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Nitrogen regulation of the xyl genes of Pseudomonas putida mt-2 propagates into a significant effect of nitrate on m-xylene mineralization in soil

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Summary
The nitrogen species available in the growth medium are key factors determining expression of xyl genes for biodegradation of aromatic compounds by Pseudomonas putida. Nitrogen compounds are frequently amended to promote degradation at polluted sites, but it remains unknown how regulation observed in the test tube is propagated into actual catabolism of, e.g. m-xylene in soil, the natural habitat of this bacterium. To address this issue, we have developed a test-tube-to-soil model system that exposes the end-effects of remediation practices influencing gene expression of P. putida mt-2. We found that NO3− compared with NH4+ had a stimulating effect on xyl gene expression in pure culture as well as in soil, and that this stimulation was translated into increased m-xylene mineralization in soil. Furthermore, expression analysis of the nitrogen-regulated genes amtB and gdhA allowed us to monitor nitrogen sensing status in both experimental systems.

Hence, for nitrogen sources, regulatory patterns that emerge in soil reflect those observed in liquid cultures. The current study shows how distinct regulatory traits can lead to discrete environmental consequences; and it underpins that attempts to improve bioremediation by nitrogen amendment should integrate knowledge on their effects on growth and on catabolic gene regulation under natural conditions.

Introduction
Approaches used in environmental biotechnology to bring about pollutant degradation, i.e. bioremediation, rely on the activities of catabolic microorganisms in complex environments. In their natural habitat, these microorganisms encounter numerous exogenous factors that can affect their growth and general metabolic activity, and even their ability to express specific genes involved in pollutant degradation (de Lorenzo, 2008). Hence, the impact of environmental factors on catabolic microorganisms is of paramount significance for the optimal exploitation of specific degrader microorganisms in bioaugmentation, where microorganisms selected for catabolic performance under laboratory conditions are brought back into their natural habitat.

However, how these properties are regulated by microorganism in the environment is at present not clear (Meckenstock et al., 2015).

Pseudomonas putida mt-2, which carries the catabolic TOL plasmid, pWW0, enabling m-xylene and toluene degradation is a safe and well-studied paradigm organism for applications in bioaugmentation (de Lorenzo et al., 2013). The catabolic xyl genes on pWW0, which are involved in m-xylene and toluene degradation, are organized in the upper and lower/meta TOL pathway operons (Fig. 1). The significance of environmental factors for xyl gene expression has been intensively studied in pure culture model systems (Velázquez et al., 2006; Del Castillo and Ramos, 2007). Some of these studies have revealed that nitrogen sources, e.g. NH4+ and NO3−, influence xyl gene expression in mt-2 (Velázquez et al., 2006; Huang et al., 2008). Expression of xyl genes of the upper TOL pathway is regulated by the sigma factor, σ54, which was initially identified as a
Nitrogen regulates xyl gene expression in soil

Fig. 1. Degradation of m-xylene and organization of the xyl structural and regulatory genes of the TOL pathway on pWW0. Enzymes of the upper pathway catalyse the sequential oxidation of one methyl group of m-xylene resulting in 3-methyl benzoate (3MB), which is further converted to tricarboxylic acid cycle intermediates by enzymes encoded in the meta pathway operon. Six promoters are involved in the expression of the two catabolic gene clusters. Transcription of the upper pathway is initiated from the $\sigma^{54}$-dependent promoter $Pu$, while the meta pathway is transcribed from $Pm$ that requires either $\sigma^{32}$ ($\sigma^\alpha$) or $\sigma^{58}$ ($\sigma^\beta$). Furthermore, two regulatory proteins, XylR and XylS, are involved in the tightly controlled expression of the entire TOL pathway that additionally requires the presence of specific effector molecules, m-xylene and 3MB, and the chromosomal-encoded HU and IHF proteins. The master regulator XylR, encoded by the xylR gene, is transcribed from two $\sigma^{70}$-dependent tandem promoters, and is involved in activation of $Pu$ and the $\sigma^{54}$-dependent $Ps1$ promoter of xylS, encoding the meta pathway regulator. XylS. In addition, xylS is constitutively expressed from the $\sigma^{38}$-dependent promoter $Ps2$. The two catabolic genes employed as proxies of TOL pathway expression in this study, xylM and xylE from the upper and meta pathway, respectively, are shown in bold.

Nitrogen specific sigma factor (Ramos et al., 1997; Cases et al., 2003). Hence, this global regulator might modulate substrate-specific induction of the TOL pathway in response to altered environmental conditions caused by the nitrogen supplements (Commichau et al., 2006; Hervás et al., 2009).

Nitrogen is an important limiting element for bacterial growth in many environments, in particular at hydrocarbon contaminated sites (van Veen et al., 1997; Jensen and Nybroe, 1999; Walecka-Hutchison and Walworth, 2006), and several studies on bioremediation of hydrocarbon contaminated soils and sediments have shown the significance of inorganic nitrogen amendments for in situ pollutant biodegradation (Lindstrom et al., 1991; Davis and Madsen, 1996; Aislabie et al., 2012). The chosen source of nitrogen to add in bioremediation studies is $NH_4^+$ that can be taken up and assimilated directly by the bacterial degraders (Walecka-Hutchison and Walworth, 2006; Komilis et al., 2010). Hence, it becomes of interest whether the different effects of $NH_4^+$ and $NO_3^-$ on catabolic gene expression by $P. putida$ mt-2, which have been revealed by well-controlled pure culture conditions, where typically one parameter was changed at a time (Velázquez et al., 2006; Hervás et al., 2008) manifest also in a complex environmental context (de Lorenzo, 2008). An additional, unsettled issue is how tightly changes in xyl gene expression are coupled to the functional outcome of the pathway, i.e. changes in m-xylene biodegradation in a soil system.

The objective of current study was to address whether the test tube effects of nitrogen on catabolic gene expression by $P. putida$ mt-2 propagate into an actual environmental scenario. To this end we established an experimental model system allowing us to compare the effect of specific nitrogen sources ($NH_4^+$ vs. $NO_3^-$) on the transcriptional dynamics of $P. putida$ mt-2 catabolic xyl genes in pure culture and after introduction to natural soil with different nitrogen regimes. In either systems, we focused on expression of xylM and xylE of the upper and lower TOL pathway, respectively, as the enzyme products of these two genes are regarded as key enzymes in the biodegradation of toluene and m-xylene (Hendrickx et al., 2006; Martínez-Lavanchy et al., 2010). In addition, sensing of the nitrogen status by $P. putida$ mt-2 cells was compared for pure culture and soil systems through expression of the amtB and gdhA genes signalling $NH_4^+$ deficiency and surplus respectively (Hervás et al., 2008). The system further allowed us to examine whether changes in gene expression in soil were translated into actual variations in biodegradation performance.

Results

Nitrogen source impact on expression of xylM and xylE in pure culture

Initially, we performed a detailed analysis of the temporal dynamics of xylM and xylE expression for exponentially growing $P. putida$ mt-2 incubated with $m$-xylene and either $NH_4^+$ or $NO_3^-$ as nitrogen source. $NH_4^+$ had an initial stimulating effect on xylM expression compared with $NO_3^-$ ($P = 0.006$) within the first hour after shifting the nitrogen source (Fig. 2). However after 3–5 h, the expression pattern shifted, and while xylM expression in
NH₄⁺ medium decreased, a delayed expression peak in NO₃⁻ medium was observed. Hence after 5 h, expression of xylM in NO₃⁻ medium was significantly higher than expression in NH₄⁺ medium ($P = 0.027$). In contrast, we observed no difference in xyle expression between the two treatments until after 3 h (Fig. 2). At this time, NO₃⁻-stimulated xyle expression compared with NH₄⁺, and after 5 h the difference to expression in NH₄⁺ medium was statistically significant ($P = 0.015$). Cultures in NH₄⁺ medium as well as cultures in NO₃⁻ medium reached stationary phase after 1–3 h according to qPCR quantification of xylM and xyle copy numbers (data not shown). Hence, the differences in expression levels were not related to differences in growth phase between cultures.

Expression of the nitrogen-regulated genes amtB and gdhA, encoding a high-affinity NH₄⁺ transporter expressed during NH₄⁺ limitation, and glutamate dehydrogenase, reported to be highly expressed in the presence of NH₄⁺, respectively (Hervás et al., 2008), was measured to determine whether the two nitrogen sources were perceived differentially by the cells (Fig. 3). In cultures incubated with NO₃⁻, amtB expression was initially more than 100-fold higher than in cultures incubated with NH₄⁺ as nitrogen source. The amtB expression then declined but remained significantly higher than for cells incubated in NH₄⁺ medium for at least 7 h (Fig. 3, insert). In contrast, gdhA expression initially peaked for cells shifted from a spent to a fresh NH₄⁺ medium, but remained low for cells grown with NO₃⁻. The expression of gdhA hereafter decreased to a low and steady level that remained highest ($P < 0.05$) for cells cultured with NH₄⁺ throughout the entire sampling period.

In conclusion, the nitrogen sources impact xyl gene expression in this pure culture system with NO₃⁻ having

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**Fig. 2.** Transcriptional dynamics of xylM and xyle by *P. putida* mt-2 incubated in the presence of *m*-xylene vapours in M9 medium with either 10 mM NH₄⁺ (green) or 10 mM NO₃⁻ (red) as sole N-source. Samples were withdrawn between 15 min and 7 h after the shift in N-source. Data are mean values of mRNA normalized to DNA copies of the corresponding genes from triplicate cultures, and error bars represent standard error of mean.

**Fig. 3.** Transcriptional dynamics of the N-regulated genes gdhA and amtB by *P. putida* mt-2 incubated in the presence of *m*-xylene vapours in M9 medium with either 10 mM NH₄⁺ (green) or 10 mM NO₃⁻ (red) as sole N-source. Insert in the right panel shows expression of amtB after 2–7 h on the same scale as expression of gdhA. Data are mean values of mRNA normalized to DNA copies of the corresponding genes from triplicate cultures, and error bars represent standard error of mean.
a delayed, but overall stimulating effect compared with NH$_4$$^+$+. Furthermore, the expression of $amtB$ and $gdhA$ provided information on the NH$_4$$^+$ availability to $P$. putida mt-2, and we consequently transferred this monitoring system for use in subsequent soil experiments.

Response of $P$. putida mt-2 to changing nitrogen-conditions in soil microcosm

A soil microcosm was established in which nitrogen limitation was brought about by pre-incubation with ground barley straw (Jensen and Nybroe, 1999) so that the influence of added nitrogen sources could be addressed with minimal interference from indigenous nitrogen pools. After incubation with straw for 7 days, the soil contained ~0.1 mmol kg$^{-1}$ soil of water soluble NO$_3^-$ and NH$_4$$^+$ as determined by chemical analyses. This soil is referred to as N-limited soil hereafter.

$P$. putida mt-2 was then introduced to the N-limited soil and exposed to $m$-xylene. Nitrogen in the form of NaNO$_3$ or NH$_4$Cl was added to reach a concentration of 10 mmol kg$^{-1}$ soil respectively. The pH of the NH$_4$$^+$-amended soil (pH 5.8) decreased slightly to below the pH of the NO$_3^-$-amended soil (pH 6.5) at the end of the 46-h incubation period.

Addition of NO$_3^-$ to the N-limited soil gave rise to an increase in expression of $amtB$ compared with that seen for the N-limited soil supplemented with NH$_4$$^+$ (Fig. 4). Expression peaked at 8–10 h incubation, i.e. later than in liquid culture (Fig. 3), and after 12 h, $amtB$ expression was again downregulated; nonetheless, the expression lasted longer than in liquid culture. In contrast, $gdhA$ expression was strongly upregulated in the N-limited soil receiving NH$_4$$^+$, but only increased slightly from the background level in NO$_3^-$-amended soil (Fig. 4). Downregulation of $gdhA$ in NH$_4$$^+$-amended soil also appeared, but the downregulation occurred slightly later than the downregulation of $amtB$ in NO$_3^-$-amended soil. The levels of $amtB$ and $gdhA$ expression were comparable, which contrast the ~20-fold higher expression of $amtB$ than $gdhA$ in pure culture (compare Figs 3 and 4).

In conclusion, sources of inorganic nitrogen in the soil directly affect expression of the selected indicator genes involved in nitrogen uptake and transformations. Although the expression patterns were not identical to those recorded for $P$. putida mt-2 in liquid culture, they reveal that soil amended with NO$_3^-$, unlike soil amended with NH$_4$$^+$, is perceived as being NH$_4$$^+$ deficient by the introduced cells.

Nitrogen source impact on expression of $xylM$ and $xylE$ and on $m$-xylene mineralization in soil

In soils receiving NO$_3^-$, expression of $xylM$ and $xylE$ peaked after 10 h of incubation, while the expression peaks in NH$_4$$^+$-amended soils appeared slightly delayed after 12 h (Fig. 5). The NO$_3^-$ amendment resulted in higher expression of both $xyl$ genes in the ascending part of the expression curves. For $xylM$ the expression was significantly ($P = 0.044$) higher (approximately two-fold) in NO$_3^-$-amended soil when comparing the peak after 10 h with the peak after 12 h in the NH$_4$$^+$-amended counterpart. For $xylE$, there was nevertheless only a tendency towards a higher expression peak in the NO$_3^-$-amended soil ($P = 0.30$).

Compared with observations made for pure cultures, the initial stimulatory effect of NH$_4$$^+$ on $xylM$ expression was not seen in soil, and there was a delay in the stimulatory effect on $xylM$ and $xylE$ expression exerted by NO$_3^-$ (compare Figs 3 and 5). Importantly, the higher $xyl$ gene expression in NO$_3^-$-amended soils was reflected in a significantly ($P = 0.047$) higher mineralization of $m$-xylene after 21 h and onwards when compared with soils amended with NH$_4$$^+$ (Fig. 5). In N-limited soil

Fig. 4. Dynamics of expression of the N-regulated genes $gdhA$ and $amtB$ by $P$. putida mt-2 inoculated into N-limited soil amended with 10 mmol kg$^{-1}$ soil NH$_4$$^+$ or NO$_3^-$ . Data are mean values of mRNA normalized to DNA copies of the corresponding genes from triplicate soil setups, and error bars represent standard error of mean.

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that did not receive NO$_3$\textsuperscript{-}/C$_0$ or NH$_4$\textsuperscript{+}, the mineralization did not differ from mineralization in NH$_4$\textsuperscript{+}-amended microcosms (data not shown).

In the current experiment, _P. putida_ mt-2 was introduced to a natural soil that may contain indigenous xylene mineralizing populations carrying xyl genes. To determine the extent to which these populations contributed to our expression and mineralization analyses, we showed that the soil contained less than 10$^3$ copies g$^{-1}$ soil of xyl genes. Finally, mineralization analysis in soil microcosm without inoculated mt-2 cells revealed that mineralization by indigenous m-xylene-degrading soil bacteria accounted for a minor part (~10%) of $^{14}$C-CO$_2$ build up (data not shown). Consequently, m-xylene mineralization could be ascribed to the inoculated _P. putida_ mt-2 cells.

In conclusion, xyl gene expression by _P. putida_ mt-2 in soil was stimulated more by NO$_3$\textsuperscript{-} than by NH$_4$\textsuperscript{+}. The same was observed for liquid cultures, however with a different temporal expression pattern. Importantly, the changes in xyl gene expression in response to the different nitrogen sources served as descriptors of corresponding changes in m-xylene mineralization in the soil system.

Discussion

Environmental conditions affecting xyl gene expression by _P. putida_ mt-2 introduced to soil has to the best of our knowledge not previously been clarified. In complex soil environments, introduced degrader bacteria are exposed to a variety of biotic and abiotic stress factors, which might not resemble situations tested individually under standard liquid culture conditions. At the current time, it remains obscure how bacterial metabolism is regulated in their natural habitat (Meckenstock _et al._, 2015), and whether regulatory concepts derived from liquid culture studies (Duetz _et al._, 1996; Commichau _et al._, 2006; Hervás _et al._, 2008; Pflüger-Grau and Görke, 2010) are valid for catabolic bacteria exposed to the plethora of challenging conditions in their natural environment. Hence, the current study integrates experiments in a pure culture system and a soil model system. Importantly, our analyses couples changes in xylM and xylE gene expression to changes in the output of the catabolic pathway in question, m-xylene biodegradation, as transcript abundance and their cognate processes is often not correlated under environmental conditions (Rocca _et al._, 2014).

Our results on xyl gene regulation by nitrogen sources in pure culture expand the results of Velázquez and colleagues (Velázquez _et al._, 2006), who determined xyl gene expression by microarray technology and by Huang and coworkers (Huang _et al._, 2008), who detected increased Pu promoter activity with NO$_3$\textsuperscript{-} as nitrogen source at single time points. Interestingly, the stimulation of xyl gene expression by NO$_3$\textsuperscript{-} observed in the current study occurred later than in previous studies emphasizing the importance of monitoring temporal dynamics of gene expression.

NH$_4$\textsuperscript{+} is considered the preferred nitrogen source for bacteria, as its assimilation is less energy-expensive as...
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compared with assimilation of NO$_3^-$ that first needs conversion into NH$_4^+$ before it is assimilated via glutamine syntethase–glutamate synthase (Magasanik, 1993; Merrick and Edwards, 1995; Leigh and Dodsworth, 2007). The physiological change following substitution of NH$_4^+$ with NO$_3^-$ might consequently be due to introduction of a poor nitrogen source, although Velázquez et al. (2006) only noted a weak induction of indicator genes for nitrogen starvation stress in P. putida mt-2 exposed to NO$_3^-$. To monitor the availability of NH$_4^+$ to mt-2 cells, we quantified the expression of two genes, $amtB$ and $gdhA$ that are under control of the major nitrogen-associated transcriptional regulator, NtrC. The $amtB$ gene encodes a high-affinity NH$_4^+$ transporter. It belongs to the NH$_4^+$ transport family of proteins ubiquitous to all bacteria, and is blocked under conditions of nitrogen excess (Coulls et al., 2002; Javelle and Merrick, 2005; Leigh and Dodsworth, 2007). Expression of $amtB$ in P. putida is stimulated under nitrogen limiting conditions established, e.g. during growth on serine (Hervás et al., 2008; Yeom et al., 2010). The glutamate dehydrogenase gene, $gdhA$, on the other hand is reported to be expressed under conditions of normal nitrogen access and is actively repressed by nitrogen limitation in P. putida (Hervás et al., 2010). Our observation of high $amtB$ and very low $gdhA$ expression in NO$_3^-$-amended cultures over the entire time-course examined here indicates that mt-2 sensed this medium as being NH$_4^+$ deficient. The opposite expression pattern recorded during growth with NH$_4^+$ demonstrates that, in concert, $amtB$ and $gdhA$ function as indicators for the cellular nitrogen status. Interestingly, the expression of $amtB$ and $gdhA$ is highly dynamic within 1–3 h where after expression is down-regulated to a steady level likely because the nitrogen fluxes have reached balance. Peaks in expression have previously been observed, e.g. for the Cupriavidus pinatubonensis tfdA gene involved in herbicide catabolism just after exposure to the substrate, and for the P. putida catalase gene katA just after exposure to hydrogen peroxide (Svenningsen et al., 2015 and unpublished observations). We speculate that a pool of enzymes is produced during the burst in gene expression that is able to carry out the requested function for an extended time period. Again, our data underline that data obtained from sampling at single timepoints in gene expression studies might lead to incorrect conclusions.

To be able to assess the nitrogen source impact on m-xylene biodegradation in soil, we reduced the readily accessible soil nitrogen pool through incubation with barley straw. Due to a C:N ratio higher than the average bacterial C:N ratio, this treatment immobilizes nitrogen in non-sterile soil (Geisseler et al., 2010). The expression of the two nitrogen-regulated genes $amtB$ and $gdhA$ showed initial peaks as discussed above for liquid cultures. However, the response was slower and we also noted subtle differences in induction levels between pure cultures and soil that could be explained by the NH$_4^+$ levels in the two systems. Hence, the weaker induction of $amtB$ in NO$_3^-$-amended soil than in liquid NO$_3^-$-amended medium as well as the weak induction of $gdhA$ in NO$_3^-$-amended soil suggest that small amounts of an easily available nitrogen source were available in the soil. Indeed, chemical analysis showed that the nitrogen-limited soil still contained 0.1 mmol kg$^{-1}$ of water soluble NH$_4^+$ after the straw pre-treatment. This NH$_4^+$ pool has been available even to the cells introduced to NO$_3^-$-amended soils. Hence, our results indicate that mt-2 cells are able to sense and respond to indigenous nitrogen pools in the soil, and they demonstrate that the $amtB$ and $gdhA$ genes are valid indicator genes for studying the bioavailability of NO$_3^-$ versus NH$_4^+$ to P. putida mt-2 in natural soil.

The higher expression of xyl genes in response to m-xylene in NO$_3^-$-amended than NH$_4^+$-amended soil was in general agreement with observations made for pure cultures. Obviously, xyl gene induction in the soil was slower than in liquid culture. This might be caused by sorption of m-xylene to soil organic matter. However, the slower response was even recorded for expression of genes involved in nitrogen metabolism as discussed above. Hence, the delay might be ascribed to the downshift that the cells experience upon transfer to the oligotrophic soil environment (van Veen et al., 1997; Koch et al., 2001) with a high complexity of potential stressors (including the m-xylene carrier hexane) that the cells need to deal with (Daurabas and Chakrabarty, 1992; Velázquez et al., 2006). Importantly, the robustness of our gene expression system permitted us to relate small differences in gene expression in soil under the two nitrogen regimes to a significant difference in total mineralization of the added m-xylene. This is important because discrepancy between the amounts of transcripts and their corresponding protein abundance and functional activity is occasionally observed (Poblete-Castro et al., 2012). Regulation of gene expression is the first and most direct cellular response to changed environmental conditions in prokaryotes. We indeed observed that xyl gene induction preceded m-xylene mineralization in soil, probably reflecting the time required for establishing a pool of catabolic enzymes in the cells. Comparable time-courses have been observed for tfdA gene expression and MCPA herbicide mineralization by C. pinatubonensis introduced to soil (Nicolaisen et al., 2008). When combined with $^{14}$C-mineralization assays, we suggest that xyl transcript analysis provides robust insight into factors controlling m-xylene biodegradation, but for future studies proteome analyses of the catabolic enzymes could be of considerable interest.

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It remains to be clarified exactly how nitrogen status affect *xyl* gene expression. The upper TOL pathway promoter *Pu* as well as the *Ps1* promoter of the *meta* pathway transcriptional regulator XylS are controlled by the sigma factor, σ^54, encoded by the *rpoN* gene (Ramos et al., 1997; Cases et al., 2003; Shingler, 2003) (Fig. 1). Velázquez and coworkers (Velázquez et al., 2006) proposed that NO\textsubscript{3}^- could increase *xyl* gene expression by a mechanism involving stimulation of the two σ^54-dependent TOL pathway promoters by increasing the amount of σ^54-bound core RNA polymerases; a condition that might occur during assimilation of NO\textsubscript{3}^- derived NH\textsubscript{4}^+ through glutamine synthetase-glutamate synthase (Moreno-Vivián et al., 1999; Velázquez et al., 2006). An alternative mechanism suggested by Aranda-Olmedo et al. (2005) is that the nitrogen phosphotransferase system, PTS\textsuperscript{Nt}, involved in nitrogen metabolism (Pflüger-Grau and Görke, 2010), also interferes with activation of the σ^54-dependent TOL pathway promoters via their effector molecules; hence, this mechanism is more related to the interplay between nitrogen starvation/metabolism and carbon metabolism. The observation that xylene mineralization was comparable in natural soil and nitrogen-depleted soil amended with NH\textsubscript{4}^+ might suggest that the higher mineralization in NO\textsubscript{3}^- amended soils is a direct response to NO\textsubscript{3}^-. However, to the best of our knowledge, a NO\textsubscript{3}^- sensing mechanism has not been described for *P. putida*.

The current methodological approach allowed us to gain insight into regulation of catabolic gene expression of *P. putida* mt-2 by environmental factors under close-to-natural soil conditions. Although temperature and water content were kept constant, the mt-2 cells introduced to a natural soil would be confronted with some spatial heterogeneity, with possible competition or collaboration from indigenous microorganisms, and with realistic indigenous pools of carbon sources and nutrients. Our study underscores that global regulation of catabolic genes acts beyond direct substrate induction. Furthermore, regulatory patterns emerge in our soil model systems that are comparable to those observed in liquid cultures. Nevertheless, we even observe noteworthy differences in terms of temporal dynamics and induction levels. Interestingly, we have seen that environmental regulation of *xyl* genes in pure culture does not correspond to regulation in soil, when changing the available carbon sources (NB Svenningsen, unpublished results). Hence, more effort could be devoted to deciphering the environmental factors that affect expression of these genes in the soil.

With the current model system in hand, we have a good basis for investigating the ‘behaviour’ of *P. putida* mt-2 under realistic conditions in different soils and considering other stressors that could influence the potential for biodegradation. From both a basic and an applied perspective, it is a key issue to understand the *in situ* environmental conditions able to stimulate the biodegradative potential of a particular organism (Cases and de Lorenzo, 2005). Although the specific nitrogen sources available for inoculated degrader bacteria will influence their growth potential, and therefore the potential for pollutant degradation on the long run, our data show that specific nitrogen sources in soil also affect the expression of catabolic genes of degrader bacteria. Hence, we suggest that attempts to improve bioremediation of pollutants from contaminated sites should integrate knowledge on environmental effects on growth as well as on catabolic gene regulation under natural conditions.

**Experimental procedures**

**Bacterial strain and growth conditions**

*Pseudomonas putida* mt-2 harbouring the TOL plasmid, pWW0 (Greated et al., 2002) that enables it to degrade among others toluene, *m* and *p*-xylene, was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany (DSM-6125). For all experiments, the strain was pre-cultured over night at 28°C with agitation at 150 r.p.m. in M9 minimal medium (6.0 g L\textsuperscript{-1} Na\textsubscript{2}HPO\textsubscript{4}, 3.0 g L\textsuperscript{-1} KH\textsubscript{2}PO\textsubscript{4}, 0.5 g L\textsuperscript{-1} NaCl, 1.6 g L\textsuperscript{-1} NH\textsubscript{4}Cl, 0.25 g L\textsuperscript{-1} MgSO\textsubscript{4}.7 H\textsubscript{2}O, 0.015 g L\textsuperscript{-1} CaCl\textsubscript{2}.2 H\textsubscript{2}O) supplemented with 5 mM Na-succinate. For pure culture experiments, the overnight cultures were diluted in fresh M9 medium as specified in following section. For soil experiments, cultures were harvested by centrifugation (5000 g, 5 min, 21°C), and cells were subsequently washed twice and re-suspended in 1× phosphate-buffered saline (PBS). Cell densities were calculated based on measurements of optical density at 600 nm with OD = 1 corresponding to 10\textsuperscript{9} cells ml\textsuperscript{-1} measured by standard CFU counting on LB agar.

**Liquid culture experiment and sampling for nucleic acid extraction**

Overnight cultures of *P. putida* mt-2 in M9 medium (OD\textsubscript{600nm} ~ 0.8) were diluted 100 times in fresh medium and exposed to vapours of *m*-xylene (Sigma-Aldrich, St. Louis, MO, USA) stemming from a 1:5 dilution in dibutyl phthalate (Sigma-Aldrich) in sealed flasks, basically as described in Velázquez et al. (2006). At OD\textsubscript{600nm} ~ 0.5, cells were washed twice in PBS before resuspending them in N-free M9 medium supplemented with either 10 mM NO\textsubscript{3}^- or NH\textsubscript{4}^+ as nitrogen source and incubated in the presence of *m*-xylene at 28°C with agitation at 150 r.p.m. The bottles were sealed and kept closed during the incubation. At 15 min, 30 min, 1, 3, 5 and 7 h
after the shift in nitrogen sources, samples were withdrawn with a sterile syringe through a septum in the cap. Cells were pelleted by centrifugation at 4°C (10,000 g, 2 min), frozen in liquid N₂ and immediately stored at −70°C until nucleic acid extraction.

Soil characteristics and soil model set-up

Agricultural soil was collected from the Ap horizon (0–34 cm) of a soil profile located 20 km west of Copenhagen at 55°40' N and 12°18' E on the experimental farm Rørrendegård of Copenhagen University. The soil was stored in closed containers at 4°C until use. A subsample of soil was used for soil characterization. The soil pH was 6.8, determined in a 1:1 soil–water suspension. The particle size distribution was 22% clay, 13% silt, 29% course sand and 36% sand. The total carbon content was 1.1% (w/w) and total nitrogen was determined by Kjeldahl digestion. The total carbon content was 1.1% (w/w) and total nitrogen was 0.13% (w/w).

Prior to the experiment, the moist soil was passed through a 2 mm mesh sieve, and a subsample of soil was heated at 105°C for 24 h to determine the water content. To obtain depletion of nitrogen, ground barley straw (2.5% w/w) was mixed into the soil, which was subsequently incubated at 20°C for 1 week in order to immobilize soil nitrogen (Jensen and Nybroe, 1999). Straw residues were then removed by passing the soil through the 2 mm mesh sieve again. Next the moisture content was adjusted to 18% of soil dry weight with sterile filtered demineralized water (MilliQ; Merck Millipore, Darmstadt, Germany), taking into account the subsequent addition of cells and nitrogen solutions. Washed stationary phase P. putida mt-2 cells were inoculated into the soil to reach a cell density of approximately 10⁸ cells g⁻¹ soil. Cells were mixed into the soil by hand in a diffusion limited soil mixing bag (Rilsan; Rotek A/S, Sønder Felding, Denmark) together with NaN₃ or NH₄Cl solutions. Nitrogen solutions were added to obtain concentrations of 10 mmol N kg⁻¹ soil. A mixture of m-xylene and ¹⁴C-labelled m-xylene (8000 dpm g⁻¹ soil) in hexane as carrier was then mixed into the soil to a concentration of m-xylene of 200 mg kg⁻¹. Subsequently, 20 g soil was distributed in triplicate into glass bottles, and glass vials containing 1 M NaOH were placed on top of the soil. From each bottle, triplicate soil samples were immediately taken, in which radioactivity was measured by liquid scintillation counting to be able to correct for loss of m-xylene evaporating during the set-up. Bottles were sealed with screw caps and incubated at 20°C in the dark.

Mineralization of m-xylene was measured during the incubation period by liquid scintillation counting of collected ¹⁴C-CO₂ in the NaOH using a Beckman LS1801 scintillation counter (Beckman Coulter, Copenhagen, Denmark) after mixing the 1 ml NaOH samples with 4 ml scintillation cocktail (Optiphase Hisafe3; Perkin Elmer, Skovlunde, Denmark) followed by incubation in the dark for 2 h. After 0, 5, 8, 10, 12, 14, 21 and 46 h of incubation, soil samples of 0.5 g were obtained. For each sampling, the screw caps were shortly removed from the bottles. Soil samples were immediately frozen in liquid N₂ and stored at −70°C until nucleic acid extraction.

In addition to the treatments described above, a control soil microcosm without inoculation of mt-2 cells, plus addition of m-xylene was prepared to test for background mineralization of m-xylene and direct capture of ¹⁴C m-xylene in NaOH traps. Furthermore, a control with inoculation of mt-2 cells but without addition of m-xylene was set up to test for if the soil itself stimulates induction of the xyl genes in soil.

Additionally, at the beginning and in the end of incubation, soil water samples were collected for determination of pH and concentrations of NH₄⁺ and NO₃⁻ associated to soil water (i.e. the directly bioavailable part). This was done by vortexing 0.15 g soil with 1.5 ml MilliQ water, followed by 1 h of shaking at 200 r.p.m. Finally, samples were centrifuged (10,000 g, 5 min) and analyses of pH and concentrations of NO₃⁻ and NH₄⁺ were conducted on the supernatants.

Nucleic acid extraction and quantitative PCR

For extraction of nucleic acids from pure culture samples, the AllPrep DNA/RNA Mini Kit (Qiagen, Manchester, UK) was used according to the manufacturer’s protocol with addition of a lysozyme pre-treatment step at room temperature for 20 min (100 µl of 1 mg ml⁻¹ in 10 mM Tris-Cl buffer, pH 8, per sample of 100 µl) as the only modification. From soil samples, DNA and RNA were co-extracted by the phenol–chloroform method as formerly described (Nicolaisen et al., 2008). Subsequent to extraction DNA was eliminated from RNA samples by treating aliquots of each nucleic acid sample with RQ1 RNase-free DNase 1 (Promega, Nacka, Sweden) according to the manufacturer’s protocol. cDNA was synthesized immediately thereafter by using 2 µl subsamples of each DNase-treated extract as template for reverse transcription (RT) with the Omniscript RT Kit (Qiagen). DNase-treated control reactions were prepared in parallel for RNA samples without addition of the reverse transcriptase (RT) to ensure the absence of DNA contamination. RT reactions were prepared with 400 ng of random hexamer primers (Promega), 4 U of SUPER RNase inhibitor (Ambion, Austin, TX, USA) and reagents provided in the kit for a final volume of 20 µl. Incubation conditions were followed as recommended by

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the manufacturer. Resulting cDNA samples were stored at –20°C until use in qPCR.

Previously published primers were used to quantify the expression of xyLM, xyle (Martinez-Lavanchy et al., 2010), amtB and gdhA (Hervás et al., 2008). qPCR reactions were prepared in 20 µl with 10 µl Brilliant II SYBR Green QPCR Master mix (Stratagene, La Jolla, CA, USA), 0.3 µM of each primer and 1 mg ml⁻¹ BSA. Thermal cycling conditions were following: an initial cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, primer annealing at the temperatures stated in (Hervás et al., 2008; Martínez-Lavanchy et al., 2010) for 45 s and an elongation step at 72°C for 1 min. Subsequently a melting curve was run. All cDNA and DNA samples were diluted 1:10 before the qPCR. To check for possible contamination of RNA samples by genomic DNA, diluted samples of RNA were analysed by qPCR as well. Ct values for pure culture samples were related to a standard curve prepared from 10-fold dilutions of DNA extracted from 1 ml of liquid culture with OD₆₀₀nm of 0.8. For soil samples, a standard curve was prepared by inoculation of 10⁰–10⁹ P. putida mt-2 cells per gram soil and subsequently extracting the DNA. Soil without inoculation of mt-2 cell had a natural background of xy genes; hence, the standard curve was not linear below 10³ gene copies per gram of soil. From the slope of the standard curves, the amplification efficiencies of the qPCRs were calculated using the formula

\[ E = \frac{10^{(-1/slope)} - 1}{1} \]

for all four gene amplified, the efficiencies were in the range 98–106%. Gene expression was calculated as mRNA normalized per DNA copy numbers as previously described (Nicolaisen et al., 2008), taking dilution steps from the DNase-treatment and RT into consideration when calculating mRNA numbers from the cDNA numbers. DNA and cDNA samples from the soil qPCRs were randomly chosen for Sanger sequencing to verify that the primers only targeted specific products in the soil.

**Statistical analysis**

All experiments were repeated independently at least twice, with each independent experiment involving triplicate samples. Mean values of such triplicates from one representative experiment are reported ± standard error of mean. Statistical significance was tested with Student’s t-test using the software PAST3.10 (University of Oslo, Norway). Data were considered significant when P < 0.05.

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**Conflict of interest**

The authors have no conflict of interest.

**References**


