Response of bacterial community during bioremediation of an oil-polluted soil

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ABSTRACT


Methods and Results: The bacterial community in laboratory soil columns during a 72-day biostimulation treatment was followed by analysing the number of total cultivable hydrocarbon-degrading bacteria, soil respiratory activity and the 16S–23S rDNA internal transcribed spacer homoduplex heteroduplex polymorphisms (ITS-HHP) of total soil bacterial DNA. ITS-HHP permits an estimate of both length and sequence polymorphism in a 16S–23S rDNA spacer population, using to advantage the homoduplex and heteroduplex fragments that are generated during PCR. The treatment, made by air sparging and biostimulation with a mineral nutrient and surfactant solution, resulted in a 39-5% decrease of the total hydrocarbon content. Within 4 days of treatment onset the bacterial community underwent a first phase of activation that led to a substantial increase in the observable diversity. Subsequently, after a 12-day period of stability, another activation phase was observed with further shifts of the community structure and an increase in the abundance and diversity of catechol-2,3-dioxygenase (C23O) genes.

Conclusions: The overall data suggest an important contribution of uncultivable bacteria to the soil bioremediation, since, during the second activation phase, the increases of the respiratory activity, bacterial diversity and C23O gene abundance and diversity were not accompanied by a corresponding increase of the cultivable bacteria number.

Significance and Impact of the Study: This study shows that successive phases of activation of bacterial populations occur during a bioremediation treatment of oil-polluted soil.

Keywords: aged oil contamination, bacterial community, bacterial diversity, catabolic genes, DNA fingerprinting, soil bioremediation.

*Dedicated to the memory of Mauro Zucchi (1965–2001).

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INTRODUCTION

The potential of bioremediation depends on the ability of microbes to adapt to new environmental conditions (Head 1998; Mishra et al. 2001). Description and understanding of factors underlying adaptation is key to defining predictable field application endpoints of this biotechnology. An investigation into how bioremediation influences the response of a soil microbial community, in terms of activity and diversity, may contribute to addressing this issue.

Bacterial communities in contaminated soils tend to be dominated by the strains that can survive toxicity and are able to utilize the contaminant itself for growth. As a response to bioremediation treatment, these populations may begin to actively degrade the pollutants and detoxify the soil, allowing other quiescent/starving populations to increase their numbers, leading to an increase of the bacterial community within the soil. Such a process can result in a quantitative increase in the number of bacterial cells and of their activity, implying a succession of leading bacterial groups and the change of the observable diversity (Øvreas et al. 1998; MacNaughton et al. 1999; Iwamoto et al. 2000; Ranjard et al. 2000b).

Modern tools in molecular biology allow us to describe such diversity. For example, total soil DNA melting profile analysis (Øvreas et al. 1998) gives an overall picture of the shift in microbial composition during a treatment, while the PCR fingerprinting of molecular chronometers permits an analytical description of the diversity (MacNaughton et al. 1999; Beaulieu et al. 2000; Ranjard et al. 2000a; Bruns et al. 2000).

During bioremediation treatment, bacterial diversity change is accompanied by an increase in the quantity and observable diversity of microbial genes involved in contaminant degradation pathways (Iwamoto et al. 2000). Catechol 2,3-dioxygenase (C23O) catalyses extradiol cleavage of the catechol aromatic ring, a convergent reaction in the bacterial degradation pathways of aromatic hydrocarbons (Elits and Bolin 1996). Several works have reported the usefulness of C23O-like genes as markers to evaluate the efficacy of bioremediation in treating soils and aquifers polluted with crude oil and oil-derived products (Hallier-Soulier et al. 1996; White et al. 1996; Wikström et al. 1996, 2000; Daly et al. 1997; Guo et al. 1997; Hosein et al. 1997; Meyer et al. 1999; Cavalca et al. 2000; Mesarch et al. 2000; Ringelberg et al. 2001).

The aim of this work was to investigate the response of a bacterial population to a bioremediation treatment of a soil with a heavy and aged crude oil contamination. In laboratory microcosms, we subjected the polluted soil to biostimulation and followed the quantitative dynamics of the cultivable oil-degrading bacterial population and C23O genes during the treatment and compared with soil respiratory activity. An assessment was made of the diversity of the bacterial population and the C23O genes during the treatment by, respectively, the analysis of 16S–23S rDNA internal transcribed spacers homoduplex heteroduplex polymorphisms (ITS-HHP; Daffonchio et al. 2000) and single-strand conformation polymorphisms (SSCP; Orita et al. 1989; Beaulieu et al. 2000) of the PCR-amplified products.

MATERIALS AND METHODS

Soil, soil microcosms and treatment

The soil used in this study was a sandy loam with only a small amount of clay. From the determination of organic carbon (Walkley and Black 1934), total nitrogen (Kjeldahl 1883) and phosphorous (Olsen and Dean 1965), we estimated a C/N ratio of 129 and a phosphate content lower than 5 mg kg⁻¹.

Sixteen soil microcosms were set up in cylindrical glass columns, 9 cm in diameter and 50 cm in height, and each was filled with 4.5 kg of sieved polluted soil. The soil used for the eight columns was spiked before filling the column with 800 ml of a mineral solution to supply the following quantities per kilogram of soil: NH₄Cl 3.55 g, (NH₄)₂SO₄ 2.22 g, K₂HPO₄ 0.79 g, KH₂PO₄ 0.61 g to reach a C : N : P ratio of 100 : 10 : 1. The surfactant Tween 80 was also added to the nutrient solution (0.05% v/v) to increase the pollutant availability to bacteria. During the 72 days of trial, these eight columns were continuously aerated (flow rate, 0.31 h⁻¹) with moist air via three vertical glass pipes perforated every 10 cm along the column length. The soil of the remaining eight columns was added with 800 ml of pure sterile water before column filling and was otherwise untreated, working as a control. In all the columns the percolating liquid was recycled to the top during the treatment.

From two columns, named main columns (one treated and one control), at successive days after treatment onset, 3-g samples from three different depths (0, 25 and 50 cm) were taken and pooled to represent the whole column. Three subsamples (1 g each) were analysed within 1 h for microbial counts and, after storing at −20°C, for molecular analyses.

For the respiratory activity at each sampling time, the entire contents of two (one treated and one control) of the remaining columns were used for analysis. In parallel, a count was made of the hydrocarbon-degrading bacteria to evaluate whether the microflora behaved like that in the main soil columns.

Analytical procedures

The total hydrocarbon content in the soil before and at the end of the treatment was determined from a pooled sample of three 50-g samples obtained from three sampling ports at
different column depths (0, 25 and 50 cm). From each pooled sample three subsamples were taken and analysed for total hydrocarbon content. The mean and standard deviation from the triplicate data were calculated. Total hydrocarbons were determined at the Stazione Sperimentale per i Combustibili (S. Donato Milanese, Milano, Italy) following the method D 3921-96 of American Standard and Test of Materials (ASTM) by infrared spectroscopy after sample extraction with 1,1,2-trichloro-1,2,2-trifluoroethane and treatment with Florisil™ (Aldrich, Milan, Italy).

The number of oil-degrading bacteria was determined by the Most Probable Number (MPN; Mills et al. 1978; Delille 2000), in triplicate, using M9 mineral medium supplemented with crude oil to a final concentration of 250 mg l⁻¹. Tubes were inoculated with 1 ml of the tenfold-based M9 serial dilutions of the soil samples and incubated at 28 °C for 30 days. Bacterial growth was evaluated by the presence of turbidity in the medium and by microscope inspection. Total heterotrophs were determined by MPN using plate count broth (DIFCO, Milan, Italy) supplemented with sterilized soil extract to a concentration of 10% (v/v).

Respiratory activity was determined according to the Pochon and Tardieux method (Pochon and Tardieux 1962), slightly modified by placing five replicate subsamples of moist soil (corresponding to 50 g oven-dried soil) in 1-l jars slightly modified by placing five replicate subsamples of moist soil (corresponding to 50 g oven-dried soil) in 1-l jars equipped with a 50-ml beaker containing 10 ml 1 M KOH to trap the evolved CO₂. The CO₂ trapped in the KOH solution was measured by back-titration with 1 M HCl to endpoint pH values of 8-00 and 4-t10, with a Radiometer DTS 800 multititration system (Radiometer, Copenhagen, Denmark). The CO₂ determination was carried out after 10 days of soil incubation in the dark at 25°C.

**Analysis of the bacterial community DNA**

For each sampling time, DNA was extracted from 2·5 g of soil of each main column and eluted in 400 µl of TE pH 8 as described by Zhou et al. (1996).

DNA was purified from humic acids by fractioning the crude extract onto Sepharose 4B (Sigma, Milan, Italy) as described by Jackson et al. (1997). The purity of DNA obtained after treatment with Sepharose 4B was tested by the amplification of 16S rDNA by PCR, using as template 2 µl of each DNA fraction and their decimal dilutions. 16S rDNA was amplified in a Perkin Elmer 2400 thermal cycler using primers S-D-Bact-0008-a-S-20 and S-D-Bact-1495-a-A-20 as previously described (Daffonchio et al. 1998). After the PCR amplificability tests, for each soil sample, the fractions 2, 3, 4 and 5 obtained from Sepharose 4B purification were pooled. DNA was precipitated with ethanol, resuspended in TE pH 8 and used for PCR experiments.

Bacterial community analysis was performed by ITS-HHP following procedures previously described (Daffonchio et al. 2000). The PCR products were separated in 0·6× MDE gel (FMC Bioproducts, Milan, Italy) and silver stained (Bassam et al. 1991). We acquired the ITS-HHP banding profiles using the Gel Doc 2000 image system and Diversity Database fingerprinting software (Biorad) as previously described (Daffonchio et al. 2000). The elaboration of the band patterns was performed according to the assumptions of Yin et al. (2000): (i) The number of bands at each treatment time was taken to represent the number of different organisms (species richness) present in the soil during the treatment. This represents an approximation as many bacteria harbour several rDNA operons per cell that may be different, thus giving a pattern of bands. (ii) The band intensity was taken to represent the number of individuals within an organism type (diversity index). The Shannon index was used to calculate the diversity index: 

$$H = -\sum\left(\frac{n_i}{N}\right) \ln\left(\frac{n_i}{N}\right)$$

where \(n_i\) is the area of each band peak and \(N\) the sum of all the band peak areas (Yin et al. 2000).

The gel band matrix obtained from the ITS-HHP profile analysis was subjected to cluster and principal component analysis (PCA; Ranjard et al. 2000b,c) using NTSYS pc 2·01 software (Rohlf 1987). A dendrogram was constructed by the unweighted pair group method with arithmetic averages and the Jaccard coefficient (Sneth and Sokal 1973).

For C23O relative quantification the purified DNA from each soil sample was tenfold-based serially diluted, and 2 µl from each dilution was used for PCR. A portion of the C23O gene was amplified using primer pair XE1–XE3, homologous to positions 406–425 and 706–725 of the xylE gene (Hallier–Soulier et al. 1996). PCR was made in a final volume of 50 µl, using the following reagents: primers 0·5 µM, dNTP 0·12 mM, 1× Promega buffer, MgCl₂ 2·5 mM and 1·5 U DNA Taq polymerase (Promega). The thermal protocol consisted of three levels of annealing temperature, five cycles at 60 °C, five cycles at 55 °C and 25 cycles at 50 °C, all for 1 min. All the cycles with annealing temperatures of 60 and 55 °C underwent denaturation for 2 min and elongation for 3 min. The 25 cycles at 50 °C annealing underwent denaturation for 40 s and elongation for 3 min (Thermal cycler: Perkin Elmer 2400). The specificity of C23O amplifications was confirmed by Southern hybridization, using xylE as probe labelled by random priming as previously described (Brusa et al. 2001).

For each treatment time, C23O fragments amplified from the first dilutions of soil DNA (5 µl) were mixed with loading buffer (0·25% xylene cyanol, 0·25% bromophenol blue, 10 mM NaOH and 95% formamide), heat denatured for 2 min at 95 °C and loaded on a 6× MDE gel (FMC Bioproducts, Milan, Italy) in a MiniProtean electrophoresis system (Biorad, Milan, Italy). The gel was
stained by silver staining (Bassam et al. 1991). The C23O PCR-SSCP profiles were analysed as described for ITS-HHP patterns.

RESULTS

Change in cultivable microflora and soil respiratory activity during the treatment

A heavily contaminated soil was obtained from a petroleum-contaminated refinery site. The level of total hydrocarbon contamination was 24.1 ± 2.9 g kg⁻¹ of dry soil. At all the soil depths (0–15, 15–30, 30–45, 60–75 and 90–105 cm), the hydrocarbon-degrading bacteria were in the same order (10⁵–10⁶ cells g⁻¹ of dry weight) as the total heterotrophic bacteria.

Soil columns were prepared to assess the response of the microflora to biostimulation treatment described in the experimental procedures. During the 72 days of treatment, the total hydrocarbon content of 24.1 ± 2.9 g kg⁻¹ of dry soil decreased to 14.6 ± 2.0 and 22.0 ± 3.0 g kg⁻¹ of dry soil in the treated and control columns, respectively, with a hydrocarbon reduction of 39.5 and 88.9%, respectively.

Both main columns showed an increase in total cultivable heterotrophic and oil-degrading bacteria within the first days of treatment initiation (Fig. 1a). The bacterial density in the control column decreased after a few days, reaching the initial levels of around 10⁵–10⁶ cells g⁻¹ (dry soil), whereas in the treated soil, after the initial peak at 10⁸–5 × 10⁸ cells g⁻¹ (dry soil), it stabilized to between 5 × 10⁷ and 10⁸ cells g⁻¹ (dry soil), showing that the biostimulation treatment activated the bacterial flora.

The increase in the number of the hydrocarbon-degrading bacteria was accompanied by an increase in the soil respiratory activity during the treatment (Fig. 1b). The untreated soil showed only a slight increase in respiratory activity after 4 days of treatment, and this activity remained stable throughout the following period (Fig. 1b). The cell numbers of the oil degraders measured in the control columns used for respiratory activity determinations showed a moderate increase, like in the main control column, and this remained stable during the rest of the treatment. The initial increase might be because of the aeration of the soil during the column filling. In the treated soil, the respiratory activity passed from less than 20 to more than 60 mg CO₂-C kg⁻¹ of dry soil in 72 days. Almost half of the increase in cumulative activity occurred during the first four treatment days, indicating that the microflora responded rapidly to the treatment. In the same period, the number of oil-degrading bacteria measured in the treated columns used for respiratory activity increased in the treated soil, as observed in the main treated column, reaching a level of around 10⁸ cells g⁻¹ of dry weight a few days after beginning treatment.

From days 4 to 16, the respiratory activity was relatively stable, being 30–35 mg CO₂-C kg⁻¹ of dry soil, and in the following treatment period (after day 16) the soil respiratory activity doubled again, but this increase was not accompanied by any further increase in the number of cultivable oil degraders. This suggests the metabolic activation of other functional populations or uncultivable oil degraders, and response to treatment on their part, although at a lower rate.

Bacterial population shift during treatment

To evaluate the effect of the treatment on the diversity of uncultivable microflora, the total undiluted DNA obtained at each sampling time during the first 52 days of the experiment was analysed for ITS-HHP (Daffonchio et al. 2000), which permits an estimate of both length and sequence polymorphism in an ITS population, using to advantage the homoduplex and heteroduplex fragments that are generated during PCR, and differentiates between closely related strains (Daffonchio et al. 2000). The soil treatment was accompanied by a notable increase in bacterial diversity (Fig. 2a). Before the treatment, the ITS-HHP profile showed a relatively low polymorphism and a main band that migrated at about 570 bp, indicating that oil contamination led to the selection of a relatively few species (Fig. 2a,b). The soil treatment determined a dramatic change in the community ITS-HHP profile that resulted in an increase in band number at day 2 of the treatment. In the following period the fingerprinting complexity remained relatively stable until day 8, and at days 16, 32 and 52 showed further changes, with additional bands.

The dendrogram resulting from the elaboration of the ITS-HHP patterns revealed two main clusters (Fig. 2c): The first, including the soil community before and after 2 days of treatment, indicates the initial change in population following biostimulation. The first subgroup of cluster two, including in close proximity the community profiles at days 4 and 8 of treatment, indicates a phase of relative stability in the community. The second sub grouping of cluster two shows the community profiles at days 16, 32 and 52 to be in close proximity, indicating a further change of the bacterial community.

Estimate of relative C23O gene abundance and diversity

To estimate the behaviour of hydrocarbon-degrading genes during the bioremediation treatment, we chose the C23O gene as representative gene of the lower pathways of aromatics and we determined the relative abundance and diversity of such a gene by PCR. For each sampling time,
the relative abundance of C23O genes in soil was determined by dividing the dilution factor of the last positive C23O PCR by the dilution factor of the last positive 16S rDNA PCR (C23O/16S rDNA ratio). The relative enrichment of C23O with respect to 16S rDNA following the treatment was shown by plotting, on a logarithmic scale, the C23O/16S rDNA ratio at different sampling times vs treatment time (Fig. 3a). At the beginning of the treatment (day 0), the amplification of C23O showed no positive signal, even though the amplification of 16S rDNA was positive until the \(10^{-2}\) dilution (C23O/16S rDNA ratio < 0.001 = 0). At day 2, a positive signal appeared also for C23O, and the C23O/16S rDNA ratio increased to 0.01, remaining stable until day 8. At day 16, the C23O/16S rDNA ratio increased to 0.1, and decreased again only at day 52. The C23O/16SrDNA ratio showed a similar trend, even though anticipated, to the curve of the soil respiratory activity with two activation phases (Fig. 3a).

In order to evaluate the diversity of C23O genes enriched during the biostimulation treatment, the amplified C23O fragments (320 bp) were analysed by SSCP (Fig. 3b). The SSCP experiments showed the enrichment of different C23O types during the treatment period. Time course of the diversity index and species richness (Fig. 3c) showed that, after appearance of PCR signal at day 2, C23O gene diversity was stable until day 8, to increase at days 16 and 32. At day 52, the C23O diversity returned to the same level of day 8. Cluster analysis discriminated two main groups of C23O SSCP patterns (Fig. 3d,e): the first included patterns at days 2, 4, 8 and 52; the second group included patterns at
days 16 and 32. SSCP profiles indicated that activation of bacteria-harbouring C23O occurred within 2 days of treatment onset. Increase of C23O diversity was observed only in parallel with the second respiratory activity increase and the second shift of total bacterial population assessed by ITS-HHP analysis, both occurred between days 8 and 16.

DISCUSSION

Several studies have been published on the response of the bacterial community to bioremediation treatment of soils polluted with crude oil or specific hydrocarbon classes (MacNaughton et al. 1999; Iwamoto et al. 2000; Ringelberg...
et al. 2001), but most of them evaluated the behaviour of the microflora at long time intervals and lacked the short time shifts of the bacterial population responding to the treatment. In this study, we were aimed to analyse the short time response of the bacterial community to the bioremediation treatment of a polluted soil, and to evaluate the successive changes occurring in the structure of the bacterial population detectable by PCR fingerprinting. We focused attention to a soil with an aged contamination of crude oil. The contamination occurred 15 years before the sampling and a comparison of the oil recovered from the cleaning wells and the original oil showed that the oil recovered from the contaminated soil was much heavier (density, 0.959 vs 0.86 g cm$^{-3}$; viscosity, 175.5 vs 40.5 cP) probably because of the volatilization and/or degradation of the lightest hydrocarbon fractions.

Biostimulation of the contaminated soil with air and mineral nutrients showed that deep changes in the abundance and diversity of bacterial community occurred during the first 2 days from the treatment onset. Both respiratory activity and the numbers of hydrocarbon-degrading bacteria sharply increased in the first 2 days of the treatment, in parallel with deep changes in the structure of the bacterial community as was shown by population fingerprinting. For bacterial community analysis, we used as target regions for the PCR the ITS which are frequently used to analyse complexity of mixed natural bacterial communities (Borneman and Triplett 1997; Borneman 1999; Fisher and Triplett 1999; Yin et al. 2000). The PCR approach on molecular chronometers of the ribosomal operon resolves the diversity also of uncultivable bacteria, even though PCR bias, differences in rDNA copy number between species and the heterogeneity of the target sequences within species are limitations for detecting all the soil bacterial diversity. For example, Ranjard et al. (2000a), who used RISA (ribosomal intergenic spacer analysis), a method based on band separation in denaturing polyacrilamide gel electrophoresis, to describe the diversity of the bacterial community of a soil spiked with mercury, found that the fingerprinting
band pattern underrepresented the PCR amplified ITS population. By cloning and sequencing single-band products they found several ITS of the same length but with different sequences belonging to phylogenetically unrelated bacteria. The approach we used may overcome this limitation as it highlights both length and sequence polymorphisms, the latter in the form of heteroduplex products formed between different ITS in a single strain (Daffonchio et al. 2000) or in different strains (Borin et al. unpublished data).

In the first 4 days from the treatment onset, the resident bacterial community in the polluted soil changed dramatically and the original populations were replaced by other more diverse populations. In the successive 10-day period, the community did not show apparent further changes and maintained the respiratory activity rate reached after 2 days from the treatment onset. These results indicate that the first days of the treatment were crucial for the establishment of a new equilibrium in the bacterial community, which allows starting the soil detoxification. After day 16, a second activation phase of the bacterial population occurred as was shown by the respiratory activity. During this period, which lasted more than 40 days, the level of cultivable bacteria, both total heterotrophs and oil degraders, remained stable, suggesting that the respiratory activity rate of cultivable bacteria increased or new uncultivable bacteria could be involved in the bioremediation process. The latter explanation is in agreement with the increase of complexity of bacterial community as revealed by ITS-HHP fingerprinting.

New bacteria could be enriched by substrates made available from the degradation of oil pollutants, such as metabolites produced by primary hydrocarbon degraders activated in the first phase of the treatment. If metabolites of oil compounds could support the growth of other bacterial populations, the genes for enzymes of the lower degradation pathways of hydrocarbons should be enriched in the latest phases of the treatment both in term of quantity and diversity. Several authors have reported the detection of different genes of the hydrocarbon-degradation pathways during bioremediation of soils and aquifers polluted with crude oil and oil-derived products (Hallier-Soulier et al. 1996; Wikström et al. 1996; Cavalca et al. 2000; Mesarch et al. 2000; Ringelberg et al. 2001). Recently, Ringelberg et al. (2001) found that genes of the lower degradation pathways of aromatics, such as C23O, increased after 2 months from the treatment onset in bioslurry reactors set up to decontaminate sediments from polycyclic aromatic hydrocarbons. We verified if in our system a similar behaviour occurred by analysing the prevalence and diversity of C23O, which has been reported as a useful marker of bacterial metabolic activity on polluting hydrocarbons (Hallier-Soulier et al. 1996; Wikström et al. 1996; Cavalca et al. 2000; Mesarch et al. 2000; Ringelberg et al. 2001). Using an approach similar to that of Ringelberg et al. (2001), we estimated the quantity of C23O-like genes in a sample based on the extinction of PCR signal in the dilutions of total soil DNA. Differently from Ringelberg et al. (2001), we based the analysis on the relative increase (or decrease) measured by PCR of C23O, a gene harboured by many aromatic hydrocarbon-degrading bacteria, with respect to 16S rDNA, a gene harboured by all bacteria, regardless of the hydrocarbon degradation ability. 16S rDNA worked as an internal standard for the relative quantification of the gene of interest. Using this approach, we found that C23O was detectable by PCR only after 2 days of treatment indicating a first phase of enrichment of this gene. In the following period the stability of the community was confirmed by a stable level of the C23O gene in soil. After day 16, the further increase of the relative quantity of C23O gene as well as the detection of different types of C23O-like genes by SSCP suggest that the lower degradation pathways of aromatics of the bacterial community were activated at a higher extent and hence catechols were available for bacteria supporting the hypothesis of the activation of a new and more complex bacterial population.

In conclusion, the overall data indicate that in our system the initial days of treatment are of major importance for establishing an active bacterial community and that the bioremediation proceeds by successive activation phases. The stability during the treatment of cultivable hydrocarbon-degrading bacteria with respect to the further increase of respiratory activity and diversity of community and of C23O gene fingerprinting profiles suggests the contribute of uncultivable bacteria to the soil bioremediation.

ACKNOWLEDGEMENTS

Partial support was given by the Italian national agency for protection of the environment (Agenzia Nazionale per la Protezione dell’Ambiente, ANPA), project ‘Risanamento di suoli della Bovisa (Milano) contaminati da idrocarburi mono- e poli-aromatici, e da altri inquinanti’. The manuscript was edited by Barbara Carey.

REFERENCES


