Addressing the role of the extrusion pump-bearing pGRT1 plasmid in toluene biodegradation by *Pseudomonas putida* DOT-T1E under real case scenarios

M. Hernandez, J. Gouveia, A. Segura, R. Muñoz and S. Villaverde

**ABSTRACT**

The role of both the plasmid pGRT1 and the solvent extrusion pump *ttgGHI* during toluene biodegradation in *Pseudomonas putida* DOT-T1E cultures was investigated in a sterile suspended growth bioreactor operated as chemostat and inoculated independently with a wild type strain, a mutant lacking the pGRT1 plasmid (*P. putida* DOT-TIE-100), and a mutant with a modified pGRT1 plasmid lacking the genes encoding the *ttgGHI* solvent efflux pump (*P. putida* DOT-TIE-28).

A similar process performance was recorded in all tested strains at 4 g tol m$^{-3}$ and dilution rates (*D*) of 0.1 h$^{-1}$. However, operation at 10 g tol m$^{-3}$ and *D* of 0.2 h$^{-1}$ revealed a much lower toluene EC (285 g m$^{-3}$ h$^{-1}$) in *P. putida* DOT-TIE-100 cultures when compared to wild type and *P. putida* DOT-TIE-28 cultures (483–498 g m$^{-3}$ h$^{-1}$), which suggests that other mechanisms rather than solvent extrusion by the *ttgGHI* efflux pump supported this superior process performance. Finally, the plasmid pGRT1 analysed exhibited a remarkable stability towards toluene harmful mediated effects, regardless the strain or toluene loading tested.

**Key words** | extrusion pump *ttgGHI*, plasmid pGRT1, *Pseudomonas putida* DOT-T1E, toluene tolerance

**INTRODUCTION**

Long term microbial stability is a critical requirement to promote the industrial application of biological methods for air pollution control (Díaz et al. 2008). However, this stability is very often challenged by the inherent toxicity and mutagenic nature of some of the target contaminants present in the off-gas treated (Song & Kinney 2005). Volatile Organic Contaminants (VOCs) such as toluene or benzene can act as uncouplers diminishing the efficacy of the respiratory chain and therefore reducing microbial metabolic activity (De Smet et al. 1978). In this context, Jones et al. (1997) reported that the prolonged exposure of *Pseudomonas putida* 54g to toluene aqueous concentrations of approx. 7 mg l$^{-1}$ caused an important fraction of the culture to die or irreversibly lose its ability to grow, whereas other cells lost their capacity to degrade toluene.

Pernicious effects caused by VOCs on bacterial communities depend not only on the nature of the toxic VOCs, but also on the specific tolerance of each individual strain (Aono & Kobayashi 1997). Among the most common mechanisms underlying VOCs tolerance are the modification of the composition of membrane fatty acids and phospholipids head groups, and VOCs extrusion via efflux pumps. Multidrug resistance efflux systems are responsible of VOCs extrusion from bacterial cytoplasm to the external medium, which reduces its intracellular pollutant concentration and therefore its harmful effects (Ramos et al. 2002). However, most of the research on the elucidation of the complex mechanisms governing VOCs tolerance has been carried out at concentrations far above those found in real biofiltration processes. Little data is thus available on the role of these tolerance mechanisms on the long term...
stability and biodegradation performance under the scenarios commonly found in real plant operation (gas concentrations lower than 10 g m\(^{-3}\)) (Revah & Morgan-Sagastume 2005).

*Pseudomonas putida* DOT-T1E, a highly tolerant toluene degrading strain whose tolerance has been mainly associated to the *ttgGHI* solvent efflux pump encoded in the pGRT1 plasmid, was used as a model microorganism (Segura et al. 2003). In this context, the long term performance and stability of a wild type *P. putida* DOT-T1E strain, a *P. putida* DOT-T1E mutant lacking the pGRT1 plasmid, and a *P. putida* DOT-T1E mutant with a modified pGRT1 plasmid in which the *ttgGHI* solvent efflux pump is not functional, were evaluated during toluene biodegradation in a continuous suspended growth bio-reactor. This study investigated the role of the tolerance provided by the pGRT1 plasmid and the *ttgGHI* solvent efflux pump on toluene biodegradation.

**METHODS**

Microorganisms and culture conditions

*P. putida* DOT-T1E, a mutant of *P. putida* DOT-T1E lacking the pGRT1 plasmid, *P. putida* DOT-T1E-100 and a *P. putida* DOT-T1E mutant with a modified pGRT1 plasmid in which the *ttgGHI* efflux pump was not functional (P. putida DOT-T1E-28) were obtained from the *Pseudomonas* Reference Culture Collection established at Estación Experimental del Zaidín (Granada, Spain). The Mineral Salt Medium (MSM) used for inocula cultivation and bioreactor operation was prepared according to Bordel et al. (2007).

Experimental design

Process stability was investigated under sterile conditions in a magnetically stirred 1-L glass bioreactor (Afora S.A, Spain) operated as a chemostat according to Díaz et al. (2008). The reactor was filled with 900 ml of sterile MSM and inoculated with 40 ml of the tested strain to attain an initial biomass concentration ranging from 16 to 28 mg DW l\(^{-1}\). The system was initially operated in batch mode and continuous operation was established 10 h following inoculation by continuous MSM supply and overflow under sterile conditions. Temperature and agitation rate were maintained constant at 25°C and 500 rpm, respectively. Toluene was supplied in the gas phase via aeration (1,100 ml min\(^{-1}\) of synthetic air filtered through a 0.2 µm Millex\(^{®}\)-FG membrane filter) by mixing a toluene-saturated stream with a toluene-free air stream at different proportions (Figure 1).

Two series of continuous experiments were carried out for each bacterial strain tested. In the first series, the system was operated at 4 g tol m\(^{-3}\) and dilution rates \(D\) of 0.1 h\(^{-1}\), while in the second series the inlet concentration was set at 10 g tol m\(^{-3}\) and \(D\) of 0.2 h\(^{-1}\). The dilution rate was calculated as the ratio of the MSM flow rate \((Q = [\text{l h}^{-1}])\) to the bioreactor volume \((V_R = [\text{l}])\) and, under chemostat operation, \(D\) represents the actual specific bacterial growth rate. Despite only one replicate per operational condition was performed, the steady states achieved were monitored for at least 7 days (7 measurements per steady state).

Analytical procedures

Toluene and CO\(_2\) analyses were performed in a Gas Chromatograph according to Bordel et al. (2007). Dissolved total organic carbon (TOC) was measured using a TOC analyzer (Shimadzu TOC-5050A, Japan) according to the manufacturer. The DOC and the temperature were determined using an O\(_2\) transmitter 4100 (Meter Toledo GmbH, Urdolf, Germany). A CRISON micropH 2002 (Crison Instruments, Barcelona, Spain) was used for pH determination. Absorbance at 650 nm was used as an indicator of microbial growth and measured using a HITACHI U2000 UV/visible spectrophotometer (Hitachi, Tokyo, Japan). A correlation between absorbance at 650 nm and biomass DW was performed according to Bordel et al. (2007) and absorbance readings directly converted to biomass concentrations. ATP was measured using a Microbial ATP kit HS (Biothema, Stockholm, Sweden) and a Microtox 500 luminometer (Azur Environmental, Carlsbad, Germany). The specific toluene respiration rate was determined in a STRATHKELVIN STRATHOX
A respirometer (Strathkelvin Instruments Limited, Glasgow, UK) according to Muñoz et al. (2009). The fraction of viable bacteria degrading toluene was determined by standard replica plating on non-selective medium (LB solid medium supplemented with rifampicin at 10 mg L\(^{-1}\)) and selective medium (MSM supplemented with toluene vapors). Cells were incubated in sealed containers for 24 h at 30°C. The presence of pGRT1 in Pseudomonas putida DOT-T1E was analyzed by amplifying a fragment of the \(ttgH\) gene. DNA was amplified using oligonucleotides srpB5 (5' GCGCTTGTCCACCGGCGGCAAT 3') and srpB21 (5' CGAATACAGCCGACACGCACC 3'). 30 PCR cycles (95°C 1 minute, 55°C 1 minute and 72°C 1 minute) were done. Amplified fragments were analyzed on 0.8% agarose gels. 10 colonies were randomly chosen from each sampling (samples taken every 24 hours) to analyzed the presence of the \(ttgH\) gene. The presence of the plasmid in Pseudomonas putida DOT-T1E-28 (which carries a streptomycin resistance cassette inserted in \(ttgH\)) was followed using streptomycin (150 mg L\(^{-1}\)) in the non-selective plates.

**RESULTS AND DISCUSSION**

**Process operation at low toluene loadings and low dilution rates**

Neither the presence of the plasmid pGRT1 nor the efflux pump therein encoded supported a superior process performance in the cultivations carried out at 4 g tol m\(^{-3}\) and \(D\) of 0.1 h\(^{-1}\) as shown by the comparable ECs and pH recorded for the three strains tested (Figure 2a, c and e). No significant differences were also recorded in the specific ATP cellular content, which ranged from 3.1 to 3.9 \(\times 10^{-6}\) mol ATP g DW\(^{-1}\) (Table 1). These values herein reported are in accordance with previous studies from Bordel et al. (2007) who measured ATP concentrations ranging from 3.6 to 4.8 \(\times 10^{-6}\) mol ATP g DW\(^{-1}\) in \(P.\ putida\) F1 cultures grown at low toluene concentrations. However, at low toluene loadings rates, the absence of the plasmid or the efflux pump (\(P.\ putida\) DOT-T1E-100 or \(P.\ putida\) DOT-T1E-28) did result in slightly higher biomass concentrations, a less intensive extracellular carbon excretion (based on TOC concentration) and a lower specific nitrogen content (0.2 \(\pm\) 0.03 g N g DW\(^{-1}\) in wild type strains vs. 0.13 \(\pm\) 0.01 g N g DW\(^{-1}\) in the two mutants tested).
The presence of the plasmid pGRT1 (either original or modified) provided the cells with lower and more stable specific respiration rates (based on in-vitro respirometric assays) when compared with cultures inoculated with the mutant P. putida DOT-T1E-100 (Table 1). Lower specific oxygen consumption rates during toluene degradation might result from either lower toluene degradation rates or a less efficient O$_2$ usage. In this context, the specific toluene consumption rates estimated from bioreactor operation and the DOC recorded (1.6 mg O$_2$ l$^{-1}$ in wild type and P. putida DOT-T1E-28 cultures vs 0.3 mg O$_2$ l$^{-1}$ in P. putida DOT-T1E-100) points out towards a more efficient O$_2$ utilization in cells harboring the plasmid. Lower specific O$_2$ consumption rates can constitute a competitive advantage, especially in oxygen limited scenarios such as the degradation of highly soluble VOCs (alcohols, ketones, etc.) and the degradation of high organic loads of moderately soluble VOCs (toluene, benzene, etc.).

Despite the well-known deleterious effects of toluene on bacterial activity, the entire cell population maintained the ability to degrade toluene throughout the assays regardless the strain used, which confirms the stability of the TOD biodegradation pathway encoded in bacterial chromosome (Mosqueda et al. 1999). Losses in the toluene degradation capacity have been described mainly in systems with plasmid-encoded degradation pathways (TOL degradation pathways encoded in the archetypical pWW0 plasmid),
being more severe the higher the toluene concentration and longer the exposure time was (Mirpuri et al. 1997; Muñoz et al. 2009). In addition, none the cells analyzed (136 colonies per day) lost the pGRT1 plasmid (original or modified), which confirms its high stability. This result was not surprising since frequencies of loss below 10^{-8} per cell per generation have been previously described in P. putida DOT T1E under a wide variety of growth conditions, including nutritional and physical stresses (Rodriguez-Hervá et al. 2007).

Several episodes of culture collapse were recorded in wild type and P. putida DOT-T1E-100 cultures. Process collapse was characterized by a severe deterioration in the ECs, biomass and CO₂ production concomitant with a sharp decrease on the specific toluene respiration rates even though it did not influence ATP cellular levels (Figure 2a and c). A pH-mediated inhibition as a result of acidic metabolite excretion was probably the reason underlying these episodes of poor process performance since Pseudomonas putida activity is commonly inhibited at pH values below 5 (Díaz et al. 2008). Hence, the low dilution rate applied (low buffer capacity supply) together with the high metabolic activity exhibited by the wild type and P. putida DOT-T1E-100 cultures (high acidic metabolite excretion) probably cause the large pH decreases observed. In this context, it must be stressed that process inhibition corresponded to slight increases in toluene inlet concentration (from 3.4 to 4.4 g m⁻³ at day 9 and from 3.7 to 4.4 g m⁻³ at day 14, scenarios where a higher excretion of acidic metabolites might be expected. However, it is impossible to establish the critical pH for process collapse, due to the rapid recovery in pH values after process collapse. For instance, pH increased from 4.18 to 6.37 in approx 6 hours as recorded during process failure at day 14 in the cultivation carried out with P. putida DOT T1E-100 (Figure 2c and d). At this point it must be stressed that P. putida DOT-T1E-28 was cultured at dilutions rates tiny higher than the other strains (0.12 vs 0.11 h⁻¹), which might have avoided sudden decreases in the cultivation pH due to the larger buffer capacity available at higher dilution rates.

### Process operation at high toluene loadings and high dilution rates

Larger variations in the performance of the 3 strains tested were recorded when operating at 10 g Tol m⁻³, where process ECs in cultures harboring the plasmid (either modified or original) were superior than those achieved in the absence of plasmid (483–498 vs 285 g m⁻³ h⁻¹) despite similar DOCs were monitored in all experimental runs (Table 1). This lower EC value recorded for DOT T1E-100 might be due either to the lower tolerance of this strain towards toluene as a result of the absence of the plasmid PGRT1 or to the lower efficiency in oxygen use (more grams of oxygen needed per gram of toluene degraded) mediated by the absence of some functions encoded in the above mentioned plasmid, as explained in the paragraph below. This superior performance suggests that other mechanisms are involved.

### Table 1: Steady state values recorded in chemostats inoculated with a wild type P. putida DOT-T1E strain, P. putida DOT T1E-100, and P. putida DOT T1E-28 at two operational conditions

<table>
<thead>
<tr>
<th>Condition</th>
<th>EC (g m⁻³ h⁻¹)</th>
<th>CO₂ (g m⁻³ h⁻¹)</th>
<th>Biomass (mg l⁻¹)</th>
<th>DOC (mg l⁻¹)</th>
<th>pH</th>
<th>ATP (mol ATP g DW⁻¹)</th>
<th>Respiration (gO₂ g DW⁻¹ h⁻¹)</th>
<th>TOC (mg l⁻¹)</th>
<th>Nitrogen content (g N/g DW⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOT-T1E</td>
<td>284 ± 22</td>
<td>503 ± 43</td>
<td>1,108 ± 182</td>
<td>1.6 ± 0.5</td>
<td>5.9</td>
<td>3.9 ± 0.5 x 10⁻⁶</td>
<td>0.47 ± 0.1</td>
<td>188 ± 33</td>
<td>0.20 ± 0.03</td>
</tr>
<tr>
<td>DOT-T1E-100</td>
<td>249 ± 21</td>
<td>558 ± 40</td>
<td>1,716 ± 73</td>
<td>0.3 ± 0.2</td>
<td>5.8</td>
<td>3.1 ± 0.6 x 10⁻⁶</td>
<td>0.72 ± 0.1</td>
<td>115 ± 30</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>DOT-T1E-28</td>
<td>263 ± 22</td>
<td>616 ± 44</td>
<td>1,668 ± 75</td>
<td>1.6 ± 0.6</td>
<td>5.8</td>
<td>3.7 ± 0.8 x 10⁻⁶</td>
<td>0.44 ± 0.1</td>
<td>90 ± 18</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>DOT-T1E</td>
<td>483 ± 25</td>
<td>677 ± 22</td>
<td>1,260 ± 36</td>
<td>0.2 ± 0.1</td>
<td>5.7</td>
<td>4.0 ± 0.6 x 10⁻⁶</td>
<td>0.51 ± 0.1</td>
<td>133 ± 25</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>DOT-T1E-100</td>
<td>285 ± 37</td>
<td>684 ± 47</td>
<td>1,354 ± 33</td>
<td>0.1 ± 0.0</td>
<td>6.2</td>
<td>4.2 ± 0.4 x 10⁻⁶</td>
<td>0.76 ± 0.0</td>
<td>80 ± 14</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>DOT-T1E-28</td>
<td>498 ± 29</td>
<td>772 ± 62</td>
<td>1,520 ± 134</td>
<td>0.1 ± 0.0</td>
<td>5.8</td>
<td>8.3 ± 1.4 x 10⁻⁶</td>
<td>0.53 ± 0.1</td>
<td>66 ± 15</td>
<td>0.13 ± 0.01</td>
</tr>
</tbody>
</table>

Values are given as the average ± standard deviation from the measurements obtained during the stationary states achieved (at least 7 days).
rather than toluene extrusion by the efflux pump ttgGHI might be involved in this enhancement. However, since the complete sequence of plasmid pGRT1 has not been elucidated yet, it is difficult to speculate about the molecular mechanisms involved in the stabilization of the process. The specific ATP contents in *P. putida* DOT-T1E and *P. putida* DOT-T1E-100 were similar and steady throughout stationary operation (4.0–4.2 × 10⁻⁶ mol ATP g DW⁻¹) but lower than those recorded in *P. putida* DOT-T1E-28 cultures (8.3 × 10⁻⁶ mol ATP g DW⁻¹). This higher specific ATP concentration was obtained at toluene aqueous concentrations of approx. 24 mg l⁻¹ (vs 12 mg l⁻¹ in *P. putida* DOT-T1E and *P. putida* DOT-T1E-100 cultures), and it is in agreement with previous studies that recorded higher specific ATP contents at higher aqueous toluene concentrations. In this context, Bordel et al. (2007) observed that the specific ATP content of *P. putida* F1 (strain that shares 99% of the genome with *P. putida* DOT-T1E) increased from 3.6 ± 0.2 × 10⁻⁶ mol ATP g DW⁻¹ at 2 mg toluene l⁻¹ to 7.4 ± 0.4 × 10⁻⁶ mol ATP g DW⁻¹ at 170 mg toluene l⁻¹. Hence, the low aqueous toluene concentrations present in the experiments carried out at low toluene concentrations (high removal efficiencies) could explain the lower specific ATP contents compared to those recorded during the second series of experiments (Table 1).

As above described in cultures operated at low toluene concentrations, the presence of the plasmid (either in its original or modified form) provided cells with lower and specific ATP contents at higher aqueous toluene concentrations. In this context, Bordel et al. (2007) observed that the specific ATP content of *P. putida* F1 (strain that shares 99% of the genome with *P. putida* DOT-T1E) increased from 3.6 ± 0.2 × 10⁻⁶ mol ATP g DW⁻¹ at 2 mg toluene l⁻¹ to 7.4 ± 0.4 × 10⁻⁶ mol ATP g DW⁻¹ at 170 mg toluene l⁻¹. Hence, the low aqueous toluene concentrations present in the experiments carried out at low toluene concentrations (high removal efficiencies) could explain the lower specific ATP contents compared to those recorded during the second series of experiments (Table 1).

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more stable specific respiration rates on toluene. This phenotype is especially important under the oxygen limiting conditions present at high toluene loading rates, which might explain the lower ECs achieved by P. putida DOT T1E-100 compared to the two other strains exhibiting a much more efficient oxygen utilization. The low oxygen concentrations recorded for the 3 P. putida strains tested (DOC = 0.1 mgO₂ l⁻¹), together with the low value of the Monod affinity constant for P. putida cultures (Kₐₒ₂ = 1.1 ± 0.47 mg O₂ l⁻¹⁻¹; Alagappan & Cowan 2004) suggest the occurrence of oxygen limiting conditions at 10 g tol m⁻³.

In addition, none of the cells in the bioreactor lost their ability to degrade toluene nor the plasmid (when present), which confirmed the high stability of both the TOD biodegradation pathway and pGRT1 plasmid. Process deterioration also occurred within the first 48 h of operation regardless the bacterial strain tested (Figure 3). However, while in wild type cultures and P. putida DOT-T1E-28 cultures the decrease in process performance was gradual and steady state operation was rapidly achieved, in P. putida DOT-T1E-100 cultures the process deterioration was sharp, lasting for approx. 3 days. The fact that pH remained at relatively high values (pH > 6) during these episodes of culture collapse, together with the absence of deterioration in the specific oxygen consumption rates (data not shown) suggest the possibility of a reversible toxic metabolite-mediated inhibitory effect rather than a pH induced inhibition. The high dilution rates employed at high toluene concentrations provided enough buffer capacity to rule out any potential pH inhibition.

**CONCLUSIONS**

In brief, this study confirmed the key role of the plasmid pGRT1 on toluene biodegradation. Thus, while the absence of the plasmid (pGRT1⁻) or the efflux pump (ttgGHI⁻) did not provide any significant enhancement on toluene ECs at low toluene loading rates (comparable ECs recorded for the three strains tested), a superior process performance was recorded at 10 g tol m⁻³ in cultures harbouring the plasmid on its original (wild type) or modified form (ttgGHI⁻), higher ECs, 483 and 498 g m⁻³ h⁻¹ respectively, compared to the case of cultures without plasmid pGRT1, lower EC, 285 g m⁻³ h⁻¹. Therefore other mechanisms encoded in the plasmid rather than the tolerance provided by the efflux pump ttgGHI were involved on the superior performance exhibited by the strains harbouring the plasmid (either original or modified). The elucidation of the exact mechanisms involved in toluene tolerance constitutes a very difficult task due to the large size of the plasmid and was out of the scope of this investigation. This plasmid supported as well lower and more stable toluene respiration rates when compared to P. putida DOT-T1E-100, which constitutes a competitive advantages in O₂ limited environments. In addition, both the plasmids pGRT1 analysed and the TOD biodegradation pathway exhibited a remarkable stability regardless of the strain or toluene loading tested.

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**REFERENCES**


