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Survival and activity of individual bioaugmentation strains

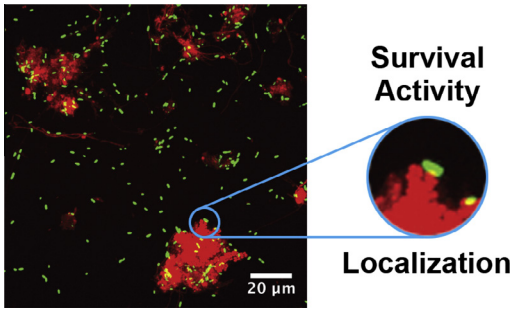
Morten S. Dueholm [a], Irina G. Marques [a], Søren M. Karst [a], Seth D'Imperio [b], Vaibhav P. Tale [b], Derrick Lewis [b], Per Halkjær Nielsen [a], Jeppe Lund Nielsen [a,*]

[a] Center for Microbial Communities, Department of Chemistry and Bioscience, Aalborg University, Fredrik Bajers Vej 7H, 9220 Aalborg East, Denmark
[b] Novozymes Biologicals, Inc., 5400 Corporate Circle, Salem, VA 24153, United States

HIGHLIGHTS

- Molecular tools are developed to study individual bioaugmentation strains.
- The tools are general applicable and can be used for *in situ* studies.
- Obtained information allows evaluation of bioaugmentation strategies.
- Valuable tool for the design of future bioaugmentation strategies.

GRAPHICAL ABSTRACT



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ABSTRACT

Successful application of bioaugmentation for enhanced degradation of environmental pollutants is often limited by the lack of methods to monitor the survival and activity of individual bioaugmentation strains. However, recent advancements in sequencing technologies and molecular techniques now allow us to address these limitations. Here a complementing set of general applicable molecular methods are presented that provides detailed information on the performance of individual bioaugmentation strains under *in situ* conditions. The approach involves genome sequencing to establish highly specific qPCR and RT-qPCR tools for cell enumerations and expression of involved genes, stable isotope probing to follow growth on the target compounds and GFP-tagging to visualize the bioaugmentation strains directly in samples, all in combination with removal studies of the target compounds. The concept of the approach is demonstrated through a case study involving degradation of aromatic hydrocarbons in activated sludge augmented with the bioaugmentation strain *Pseudomonas monteilii* SB3078.

1. Introduction

Bioremediation is an environmental friendly and economic approach for the cleanup of soil and water contaminated with heavy metals and/or organic pollutants (Tyagi et al., 2011). The diverse metabolic capacity of microorganisms combined with their ability to adapt to various environments represent the backbone of this technology, and allows the conversion of toxic substances into harmless or, at least, less harmful compounds (El Fantroussi and Agathos, 2005). The combined capacity of catabolic pathways for a given compound within the microbial community determines the theoretical limitation for the bioremediation efficiency (El Fantroussi and Agathos, 2005; Thompson et al., 2005; Tyagi et al., 2011). Improved bioremediation may consequently be obtained by bioaugmentation with catabolically relevant microorganism or a consortium of such organisms.

Although bioaugmentation seems simple in principle, many attempts with bioaugmentation have failed due to poor survival or low activity of the bioaugmentation strains *in situ*

* Corresponding author. Tel.: +45 9940 8506; fax: +45 9814 1808.
E-mail address: jln@bio.aau.dk (J.L. Nielsen).

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(El Fantroussi and Agathos, 2005; Thompson et al., 2005). However, due to the lack of techniques to monitor these parameters it is seldom known why the bioaugmentation strains perform poorly. The recent advancements in sequencing technologies and molecular techniques overcomes these limitations (Padmanabhan et al., 2013).

The aromatic hydrocarbons benzene, toluene, ethylbenzene and xylene isomers (BTEX) are present at high concentrations in crude oil and petrochemical products such as gasoline (Jo et al., 2008; Trusek-Holownia and Noworyta, 2012). BTEX compounds are consequently found in the 10 mg/L concentration range in processed water from the oil industry (Faksness et al., 2004). Similar levels may consequently be encountered in the wastewater treatment plants (WWTP) during peak loads. Exposure to even low concentrations of BTEX compounds are associated with human health problems, which include damage of the nervous system and leukemia (Costa et al., 2012; Mathur et al., 2007; Shen, 1998). Benzene, toluene and ethylbenzene are listed as priority pollutants by the U.S. Environmental Protection Agency (USEPA, 1996) and benzene is considered a priority pollutant by the European Commission (EC, 2008). There are accordingly strict requirements for the effluent limit of total BTEX after wastewater treatment. In order to meet these requirements there is for some WWTP a need to temporally improve the BTEX degradation. This improvement may be obtained through bioaugmentation.

Pseudomonas monteilii SB3078 is part of the commercial bioaugmentation product BioRemove™2300 (Novozymes Biologicals Inc.) for enhanced overall biodegradation of petrochemical hydrocarbons in WWTPs. The specific role of SB3078 is the degradation of BTEX compounds, which occur via a toluene degradation (tod) pathway encoded by the *todXFC$_1$C$_2$BADEGIH* operon, which is similar to that in *Pseudomonas putida* F1 (Choi et al., 2003; Cho et al., 2000; Dueholm et al., 2014).

Here we present a general applicable multiphasic methodological approach that provides detailed information on the performance of individual bioaugmentation strains under *in situ* conditions. The approach combines a simple method for designing strain-specific quantitative polymerase chain reaction (qPCR) cell enumeration and reverse transcription (RT)-qPCR activity assays with DNA stable isotope probing (SIP) and enhanced green fluorescent protein (eGFP)-tagging of bioaugmentation strains for direct fluorescence microscopy imaging. The quality of the approach is demonstrated through a case study involving bioaugmentation of activated sludge with SB3078 for enhanced BTEX degradation.

Application of the approach confirmed the bioaugmentation potential of SB3078 for benzene, toluene, and ethylbenzene degradation in activated sludge. However, it also identifies two fundamental problems associated with direct application of SB3078 as planktonic pure cultures. These are the loss of the bioaugmentation cells by wash out during the secondary settling and low activity due to nutrient scarcity. Access to such information will allow better design of future bioaugmentation strategies.

2. Methods

2.1. Microorganism and media

P. monteilii SB3078 was obtained from Novozymes Biological, Inc. (Salem, VA). The microorganism was routinely grown in LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.0) or M9 minimal salts (12.8 g/L Na_2HPO_4 $7H_2O$, 3.0 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, 2 mM $MgSO_4$, pH 7.4) supplemented with 10 g/L D-glucose at 25 °C with 200 rpm of agitation. The ability of SB3078 to assimilate the individual BTEX compounds was determined by washing and diluting an active culture of SB3078 in M9 minimal salts to an optical density at 600 nm (OD_{600}) of 0.01. Aliquots of 2 mL culture were then transferred to 10 mL crimp sealed flasks and supplemented with 250 μg/mL of carbon source. The cultures where grown over night (25 °C, 150 rpm) after which OD_{600} was measured.

Activated sludge was obtained from two WWTPs on the same day as the experiments were conducted. Aalborg West (AAW) WWTP (Aalborg, Denmark; 275,000 population equivalents), which is treating domestic wastewater with 30% industrial contribution and Fredericia Central (FC) WWTP (Fredericia, Denmark; 420,000 population equivalents), which is treating domestic wastewater with 75% industrial contribution (primarily from the oil industry). Both WWTPs carry out biological phosphorous removal. The concentration of suspended solids (SS) in the activated sludge was determined using a HB43-S moisture analyzer (Mettler Toledo) prior to each experiment.

2.2. DNA and RNA purification

Samples for DNA extraction were obtained by pelleting 500 μL of activated sludge (SS ≈ 2 g/L) by centrifugation (21,100*g*, 5 min), removing the supernatant and storing the pellet at −18 °C. DNA was purified from the samples using the FastDNA Spinkit for Soil (MP Biologicals) according to manufacturer's recommendations and the concentration was determined using the Quant-iT PicoGreen assay (Life Technologies) or the Qubit dsDNA HS assay kit (Life Technologies). DNA purity was confirmed using the A_{230}/A_{260} and A_{260}/A_{280} ratios on a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific). The DNA did not contain PCR inhibitors as determined by qPCR of diluted samples.

Samples for RNA extraction were made by mixing 500 μL of pure culture ($OD_{600} \approx 1$) or activated sludge (SS ≈ 2 g/L) with 500 μL RNAlater (QIAGEN) and storing the samples at 4 °C. Total RNA was isolated from the samples using the PowerLyzer UltraClean Tissue & Cells RNA Isolation Kit (MO-BIO) according to the manufacturer's recommendations with some minor modifications. Briefly, the supplied beating tubes were replaced with lysing matrix E tubes (MP Biologicals) and bead beating was performed using a Fast Prep 120 (Bio101) bead beater (2 × 45 s, 6 ms^{-1}). An on-column as well as an in-solution DNase I treatment was performed using the On-Spin Column DNase I Kit (MO-BIO) in order to remove genomic DNA contaminations. An RNA clean up was performed after the in-solution DNase I treatment using the same RNA isolation kit. The RNA concentration was determined using the Qubit RNA BR assay kit (Life Technologies). RNA integrity was confirmed using a 2200 TapeStation system (Agilent Technologies) with the R6K ScreenTape (Agilent Technologies). The RNA did not contain reverse transcriptase or PCR inhibitors as determined by RT-qPCR of samples diluted before and after cDNA synthesis, respectively.

2.3. cDNA synthesis

cDNA was synthesized from the purified total RNA using the AffinityScript qPCR cDNA synthesis kit (Agilent Technologies) using the supplied random nonamers primers according to manufacture's recommendations. Reverse transcriptase negative controls were prepared by omitting the AffinityScript RT/RNase Block enzyme mix.

2.4. qPCR assay

A strain specific primer and hydrolysis probe set for quantification were developed using the CLC Main Workbench 6.0 based on a unique genomic region (Table 1) that was identified by splitting the complete SB3078 genome into 1000 bp fragments