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Toluene-degrading Antarctic *Pseudomonas* strains from fuel-contaminated soil

Roberta L. Farrell,[a,*] Phillippa L. Rhodes,[a,1] and Jackie Aislabie[b]

[a] *Department of Biological Sciences, University of Waikato, Private Bag 3105, Hamilton, New Zealand*
[b] *Landcare Research, Private Bag 3127, Hamilton, New Zealand*

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Abstract

Two psychrotolerant toluene-degrading *Pseudomonas* spp. were isolated from JP8 jet-fuel-contaminated soils, Scott Base, Antarctica. Isolates metabolized *meta*-toluate as sole carbon source at temperatures ranging from 6 to 30 °C. Large plasmids (>64 kb) were isolated from both isolates. Sequence analysis of PCR products amplified using *xylB* (the gene encoding benzyl alcohol dehydrogenase) primers revealed that isolates 7/167 and 8/46 were 100% and 92% homologous, respectively, to the *xylB* gene of the *meta*-cleavage toluene degradative pathway encoded by the TOL plasmid (pWWO) of *Pseudomonas putida* mt-2. Assays of cell-free extracts of 7/167 and 8/46 demonstrated activity of catechol 2,3-dioxygenase, benzyl alcohol dehydrogenase, and benzaldehyde dehydrogenase, indicating that the isolates use the *meta*-cleavage pathway enzymes of toluene degradation typical of TOL type plasmids. As both isolates are able to grow at 6 °C ex situ it is feasible that they would be able to metabolize toluene in the Antarctic soils from where they were originally isolated.

Keywords: *Pseudomonas*; Antarctica; Bioprospecting; Biodegradation; Bioremediation; Fuel; Toluene; Catechol; Psychrotolerant; Plasmid

Indigenous Antarctic biological communities are vulnerable to the impact of human activities occurring in Antarctica. A source of human impact is the pollution of Antarctic sites by fuel. Fuels (hydrocarbons), used to support the scientific research bases in Antarctica, are the most widespread pollutants in Antarctica [1]. Fuel spillage is associated with handling and storage of fuels [2]. Fuel contamination is toxic to the marine life and the limited terrestrial biota of Antarctica [2–4]. The majority of fuel used at Scott Base, New Zealand's year-round scientific research presence in Antarctica, is JP8 jet-fuel. JP8 is composed of low molecular weight *n*-alkanes (C8–C15) and aromatic hydrocarbons such as toluene and naphthalene.

Microbial metabolism (degradation) of aromatic hydrocarbons as an energy and carbon source has been intensively studied to develop application technologies to remove these toxic compounds from contaminated environments. Bioremediation has been proposed as such a technology to clean-up fuel-contaminated sites in Antarctica [5]. A successful bioremediation strategy involves either encouraging the native microbial community to degrade the pollutant more rapidly, or addition of effective degradative microbes in the absence of native degrading microbes [6]. Due to the prohibition of the introduction of foreign organisms to Antarctica (Antarctic Conservation Act, 1978, sect 670-4, cited in [7]), the development of a bioremediation program in Antarctica must use either indigenous hydrocarbon-degrading organisms in situ or isolated as a result of bioprospecting. Additionally, bioprospecting research that looks for a useful application, process or product in nature is often most successful when matching the search in an environment with the desired parameters. A number of indigenous hydrocarbon-degrading *Pseudomonas* species have been isolated from fuel-contaminated soils in Antarctica [8–11]. The microbial metabolism of hydrocarbons has been intensively

* Corresponding author. Fax: +64-7-838-4976.
E-mail address: r.farrell@waikato.ac.nz (R.L. Farrell).
[1] Present address: Food Science Postgraduate Programme, Department of Chemistry, University of Auckland, Private Bag 92019, Auckland, New Zealand.

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studied [12]; however, as yet, little is known about the degradative genes or the mechanism of hydrocarbon degradation utilized by indigenous microbes existing in fuel-contaminated Antarctic soils. Whether these microorganisms were introduced to Antarctica by human activity, or present endemically, is unclear, but a number of Antarctic-isolated bacterial species show lower optimum growth temperatures to non-Antarctic species; for example, Antarctic *Methanococcoides burtonii* has a growth temperature of 23 °C whereas the non-Antarctic species has a temperature optimum of 35 °C [13]. In this paper we describe substrates able to sustain growth as sole carbon sources, the temperature growth ranges, and the likely degradative mechanism utilized by two toluene-degrading Antarctic *Pseudomonas* isolates.

Materials and methods

Bacterial strains. The toluene-degrading isolates, 7/167 and 8/46, used in this study were isolated as described elsewhere [11]. Isolate 7/167 was isolated from a soil at a fuel leak near the Scott Base kitchen and 8/46 was isolated from a site of a leaking pipeline to Williams Field. Isolates were maintained on carbon-free Bushnell Haas Broth (Difco) solidified with 1.6% purified agar (Oxoid) (BHA) and supplemented with 5 mM *meta*-toluate (Aldrich) [14] or 100 μl toluene (BDH scintillation grade) added as a vapor in the lid of the petri plate. Isolates were checked for their reaction to Gram stain and characterized by use of API 20 NE test strips (bioMerieux).

Substrate screening of aromatic hydrocarbon-degrading isolates. Isolates 7/167 and 8/46, grown on JP8 jet-fuel vapors, were tested for growth on carbon-free Bushnells Haas agar with vapors of the following supplied as a carbon source: benzene, toluene, *o*-xylene, *m*-xylene, *p*-xylene, 1,2,4-trimethylbenzene, ethylbenzene, naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, heptane, dodecane, undecane, and tridecane, according to established procedures [15]. Degradation of phenanthrene was detected using agar overlayer plates [16]. Inoculated plates were incubated at 16 °C for at least 1 month and bacterial growth was assessed by comparison with inoculated carbon-free control plates. All substrates were from Aldrich Chemical and were >98% pure.

PCR amplification of a toluene degradative gene. Total DNA isolated from 7/167 and 8/46 was assessed for the presence of three TOL plasmid encoded *xyl* genes that are involved in the *meta*-cleavage pathway of toluene degradation (Fig. 1). Toluene degradation genes *xylB* (benzyl alcohol dehydrogenase), *xylE* (catechol 2,3-dioxygenase), and *xylC* (benzaldehyde dehydrogenase) were selected for study in the Antarctic isolates and in the positive control isolate *Pseudomonas putida* (*arvilla*) mt-2 (kindly provided by P. Williams, Bangor, Wales). These proteins are encoded by the well-characterized TOL plasmid pWWO (http://www.ncbi.nlm.nih.gov/htbin-post/Entrez/nucleotide.html) and degrade toluene through the substrate induced *meta*-toluene degradative pathway [17]. Total DNA extracts were used for PCR to detect plasmid and/or chromosomally encoded genes involved in the toluene degradative pathway. Specific primers were designed from the DNA sequence of the *xylB*, *xylE*, and *xylC* genes of the pWWO TOL plasmid. DNA amplification was performed in a total volume of 50 μl in 0.2 ml-Eppendorf tubes using an Eppendorf Mastercycler gradient PCR machine. Polymerase chain reactions were generated according to established protocol [18] using 1× PCR $MgCl_2$ Buffer (Boehringer–Mannheim), 200 μM each deoxynucleoside triphosphate, 0.2 μM each primer, 10 ng template DNA, and 1 U *Taq* DNA polymerase (Boehringer–Mannheim). During the 40 cycles run for PCR, DNAs were denatured at 94 °C for 1 min, primers were annealed at 50 °C for 1 min, and DNAs were extended at 72 °C for 1 min. PCR products were visualized on a 1% (w/v) agarose gel amended with ethidium bromide. Positive control *P. putida* (*arvilla*) mt-2 DNA and negative control (without DNA) were run in parallel with the experiment.

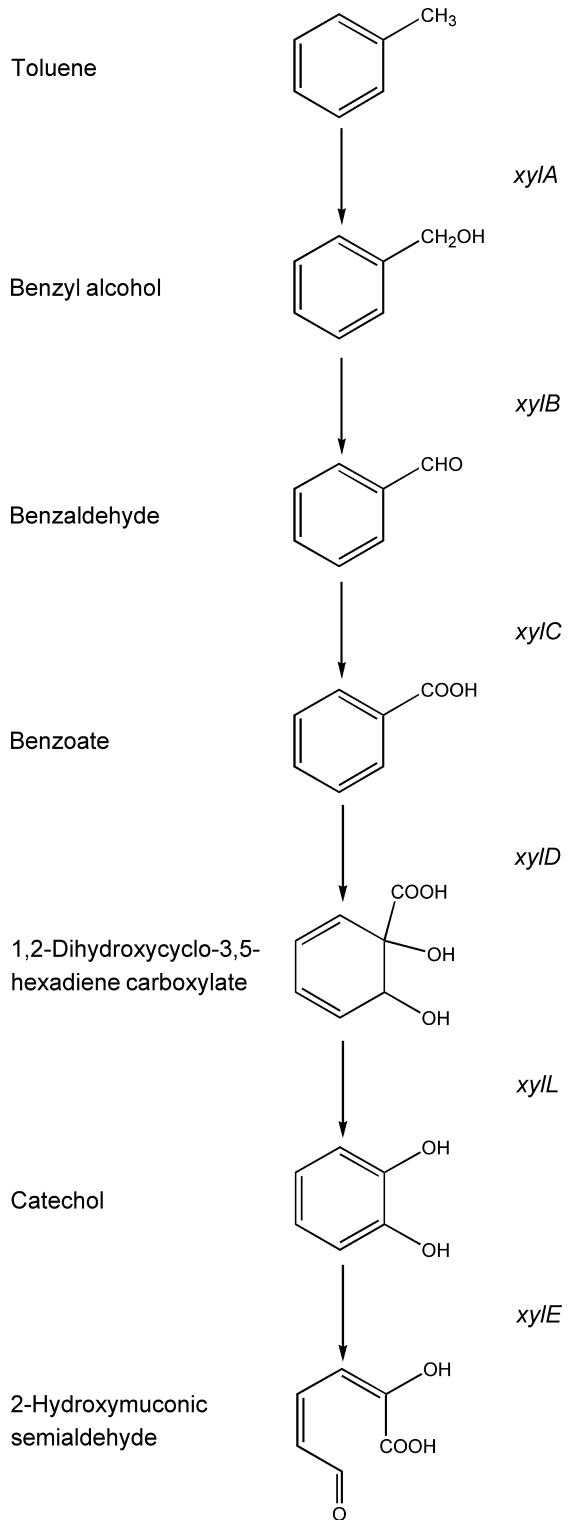

Fig. 1. Toluene degradative pathway encoded by the pWWO plasmid in *Pseudomonas putida* (*arvilla*) mt-2. Gene abbreviations in italics.

Toluene degradative enzyme assays. Isolates were assayed for the presence of four enzymes known to catalyze toluene degradation through the *meta*-cleavage pathway. Two ring cleavage enzymes, catechol 2,3-dioxygenase (*meta*-cleavage) and catechol 1,2-dioxygenase (*ortho*-cleavage), were assayed based on the protocols [19,20], respectively. Benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase were assayed according to protocol [21]. Cell-free extracts were prepared in duplicate. Bacterial cultures were grown to log phase in 100 ml of Bushnell Hass broth amended with 5 mM *meta*-toluate at 15 °C. Cells were harvested by centrifugation at 6000 rpm for 5 min at 4 °C. The cell pellet was washed twice with 50 mM phosphate buffer, pH 7.5, and recentrifuged. The washed pellet was resuspended in 50 mM phosphate buffer, pH 7.5, containing 10% acetone to give 0.5 g wet weight/ml. The cell suspension was lysed by sonication on ice three times for 30 s with holding on ice between sonications. Cell debris was removed by centrifugation at 10,000 rpm for 15 min. The supernatant (cell-free extract) was decanted and used for enzyme assays. Cell-free extracts were assayed in duplicate in a total volume of 770 μl (70 μl of cell-free extract, 700 μl of 50 mM phosphate buffer containing 10% acetone inclusive of required substrate) at room temperature on an Ultraspec 3000 spectrophotometer (Pharmacia Biotech). Catechol 2,3-dioxygenase and catechol 1,2-dioxygenase were assayed continuously for 180 s at 375 nm with 10 mM catechol as a substrate, and the latter enzyme was assayed after inactivation of catechol 2,3-dioxygenase with the addition of 0.01% hydrogen peroxide. Benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase were assayed continuously for 360 s at 340 nm with 7.5 μM NAD (Boehringer–Mannheim) and 8 μM benzyl alcohol (BDH) or 1.2 μM benzaldehyde (BDH), respectively. Boiled enzyme controls were run in parallel. Enzyme activity results are expressed as micromole product produced per minute per milligram of total protein.

Isolation of plasmid DNA. Plasmid DNA was isolated from 7/167 and 8/46 using established protocols [22,23]. DNA collected using the methods was analyzed for presence of plasmid and chromosomal DNA by electrophoresis of DNA on a 1% (w/v) agarose gel in TAE buffer with and without restriction endonuclease digestions of the DNA with *Xho*I and *Sma*I restriction enzymes (Boehringer–Mannheim) [24].

Results

Isolate identification

Antarctic isolates 7/167 and 8/46 were Gram negative, motile rods, 1–2 μm long, and 1 μm wide. Bacterial colonies were large, mucoid, and creamy yellow in color. Isolates tested were aerobic, oxidase positive, used arginine as a carbon source, and did not produce indole and were identified as *Pseudomonas* species.

Growth characteristics

Antarctic isolates 7/167 and 8/46 were able to grow on toluene vapor or *meta*-toluate as a sole carbon source over a temperature range of 6–30 °C. The optimum growth temperature ranged between 20 and 25 °C for 7/167 and appeared to be 15 °C for 8/46, though this determination requires further study (MSc Thesis Phillippa Rhodes, The University of Waikato, 1999). Neither isolate could grow at 35 °C but both were able to grow at 30 °C. Carbon-free controls showed no growth of isolates at any temperature, indicating isolates were growing at the expense of *meta*-toluate as a carbon source.

Substrate specificity of isolates

Both isolates were able to grow on JP8 jet-fuel and a wide range of pure hydrocarbons typical of those which occur in JP8, including mono- and diaromatic compounds and linear alkanes (Table 1). The isolates were able to utilize similar compounds except that isolate 8/46 degraded benzene and 7/167 did not, whereas isolate 7/167 was able to grow on 2-methylnaphthalene and 8/46 did not.

Isolation and size determination of plasmid DNA

Plasmid DNA was isolated from 7/167 and 8/46. Isolate 7/167 showed a DNA pattern indicative of more than one plasmid being isolated. Isolate 8/46 showed a more typical plasmid DNA pattern of a single plasmid. Digestion of 7/167 and 8/46 plasmid DNA with *Xho*I and *Sma*I indicated that each of the isolates contained at least one large plasmid (>64 kb) (MSc Thesis Phillippa Rhodes, The University of Waikato, 1999).

PCR amplification of a toluene degradative gene

PCR analysis of 7/167 and 8/46 total DNA resulted in the amplification of the *xylB* DNA fragments of similar size generated from the *xylB* positive control *P. putida* mt-2. Nucleotide sequence alignment of the 7/167 and

Table 1
Substrate specificities of toluene-degrading bacteria isolated from soil around Scott Base, Antarctica (+ means growth; − means no growth)

Hydrocarbon(s) tested for growth	Isolate	
	7/167	8/46
Substrate specificity of isolates		
JP8	+	+
Monoaromatics		
Benzene	−	+
Toluene	+	+
o-Xylene	−	−
m-Xylene	+	+
p-Xylene	+	+
1,2,4-Trimethylbenzene	+	+
Ethylbenzene	−	−
Polyaromatic		
Naphthalene	−	−
1-Methylnaphthalene	+	+
2-Methylnaphthalene	+	−
Phenanthrene	−	−
Linear alkanes		
Heptane	+	+
Undecane	+	+
Tridecane	+	+
Dodecane	+	+

Table 2
Enzyme activities of the cell-free extracts of Antarctic isolates 7/167 and 8/46

Growth phase of isolate	Isolate 7/167		Isolate 8/46	
	Early log phase	Late log phase	Early log phase	Late log phase
Enzymes assayed				
Catechol 2,3-dioxygenase	0.238 ± 0.031	0.16 ± 0.011	0.138 ± 0.057	0.075 ± 0.010
Benzyl alcohol dehydrogenase	0.0017 ± 0.0019	0.0011 ± 0.002	0.0031 ± 0.002	0.0008 ± 0.002
Benzaldehyde dehydrogenase	0.0031 ± 0.002	0.0008 ± 0.002	0.0012 ± 0.0002	0.0012 ± 0.0002

Number of replicates, 4. Relative specific activity in μmol/min/mg total protein ± standard deviation.

8/46 *xylB* PCR fragments revealed 100% and 92% sequence homology, respectively, to the *xylB* gene encoded by the pWWO plasmid of *P. putida* mt-2. When amplified with the *xylE* primers only 7/167 DNA produced a PCR product the same size as positive control *P. putida* mt-2 DNA, although not enough of the PCR product was produced to give a definitive DNA sequence. Isolate 8/46 DNA was not amplified with the *xylE* primers. In neither of the isolates was DNA amplified with the *xylC* primers, whereas the positive control *P. putida* mt-2 DNA produced a PCR fragment of the expected size. In all cases negative controls (without DNA) showed no PCR products.

Toluene degradative enzyme assays

Cell-free extracts from 7/167 and 8/46 had catechol 2,3-dioxygenase activity when assayed with catechol as a substrate (Table 2) and showed no catechol 1,2-dioxygenase activity. Benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase assay results confirm the presence and activity of both these enzymes in cell-free extracts of both Antarctic isolates (Table 2); benzyl alcohol dehydrogenase was present in 7/167 significantly only in the late log phase of growth. Overall, the cell-free extracts of both isolates showed higher enzyme activity with catechol as a substrate than with benzyl alcohol dehydrogenase or benzaldehyde dehydrogenase. No enzyme activity was observed in the boiled enzyme controls.

Discussion

The Antarctic isolates in this study are able to degrade toluene at low temperatures. The isolates, identified as belonging to the genus *Pseudomonas*, were isolated from fuel-contaminated Antarctic soils and are potential candidates for development of a bioremediation program to decontaminate these soils. The bioremediation of Antarctic soils requires not only the use of indigenous organisms but requires the organism to metabolize the contaminant to non-toxic by-products under extreme environmental conditions. The isolates in this study are able to metabolize *meta*-toluate (an acid derivative of toluene) at temperatures similar to temperatures that occur in the surface (−4.7 to 17.8 ° C) and subsoil (7.5 °C) at Scott Base, Antarctica [25]. As both isolates are able to grow at 6 °C it is feasible that they would be able to metabolize toluene in the soils at Scott Base from where they were originally isolated. The organisms are deemed psychrotolerant, defined here as cold tolerant bacteria capable of growth at temperatures at or less than 7 °C [26]. The enhanced numbers of culturable Antarctic hydrocarbon-degrading bacteria in oil-contaminated soil compared to pristine soils [8] also suggest that hydrocarbon degraders are capable of growth on oil components in Antarctic soils.

Confirmation of the presence of *xyl* genes, by sequence analysis PCR products amplified from 7/167 and 8/46 DNA, showed the sequence of the *xylB* fragment of 7/167 and 8/46 to be 100% (over 224 bp) and 92% (over 86 bp) homologous, respectively, to a 240 bp region of *xylB* encoded by the TOL plasmid, pWWO, of *P. putida* mt-2. The DNA in neither isolate had homology to the *xylC* gene of pWWO, and only 7/167 showed a PCR fragment of the same size as the fragment amplified from pWWO with *xylE* primers. A TOL plasmid was isolated from a *Pseudomonas* strain that carried genes for two non-homologous *meta*-cleavage dioxygenases [27]. Although the Antarctic isolates use the *meta*-cleavage pathway enzymes to degrade toluene they do not have complete homology to the *xyl* genes and perhaps contain different versions of these genes.

Given that the isolates under investigation are active at low temperatures and are found naturally occurring in the soil at Scott Base, an understanding of how they metabolize toluene would provide further information of their potential to clean up contaminated soil in Antarctica. Toluene degradation by pseudomonads is generally encoded by the TOL group of plasmids and involves degradation of toluene through the *meta*-cleavage pathway to acetaldehyde and pyruvate [17]. Psychrotolerant *Pseudomonas* spp. able to degrade toluene have been isolated from other cold climate soils. Study of these isolates showed that they exhibited components of the *meta*-cleavage toluene degradation pathway, more specifically the presence of a *xylE* gene [28] and activity of its product, catechol 2,3-dioxygenase [29]. When assayed for the presence of *meta*-cleavage pathway enzymes, both Antarctic isolates 7/167 and 8/46

demonstrated such activity. Enzyme assay results from 7/167 and 8/46 cell-free extracts revealed that both benzyl alcohol dehydrogenase (*xylB* gene) and benzaldehyde dehydrogenase (*xylC* gene) are induced and active in the *meta*-toluate grown isolates. Enzyme assay results of two ring cleavage enzymes indicated that the *meta*-ring cleavage enzyme catechol 2,3-dioxygenase, but not the *ortho*-ring cleavage enzyme catechol 1,2-dioxygenase, is active in these isolates.

The *xyl* genes of the *meta*-cleavage toluene degradation pathway are typically plasmid encoded [17]. Large metabolic plasmids (>64 kb) were isolated from both Antarctic isolates. The 7/167 presumptive plasmid DNA isolated from a cesium gradient exhibited an atypical plasmid pattern with four distinct bands of DNA observed when submitted to electrophoresis on an agarose gel, possibly indicative of multiple plasmids. *Pseudomonas* strains harboring multiple plasmids have been discovered [30]. A psychrotolerant hydrocarbon-degrading *Pseudomonas* species was isolated from the Arctic that contained a naphthalene- and an alkane-degrading plasmid [18]. It is possible that isolate 7/167 had two degradative plasmids that would make it a good potential candidate for use in bioremediation, although further investigation of this isolate is needed. Given the presence of large plasmids together with *xylB* gene homology and the activity of *meta*-cleavage pathway enzymes in these two psychrotolerant Antarctic *Pseudomonas* species, 7/167 and 8/46, it is likely that they degrade toluene through a pathway analogous to the toluene degradative pathway encoded by the TOL plasmid, pWWO, in the mesophilic bacterium *P. putida* mt-2. These Antarctic toluene-degrading isolates can metabolize toluene at temperatures similar to those that occur in soils at Scott Base, Antarctica, over the summer months. These isolates therefore have the potential to be used for bioremediation to clean-up JP8 jet-fuel-contaminated sites at Scott Base, Antarctica.

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