metabolized. 2: Toluene 2,3-dioxygenase (TDO-pathway; *P. putida* F1) forms toluene-*cis*-dihydrodiol (e) and 3-methylcatechol (f), which is converted into 2-hydroxy-6-oxohepta-2,4-dienoate (g) by the 3-methylcatechol 2,3-dioxygenase. 3: Toluene 2-monooxygenase (T2MO-pathway; *B. cepacia* G4) catalyses the initial monooxygenation to *o*-cresol (h), which is first converted to 3-methylcatechol (i) and finally to 2-hydroxy-6-oxohepta-2,4-dienoate (j). 4: Toluene 4-monooxygenase (T4MO-pathway; *P. mendocina* KR1) and toluene *para*-monooxygenase (former toluene 3-monooxygenase; *R. pickettii* PK01) convert toluene to *m*-cresol (l) and *p*-cresol (i). The toluene *para*-monooxygenase produces more *m*-cresol than the toluene 4-monooxygenase although not more than 10%, whereas most of the toluene is converted to *p*-cresol by both enzymes. *m*- and *p*-cresol are further converted to protocatechuate (j) and 3-carboxy-*cis*,*cis*-muconate (k).