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Expression of alkane monooxygenase (*alkB*) genes by plant-associated bacteria in the rhizosphere and endosphere of Italian ryegrass (*Lolium multiflorum* L.) grown in diesel contaminated soil

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Bacterial alkane degradation genes are expressed in the rhizosphere and in the plant interior.

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ABSTRACT

For phytoremediation of organic contaminants, plants have to host an efficiently degrading microflora. To assess the role of endophytes in alkane degradation, Italian ryegrass was grown in sterile soil with 0, 1 or 2% diesel and inoculated either with an alkane degrading bacterial strain originally derived from the rhizosphere of Italian ryegrass or with an endophyte. We studied plant colonization of these strains as well as the abundance and expression of alkane monooxygenase (*alkB*) genes in the rhizosphere, shoot and root interior. Results showed that the endophyte strain better colonized the plant, particularly the plant interior, and also showed higher expression of *alkB* genes suggesting a more efficient degradation of the pollutant. Furthermore, plants inoculated with the endophyte were better able to grow in the presence of diesel. The rhizosphere strain colonized primarily the rhizosphere and showed low *alkB* gene expression in the plant interior.

1. Introduction

Phytoremediation combines the use of plants and their associated microorganisms to degrade toxic organic contaminants (Pilon-Smits, 2005). The efficiency of a phytoremediation process depends largely on the presence and activity of the plant-associated microflora carrying degradation genes required for enzymatic break-down of organic pollutants. The rhizosphere as well as the plant apoplast have been reported to host degrading bacteria (Siciliano et al., 2001), although comparably little is known about degradation activities of endophytes (Newman and Reynolds, 2005). Endophytes are defined as bacteria that reside within plant tissue without conferring pathogenicity and frequently show plant growth-promoting activities (Sessitsch et al., 2004; Ryan et al., 2008). There is increasing interest in the role of endophytes in phytoremediation applications as they can serve as vector delivering biodegradative capacities inside the plant (Barac et al., 2004). However, it is not fully understood, whether potentially degrading endophytes are active once inside the plant and thereby contribute to the detoxification of the pollutant and better survival of the plant under toxic conditions.

Highly diverse alkane degraders containing alkane monooxygenase genes (*alkB*) have been isolated from the plant environment (van Beilen et al., 2002; Hamamura et al., 2005; Kaimi et al., 2007). The abundance of *alkB* genes has been assessed in the rhizosphere and in the root interior (Siciliano et al., 2001; Whyte et al., 2002), but *alkB* gene expression under natural conditions has been rarely addressed (Powell et al., 2006).

The aim of this study was to analyze plant colonization and activities of two strains, the endophyte *Pseudomonas* sp. strain ITRI53 and the rhizosphere strain *Rhodococcus* sp. ITRH43, isolated from Italian ryegrass (Andria et al., submitted for publication). This study shows efficient plant colonization by the endophyte and high *alkB* gene expression inside the plant, particularly in the presence of high diesel oil concentrations.

2. Materials and methods

2.1. Bacterial strains and plant experiment

Pseudomonas sp. strain ITRI53 and *Rhodococcus* sp. strain ITRH43 were isolated from the root interior and the rhizosphere of Italian ryegrass, respectively. Both strains have the capacity to degrade alkanes and contain identical or highly similar alkane monooxygenase (*alkB*) gene sequences (Andria et al., submitted for publication). Strains were cultivated in 10% Luria Bertani broth amended with 1% (v/v) filter-sterilized diesel at 30 °C. Cells were harvested by centrifugation and resuspended in sterile 0.9% NaCl solution.

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For the plant experiment Magenta boxes were filled with 72.5 g air-dried soil mixed with 40% (v/v) sand and sterilized by 30 kGy γ-radiation. Before sowing, the soil was amended with either 0%, 1% or 2% (v/v) filter-sterilized diesel and then mixed with 25 ml inoculant suspension (app. 10^8 CFU/ml) containing either strain ITRI53, strain ITRH43 or sterile 0.9% NaCl solution. Control treatments with the amendment of diesel but without bacterial inoculation were included. Seeds of Italian ryegrass were surface-sterilized in a 20% (v/v) NaOCl for 10 min, washed 5 times with sterile water and were germinated on sterile filters at 25 °C. Twenty plantlets were placed in each box and each treatment was triplicated. Plants were grown at 25 °C in a sterile environment and subjected to a cycle of 16 h light and 8 h dark for 2 months. Plants were watered with equal amounts of sterile water.

2.2. Sampling and extraction of DNA and RNA

After 2 months, shoots were cut from 2 cm above soil and the remaining plants were harvested to obtain root and rhizosphere samples. Rhizosphere soil was collected by gently sampling the soil closely attached to the root surface. Subsequently, the roots were washed several times in sterile water. Roots and shoots (1 g) were surface-sterilized as described by Reiter et al. (2003), replacing distilled sterile water by DEPC-treated water. Sterility was checked by plating on Tryptic Soy Agar plates (TSA, Merck).

DNA from rhizosphere was extracted by using FastDNA Spin Kit for soil (Qbiogene), whereas RNA was isolated with the FastRNA Pro Soil-Direct Kit (MP Biomedicals). Roots and shoots were briefly ground in liquid N_2 and microbial cells were disrupted by bead-beating (Reiter et al., 2003). For isolation of DNA and RNA the DNeasy Plant Mini Kit and RNeasy Plant Mini Kit (Qiagen) were used. In RNA preparations genomic DNA was eliminated by DNase I enzyme (Ambion) digestion and potential presence of contaminating DNA was checked by PCR amplification of 16S rDNA (Rasche et al., 2006).

2.3. Abundance of inoculant strains

Surface-sterilized roots and shoots (1 g) were cut into small pieces. Rhizosphere soil as well as shoot and root pieces were suspended in 2 ml of 0.9% (w/v) NaCl solution and shaken at 180 rpm for 30 min. After plant and soil particles settled, the aqueous phase (100 μL) of 10^{-3} dilutions was plated on 10% TSA in duplicates and incubated at 30 °C for 2 days to determine CFU/g dry soil or plant material. Ten colonies were randomly picked and the identity of isolates with the inoculant strain was confirmed by restriction fragment length polymorphism (RFLP) analysis of the 16S–23S rRNA intergenic spacer region (IGS) (Rasche et al., 2006). Similarly, *alkB* genes were amplified (Whyte and Greer, 1996), digested with AluI (Invitrogen), electrophoresed on 1.5% agarose gels and compared with the profiles obtained from the inoculant strains. Isolates and inoculant strains had identical restriction patterns.

2.4. Quantitative analysis of the abundance and expression of alkB genes

Reverse transcription was performed with 10–20 ng RNA, the specific primer PpalkB-for (Bustin, 2000) and Superscript II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Abundance and expression of *alkB* genes were quantified by real-time PCR using an iCycler IQ (Biorad). Specific qRT-PCR primers were designed based on the conserved region within the *alkB* of the inoculant and closely related strains (Genbank accession number FJ014898–FJ014915, AJ233397, AJ344083, AJ250560 and AY034587). The specificity of the designed primers RTalkB-f (5′-ATCCGCCTGAGGAAGTAGTG-3′) and RTalkB-r (5′-CGGCCACTTCTTTATTGAGC-3′) was checked by using Primer 3 and BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Standards for qRT-PCR were generated by serial dilution of stocks containing purified PpalkB PCR products from strain ITRI53 DNA. Analyses were performed in triplicates and gene copy numbers were calculated as described by Powell et al. (2006). Reaction mixtures (25 μl) contained 12.5 μl of Q Mix (Biorad), 2 μl 10 mg/ml BSA, 0.8 μl DMSO, 0.5 μl 2 μM of each primer, 25–50 ng of DNA/cDNA template and RNase-free water. Thermal cycling conditions were: 4 min at 94 °C followed by 40 cycles of 94 °C for 20 s, 60 °C for 30 s, 72 °C for 45 s followed by a melt curve from 50 to 100 °C. Besides melt curve analysis, PCR products were examined on 2% agarose gels. No primer-dimers were detected. Serial dilutions of DNA and cDNA were spiked with 10^6 copies of amplified *alkB* genes to check for real-time PCR inhibition (López-Gutiérrez et al., 2004). Highly linear standard curves (r^2 values > 0.95, PCR efficiency > 98%) over the dilution range and a detection limit of 10^1 copies were obtained indicating no PCR inhibition. The *alkB* gene copy numbers were quantified relative to a standard curve of positive control and were normalized to the copy number of control plants. Statistical analysis was based on Least Significant Difference tests using SAS software (SAS Institute Inc., Cary, NC, USA) and regression analysis using Microsoft Excel.

3. Results and discussion

Colonization of alkane degrading inoculant strains was determined by cultivation and a cultivation-independent approach. Both approaches clearly showed that strain ITRI53, originally isolated from

Table 1
Mean values of Colony Forming Unit (CFU), *alkB* genes abundance and *alkB* genes expression in rhizosphere (coded RH), root interior (coded RI) and shoot interior (coded SI) of italian ryegrass inoculated either by endophytic bacteria *Pseudomonas* sp. ITRI53 or by rhizosphere bacteria *Rhodococcus* sp. ITRH43 in association with diesel amendment 0%, 1% and 2%. The standard error of the distribution is presented in parentheses.

Diesel (v/v)	CFU/g dry weight[a]			*alkB* genes abundance[b] (copies/g dry weight)			*alkB* genes expression[c] (copies/g dry weight)			Ratio *alkB* expression/abundance[d]		
	RH	RI	SI	RH	RI	SI	RH	RI	SI	RH	RI	SI
Bacterial inoculant: *Pseudomonas* sp. ITRI53												
0%	2.4E+05^{A} (1.0E+05)	3.8E+06^{B} (9.8E+04)	5.0E+07^{B} (3.5E+05)	1.2E+04^{AB} (6.8E+029	1.0E+04^{A} (9.8E+02)	1.3E+02^{B} (3.5E+01)	8.7E+03^{A} (6.0E+02)	4.7E+03^{B} (9.3E+01)	8.9E+01^{A} (2.7E+01)	0.7^{A}	0.5^{A}	0.5^{A}
1%	1.3E+06^{A} (2.9E+05)	8.0E+06^{B} (4.4E+05)	4.6E+07^{B} (9.8E+05)	1.8E+05^{A} (1.7E+03)	1.4E+04^{B} (4.4E+03)	9.3E+02^{C} (9.8E+01)	1.3E+05^{A} (9.0E+039	1.2E+04^{A} (3.4E+03)	1.0E+03^{B} (9.2E+01)	0.7^{A}	0.8^{A}	1.1^{B}
2%	8.1E+05^{A} (6.8E+05)	1.0E+07^{B} (3.5E+06)	2.4E+07^{C} (6.6E+05)	1.6E+05^{A} (3.8E+03)	2.1E+04^{B} (3.5E+03)	8.6E+02^{C} (6.6E+01)	9.6E+04^{A} (1.1E+04)	1.7E+04^{A} (6.4E+02)	1.2E+03^{B} (6.3E+01)	0.6^{A}	0.8^{A}	1.3^{B}
Bacterial inoculant: *Rhodococcus* sp. ITRH43												
0%	2.0E+06^{A} (4.6E+05)	1.8E+06^{A} (1.2E+05)	3.2E+02^{B} (1.0E+01)	2.6E+04^{A} (9.8E+01)	2.92E+02^{B} (1.2E+02)	6.16E+01^{C} (1.2E+01)	9.2E+03^{A} (1.6E+02)	2.0E+02^{A} (5.2E+00)	6.5E+01^{B} (4.9E+01)	0.4^{A}	0.2^{A}	1.1^{B}
1%	7.1E+06^{A} (6.8E+05)	6.0E+04^{B} (0.1E+00)	4.6E+02^{C} (1.3E+01)	1.1E+05^{A} (6.8E+03)	9.0E+02^{B} (6.9E+01)	6.48E+01^{B} (7.29E+00)	5.2E+04^{A} (5.2E+02)	1.3E+02^{A} (3.9E+01)	6.2E+01^{B} (3.2E+01)	0.5^{A}	0.5^{A}	1.0^{B}
2%	8.0E+06^{A} (4.3E+05)	6.0E+06^{B} (4.8E+05)	2.6E+06^{C} (5.9E+05)	1.2E+05^{A} (1.1E+04)	1.1E+04^{A} (1.2E+03)	6.4E+02^{B} (5.9E+01)	1.9E+05^{A} (5.1E+03)	1.3E+03^{B} (9.9E+01)	1.3E+03^{B} (2.2E+02)	1.6^{A}	0.1^{B}	2.0^{A}

Significant difference test compared mean values for each parameter in different plant environments (RH, RI and SI) in certain diesel concentration (read within rows). Means followed by the same letters are not significantly different according to Least Significant Difference test ($P < 0.05$).

[a] Colony Forming Unit per gram dry weight of environmental samples.

[b] Gene copy number per gram dry weight of environmental samples resulted from q-RT PCR of extracted DNA. PCR efficiency 98% for *Pseudomonas* sp. and *Rhodococcus* sp.

[c] Gene copy number per gram dry weight of environmental samples resulted from q-RT PCR of reverse-transcribed extracted mRNA. PCR efficiency 95% for *Pseudomonas* sp. and *Rhodococcus* sp.

[d] Ratio between reverse-transcribed mRNA and DNA gene copy number per gram weight of environmental samples.

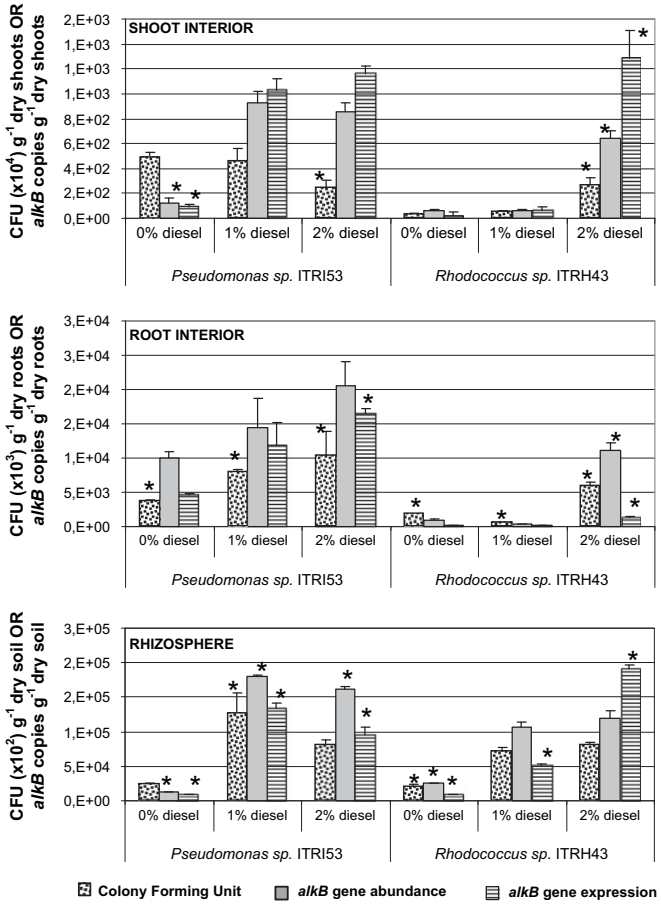

Fig. 1. Mean values of Colony Forming Unit (CFU), *alkB* genes abundance and *alkB* genes expression of endophyte *Pseudomonas* sp. ITRI53 and rhizosphere bacteria *Rhodococcus* sp. ITRH43 at rhizosphere, root and shoot interior of Italian ryegrass, in presence of 0%, 1% and 2% of diesel. Error bars represent standard error. The asterisk (*) shows significant difference between means for every parameter (CFU, *alkB* abundance and *alkB* expression) in different diesel concentration for the same inoculant. Means followed by the same letters are not significantly different according to Least Significant Difference test ($P < 0.05$).

the root interior of Italian ryegrass, better colonized all plant compartments than strain ITRH43, originally isolated from the Italian ryegrass rhizosphere (Table 1, Fig. 1). Primarily the plant interior without or at low diesel concentrations was better colonized by strain ITRI53, whereas differences in the rhizosphere or in plants grown in 2% diesel were less pronounced. This is in agreement with Rosenblueth and Martínez-Romero (2006), who postulated that endophytes are generally better able to colonize plant tissues than rhizosphere isolates. Strain ITRH43 entered the plant at certain conditions, however, endophyte strain ITRI53 was better adapted to grow in the plant interior. The diesel concentration influenced endophytic plant colonization, which was most efficient in the presence of high (2%) diesel concentrations. As the experiment was performed under sterile conditions, these observations cannot be explained by competition effects. Rhizosphere colonization of strain ITRI53 in the absence of a selective pressure was low, but endophytic colonization was better, which might be due to the presence of alkanes potentially produced by Italian ryegrass (Marseille et al., 1999).

The comparison of results obtained by cultivation and by quantifying *alkB* gene copies suggests that with increasing diesel concentration the number of culturable bacteria decreased. It might be that due to the stress conditions encountered the number of viable-but-non-culturable bacteria increased. Similarly, *Pseudomonas frederiksbergensis* cells entered a viable-but-non-culturable state in a mercury contaminated soil (Johnsen et al., 2003).

Table 2
Average number of grown plantlets after 2 months ($n = 3$).

Bacterial inoculant	No diesel	Diesel 1%	Diesel 2%
Pseudomonas sp. ITRI53	20	20	16
Rhodococcus sp. ITRH43	20	15	9

We observed that plants inoculated with strain ITRI53 showed better growth and survival in the presence of diesel. All (20) plants were able to grow in un-polluted soil, whereas less plantlets were able to grow in the presence of diesel (Table 2). Only 15 and 9 plants inoculated with strain ITRH43 survived in the presence of 1% and 2%, diesel, respectively, but 20 and 15 plants, respectively, survived when inoculated with strain ITRI53. Diesel oil is toxic to plants even at low levels (Adam and Duncan, 2003) and strain ITRI53 protected plants more efficiently than strain ITRH43. It might be that strain ITRI53, which belongs to the genus *Pseudomonas*, increased stress tolerance in the plant as many pseudomonads have been identified so far with plant growth-promoting properties (Compant et al., 2005). Furthermore, strain ITRI53 colonized plants better and might have degraded alkanes more efficiently and thereby protected the plants.

Both strains principally expressed *alkB* genes indicating an active role in the degradation of the pollutant and *alkB* gene expression was found in all compartments (Table 1, Fig. 1). The differences between strains ITRI53 and ITRH43 in regard to *alkB* gene expression followed essentially the same patterns as *alkB* abundance data. However, both strains showed higher *alkB* gene expression in relation to *alkB* gene abundance in the shoot interior than in the root interior indicating that root and shoot metabolites influenced *alkB* gene expression. It appears that the endophyte strain maintained its degrading activity in the rhizosphere and in the plant interior independently from the diesel concentration, whereas the rhizosphere strain needed a higher diesel concentration as selective pressure to maintain its degradation activity within plant tissues, but not in the rhizosphere. However, for both inoculants, *alkB* gene abundance and expression were the highest in rhizosphere (Table 1) indicating that root exudates provide nutrients for bacterial growth and co-metabolites for alkane degradation (Olson et al., 2003). In conclusion, we showed that bacterial *alkB* genes can be expressed not only in the rhizosphere but also *in planta*. The endophyte strain tested was superior than the rhizosphere strain regarding colonization and *alkB* gene expression. Furthermore, inoculation of the endophyte strain resulted in better survival of plants which might be due to plant growth-promoting and/or alkane degrading activities. Such differences in endophytic populations might be an important factor that could help to explain the huge differences among plant species and even cultivars in sensitivity/tolerance to hydrocarbon contaminants in soils. Further studies will address the application potential of endophytes in phytoremediation applications.

References

Adam, G., Duncan, H., 2003. The effect of diesel fuel on common vetch (*Vicia sativa* L.) plants. Environmental Geochemistry and Health 25, 123–130.

Andria, V., Yousaf, S., Reichenauer, T.G., Smalla, K., Sessitsch, A. Phylogenetic and functional diversity of alkane degrading bacteria associated with Italian ryegrass (*Lolium multiflorum*) and Birdsfoot trefoil (*Lotus corniculatus*) in a petroleum oil-contaminated environment, submitted for publication.

Barac, T., Taghavi, S., Borremans, B., Provoost, A., Oeyen, L., Colpaert, J.V., Vangronsveld, J., van der Lelie, D., 2004. Engineered endophytic bacteria improve phytoremediation of water-soluble, volatile, organic pollutants. Nature Biotechnology 22, 583–588.

Bustin, S.A., 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. Journal of Molecular Endocrinology 25, 169–193.

Compant, S., Duffy, B., Nowak, J., Clément, C., Barka, E.A., 2005. Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. Applied and Environmental Microbiology 71, 4951–4959.

Hamamura, N., Olson, S.H., Ward, D.M., Inskeep, W.P., 2005. Diversity and functional analysis of bacterial communities associated with natural hydrocarbon seeps in acidic soils at Rainbow Springs, Yellowstone National Park. Applied and Environmental Microbiology 71, 5943–5950.

Johnsen, K., Ekelund, F., Binnerup, S.J., Rasmussen, L.D., 2003. Mercury decreases culturability of *Pseudomonas frederiksbergensis* JAJ 28 in soil microcosms. Current Microbiology 47, 0125–0128.

Kaimi, E., Mukaidani, T., Tamaki, M., 2007. Screening of twelve plant species for phytoremediation of petroleum hydrocarbon-contaminated soil. Plant Production Science 10, 211–218.

López-Gutiérrez, J.C., Henry, S., Hallet, S., Martin-Laurent, F., Catroux, G., Philippot, L., 2004. Quantification of a novel group of nitrate-reducing bacteria in the environment by real-time PCR. Journal of Microbiological Methods 57, 399–407.

Marseille, F., Disnar, J.R., Guillet, B., Noack, Y., 1999. n-Alkanes and free fatty acids in humus and A1 horizons of soils under beech, spruce and grass in the Massif-Central (Mont-Lozère), France. European Journal of Soil Science 50, 433–441.

Newman, L.A., Reynolds, C.M., 2005. Bacteria and phytoremediation: new uses for endophytic bacteria in plants. Trends in Biotechnology 23, 6–8.

Olson, P.E., Reardon, K.F., Pilon-Smits, E.A.H., 2003. Ecology of rhizopshere bioremediation. In: McCutcheon, S.C., Schnoor, J.L. (Eds.), Phytoremediation: Transformation and Control of Contaminants. John Wiley & Sons, New Jersey, pp. 317–353.

Pilon-Smits, E.A.H., 2005. Phytoremediation. Annual Review of Plant Biology 56, 15–39.

Powell, S.M., Ferguson, S.H., Bowman, J.P., Snape, I., 2006. Using real-time PCR to assess changes in the hydrocarbon-degrading microbial community in Antarctic soil during bioremediation. Microbial Ecology 52, 523–532.

Rasche, F., Velvis, H., Zachow, C., Berg, G., van Elsas, J.D., Sessitsch, A., 2006. Impact of transgenic potatoes expressing anti-bacterial agents on bacterial endophytes is comparable with the effects of plant genotype, soil type and pathogen infection. Journal of Applied Ecology 43, 555–566.

Reiter, B., Wermbter, N., Gyamfi, S., Schwab, H., Sessitsch, A., 2003. Endophytic *Pseudomonas* spp. populations of pathogen-infected potato plants analysed by 16S rDNA- and 16S rRNA-based denaturating gradient gel electrophoresis. Plant and Soil 257, 397–405.

Rosenblueth, M., Martínez-Romero, E., 2006. Bacterial endophytes and their interactions with hosts. Molecular Plant–Microbe Interactions 19, 827–837.

Ryan, R.P., Germaine, K., Franks, A., Ryan, D.J., Dowling, D.N., 2008. Bacterial endophytes: recent developments and applications. FEMS Microbiology Letters 278, 1–9.

Sessitsch, A., Reiter, B., Berg, G., 2004. Endophytic bacterial communities of field-grown potato plants and their plant-growth-promoting and antagonistic abilities. Canadian Journal of Microbiology 50, 239–249.

Siciliano, S.D., Fortin, N., Mihoc, A., Wisse, G., Labelle, S., Beaumie, D., Ouellette, D., Roy, R., Whyte, L.G., Banks, M.K., Schwab, P., Lee, K., Greer, C.W., 2001. Selection of specific endophytic bacterial genotypes by plants in response to soil contamination. Applied and Environmental Microbiology 67, 2469–2475.

van Beilen, J.B., Smits, T.H.M., Whyte, L.G., Schorcht, S., Röthlisberger, M., Plaggemeier, T., Engesser, K.H., Witholt, B., 2002. Alkane hydroxylase homologues in Gram-positive strains. Environmental Microbiology 4, 676–682.

Whyte, L.G., Greer, C.W., 1996. Assessment of the biodegradation potential of psychrotrophic microorganisms. Canadian Journal of Microbiology 42, 99–106.

Whyte, L.G., Schultz, A., van Beilen, J.B., Luz, A.P., Pellizari, V., Labbe, D., Greer, C.W., 2002. Prevalence of alkane monooxygenase genes in Arctic and Antarctic hydrocarbon-contaminated and pristine soils. FEMS Microbiology Ecology 41, 141–150.