Environmental Pollution 158 (2010) 3618–3625

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

2,6-Dichlorobenzamide (BAM) herbicide mineralisation by *Aminobacter* sp. MSH1 during starvation depends on a subpopulation of intact cells maintaining vital membrane functions

Ole R. Sjøholm [a], Ole Nybroe [a], Jens Aamand [a,b], Jan Sørensen [a,*]

[a] Department of Agriculture and Ecology, Section of Genetics and Microbiology, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark
[b] Department of Geochemistry, Geological Survey of Denmark and Greenland, Øster Voldgade 10, 1350 Copenhagen K, Denmark

The intact cell population of Aminobacter MSH1 mineralises BAM at a constant rate independent of growth or extended starvation in mineral solution and natural groundwater.

ARTICLE INFO

Article history:
Received 4 March 2010
Received in revised form
4 August 2010
Accepted 5 August 2010

Keywords:
Mineralisation
2,6-Dichlorobenzamide
Groundwater
Flow cytometry
Single-cell activity

ABSTRACT

Mineralisation capability was studied in the 2,6-dichlorobenzamide (BAM)-degrading *Aminobacter* sp. MSH1 under growth-arrested conditions. Cells were starved in mineral salts (MS) solution or groundwater before ^{14}C-labelled BAM (0.1 mM) was added. Cell physiology was monitored with a panel of vitality stains combined with flow cytometry to differentiate intact, depolarised and dead cells. Cells starved for up to 3 weeks in MS solution showed immediate growth-linked mineralisation after BAM amendment while a lag-phase was seen after 8 weeks of starvation. In contrast, cells amended with BAM in natural groundwater showed BAM mineralisation but no growth. The cell-specific mineralisation rate was always comparable (10^{-16} mol C intact cell^{-1} day^{-1}) independent of media, growth, or starvation period after BAM amendment; lower rates were only observed as BAM concentration decreased. MSH1 seems useful for bioremediation and should be optimised to maintain an intact cell subpopulation as this seems to be the key parameter for successful mineralisation.

1. Introduction

The pesticide 2,6-dichlorobenzonitrile (dichlobenil) and its degradation product 2,6-dichlorobenzamide (BAM) are among the most frequently encountered groundwater pollutants in Denmark, Sweden, Germany and the Netherlands (Holtze et al., 2008; Brüsch, 2004) in spite of the fact that its use has been discontinued in some of these countries. Environmental samples typically show limited or no mineralisation of BAM (Montgomery et al., 1972; Clausen et al., 2007; Holtze et al., 2007). In a few cases, however, soils capable of mineralising BAM have been localised and from such soils the *Aminobacter* strains MSH1 and ASI1 capable of BAM mineralisation have been isolated (Simonsen et al., 2006; Sørensen et al., 2007).

In Denmark, groundwater represents an important drinking water resource and BAM concentrations above EU threshold limits have led to the closure of several drinking water wells. It has therefore been suggested to use *Aminobacter* MSH1 for bioremediation of polluted groundwater (Sørensen et al., 2007) for example by a bioaugmentation strategy in which the bacterium is inoculated to sand filters; the latter are already present in most waterworks where they are used for removal of unwanted natural compounds like methane, hydrogen sulphide and iron species. The filters present a challenging environment with very limited nutrient availability. The survival and BAM mineralisation potential of the inoculum must therefore be considered since several studies have reported drastic reductions in viable and active bacteria during starvation (Fusconi et al., 2007; Leung et al., 2005; Tadonleke et al., 2009).

The aim of the current study was to evaluate the cell physiology and BAM mineralisation potential in *Aminobacter* MSH1 during starvation conditions designed to simulate bioremediation conditions. First we investigated the effect of medium composition on BAM mineralisation capacity of *Aminobacter* MSH1. Secondly we followed the performance of MSH1 under starvation in both mineral medium and natural groundwater. In the experiments, ^{14}C-labelled BAM was used to monitor mineralisation and a panel of fluorescent vitality stains and flow cytometry to monitor subpopulations of depolarised, dead (permeabilised) and intact (polarised and non-permeable) cells.

* Corresponding author.
E-mail address: jan@life.ku.dk (J. Sørensen).

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doi:10.1016/j.envpol.2010.08.006

2. Material and methods

2.1. Chemicals

Analytical grade BAM (99.5% purity) and [Ring-U-^{14}C]-BAM (25.2 mCi mmol^{-1}, >95% radiochemical purity) were purchased from Dr. Ehrenstorfer GmbH and Izotop, respectively. Stock solutions of 5 mg ml^{-1} (26.3 mM) and 400,000 dpm ml^{-1} (7.2 μM) for unlabelled and labelled BAM, respectively, were made in 99.5% dimethylsulfoxide (Merck).

2.2. Bacteria

Aminobacter sp. MSH1 (Genbank DQ401867) isolated from a former plant nursery in Hvidovre, Denmark (Simonsen et al., 2006; Sørensen et al., 2007) and able to mineralise BAM was used throughout this study.

2.3. Culture experiments

Experiment 1 was designed to determine whether maintenance of physiological state and capability of BAM mineralisation in *Aminobacter* MSH1 was independent of preceding growth conditions in terms of C- and N-source and presence of BAM. MSH1 grown on R2A agar plates (Difco) was inoculated into a C- and N-free mineral salts (MS) solution supplemented with 4 mM glucose (G), 4.5 mM NH_4NO_3 (N) and 0.1 mM BAM (B) in three combinations: GB, NB or GNB. The MS solution (Sørensen and Aamand, 2003) consisted of a phosphate buffer (pH 7.2) supplemented with Ca, Mg, K, Na, Mn, Fe, S, Zn, Cu, Co, Mo and B. MSH1 cells were cultured for up to 7 days on a rotary shaker (150 rpm) at 20 °C. The stationary-phase cells were harvested, washed twice in MS solution and re-suspended in MS solution.

Experiment 2 was performed to study maintenance of physiological state of MSH1 and its capability of BAM mineralisation during growth-arresting, starvation conditions. MSH1 was cultured to stationary phase (OD_{600} 1.4) in R2B medium (Difco) with shaking (150 rpm) at 20 °C and harvested after 3 days. The stationary-phase cells were washed twice in MS or groundwater and re-suspended in the C- and N-free MS solution or in natural groundwater collected from Islevbro waterworks (Hvidovre, Denmark); the latter contained (mg l^{-1}): total solids (600), non-volatile organics (2.1), nitrate (1.6), ammonium (<0.01), phosphorous (<0.01) and iron (<0.005). BAM was found at a concentration of 0.015 μg l^{-1} in the groundwater well below the EC limit of 0.1 μg l^{-1}. Further chemical data are available online at http://www.ke.dk/portal/pls/portal/docs/578002.PDF.

In both experiments 1 and 2, suspensions of the washed, stationary-phase cells were adjusted to a final density of 2×10^6 cells ml^{-1} and 25-ml suspensions in MS or groundwater were distributed in triplicate Erlenmeyer flasks. All flasks were equipped with a small (8 ml) glass vial (base trap) containing 2 ml 0.5 M NaOH. The flasks were closed with airtight glass stoppers and incubated on a rotary shaker (150 rpm) at 20 °C in the dark. The growth-arresting starvation conditions were maintained in the flasks until unlabelled (0.1 mM) and ^{14}C-labelled (20,000 dpm) BAM were added after 0, 1, 3 or 8 weeks in the MS treatments and after 0, 1 or 3 weeks in the groundwater treatments.

To measure $^{14}CO_2$ released from the BAM mineralisation at regular intervals, the flasks were temporarily opened and the NaOH solution exchanged, using a laminar flow bench. For isotope analyses, the NaOH samples were mixed with 10-ml Wallac Optiphase Hisafe 3 scintillation cocktail (Wallac Oy) and counted for 10 min in a Wallac 1409 liquid scintillation counter. The $^{14}CO_2$ measurements were corrected for background radioactivity and quenching.

2.4. Cell staining and flow cytometry

When base traps were exchanged, aliquots of 100 μl cell suspension were also taken to observe changes of physiological state and growth of *Aminobacter* MSH1. Our protocol to discriminate several physiological states of cells by differential staining and flow cytometry (Nielsen et al., 2009) was here modified to detect: Total cells, depolarised cells and permeabilised (dead) cells. First, each 100-μl sample was prepared for staining when mixed with 870 μl MS solution containing 5 mM EDTA and 4 mM glucose for outer membrane permeabilisation and polarisation respectively (Leive and Kollin, 1967; Nebe-von-Caron et al., 2000; Shapiro, 2003). The MS solution also contained 2×10^5 ml^{-1} (2 μm diameter) yellow-green Fluospheres-carboxylate microspheres (F-8827; Molecular Probes) as internal standards for flow control in the flow cytometer.

Cells were first stained to detect the dead and depolarised cell subpopulations. Dead (permeable) cells were detected with propidium iodide (PI) (Hudson et al., 1969; Crissman and Steinkamp, 1973; Haugland, 1999) added to a final concentration of 10 μg ml^{-1} while depolarised cells were detected with bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$ or BOX) (Invitrogen, Carlsbad, CA) (Novo et al., 2000; Wilson and Chused, 1985; Tadonleke et al., 2009) added to a concentration of 10 μg ml^{-1}; recordings were made after 30 min incubation in the dark. Total cell numbers were determined by further addition of SyBr green (Invitrogen, Carlsbad, CA) to the samples (Noble and Fuhrman, 1998; Tadonleke et al., 2005); recordings were made 10 min after addition of the stain. Finally, the subpopulation of cells that was both non-permeable and polarised was calculated by difference, i.e. total cell number minus combined subpopulations of depolarised and dead cells. This subpopulation is referred to as intact cells.

Flow cytometry was performed with a FACSCalibur instrument (Becton Dickinson) equipped with 488-nm excitation from a 15 mW argon–ion laser. The channels forward scatter (FSC), side scatter (SSC) and fluorescence channels FL1 (530/30 BP) and FL2 (661/16 BP) were used for detection. Threshold was set for SSC and compensation was used as required. Carrier liquid used was 0.22 μm filtered MilliQ water. Samples were prepared to a final volume of 1 ml and measured for 30 s at low flow speed (12 ± 3 μl min^{-1}) with event counts below 2000 s^{-1}.

3. Results

3.1. Influence of preceding growth conditions on BAM mineralisation

When *Aminobacter* strain MSH1 was inoculated into MS solution supplemented with glucose + BAM (GB), NH_4NO_3 + BAM (NB), or glucose + NH_4NO_3 + BAM (GNB), total cell numbers increased from the initial 6×10^6 ml^{-1} and stationary phase was reached after 3–6 days in all three batch cultures. Final cell densities (total cell numbers) were 6×10^7 ml^{-1} (GB and NB) and 6×10^8 ml^{-1} (GNB; Fig. 1A, left). Hence, the 0.1 mM BAM seemed to support limited growth comprising either an N- or C-source in combination with an additional C (glucose) or N (NH_4NO_3) source, respectively. The highest growth was obtained when both additional C and N sources were added together with BAM.

When cells from the three above precultures were harvested (Day 0 on Fig. 1A) and re-suspended in MS solution supplemented with 0.1 mM BAM, they showed similar patterns of subsequent growth on BAM now representing the sole C- and N-source. Initial inoculum density (total cell number) was here $2–5 \times 10^6$ ml^{-1}, reaching 2×10^7 ml^{-1} after 7 days (Fig. 1A, right). Maximum abundance was therefore smallest when BAM was the sole C- and N-source, without additional NH_4NO_3 or glucose. Based on the staining protocol for flow cytometry, it was demonstrated that a majority of the total cells were intact (Fig. 1B); depolarised and dead cells accounted for only approx. 5% and 1%, respectively (data not shown).

The progress curves for BAM mineralisation (accumulating ^{14}C-CO_2 in % of added ^{14}C-BAM) had a shape similar to the growth curves and there was a high correlation between mineralisation and intact cell numbers as r^2 values were 0.90, 0.98 and 0.96 for GNB, GB and NB, respectively (Fig. 1C). Mineralisation was therefore growth-coupled and associated with the intact, proliferating cell subpopulation. Assuming the intact cell subpopulation accounted for all BAM mineralisation, we could calculate the cell-specific mineralisation rates (mol C intact cell^{-1} day^{-1}) at any sampling point during the incubations (Fig. 1C). For all treatments, the cell-specific mineralisation rates were highest (about 10^{-16} mol C intact cell^{-1} day^{-1}) in the early growth phase at Day 4. They subsequently decreased 1.5 log units during the increase in the intact cell subpopulation and another log unit from day 7 to 14 while the intact cell number remained constant. The decrease in cell-specific mineralisation is most likely caused by the increase in intact cells competing for substrate and perhaps by the quantity and quality of the remaining BAM molecules or breakdown products.

3.2. Starvation conditions in mineral salts solution

Since the preceding growth conditions had no effect on physiological state or capability of BAM mineralisation in the MSH1 cultures, we undertook all subsequent experiments with cells pre-cultured in R2B medium. A control treatment (never to receive BAM) was first established to detect changes of cell physiological state during the 8 weeks of progressive starvation in MS solution. In other flasks, the starvation conditions were released by the