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A new fluorescent oligonucleotide probe for *in situ* detection of s-triazine-degrading *Rhodococcus wratislaviensis* in contaminated groundwater and soil samples

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ABSTRACT

A bacterial strain (FPA1) capable of using terbuthylazine, simazine, atrazine, 2-hydroxysimazine, deethylatrazine, isopropylamine or ethylamine as its sole carbon source was isolated from a shallow aquifer chronically contaminated with s-triazine herbicides. Based on its 16S rDNA sequence analysis, the strain FPA1 was identified as *Rhodococcus wratislaviensis*. The disappearance time of 50% of the initial terbuthylazine concentration in the presence of this strain (DT_{50}) was 62 days. This strain was also able to mineralise the [U-ring ^{14}C] triazine-ring, albeit at a slow rate. A 16S rRNA target oligonucleotide probe (RhLu) was designed, and the FISH protocol was optimised, in order to detect *R. wratislaviensis* in s-triazine-contaminated sites. The RhLu probe gave a positive signal (expressed as % of total DAPI-positive cells) in both the groundwater ($2.19 \pm 0.41\%$) and soil ($2.10 \pm 0.96\%$) samples analysed. Using the RhLu probe, *R. wratislaviensis* can be readily detected, and its population dynamics can be easily monitored, in soil and in water ecosystems contaminated with s-triazine. To the best of our knowledge, this is the first report showing the isolation, from groundwater, of a bacterial strain able to degrade s-triazines.

1. Introduction

s-Triazines are among the most commonly used herbicides in the world. In recent years, concerns about the persistence, mobility and toxicity of triazines and their metabolites have been growing, owing to the detection of residual concentrations of these herbicides in groundwater. In Italy and Spain, a considerable number of monitored aquifers are contaminated by simazine, terbuthylazine and their deethylate metabolites, with concentrations greater than 0.1 μg l^{-1},

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which is the maximum admissible concentration under the EC drinking-water legislation (EC 98/83EEC). Since the number of aquifers that cannot be used for drinking purposes is increasing due to contamination, there is a need to study the natural capacity of groundwater to recover from pesticide contamination. However, until now, the presence in groundwater of bacterial strains with potential degrading ability had not been thoroughly studied.

Natural attenuation of contaminated groundwater has been achieved *in situ* (Williams et al., 2003; Tuxen et al., 2002) or in the laboratory setting (using indigenous bacteria from contaminated sites) only for a limited number of pesticides (Harrison et al., 2003; Johnson et al., 2000; Mirigain et al., 1995; Pucarevic et al., 2002) and never against *s*-triazines. Furthermore, in these studies, biological degradation occurred only when aquifer pesticide concentrations exceeded 40 $\mu g\ l^{-1}$. Although there are many studies showing biotic and abiotic triazine degradation in soil and surface water (Di Corcia et al., 1999; Barra Caracciolo et al., 2005a,b), to our knowledge, their degradation in groundwater remains to be explored.

Groundwater bacterial populations are adapted to physical and chemical environmental conditions that are very different from those of surface ecosystems (de Lipthay et al., 2003). Examples of these are: the absence of light, low carbon and oxygen availability, and relatively low temperatures. The use of fluorescence *in situ* hybridization (FISH) allows for the direct and selective detection of both cultivable and uncultivable bacteria in environmental samples, even in particular ecosystems like groundwater (Detmers et al., 2004). Furthermore, fluorescently-labelled 16S rRNA-targeted oligonucleotide probes can be used to detect microorganisms at different levels of taxonomic specificity (Amann et al., 1995).

We describe the isolation of a terbuthylazine-degrading bacterium, *Rhodococcus wratislaviensis* strain FPA1, from a contaminated aquifer. To the best of our knowledge, this is the first time that these bacteria have been found in groundwater. We also designed a 16S rRNA-targeted oligonucleotide probe, and applied it to environmental samples using an optimised FISH protocol to monitor the attenuation capability of this bacterial strain in a soil-groundwater system.

2. Materials and methods

2.1. Field site, groundwater and soil sampling

The field site near Assisi (PG, Central Italy) is an intensive agriculture area with a shallow alluvial aquifer (water table at 12 m depth, geochemical facies alkaline-bicarbonate) of intrinsic vulnerability (Daly et al., 2002). Field parameters were: 15 °C, pH 6.87, dissolved oxygen 9.01 $mg\ l^{-1}$, Eh 210 mV and conductibility 930 $\mu S\ cm^{-1}$ (determined at the sampling point by using portable meters from WTW Instruments). According to the Umbria Regional Environmental Agency's monitoring surveys (2000–2008), terbuthylazine, a frequently used pesticide, and its metabolite, desethyl-terbuthylazine, are commonly found in this groundwater (>0.1 $\mu g\ l^{-1}$ parametric value). It is also common to find significant nitrate contamination at this site (>100 $mg\ l^{-1}$).

Groundwater samples were collected with a sterile sampler (bailer) from a well and then placed directly into sterile polyethylene bottles. Samples were kept at 4 °C until laboratory processing, which was performed within 5 h of sampling. The dissolved organic carbon (DOC), which was measured using the Total Organic Carbon Analyzer (Shimadzu model TOC500A- ASI-5000A) in accordance with the manufacturer's instructions, was 0.56 $mg\ l^{-1}$.

Agricultural soil samples were collected from the surface horizon (0–20 cm depth) of the same area. The soil was salty-clay, with 0.46% organic carbon content, 0.13% nitrogen content and a pH of 7.

2.2. Bacterial abundance, cell viability and microbial characterization in groundwater samples

The bacterial abundance (No. bacteria ml^{-1}) was determined in four fixed sub-samples (5 ml each) by direct count using DAPI as the DNA stain agent (Barra Caracciolo et al., 2005a,b,c). The cell viability, expressed as the percentage of live cells compared to total (sum of live and dead) cells, was determined in four fresh sub-samples (5 ml) using a two-dye fluorescent bacterial viability kit (Kit Live/Dead® Bacterial Viability Kit, BacLight™; Alonso et al., 2002). Finally, the phylogenetic composition of the indigenous bacterioplancton was analysed in sub-samples (5 ml) by applying the FISH technique using Cy3-labelled commercially-synthesised oligonucleotide probes against different taxa: ARCH915 (*Archaea* domain), EUB338I-III (*Bacteria* domain), ALF1B (α-*Proteobacteria*), BET42a (β-*Proteobacteria*), GAM42a (γ-*Proteobacteria*), HGC69A (*Actinobacteria*, high G + C content Gram-positive bacteria), Pla46 and Pla886 (*Planctomycetes*), CF319a (*Cytophaga–Flaviobacterium* cluster phylum CFB), LGC354a (*Firmicutes* with low G + C content), EPS710 (*Epsilonbacteria*) and SRB385 (some sulfate-reducing bacteria of *Deltaproteobacteria*, other *Deltaproteobacteria* and Gram-positive bacteria),(Biomers.net, Ulm, Germany). Further details on these probes are available at probeBase (http://www.microbial-ecology.de/probebase/; Loy et al., 2003). For the FISH analysis, each groundwater subsample was filtered through a 0.2 μm polycarbonate membrane, fixed by using a 70, 90 and 95% (v/v) ethanol series (10 min each, at room temperature), and then air-dried. FISH of the fixed cells was performed following published protocols (Pernthaler et al., 2001; Barra Caracciolo et al., 2005b; Amalfitano et al., 2008). The average number of probe-labelled cells was calculated as the percentage of the total number of DAPI-positive cells. Cells were counted from 10 to 20 randomly selected fields on each filter section corresponding to 500–1000 stained cells.

2.3. Bacterial strain isolation and characterization

In order to isolate terbuthylazine-degrading bacteria, groundwater aliquots (1 ml) were serially diluted in PBS (9 ml) and plated on minimal medium MB plates (Gerhardt et al., 1981). Cultures were supplemented with 100 $\mu g\ l^{-1}$ terbuthylazine as the sole carbon source and incubated both at 15 °C (groundwater temperature) and 28 °C. The resulting isolate