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Growth rate and nutrient limitation affect the transport of *Rhodococcus* sp. strain DN22 through sand

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Abstract

Rhodococcus strain DN22 grows on the nitramine explosive RDX as a sole nitrogen source, and is potentially useful for bioremediation of explosives-contaminated soil. In order for strain DN22 to be effectively applied *in situ*, inoculum cells must reach zones of RDX contamination via passive transport, a process that is difficult to predict at field-scale. We examined the effect of growth conditions on the transport of DN22 cells through sand columns, using chemostat-grown cultures. Strain DN22 formed smaller coccoid cells at low dilution rate (0.02 h^{-1}) and larger rods at high dilution rate (0.1 h^{-1}). Under all nutrient limitation conditions studied, smaller cells grown at low dilution rate were retained more strongly by sand columns than larger cells grown at high dilution rate. At a dilution rate of 0.05, cells from nitrate-limited cultures were retained more strongly than cells from RDX-limited or succinate-limited cultures. Breakthrough concentrations (C/C_0) from sand columns ranged from 0.04 (nitrate-limited, $D = 0.02$ h^{-1}) to 0.98 (succinate-limited, $D = 0.1$ h^{-1}). The observed strong effect of culture conditions on transport of DN22 cells emphasizes the importance of physiology studies in guiding the development of bioremediation technologies.

Introduction

The nitramine compound RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) is one of the most widely used military explosives, and is a problem contaminant at munitions-manufacturing sites worldwide (Hawari 2000). RDX is toxic (Burton et al. 1994; Kaplan 1965; Schneider 1977), persistent, and mobile in groundwater (Sheremata et al. 2001; Singh et al. 1998). Cleanup of the potentially large contaminant plumes associated with RDX contamination is difficult, although bioremediation of RDX via composting and soil–slurry systems (Axtell et al. 2000; Shen et al. 1997) has been successful at pilot and field scale.

Many microorganisms can cometabolically reduce the nitro-groups of RDX and growth on RDX as a nitrogen source has been seen in aerobic (Binks et al. 1995; Coleman et al. 1998) and anaerobic (Zhao et al. 2003) bacteria, suggesting that addition of RDX-degraders (bioaugmentation) to soil could enhance RDX degradation. Bioaugmenatation with RDX degraders has been successfully used at microcosm-scale (Coleman et al. 1998), but application *in situ* at large-scale faces several challenges, for example the need to distribute degradative cells evenly throughout the contaminated zone. Successful bioremediation in such cases will depend on understanding and optimising bacterial chemotaxis (Parales & Harwood 2002) and transport (Chen & Strevett 2001; Gannon et al. 1991; Huysman & Verstraete 1993; Johnson et al. 1996; Li & Logan 1999; Macleod et al. 1988) processes, in addition to knowledge of degradative pathways and enzymes.

Rhodococcus sp. strain DN22 was isolated from explosives-contaminated soil by enrichment on

RDX as the sole nitrogen source (Coleman et al. 1998), and has potential use in the bioremediation of RDX-contaminated sites. The pathway, enzymology and genetics of RDX degradation in this (Coleman et al. 2002; Fournier et al. 2002) and similar (Seth-Smith et al. 2002) bacteria are beginning to be understood, but the factors affecting the transport and behaviour of DN22 in the environment are largely unknown. The effect of cell morphology and hydrophobicity on transport are particularly relevant issues for *Rhodococcus* strains, which are pleomorphic and contain hydrophobic mycolic acids in the cell wall (Finnerty 1992).

We investigated the effect of various culture conditions (growth rate, nutrient limitation) on the transport of DN22 cells through sand columns, and looked for correlations between culture conditions, cell morphology, cell surface hydrophobicity, and transport efficiency with the aim of identifying growth conditions yielding mobile cells that may be more effective when inoculated at a contaminated site.

Materials and Methods

Bacterial strain and media

A rifampicin resistant derivative of *Rhodococcus* strain DN22, designated DN22rr (Coleman et al. 1998), was used for all experiments. The minimal media used are derivatives of minimal base E (Owens 1969), and have been described previously (Coleman et al. 1998). In brief, MSN contained 20 mM succinate and 2 mM KNO_3, MSC medium contained 4.4 mM succinate and 3 mM KNO_3 and MSR contained 20 mM succinate and 0.2 mM RDX. Solid media contained 15 g l^{-1} technical grade agar (Oxoid), and were sterilized by autoclaving, while liquid medium containing RDX was sterilised by filtration (0.22 μm pore size).

Chemostat growth of strain DN22

Chemostats were set up using three different conditions – carbon limitation on succinate (MSC medium), nitrogen limitation on nitrate (MSN medium), and nitrogen limitation on RDX (MSR medium). All chemostat cultures were incubated at 25 °C, mixed at 750 rpm with a magnetic stirrer, and aerated with filtered air at 600 cc min^{-1}. For each nutrient condition, three dilution rates (D) were sequentially examined; 0.10 h^{-1} (fast), 0.05 h^{-1} (intermediate), and 0.02 h^{-1} (slow). In the case of RDX limitation, $D = 0.08$ h^{-1} was used instead of $D = 0.1$ h^{-1} as the fast dilution rate because DN22 could not grow at the faster dilution in MSR medium. Three volumes were allowed to pass through the chemostat after each dilution rate change before samples were taken for analysis.

The maximum growth rate of DN22 was determined by washout kinetics (Jannasch 1969) using the equation: $\mathrm{Log_e}X_1 - \mathrm{Log_e}X_2 = (D - \mu_{max})(t_2 - t_1)$ (Tempest 1970), where X_1 and X_2 are cells/ml at time t_1 and t_2, D = Dilution rate and μ_{max} = maximum specific growth rate. The washout experiment was done in a nitrate-limited chemostat at $D = 0.15$, the decrease in OD_{530} was monitored over time, and the data converted to cells/ml by reference to a standard curve constructed from microscopic counts (1 OD_{530} unit = 5.0×10^8 cells ml^{-1}).

Cell size measurement

At each dilution rate and under each nutrient limiting condition, cells harvested from the chemostat were air dried on glass slides, and examined using phase contrast microscopy (Olympus BH, Olympus Japan) and photographed (Wild MPS 52, Wild Leitz, Heerbrugg, Switzerland). Ratios of cell length to cell width were calculated as a means of quantifying whether cultures were more coccoid or more rod-like. In each experiment ten cells were counted.

Transport of DN22 through sand columns

Sand column apparatus consisted of 10-ml, 15 mm diameter polypropylene syringe barrels packed at 1.60 g cm^{-3} with sterile sieved (200 μm–2 mm) acid-washed sand (16 g), and capped with filter paper (Whatman 541) at each end. The pore volume of the packed sand was 0.387 cm^3 g^{-1}. Sterile water was pumped through the columns from bottom to top at 2.5 cm h^{-1} (0.71 Pore volumes h^{-1}), and cells (3.5 ml at 2.8×10^8 cells ml^{-1}) in phosphate buffer (10.7 mM K_2HPO_4, 9.3 mM KH_2PO_4, pH 7) were added to the column via a three-way valve. This inoculum size correlates to $\sim 5 \times 10^7$ cells g^{-1} sand, similar to cell concentrations used in previous bioremediation trials (Coleman et al. 1998). Fractions of the column effluent were collected at

intervals, and dilution-plated on MSN agar containing rifampicin (30 μg ml^{-1}) using the Miles and Misra drop technique (Miles & Misra 1938) with five replicates. Colonies appearing after 5 days were counted, and % transport calculated as cells in eluate (C) divided by the number of initial cells (C_0), also determined by viable count.

Bacterial adherence to hydrocarbons (BATH) assay

Cell surface hydrophobicity was measured by the BATH technique (Dillon et al. 1986). Chemostat-grown cells were washed twice in PUM buffer (97 mM K_2HPO_4, 56 mM KH_2PO_4, 30 mM urea, 0.8 mM $MgSO_4$), and resuspended in the same buffer to OD_{400} of 0.750. Cell suspension (1.2 ml) was vortexed with hexadecane (0.05, 0.10, 0.15 and 0.20 ml) for 2 min, allowed 30 min to equilibrate, then the OD_{400} of the aqueous phase was measured and compared to hexadecane/water controls.

Results

Effect of dilution rate on cell morphology

Maximum dilution rate was determined to be 0.17 h^{-1} based on washout kinetics. DN22 grew as coccoid cells at $D = 0.02$ h^{-1}, but elongated into rods at $D = 0.05$ h^{-1} and $D = 0.1$ h^{-1} (Figure 1). At the same dilution rates, strain DN22 cells grown under carbon limitation were shorter than cells grown under nitrogen limitation with RDX or cells grown under nitrogen limitation with KNO_3 (Figure 1).

Transport of DN22 through sand columns

DN22 cells grown under different culture conditions were added to sand columns, eluted with water, and the cumulative viable count in the effluent determined at intervals. Cells grown under carbon limitation were retained the least on the sand columns (Figure 2a). Breakthrough concentrations are expressed as C/C_0, where C is the effluent cell concentration and C_0 the influent cell concentration. When grown at $D = 0.1$ h^{-1}, a breakthrough concentration 0.98 of succinate-limited DN22 cells passed through the sand column, compared to 0.45 and 0.64 of cells grown at $D = 0.05$ h^{-1} and 0.02 h^{-1}, respectively. Cells grown under nitrate-limitation (Figure 2b) showed the highest retention on sand

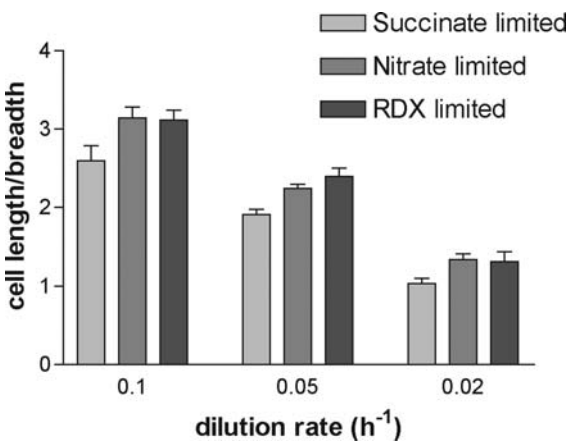

Figure 1. Comparison of cell morphologies for DN22 cells grown under conditions of succinate-limitation, nitrate-limitation, and RDX-limitation at various dilution rates. The highest growth rate tested with RDX-limited cultures was $D = 0.08$, rather than $D = 0.1$. Data are averages, and error bars represent standard deviations.

columns. When grown at $D = 0.1$ h^{-1}, a breakthrough concentration of 0.54 nitrate-limited cells passed through the column, but at $D = 0.05$ h^{-1} and $D = 0.02$ h^{-1}, concentrations of only 0.07 and 0.04 of nitrate-limited cells were eluted, respectively. DN22 cells grown under RDX limitation (Figure 2c) showed intermediate retention in columns. When grown at $D = 0.08$ h^{-1}, a breakthrough concentration of 0.69 RDX-limited cells passed through the sand columns, compared to concentrations of 0.70 and 0.41 of cells grown at $D = 0.05$ h^{-1} and $D = 0.02$ h^{-1}, respectively. Despite an overall trend toward more efficient transport of longer cells from fast-growing cultures, the type of nutrient limitation imposed on cultures also strongly affected transport, suggesting that cell morphology is not the only factor involved.

Cell surface hydrophobicity of DN22

Nocardioform actinomycetes such as DN22 typically have a hydrophobic cell surface which can change depending on culture conditions (Iwabuchi et al. 2003; Singer & Finnerty 1990; Sokolovska et al. 2003; Stratton et al. 2002). We used the BATH assay to examine whether the variable column transport observed with DN22 cells was due to changes in cell surface hydrophobicity, but this assay method did not detect any significant hydrophobicity under any growth conditions. Negative (*E. coli*) and positive (*S. aureus*) controls

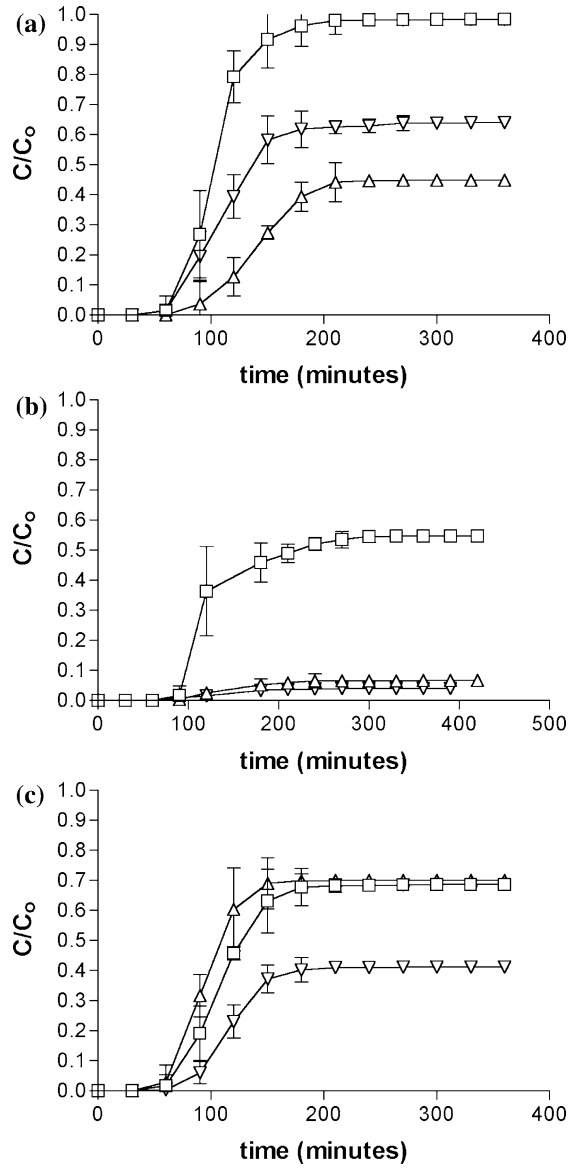

Figure 2. (a) Breakthrough concentrations (C/C_0) of DN22 cells grown under succinate-limited conditions at dilution rates of 0.1(□), 0.05 (△), and 0.02 h^{-1} (▽) and passed through sand columns. Data points are averages of three replicates. Error bars represent standard deviations of the means. (b) Breakthrough concentrations (C/C_0) of DN22 cells grown under nitrate-limited conditions at dilution rates of 0.1 (□), 0.05 (△), and 0.02 h^{-1} (▽) and passed through sand columns. Data points are averages of three replicates. Error bars represent standard deviations of the means. (c) Breakthrough concentrations (C/C_0) of DN22 cells grown under RDX-limited conditions at dilution rates of 0.08 (□), 0.05 (△), and 0.02 h^{-1} (▽) and passed through sand columns. Data points are averages of three replicates. Error bars represent standard deviations of the means.

behaved as expected in the BATH assay, with 83% of *S. aureus* cells adhering to the organic phase, and approximately 4% of *E. coli* adhering to the organic phase (data not shown).

Discussion

The change in morphology of strain DN22 from cocci to rods as growth rate increases is typical of nocardioform actinomycetes e.g. *Arthobacter globiformis* (Luscombe & Gray 1971, 1974) and *Rhodococcus equi* (Fuhrmann et al. 1997). Our finding that rods of DN22 were more mobile than cocci is in contrast to most previous results (Gannon et al. 1991; Macleod et al. 1988; Weiss et al. 1995). Gannon et al. (1991) tested a range of gram positive and gram negative bacteria (*Enterobacter*, *Pseudomonas*, *Bacillus*, *Achromobacter*, *Flavobacterium*, and *Arthrobacter*) and found that cocci were transported more readily than rods in loam soil. Both Macleod et al. (1988) and Weiss et al. (1995) reported that smaller cells travelled further through artificial rock cores and porous media, respectively. However, our results indicating slower transport of smaller, slowly growing or stationary phase cells are in agreement with some more recent studies of marine bacteria attaching to sediment (Heise & Gust 1999) and cultures of *Bacillus*, *Escherichia* and *Pseudomonas* in sand and silica (Chen & Strevett 2001). It should be noted that our comparisons to such studies are tentative due to the phylogenetically different bacteria used, and the use of batch culture and complex media, which may produce different physiological conditions to those in the present study.

The effect of cell surface hydrophobicity on bacterial transport is controversial, with one study suggesting no effect (Gannon et al. 1991), and another showing that hydrophobic cells travelled through soil 2–3 times slower than hydrophilic cells (Huysman & Verstraete 1993). While we obtained clear evidence for an effect of growth conditions on transport, we did not find any link between hydrophobicity and transport due to our inability to measure DN22 hydrophobicity using BATH assays. This result was unexpected due to prior research suggesting that rhodococci are

hydrophobic (Fuhrmann et al. 1997; Iwabuchi et al. 2003; Stratton et al. 2002), but may be resolved by the observations of Iwabuchi et al. (2003), who found that rhodococci with 'rough' colony morphotypes produced little extracellular polysaccharide (EPS) and adhered strongly to hydrocarbons, while mucoid strains produced more EPS and did not adhere strongly to hydrocarbons. These observations provide a plausible hypothesis for the lack of response of strain DN22 in the BATH assay, as DN22 produces highly mucoid colonies. Further analysis of hydrophobicity in DN22 is warranted, e.g. by use of the contact-angle test.

Our observation of a correlation between nitrate-limited conditions and DN22 retention on sand columns is intriguing due to the known link between nitrogen-limitation and biosurfactant/EPS production in *Rhodococcus* strains (Kim et al. 1990; Lang & Philp 1998; Ramsey 1988; Singer & Finnerty 1990) and other actinomycetes (Arino et al. 1998). It is puzzling however that in the case of DN22, the N-limited conditions decreased transport, given that earlier studies using rhamnolipids (Bai et al. 1997), Tween 20 (Streger et al. 2002) or SDS (Powelson & Mills 1998) showed increased bacterial transport upon surfactant addition. While we have no data thus far on the possible biosurfactants produced by DN22, such investigations are warranted due to their potential impact on cell transport through soil, RDX transport into cells, and RDX mobility in soil.

Understanding the behaviour of strain DN22 cells in environmental matrices has broad relevance due to the importance of rhodococci in the biodegradation of many pollutants (Allpress & Gowland 1999; Armstrong & Patel 1993; Gilan et al. 2004; Horn et al. 2003; Prieto et al. 2002; Schafer et al. 1996; Sharma & Pant 2000; Yoon et al. 2000). The strong effects of culture conditions and growth rate on transport that we observed in strain DN22 suggest that the transport behaviour of many other environmentally relevant rhodococci is also likely to affected by the physiological state of the cells. Control of growth rate and nutrient limitation may be useful approaches for optimising the transport of rhodococci in bioaugmentation- and biostimulation-based remediation processes. Our results indicate that cells from rapidly growing, succinate-limited cultures would provide inocula with maximum penetration through sandy soils or sediments.

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