Journal of Applied Microbiology 2002, **92**, 1058–1065

Survival and naphthalene-degrading activity of *Rhodococcus* sp. strain 1BN in soil microcosms

L. Cavalca[1], M. Colombo[1], S. Larcher[1], C. Gigliotti[2], E. Collina[3] and V. Andreoni[1]

[1]Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche and [2]Dipartimento di Produzione Vegetale, Università degli Studi di Milano and [3]Dipartimento di Scienze dell'Ambiente e del Territorio, Università degli Studi di Milano-Bicocca, Milano, Italy

2001/164: received 3 July 2001, revised 11 December 2001 and accepted 1 February 2002

L. CAVALCA, M. COLOMBO, S. LARCHER, C. GIGLIOTTI, E. COLLINA AND V. ANDREONI. 2002.

Aims: The survival and activity of *Rhodococcus* sp. strain 1BN, inoculated into naphthalene-contaminated sandy-loam soil microcosms, were studied using classical and molecular methods.

Methods and Results: The naphthalene-degrading activity of 1BN in microcosms was examined through viable counts, CO_2 production and naphthalene consumption, while its survival after inoculation was monitored by detecting the contemporary presence of alkane and naphthalene degradative genes and by analysing the 16S rDNA specific restriction profile. The inoculation of 1BN did not significantly enhance naphthalene degradation in the naphthalene-contaminated native soil, where 1BN maintained its catabolic activity also when in the presence of indigenous microflora. Instead the rate of naphthalene degradation by the inoculated 1BN was greater in sterile naphthalene-contaminated soil. The level of 1BN was only slightly higher after inoculation regardless of whether indigenous naphthalene-degrading bacteria were present or not and 1BN remained viable even when the substrate was depleted.

Conclusions: This study documents the colonization and growth of 1BN in a non-sterile, naphthalene-added, sandy-loam soil having an active indigenous naphthalene-degrading population.

Significance and Impact of the Study: An active and well-established naphthalene-degrading bacterial population in the native soil did not hamper the survival of the introduced 1BN that, through its activity, enhanced the mineralization rate of naphthalene.

INTRODUCTION

The reclamation of polluted soils through *in situ* bioremediation is particularly attractive for both environmental and economic reasons. Decontamination can be achieved by opportunely stimulating indigenous bacteria to degrade the pollutant (i.e. biostimulation) (Margesin and Schinner 1997) or by seeding selected micro-organisms with the desired catabolic traits (bioaugmentation) (Middledorp *et al.* 1990; Miethling and Karlson 1996; Zallo *et al.* 1996; Straube *et al.* 1999).

There have been both successful and failed attempts at demonstrating the potential of bioaugmentation in soils (Vogel 1996): the effects of microbial seeding for the degradation of polycyclic aromatic hydrocarbons (PAH; Straube *et al.* 1999) and 2,4-dichlorophenoxyacetic acid (Roane *et al.* 2001) in soil appear positive while such seeding has proved ineffective in soils contaminated with oil and PAH (Goldstein *et al.* 1985; Aamand *et al.* 1995; Nerella *et al.* 1995; Hughes *et al.* 1997). As degrading strains can be poor survivors, or lose catabolic activity when inoculated into mixed microbial ecosystems (McClure *et al.* 1991; Watanabe *et al.* 1998), bioaugmentation requires inocula that, besides degrading pollutants in pure culture, are also able to survive in non-sterile soil for long periods of time (Schwartz *et al.* 2000). Bioaugmentation has also proved successful when there is the transfer of genetic information from the introduced donor strain to the competitive, indigenous bacterial population of soil (Akkermans 1994; Focht *et al.* 1996); such transfer represents a valuable approach to broadening the soil biodegradation potential.

Correspondence to: V. Andreoni, Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, Universita` degli Studi di Milano, via Celoria 2, 20133 Milano, Italy (e-mail: vincenza.andreoni@unimi.it).

It is apparent that for successful bioaugmentation there is the need to identify, and properly manage, the environmental conditions controlling the survival and activity of introduced micro-organisms. Microcosms, currently used to study biodegradation and the fate and effect of introduced micro-organisms (Wagner-Döbler *et al.* 1992), can lead to a better understanding of the effect of factors controlling soil microbial inoculation, especially with regard to indigenous micro-organisms and to study on the capability of inoculated micro-organisms to degrade certain chemicals.

The monitoring of introduced and indigenous strains can be achieved using probes based on conserved housekeeping genes (Heyndrickx *et al.* 1996) and catabolic gene probes (Herrick *et al.* 1997). Genetic databases now include not only the well-known genes coding for degradative systems of Proteobacteria but also genes for naphthalene dioxygenase (Larkin *et al.* 1999) and alkane hydroxylase (Smits *et al.* 1999) of Gram-positive bacteria. Such probes have widened the possibility of describing the biodiversity of degrading microbial communities in polluted environments.

The objective of the present work was to evaluate the survival and degrading activity of the *Rhodococcus* sp. strain 1BN (Andreoni *et al.* 2000) inoculated into sandy-loam soil microcosms contaminated with naphthalene. The naphthalene-degrading activity of 1BN was examined in the presence and absence of indigenous naphthalene-degrading bacteria. Specific polymerase chain reaction (PCR) primers for alkane hydroxylase (*alk*1BN) and naphthalene dioxygenase (*nar*A) genes and a 16S rDNA restriction profile analysis were used to monitor the survival of 1BN when introduced into a soil and to distinguish it from native, naphthalene-degrading cultivable bacteria.

MATERIALS AND METHODS

Bacterial strain utilized

1BN was isolated from a contaminated soil and was selected for its ability to utilize several aromatic and aliphatic substrates as carbon and energy sources (Andreoni *et al.* 2000). Naphthalene dioxygenase and *alk*1BN genes, not apparent on any visible plasmid, were found in the strain. Throughout subsequent transfers 1BN was maintained in 20 ml M9 liquid mineral medium (Kunz and Chapman 1981) with 10 mg naphthalene, added in crystalline form, and frequently checked for purity by plating on 10% strength tryptone soy agar medium (0·1× TSA).

Soil samples

The soil samples were collected from a former industrial area near Lodi (Northern Italy) with a 30-year history of industrial pollution. The site presented widespread contamination by both organic and inorganic compounds, the highest concentrations of the former being naphthalene that had been the raw material for phthalic anhydride production.

The soil samples used in the study were collected over a 2-year period: in 1997 soil samples were collected to select hydrocarbon-degrading bacteria and 2 years later to set up soil microcosm experiments. In this latter period, in order to obtain a representative sample, 50 random samples were taken from the top layer (0–10 cm) of an area 200 m from the formerly sampled area. The samples were mixed, placed in polyethylene bags and transported to the laboratory. The soil was sieved (4 mm) and, after determining the water-holding capacity, stored moist at 4°C.

Soil characteristics

The soil pH was measured with a potentiometer in water (1 : 2·5 w/v) and the water content was determined as the weight loss after drying the moist samples in an oven (105°C for 24 h). Soil organic C was determined by wet oxidation with 0·167 mol l^{-1} $K_2Cr_2O_7$ and subsequent titration with 0·1 mol l^{-1} $(NH_4)_2Fe(SO_4)_2.6H_2O$ (Walkley and Black 1934) and total N was determined with Kjeldahl digestion. The N digestion and distillation were performed with Buchi 426 and 315 apparatus, respectively (Buchi Laboratoriums-Technik AG, Zurich, Swizerland). The measurement of exchangeable phosphorus (P_2O_5 exc.), cation exchange capacity (CEC) and soil texture was carried out according to Italian regulations (Ministero delle Politiche Agricole e Forestali 1999). The naphthalene content in the soil was determined as described below in Analytical Procedures. The presence of indigenous heterotrophic and naphthalene-degrading bacteria in native soil was checked through viable counts of these micro-organisms. The microbial cells were separated from the soil particles by adding 27 ml of a sterile extracting solution ($Na_4P_2O_7$, 2 g l^{-1}) to 3 g soil in 250-ml flasks and then incubating on an alternating shaker for 1 h. After a 10-min sedimentation period, appropriate 10-fold dilutions in physiological solution were plated in duplicate onto 0·1× TSA medium for a total heterotrophic micro-organism count. The number of naphthalene-degrading micro-organisms was determined by spreading the dilutions onto a selective medium obtained by the addition of 13 g l^{-1} agar to M9 mineral medium supplemented with 0·1% strength tryptone soy broth (0·001× TSB) and then adding crystalline naphthalene on the internal surface of the plate cover (selective M9-naphthalene agar medium); 100 μg ml^{-1} cycloheximide were then added to the medium to inhibit the growth of eukaryotes. The plates were incubated at 30°C for 10 d and then counted.

Experimental design

The ability of the introduced 1BN to degrade naphthalene was investigated through laboratory microcosm studies.

Six series of different microcosms were prepared using the collected soil: (i) native soil (S) to determine soil basal respiration; (ii) native soil with naphthalene added (S-N) to evaluate the intrinsic ability of the soil to biodegrade naphthalene; (iii) native soil inoculated with 1BN (S-1BN) to determine the basal respiration of this soil system and evaluate the survival of 1BN in the soil; (iv) native soil inoculated with 1BN and with naphthalene added (S-1BN-N) to check whether the strain accelerated naphthalene degradation when competing with indigenous micro-organisms; (v) sterilized native soil inoculated with 1BN (SS-1BN) to determine the basal respiration of this system and (vi) sterilized native soil with both 1BN and naphthalene added (SS-1BN-N) to evaluate the naphthalene degradation rate in the absence of competition from indigenous soil-degrading bacteria.

Five replicates were set up for each microcosm series, three for soil respiration determinations and two for naphthalene consumption, microbial viable counts and soil dry weight determinations.

Preparation of soil microcosms

Microcosms were set up in 1-l hermetically sealed glass bottles. Each microcosm consisted of 80 g native or sterile soil corresponding to 70 g soil dry weight (dw). The sterile soil was sterilized three times by autoclaving 500 g soil for 30 min at 121°C followed by incubation for 24 h at 37°C. Crystalline naphthalene (5 g kg^{-1} dry soil) was added for the soil microcosm when required.

1BN was grown at 30°C in nutrient broth for microcosm seeding. When the culture reached the late logarithmic phase of growth, the cells were collected by centrifugation at 22 000 *g* for 15 min at 4°C, washed twice with sterile physiological solution (NaCl, 9 g l^{-1}) and suspended in sterile distilled water to obtain a cell suspension of approx. $1{\cdot}5 \times 10^9$ colony-forming units (cfu) ml^{-1}. When required, 5 ml of this cell suspension were inoculated into each microcosm, to give approx. $2{\cdot}0 \times 10^8$ cfu g^{-1} dw (Matthias *et al.* 1998), and thoroughly mixed with the soil. The water content of the soil was adjusted to 60% water-holding capacity. The soil pH was not adjusted and no additional nutrients were used.

Microcosms were incubated in the dark at 25°C for 50 d and analysed periodically.

Analytical procedures

Determination of soil naphthalene concentration. Soil (2·5 g) taken from each microcosm was added, in a tube, to 20 ml dichloromethane and 2·5 g anhydrous sodium sulphate. Deuterated naphthalene, used as an internal standard, was added in appropriate amounts in the range 0·5–15 μg. The tube was sealed with a Teflon stopper and held for 1 h in an ultrasonic bath (MT2210; Branson, Milan, Italy) at 47 kHz. The extract was then filtered, reduced and cleaned up on a silica column. The purified extract was analysed with a gas chromatograph (HP 5890; CP-Sil 8 CB column; Chrompack; Milan, Italy) coupled to a mass selective detector (HP 5970). The temperature programme was 1 min at 60°C, 20°C min^{-1} up to 280°C and 4 min at 280°C.

Determination of respiration activity. Respiration activity was determined according to a modified Pochon and Tardieux (1962) method. Each microcosm was equipped with a 50-ml beaker containing 20 ml 1 mol l^{-1} KOH; the evolved CO_2 was trapped in the KOH solution and measured by back-titration with 0·5 mol l^{-1} HCl.

Chemicals

All chemicals were of analytical grade and purchased from Sigma-Aldrich or Merck unless otherwise stated.

Characterization of isolates to estimate the survival of *Rhodococcus* sp. strain 1BN in soil microcosms

The characterization was performed on an appropriate number of colonies (Bianchi and Bianchi 1982) isolated from naphthalene-degrading count plates: (i) from the uninoculated native soil microcosm (S-N), to exclude the presence of indigenous 1BN micro-organisms, and (ii) from the S-1BN-N microcosm at the 10th and 22nd day of the experiment to evaluate the percentage of 1BN on the cultivable naphthalene-degrading bacteria of native soil. After three subsequent transfers of the isolates onto selective M9-naphthalene liquid medium to confirm their ability to grow on the compound, the strains were characterized by 16S rDNA restriction analysis, obtained with *Hha*I enzyme, and by PCR amplification of *nar*A, *ndo*B and *alk*1BN catabolic genes. The molecular characterization of strains in the S-1BN-N microcosm immediately after inoculation was not performed as the cellular density of the introduced 1BN was determined by viable plate count.

DNA extraction

Colonies with an 1BN-like morphology were grown overnight in Rich Medium with added glycine and glycerol according to Yamada and Komagata (1970). The DNA was extracted by sodium dodecyl sulphate–lysozyme lysis, purified by phenol : chloroform : isoamylalcohol (25 : 24 : 1)

treatment and resuspended in tris-EDTA buffer, pH 8·0. DNA extraction of all the other strains isolated from S-1BN-N microcosms, maintained on selective M9-naphthalene agar medium, was conducted by boiling a loop of culture in 500 μl 3 mg ml^{-1} bovine serum albumin for 15 min (Sambrook *et al*. 1989).

16S rDNA restriction analysis

The amplification of small ribosomal subunit genes (16S rDNA) was performed by using P27 forward primer (5′-AGA GTT TGA TCC TGG CTC AG-3′) and P1495 reverse primer (5′-CTA CGG CTA CCT TGT TAC GA-3′) in a reaction volume of 50 μl. The thermal profile, carried out in a PCR Sprint apparatus (Hybaid, Ashford, UK), was as follows: denaturation at 95°C for 3 min, 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 2 min followed by a final extension step at 72°C for 15 min.

An appropriate volume of the amplified products was digested separately with 10 U *Hha*I restriction endonuclease (Pharmacia Applied Biotech, Uppsala, Sweden), according to the manufacturer's instructions. Restriction products were separated on 3% agarose gel, visualized by a standard procedure (Sambrook *et al*. 1989) and photographed (Polaroid MP-4; Polaroid, Cambridge, USA). The fragment size was estimated by a linear regression equation between the molecular mass of a 50-bp DNA ladder (Pharmacia Applied Biotech) and the log of the distance covered by fragments within the same gel run.

Polymerase chain reaction amplification of alkane hydroxylase and naphthalene dioxygenase gene families

Different sets of PCR primers were chosen in order to amplify *alk*1BN and naphthalene dioxygenase genes from either Gram-positive (*nar*A) or Gram-negative (*ndo*B/*nah*AC) strains. The forward and reverse primers of *alk*1BN and *nar*A genes have been reported elsewhere (Andreoni *et al*. 2000) and primers for the amplification of *ndo*B/*nah*AC genes, designed upon the consensus sequence of *Pseudomonas putida* strains (accession nos. M23914 and M83949, respectively), were the following: 5′-CAC TCA TGA TAG CCT GAT TCC TGC CCC CGG CG-3′ for the forward and 5′-CCG TCC CAC AAC ACA CCC ATG CCG CTG CCG-3′ for the reverse.

The following reagents were present in a final volume of 50 μl: 2·0 mmol l^{-1} $MgCl_2$, 0·2 mmol l^{-1} dNTPs (Amersham Pharmacia Biotech, Uppsala, Sweden), 0·2 μmol l^{-1} of each set of primer (Amersham Pharmacia Biotech), 1 U DNA *Taq* polymerase (Bioline, London, UK) and 1 μl extracted DNA. The thermal profile of the PCR amplifications, carried out in a PCR Sprint apparatus (Hybaid), was as follows: denaturation at 94°C for 3 min, 35 cycles at 94°C for 40 s, 55°C for 40 s, 72°C for 1 min 20 s followed by a final extension step at 72°C for 10 min.

RESULTS

Soil characteristics

In accordance with Italian regulations (Ministero delle Politiche Agricole e Forestali), the native soil was characterized by a sandy-loam texture, neutral reaction, good quantity of organic matter, quite high C/N ratio, middle CEC and an adequate amount of P_2O_5 exc. (Table 1). The soil had a naphthalene content of 19 mg kg^{-1} dw and a high number of naphthalene-degrading micro-organisms ($6{\cdot}2 \times 10^6$ cfu g^{-1} dw).

Naphthalene mineralization in the microcosms

The results obtained using the S-N microcosms showed that, after 4 d of incubation, the initial naphthalene content of the soil (5000 mg kg^{-1} dw) was reduced by about 65% (Fig. 1), with concomitant CO_2 production (Fig. 2) and growth of heterotrophic and naphthalene-degrading bacteria of one order of magnitude (Table 2). By the 22nd day of incubation, naphthalene degradation was almost complete and the heterotrophic and naphthalene-degrading bacteria were present at $2{\cdot}8 \times 10^8$ and $1{\cdot}8 \times 10^8$ cfu g^{-1} dw, respectively.

In the S-1BN microcosms, the introduced strain declined slightly within the first 4 d of incubation, from $2{\cdot}6 \times 10^8$ to approx. $5{\cdot}4 \times 10^7$ cfu g^{-1} dw, remaining almost stable up to the 22nd day of the experiment (Table 2). However, after 22 d of incubation, the number of naphthalene-degrading micro-organisms in the S-1BN microcosms was still higher

Table 1 Characteristics of native soil (dw)

Soil texture	pH (H_2O)	Organic carbon (g kg^{-1})	C/N	CEC (cmol(+) kg^{-1})	P_2O_5 exc. (mg kg^{-1})	THM	NDM (cfu g^{-1})
Sandy-loam	7·2	34	14·4	10·5	29	$1{\cdot}3 \times 10^7$	$6{\cdot}2 \times 10^6$

THM, Total heterotrophic micro-organisms; NDM, naphthalene-degrading micro-organisms; CEC, cation exchange capacity; P_2O_5 exc., exchangeable phosphorus; dw, dry weight; cfu, colony-forming units.
Each value is the mean of two determinations.

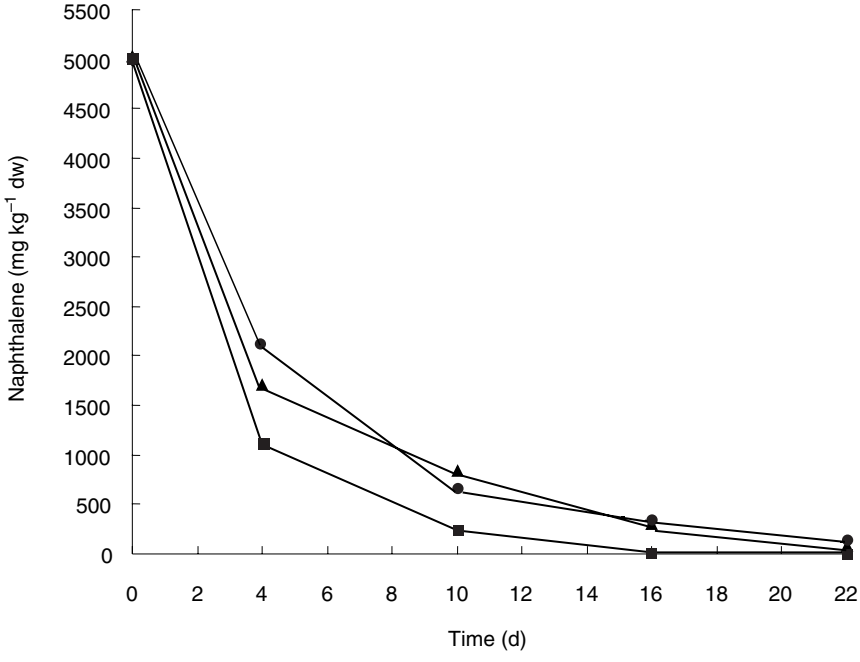

Fig. 1 Naphthalene degradation in soil microcosms (▲, S-N; ● S-1BN-N; ■, SS-1BN-N; see Materials and Methods). Each value is the mean of two determinations

than that observed in the native soil (Tables 1 and 2), evidencing the ability of 1BN to survive in the soil.

The naphthalene consumption in the S-1BN-N microcosms was not significantly different from that measured in the S-N microcosms (Fig. 1). CO_2 production was, however, until the 10th day of incubation, higher in the S-1BN-N than in the S-N microcosms (Fig. 2) then, in correspondence with the almost complete consumption of naphthalene, the CO_2 levels became comparable in the two microcosms. Derivatives of the parent compound were not mineralized to CO_2 in the S-N microcosms as rapidly as in the presence of 1BN, suggesting that the metabolites of naphthalene were probably accumulated longer in the absence of the inoculated strain. The number of naphthalene-degrading bacteria did not change significantly (Table 2).

The naphthalene consumption and CO_2 production rates in the SS-1BN-N microcosms were the highest observed in any of the monitored microcosms and the naphthalene

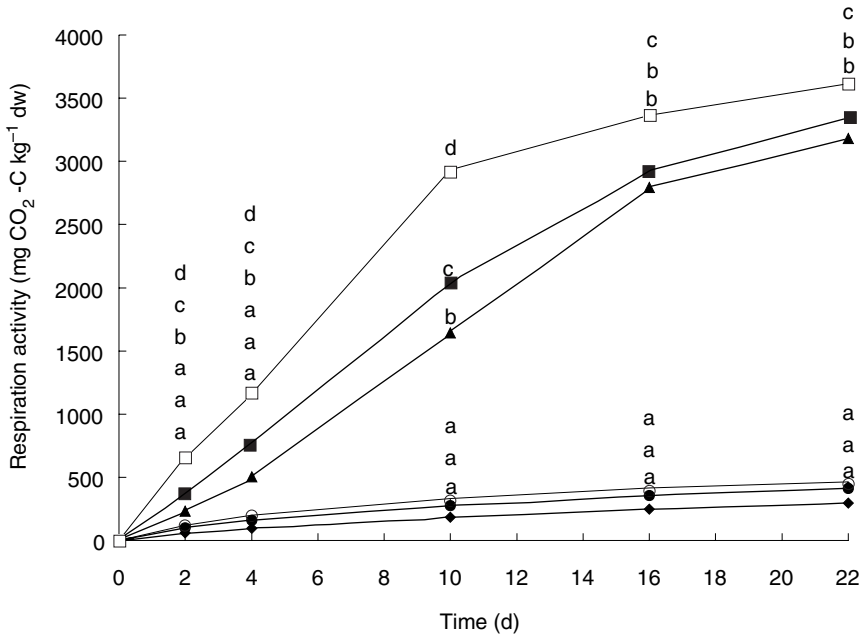

Fig. 2 CO_2 production in soil microcosms (◆, S; ●, S-1BN; ▲, S-N; ■, S-1BN-N; ○, SS-1BN; □, SS-1BN-N; see Materials and Methods). Values are the mean of three determinations. The same letter indicates values not significantly different at the 5% level according to Tukey's multiple range test

Table 2 Viable cells (cfu g^{-1} dw) of total heterotrophic and naphthalene-degrading micro-organisms in soil microcosms

	Incubation time (d)						
Microcosm	0	2	4	10	16	22	50
S-N	$1{\cdot}3 \times 10^{7}$*	$4{\cdot}9 \times 10^{7}$	$1{\cdot}0 \times 10^{8}$	$1{\cdot}1 \times 10^{8}$	$1{\cdot}4 \times 10^{8}$	$2{\cdot}8 \times 10^{8}$	nd
	$6{\cdot}2 \times 10^{6}$†	$6{\cdot}5 \times 10^{7}$	$1{\cdot}1 \times 10^{8}$	$2{\cdot}1 \times 10^{8}$	$2{\cdot}8 \times 10^{8}$	$1{\cdot}8 \times 10^{8}$	nd
S-1BN	$3{\cdot}7 \times 10^{8}$*	$9{\cdot}4 \times 10^{7}$	$7{\cdot}2 \times 10^{7}$	$6{\cdot}4 \times 10^{7}$	$5{\cdot}5 \times 10^{7}$	$4{\cdot}6 \times 10^{7}$	nd
	$2{\cdot}6 \times 10^{8}$†	$9{\cdot}0 \times 10^{7}$	$5{\cdot}4 \times 10^{7}$	$5{\cdot}6 \times 10^{7}$	$2{\cdot}8 \times 10^{7}$	$3{\cdot}0 \times 10^{7}$	nd
S-1BN-N	$3{\cdot}7 \times 10^{8}$*	$1{\cdot}7 \times 10^{8}$	$2{\cdot}5 \times 10^{8}$	$3{\cdot}3 \times 10^{8}$	$5{\cdot}3 \times 10^{8}$	$5{\cdot}1 \times 10^{8}$	$2{\cdot}0 \times 10^{8}$
	$2{\cdot}6 \times 10^{8}$†	$9{\cdot}8 \times 10^{7}$	$2{\cdot}6 \times 10^{8}$	$2{\cdot}4 \times 10^{8}$	$2{\cdot}6 \times 10^{8}$	$1{\cdot}2 \times 10^{8}$	$2{\cdot}5 \times 10^{7}$
SS-1BN-N	$3{\cdot}7 \times 10^{8}$*	nd	nd	$9{\cdot}8 \times 10^{8}$	nd	$2{\cdot}1 \times 10^{9}$	$5{\cdot}2 \times 10^{8}$
	$2{\cdot}6 \times 10^{8}$†	nd	$1{\cdot}0 \times 10^{9}$	$1{\cdot}2 \times 10^{9}$	nd	$1{\cdot}9 \times 10^{9}$	$4{\cdot}3 \times 10^{8}$

*Total heterotrophic micro-organisms.
†Naphthalene-degrading micro-organisms.
dw, Dry weight; cfu, colony-forming units; nd, not determined.
Each value is the mean of two determinations.
See Materials and Methods for definitions of microcosms.

consumption was almost completed in 16 d of incubation (Figs 1 and 2). 1BN grew rapidly on naphthalene in the absence of competition with an active indigenous soil microflora. In fact, in 4 d of incubation the naphthalene-degrading micro-organisms, composed entirely of 1BN, increased to $1{\cdot}0 \times 10^{9}$ cfu g^{-1} dw, reducing the initial naphthalene content of the soil by about 80%. 1BN remained viable even when the naphthalene was completely depleted (Table 2).

Survival of *Rhodococcus* sp. strain 1BN in the S-1BN-N microcosms

Because of the difficulty of discriminating, by morphological characteristics, 1BN and the other naphthalene degraders naturally present in the soil, the PCR amplifications of *nar*A and *alk*1BN catabolic genes and a 16S rDNA-*Hha*I restriction profile analysis (Fig. 3) were used as tools to check for the possible presence of 1BN in the native soil and to measure the percentage of 1BN in the S-1BN-N-inoculated microcosm.

Fifty strains were isolated from selective M9-naphthalene agar plates in the uninoculated S-N microcosms. Thirty-six strains were positive to PCR amplification of *ndo*B or *nar*A genes for naphthalene dioxygenase, indicating that an indigenous naphthalene-degrading population was present in the native soil. None of the strains presented the 16S rDNA-*Hha*I profile of 1BN or the *alk*1BN gene, hence the native soil used for microcosm experiments did not contain any 1BN.

The 16S rDNA restriction analyses with *Hha*I enzyme and PCR amplification of *alk*1BN, *nar*A and *ndo*B genes were performed on 60 and 44 colonies isolated, respectively, from selective M9-naphthalene agar plates at the 10th and 22nd day of the experiment from the S-1BN-N microcosms;

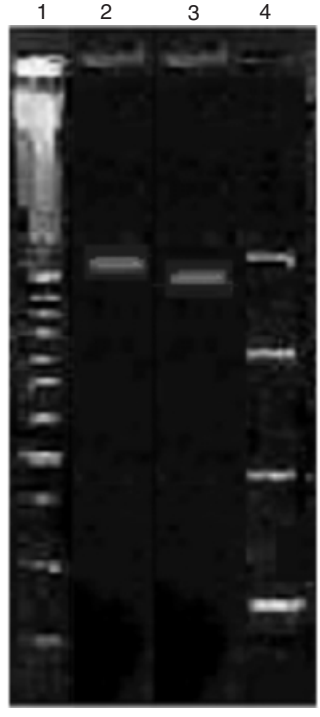

Fig. 3 Molecular markers to detect *Rhodococcus* sp. strain 1BN in the soil microcosm S-1BN-N (see Materials and Methods). Lanes: 1, 50-bp ladder; 2, *nar*A gene-amplified fragment (650 bp); 3, *alk*1BN gene-amplified fragment (550 bp); 4, 16S rDNA-*Hha*I restriction pattern

nine different 16S rDNA-*Hha*I patterns were present, one of which corresponded to that of 1BN. The 1BN-like 16S rDNA haplotypes were also positive to both *nar*A and *alk*1BN genes and were considered to be the inoculated 1BN. At the 10th day of incubation, 1BN constituted 8·3% of the whole cultivable naphthalene-degrading population;

by the 22nd day of incubation, the strain was still viable and formed 13·6% of the degraders.

This analysis of the different naphthalene catabolic systems present in the S-1BN-N microcosms revealed that some naphthalene degraders carry the *ndo*B gene and some the *nar*A gene; after 10 d of incubation 33 strains carried the *ndo*B gene (55%) and 27 strains the *nar*A gene (45%) whereas after 22 d 15 strains carried the *ndo*B gene (34·1%) and 29 the *nar*A gene (65·9%), revealing that the different naphthalene catabolic systems were present in different proportions over the time of incubation.

DISCUSSION

This study documented the growth and naphthalene-degrading activity of 1BN when inoculated into a sandy-loam soil polluted with freshly added naphthalene and containing a well-established, and competitive, bacterial indigenous naphthalene-degrading population.

Neither the uninoculated S-N nor the inoculated S-1BN-N microcosms were observed to have a lag period before naphthalene degradation and degradation occurred to the same extent, confirming that the introduction of strains at a high population density does not always result in an enhancement of xenobiotic degradation, as also reported by Kästner *et al*. (1998). The presence in soil of a large indigenous degrading microflora, nutrient availability and different kinetics of substrate utilization could explain the failure of inoculated strains to enhance the biodegradation of a pollutant (Watanabe *et al*. 1998; Schwartz *et al*. 2000). The inoculated 1BN suffered initially, probably because of the better adaptation of the indigenous naphthalene-degrading population to the soil characteristics. Despite this, the inoculation of soil with 1BN could have enhanced a faster mineralization of naphthalene metabolites, as evidenced by the higher CO_2 production in the S-1BN-N microcosms compared with the S-N microcosms. While Schwartz *et al*. (2000) reported a decline in inoculated strains of three to four orders of magnitude, 1BN in S-1BN-N microcosms declined initially by about one order of magnitude and was still present after 22 d, indicating that it was effective in colonizing the experimental ecosystem, maintaining itself and degrading actively.

The native naphthalene-degrading population could be grouped according to the presence of the two naphthalene catabolic systems retrieved, *ndo*B (Resnick *et al*. 1996) and *nar*A (Larkin *et al*. 1999). In the S-1BN-N microcosms the proportion of these two systems changed during the course of the experiment, suggesting that the naphthalene concentration in the microcosms influenced the population dynamics of indigenous and allochthonous 1BN-degrading bacteria, naphthalene being the only apparently changed factor.

In the first 10 d of incubation, when the naphthalene concentration was high (above 700 mg kg^{-1} dw), *ndo*B-bearing strains were the most abundant but, when the naphthalene in the soil was almost depleted (below 100 mg kg^{-1} dw), the group of *ndo*B-bearing strains declined while the group of *nar*A-bearing strains, also comprising 1BN, increased. This could reflect a different ability of the latter microbial group to adapt better to substrate-limiting conditions and survive longer. Rhodococci-like micro-organisms are considered to persist longer than Gram-negative bacteria, although the latter are recognized as faster degraders in non-substrate-limiting conditions (Juteau *et al*. 1999).

If degradation potential is inherent in the indigenous soil microflora, the addition of a specialist strain might only reduce the time necessary for biodegradation. In particular, the use of 1BN in soil bioremediation processes could be advantageous given its various degradation abilities also towards medium- (C_6) and long-chain (C_{16}–C_{28}) alkanes, benzene and toluene, both alone and when present in mixtures. The results suggest that 1BN can be used in the bioaugmentation of soils where there are few, or no, indigenous degrading micro-organisms or where contamination levels are low, requiring strains able to survive for long periods of time in such environments and bearing stable catabolic genes coding for oxygenase systems with high affinity for the pollutant.

ACKNOWLEDGEMENTS

This study was supported by the Italian National Research Council (CNR), Progetto Strategico Ambiente e Territorio.

REFERENCES

Aamand, J.G., Bruntse, G., Jepsen, M., Jorgensen, C. and Jensen, B.K. (1995) Degradation of PAHs in soil by indigenous and inoculated bacteria. In *Bioaugmentation for Site Remediation* ed. Hinchee, R.E., Fredrickson, J. and Alleman, B.C. pp. 121–127. Columbus, OH: Battelle Press.

Akkermans, A.D.L. (1994) Application of bacteria in soils: problems and pitfalls. *FEMS Microbiological Review* **15**, 185–194.

Andreoni, V., Bernasconi, S., Colombo, M., van Beilen, J.B. and Cavalca, L. (2000) Detection of genes for alkane and naphthalene catabolism in *Rhodococcus* sp. 1BN. *Environmental Microbiology* **2**, 572–577.

Bianchi, M.A. and Bianchi, A.J. (1982) Statistical sampling of bacterial strains and its use in bacterial diversity measurement. *Microbial Ecology* **8**, 61–69.

Focht, D.D., Searles, D.B. and Koh, S.C. (1996) Genetic exchange in soil between introduced chlorobenzoate degraders and indigenous biphenyl degraders. *Applied Environmental Microbiology* **62**, 3910–3913.

Goldstein, R.M., Mallory, L.M. and Alexander, M. (1985) Reasons for possible failure of inoculation to enhance biodegradation. *Applied Environmental Microbiology* **50**, 977–983.

Herrick, J.B., Stuart-Keil, K.G., Ghiorse, W.C. and Madsen, E.L. (1997) Natural horizontal transfer of a naphthalene dioxygenase gene between bacteria native to a coal tar-contaminated field site. *Applied Environmental Microbiology* **63**, 2330–2337.

Heyndrickx, M., Vauterin, L., Vandamme, P., Kersters, K. and De Vos, P. (1996) Applicability of combined amplified ribosomal DNA restriction analysis (ARDRA) patterns in bacterial phylogeny and taxonomy. *Journal of Microbiological Methods* **26**, 247–259.

Hughes, J.B., Beckles, D.M., Chandra, S.D. and Ward, C.H. (1997) Utilization of bioremediation processes for the treatment of PAH-contaminated sediments. *Journal of Industrial Microbiology* **18**, 152–160.

Juteau, P., Larocque, R., Rho, D. and LeDuy, A. (1999) Analysis of the relative abundance of different types of bacteria capable of toluene degradation in a compost biofilter. *Applied Microbiology and Biotechnology* **52**, 863–868.

Kästner, M., Breuer-Jammali, M. and Mahro, B. (1998) Impact of inoculation protocols, salinity, and pH on the degradation of polycyclic aromatic hydrocarbons (PAHs) and survival of PAH-degrading bacteria introduced into soil. *Applied and Environmental Microbiology* **64**, 359–362.

Kunz, D.A. and Chapman, P.J. (1981) Catabolism of pseudocumene and 3-ethyltoluene by *Pseudomonas putida (arvilla)* mt-2: evidence for new functions of the TOL (pWW0) Plasmid. *Journal of Bacteriology* **146**, 179–191.

Larkin, M.J., Allen, C.C.R., Kulakov, L.A. and Lipscomb, D.A. (1999) Purification and characterization of a novel naphthalene dioxygenase from *Rhodococcus* sp. strain NICMB12038. *Journal of Bacteriology* **181**, 6200–6204.

Margesin, R. and Schinner, F. (1997) Efficiency of indigenous and inoculated cold-adapted soil micro-organisms for biodegradation of diesel oil in Alpine soils. *Applied and Environmental Microbiology* **63**, 2660–2664.

Matthias, K., Jammali, M.B. and Mahro, B. (1998) Impact of inoculation protocols, salinity, and pH on the degradation of polycyclic aromatic hydrocarbons (PAHs) and survival of PAH-degrading bacteria introduced into soil. *Applied and Environmental Microbiology* **64**, 359–362.

McClure, N.C., Fry, J.C. and Weightman, A.J. (1991) Survival and catabolic activity of natural and genetically engineered bacteria in a laboratory-scale activated-sludge unit. *Applied and Environmental Microbiology* **57**, 366–373.

Middledorp, P.J.M., Briglia, M. and Salkinoja-Salonen, M. (1990) Biodegradation of pentachlorophenol in natural soil by inoculated *Rhodococcus chlorophenolicus*. *Microbiology Ecology* **20**, 123–139.

Miethling, R. and Karlson, U. (1996) Accelerated mineralization of pentachlorophenol in soil upon inoculation with *Mycobacterium chlorophenolicum* PCP-1 and *Sphingomonas chlorophenolica* RA2. *Applied and Environmental Microbiology* **62**, 4361–4366.

Ministero delle Politiche Agricole e Forestali (1999) Decreto Ministeriale, 13 September 1999. Approvazione dei 'Metodi ufficiali di analisi chimica del suolo'. (Suppl.) Ord. no. 185. *Gazzetta Ufficiale della Repubblica Italiana* **248**, 21 October 1999.

Nerella, S., Wright, A.L. and Weaver, R.W. (1995) Microbial inoculants and fertilization for bioremediation of oil in wetlands. In *Bioaugmentation for Site Remediation* ed. Hinchee, R.E., Fredrickson, J. and Alleman, B.C. pp. 31–38. Columbus, OH: Battelle Press.

Pochon, J. and Tardieux, P. (1962) *Techniques d'Analyse en Microbiologie du Sol* St. Maudé, Seine, France: de la Tourelle.

Resnick, S.M., Lee, K. and Gibson, D.T. (1996) Diverse reactions catalyzed by naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816. *Journal of Industrial Microbiology and Biotechnology* **17**, 438–457.

Roane, T.M., Josephson, K.L. and Pepper, I.L. (2001) Dual-bioaugmentation strategy to enhance remediation of cocontaminated soil. *Applied and Environmental Microbiology* **67**, 3208–3215.

Sambrook, J., Fritsch, E. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. New York: Cold Spring Harbor Laboratory Press.

Schwartz, E., Trinh, S.V. and Scow, K.M. (2000) Measuring growth of a phenantrene-degrading bacterial inoculum in soil with a quantitative competitive polymerase chain reaction method. *FEMS Microbiology Ecology* **34**, 1–7.

Smits, T.H.M., Röthlisberger, M., Witholt, B. and van Beilen, J.B. (1999) Molecular screening for alkane hydroxylase genes in Gram-negative and Gram-positive strains. *Environmental Microbiology* **1**, 307–316.

Straube, W.L., Jones-Meehan, J., Pritchard, P.H. and Jones, W.R. (1999) Bench-scale optimization of bioaugmentation strategies for treatment of soils contaminated with high molecular weight polyaromatic hydrocarbons. *Resource Conservation and Recycling* **27**, 27–37.

Vogel, T.M. (1996) Bioaugmentation as a soil bioremediation approach. *Current Opinion Biotechnology* **7**, 311–316.

Wagner-Döbler, I., Pipke, R., Timmis, K.N. and Dwyer, D.F. (1992) Evaluation of aquatic sediment microcosms and their use in assessing possible effects of introduced micro-organisms on ecosystem parameters. *Applied and Environmental Microbiology* **58**, 1249–1258.

Walkley, A. and Black, I.A. (1934) An examination of Degtjareff method for determining soil organic matter and a proposed modification of the chromic acid titration method. *Soil Science* **37**, 29–38.

Watanabe, K., Yamamoto, S., Hino, S. and Harayama, S. (1998) Population dynamics of phenol-degrading bacteria in activated sludge determined by *gyr*B-targeted quantitative PCR. *Applied and Environmental Microbiology* **64**, 1203–1209.

Yamada, K. and Komagata, K. (1970) Taxonomic studies on Coryneform bacteria III. DNA base composition of Coryneform bacteria. *Journal of General and Applied Microbiology* **16**, 215–224.

Zallo, M., Susaeta, L., Bargos, T. and Gorostiza, I. (1996) Evaluation of the performance of two commercial starters on the bioremediation of soils contaminated with mineral oil and HCH (hexachlorocyclohexane). *Biotechnology Letters* **18**, 339–342.