Degradation and mineralization of nano-molar concentrations of the herbicide dichlobenil and its persistent metabolite 2,6-dichlorobenzamide by Aminobacter spp. isolated from dichlobenil-treated soils

Sebastian R. Sørensen,* Maria S. Holtze, Allan Simonsen and Jens Aamand

Running title: BAM mineralization by Aminobacter spp.

Department of Geochemistry, Geological Survey of Denmark and Greenland (GEUS), Øster Voldgade 10, DK-1350 Copenhagen K, Denmark

* Corresponding author. Mailing address: Department of Geochemistry, Geological Survey of Denmark and Greenland, Øster Voldgade 10, DK-1350 Copenhagen K, Denmark. Phone: +45 3814 2317. Fax: +45 3814 2050. E-mail: srs@geus.dk
ABSTRACT

2,6-dichlorobenzamide (BAM), a persistent metabolite from the herbicide 2,6-dichlorobenzonitrile (dichlobenil), is the pesticide residue most frequently detected in Danish groundwater. A BAM-mineralizing bacterial community was enriched from dichlobenil-treated soil sampled from the courtyard of a former plant nursery. A BAM-mineralizing bacterium (designated MSH1) was cultivated and identified by 16S rRNA gene sequencing and fatty acid analysis as closely related to members of the genus Aminobacter, including the only cultured BAM-degrader, Aminobacter sp. ASI. Strain MSH1 mineralized 15 – 64% of the added \([\text{ring}-\text{U}-^{14}\text{C}]\text{BAM}\) to \(^{14}\text{CO}_2\) with initial BAM concentrations in the range of 7.9 nM to 263.1 µM provided as sole carbon, nitrogen and energy source. A quantitative enzyme-linked immunoassay analysis with antibodies against BAM revealed residue concentrations of 0.35 – 18.05 nM BAM following incubation for 10 days, corresponding to a BAM depletion of 95.6 to 99.9%. In contrast to the Aminobacter sp. ASI1, strain MSH1 also mineralized the herbicide itself along with several metabolites including ortho-chlorobenzonitrile, ortho-chlorobenzoic acid and benzonitrile making it the first known dichlobenil-mineralizing bacterium. Aminobacter type strains not previously exposed to dichlobenil or BAM were capable of degrading non-chlorinated structural analogs. Combined these results suggest that closely related Aminobacter strains may have a selective advantage in BAM-contaminated environments as they are able to use this metabolite or structurally related compounds as a carbon and nitrogen source.
INTRODUCTION

Millions of tons of xenobiotic compounds are each year applied globally as pesticides in agricultural production as well as on consolidated urban areas, along railways and roads and within farmyards. As an outcome of this extensive environmental input natural water in rivers, lakes and aquifers has been contaminated with trace amounts of pesticide residues. In Denmark, where over 99% of the drinking water originates from groundwater, the detection of pesticide residues above the EC threshold limit of 0.1 µg l\(^{-1}\) has resulted in the costly closure of numerous groundwater abstraction wells (29). Not only the pesticides themselves are monitored, selected stable metabolites are also included and often these are detected more frequently than the pesticide itself (3). The most commonly encountered pesticide residue in Danish groundwater is 2,6-dichlorobenzamide (BAM). BAM is a metabolite produced from partial degradation of the benzonitrile herbicide 2,6-dichlorobenzonitrile (dichlobenil) (1,9,16) and is often highly persistent in the environment. Dichlobenil is a broad-spectrum herbicide mostly used on non-agricultural areas as well as in plant nurseries and fruit orchards. This herbicide was banned for use in Denmark in 1997, but BAM is still the main pesticide residue in Danish groundwater, with 19.7% of the abstraction wells analyzed in 2003 having detectable BAM concentrations and 8.1% of the wells containing BAM concentrations exceeding the EC threshold limit of 0.1 µg l\(^{-1}\) for drinking water (3). Similar results have been reported in 2003 in a monitoring program from Sweden (13) and BAM has additionally been detected in groundwater in the Netherlands, Germany and Italy (18,42,44).

A potential for partial degradation of the herbicide dichlobenil to BAM has been measured in various soils and subsurface sediments, with estimated half-lives ranging from 106 – 2079 days (6,8,16,41). Also 2,6-dichlorobenzoic acid, another known metabolite from dichlobenil, has been measured in dichlobenil-treated soils (8,24) and in groundwater samples (3,18). Two additional
metabolites, ortho-chlorobenzamide and ortho-chlorobenzoic acid, have been detected in laboratory
experiments with dichlobenil-treated soils (8). The metabolite BAM appears to be much more
persistent than dichlobenil itself and several studies have reported no apparent degradation of BAM
in soils (1,6,8,16,40,41), aquifer sediments (2,6,36,37) or bacterial isolates (9,43). Little is therefore
known about the environmental degradation of BAM.

We are interested in using degradative bacteria for remediation of groundwater contaminated
with low concentrations of BAM, and we have therefore recently initiated a large-scale screening of
dichlobenil-treated areas to locate soils capable of rapid mineralization of BAM (10,25). These
efforts have pinpointed six soils that have a potential for mineralization of \([\text{ring-}\text{U-}^{14}\text{C}]\text{BAM}\) to
\(^{14}\text{CO}_2\), out of a total of 79 samples screened for mineralization activity, obtained from 39 different
Danish locations previously exposed to dichlobenil. One of the soils with a potential for BAM
mineralization, but with no evident dichlobenil mineralization, has recently been used to enrich and
isolate a BAM-mineralizing bacterium identified as an *Aminobacter*, designated strain ASI1 (25). A
second soil sampled from the courtyard of a former plant nursery located above a BAM-
contaminated aquifer had a unique ability to mineralize both BAM and \([\text{ring-}\text{U-}^{14}\text{C}]\text{dichlobenil}\) to
\(^{14}\text{CO}_2\) (25) and we later succeeded in obtaining stable BAM-mineralizing enrichment cultures (8),
but isolation of degradative bacteria was not achieved. In this study we revived one of these
enrichment cultures and isolated a BAM- and dichlobenil-mineralizing *Aminobacter* sp. (designated
strain MSH1). This strain is closely related to our BAM-mineralizing *Aminobacter* sp. ASI1, and
these two strains along with selected *Aminobacter* type strains were compared with regards to their
taxonomy, degradative capacity toward dichlobenil and related compounds as well as their ability to
degrad and mineralize low concentrations of BAM and dichlobenil.
MATERIALS AND METHODS

Dichlobenil and metabolites. Analytical grade dichlobenil (CAS RN 001194-65-6) (99.5% purity, 18 mg l\(^{-1}\) water solubility at 20°C), BAM (CAS RN 002008-58-4) (95.5% purity, 2,730 mg l\(^{-1}\) water solubility at 23°C), 2,6-dichlorobenzoic acid (CAS RN 000050-30-6) (99.5% purity, 14,100 mg l\(^{-1}\) water solubility at 20°C), benzonitrile (CAS RN 000100-47-0) (99.5% purity, water solubility 2,000 mg l\(^{-1}\) at 25°C), benzoic acid (CAS RN 000065-85-0) (99.5% purity, water solubility 3,400 mg l\(^{-1}\) at 25°C) were all purchased from Dr. Ehrenstorfer (Augsburg, Germany). Ortho-chlorobenzonitrile (CAS RN 000873-32-5) (99% purity, water solubility unknown), ortho-chlorobenzamide (CAS RN 000609-66-5) (97% purity, water solubility unknown), ortho-chlorobenzoic acid (CAS RN 000118-91-2) (98% purity, water solubility 2,090 mg l\(^{-1}\) at 25°C) were acquired from ABCR GmbH & Co (Karlsruhe, Germany). Benzamide (CAS RN 027208-38-4) (99.3% purity, water solubility unknown) was obtained from Acros Amresco ICN Biomedicals (Irvine, CA). \([{\text{ring}}\-{\text{U}}\-^{14}\text{C}]\)dichlobenil (28.3 mCi mmol\(^{-1}\)) \((^{14}\text{C}\)-dichlobenil) and \([{\text{ring}}\-{\text{U}}\-^{14}\text{C}]\)BAM (25.2 mCi mmol\(^{-1}\)) \((^{14}\text{C}\)-BAM) were purchased from International Izotop (Budapest, Hungary). Radiochemical purities better than 95% were verified for the \(^{14}\text{C}\)-dichlobenil and \(^{14}\text{C}\)-BAM in our laboratory by a TLC-based assay. Both these \(^{14}\text{C}\)-tracers are labeled in the ring structure, which enables us to conclude that mineralization is occurring whenever more than 5% of the initially added \(^{14}\text{C}\)-tracer is recovered as \(^{14}\text{CO}_2\).

Enrichment and isolation of a BAM-mineralizing bacterium. A BAM-mineralizing enrichment culture, designated 2-MS-V, derived from dichlobenil-treated soil sampled from the courtyard of a former plant nursery located above a BAM-contaminated aquifer near Hvidovre in Denmark (10) served as the inoculum for the current enrichment procedure. A 50% glycerol stock solution of enrichment culture 2-MS-V was removed from storage at – 80°C and washed twice in
sterile phosphate buffer before inoculation into sterilized 100-ml glass flasks containing 25.0 ml of
an autoclaved mineral salt solution (MS) (26). BAM and $^{14}$C-BAM were added from a 5,000-mg l$^{-1}$
stock solution in HPLC-grade dimethylsulfoxide (Merck, Darmstadt, Germany) directly to the
media providing 263.12 µM BAM (50-mg l$^{-1}$) as sole source of carbon, nitrogen and energy and
10,000 DPM $^{14}$C-BAM as a tracer for detection of $^{14}$CO$_2$ production. $^{14}$C-BAM mineralization to
$^{14}$CO$_2$ was followed as described below. When the mineralization of BAM was completed, 10% vol.
was transferred to fresh BAM-containing MS medium to a final volume of 25.0 ml. After 10 sub-
culturings of the enrichment culture over a period of approximately 500 days, plating on R2A
(Difco Laboratories, Detroit, MI) was performed. The plates were incubated for up to one month at
20°C and dominant colonies were picked, streaked for purity and screened for BAM degradation in
MS medium.

**Characterization of strains MSH1 and ASI1.** One isolate, designated strain MSH1, and the
recently isolated BAM-degrading *Aminobacter* sp. ASI1 (25) were both characterized and identified
by Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany (DSMZ)
by analysis of the cellular fatty acids and different physiological tests. Additionally, almost complete
16S rRNA gene sequences were determined for strains MSH1 (1463 bp sequenced) and ASI1 (1485
bp sequenced) within the current study. Genomic DNA extraction, PCR amplification of the 16S
rRNA gene and purification of the PCR products was performed as described previously (21).
Sequence data were compared to 16S rRNA gene sequences deposited in the GenBank database.
Sequences from closely related type strains were obtained and manually aligned using the AE2
alignment editor (14). Strains MSH1 and ASI1 were also tested for resistance to the antibiotics
ampicillin, chloramphenicol, kanamycin, penicillin G, streptomycin, rifampicin, nalidixin and
tetracycline in $1/10$ strength Luria-Bertani media (LB) (Difco Laboratories, Detroit, MI). All
antibiotics were purchased from ICN Biomedicals Inc, (Aurora, OH).
Bacterial strains and cultivation conditions. Three type strains from the genus *Aminobacter*, *A. aminovorans* DSM 7048\textsuperscript{T}, *A. aganoensis* DSM 7051\textsuperscript{T} and *A. niigataensis* DSM 7050\textsuperscript{T} (38), were obtained from DSMZ and tested for degradation of dichlobenil, its metabolites and related structural analogs. These were cultured in MS medium supplemented with 5.0 g l\textsuperscript{-1} Proteose Peptone No. 3 (Difco Laboratories) and 3.0 g l\textsuperscript{-1} Yeast Extract (Difco Laboratories) (MS-C) prior to the degradation experiments. The strain MSH1 and ASI1 were either pre-grown in MS with 25 – 50 mg l\textsuperscript{-1} BAM (132.5 – 263.1 µM) or washed from R2A plates prior to the degradation and mineralization experiments.

Degradation and mineralization experiments. Mineralization of \textsuperscript{14}C-BAM in MS was followed by inserting a 10-ml glass tube containing 2.0 ml 0.5 M NaOH to trap \textsuperscript{14}CO\textsubscript{2} into the 100-ml culture flasks following inoculation. The final volume of liquid media in the culture flasks was 25 ml. The NaOH was replaced at regular intervals in a laminar flow bench, mixed with 10 ml Wallac OptiPhase HiSafe 3 scintillation cocktail (Turku, Finland) and counted for 10 min in a Wallac 1409 liquid scintillation counter. Correction for evaporation of \textsuperscript{14}C-dichlobenil into the NaOH trap was necessary due to its high vapor pressure (31) potentially leading to an overestimation of the mineralization extent. Therefore, 2.2 ml 0.5 M NaOH served as the base trap in the dichlobenil mineralization experiments, with 1.0 ml used for measuring the total \textsuperscript{14}C trapped in the NaOH trap by scintillation counting and another 1.0 ml was used for estimating the amount of \textsuperscript{14}C-dichlobenil evaporated into the trap. The trapped \textsuperscript{14}C-dichlobenil was quantified by acidifying the 1.0 ml base solution with 1.5 ml 1 M HCl, allowing the \textsuperscript{14}CO\textsubscript{2} to evaporate during incubation for one hour followed by scintillation counting as described above. The dichlobenil mineralization was calculated by subtracting the evaporated \textsuperscript{14}C-dichlobenil from the total \textsuperscript{14}C amount measured. Mineralization experiments with BAM and dichlobenil in different initial concentrations were prepared by adding 250 µl of different stock solution strengths containing a mixture of \textsuperscript{14}C and
unlabeled compound in dimethylsulfoxide. The lowest concentrations tested were 7.9 nM BAM and 8.7 nM dichlobenil corresponding to the addition of only $^{14}$C-labeled compound equal to approximately 10,000 DPM per flask. The highest dichlobenil concentration tested, 290.7 µM, was above the solubility of dichlobenil and crystals were visible at the initiation of the experiment.

Degradation of dichlobenil, BAM and seven other dichlobenil metabolites and structural analogs by the *Aminobacter* strains was tested in 5.0 ml MS medium supplemented with 50.0 µM of each compound individually. 4.9 ml MS medium was inoculated with 0.1 ml of a washed cell suspension having an OD$_{600nm}$ of 0.5 and sub-samples were analyzed by the high-performance liquid chromatography (HPLC) method developed by Holtze et al. (8) following incubation for 14 days. The quantification was performed using a Hewlett-Packard Series 1050 HPLC System equipped with a UV detector (Phenomenex, Chire, UK) as described by Holtze et al. (8). Prior to the HPLC analysis, 750 µl sub-samples of the media were filtered through a 0.2 µm PTFE membrane 17 mm syringe filter (Titan Filtration Systems, Sun SRI, Wilmington, NC) and the last 250 µl were collected for analysis. The HPLC detection limits for the nine compounds were < 1 µM (0.035 – 0.159 mg l$^{-1}$) by direct HPLC analysis of the filtered liquid media (8). The media samples for HPLC measurements were stored at – 15°C for up to two weeks before analysis. All degradation experiments were conducted at 20°C in the dark and sterile controls were included.

Specific enzyme-linked immunosorbent assay (ELISA) for quantification of residual BAM concentrations. A highly specific and sensitive ELISA-assay developed by Bruun et al. (4) for detection of BAM in groundwater samples was used for quantifying the residual BAM concentrations in sub-samples of the culture media following the mineralization experiments. This ELISA-assay has a detection limit of 0.02 µg l$^{-1}$, equal to 0.105 nM BAM, and is very specific with only negligible cross-reactions with dichlobenil and BAM metabolites as well as structural analogs (4). Media samples for ELISA where stored at – 15°C for up to two weeks before analysis.
Nucleotide sequence accession numbers. The 16S rRNA sequences for *Aminobacter* sp. MSH1 and *Aminobacter* sp. ASI1 have been deposited in GenBank database under accession numbers DQ401867 and DQ401866, respectively.

RESULTS

Isolation and characterization of a BAM-mineralizing bacterium. A previously described BAM-mineralizing mixed bacterial enrichment culture was successfully revived and used for enrichment and isolation of the BAM-mineralizing bacterial strain MSH1. Isolate MSH1 is a gram-negative irregular motile rod with a width of 0.6 – 0.8 µm and a length of 2.0 – 3.5 µm after growth in nutrient-rich broth. It is oxidase, catalase and urease positive. MSH1 did not hydrolyze esculin, gelatine, tween 80, DNA or starch. It is negative in tests for nitrate reduction and production of H₂S. When grown on R2A agar its colony morphology is similar to that of *Aminobacter* sp. ASI1 with white colonies having a characteristic reddish-brown center appearing after 4 – 5 days at 20°C. MSH1 used glucose, D-arabinose, mannose, maltose, N-acetyl-glucosamin, acetate, D-arabitol, D-fructose, fumarate, pyruvate, L-rhamnose and D-sucrose as growth substrates. In contrast, no growth was detectable with L-arabinose, malat, adipate, citrate, caprate, gluconate, 2-OH-valerate, lactose and lactulose. Both MSH1 and ASI1 were insensitive to the antibiotic streptomycin (50 mg l⁻¹) but sensitive to ampicillin, chloramphenicol, kanamycin, penicillin G, rifampicin, nalidixin and tetracycline. It was not possible to distinguish MSH1 from *Aminobacter* sp. ASI1 based on the different physiological tests mentioned above. Additionally, whole-cell fatty acid profiles were prepared for isolate MSH1 and compared to previously published data for *Aminobacter* sp. ASI1 (25). For both strains the dominant fatty acid was 18:1ω7c with 75.0% in MSH1 and 67.8% in ASI1. The second most dominant fatty acid in MSH was 16:0 with 8.3% in comparison to 7.2% in
ASI1. In contrast, 11-methyl-18:1\textsubscript{ω7c} was the second most dominating fatty acid in ASI1 with 10.6\% while MSH1 only had 1.2\% of this fatty acid.

The 1463 bp 16S rRNA gene sequence obtained from isolate MSH1 is identical to the 16S rRNA gene sequences from \textit{A. aganoensis} DSM 7051\textsuperscript{T} and \textit{A. niigataensis} DSM 7050\textsuperscript{T}. It has 99.5 – 99.8\% similarity to the three remaining members of the genus \textit{Aminobacter}: \textit{A. aminovorans}, \textit{A. lissarensis} and \textit{A. ciceronei}. Similarities of 96.8 – 97.8\% are apparent with different type strains from the genera \textit{Pseudaminobacter} and \textit{Mesorhizobium}. 99.6\% similarity to the 1485 bp 16S rRNA gene sequence obtained from the recently described BAM-mineralizing \textit{Aminobacter} sp. strain ASI1 was observed. A phylogenetic tree showing the affiliation of strains MSH1 and ASI1 with closely related type strains is presented in Figure 1.

**Mineralization of BAM and dichlobenil by strains MSH1 and ASI1.** Strain MSH1 was tested for mineralization of BAM in concentrations ranging from 7.9 nM to 263.1 µM (Fig. 2A). With the lowest concentration, 14.7 ± 3.7\% of the added \textsuperscript{14}C-BAM was mineralized to \textsuperscript{14}CO\textsubscript{2} within 10 days. Increasing the BAM concentration to 534.1 nM gave a higher extent of mineralization with 31.4 ± 6.6\% measured as \textsuperscript{14}CO\textsubscript{2} within the same period. In contrast, a longer lag-phase before the onset of rapid mineralization was measured with the three highest BAM concentrations of 5.3 – 263.1 µM and at the end of the experiment 53.4 – 64.4 \% was mineralized (Fig. 2A). Generally, a similar pattern was observed with the mineralization of dichlobenil, the lowest concentrations having the lowest extent of mineralization (Fig. 2B). However, compared to the mineralization of BAM, dichlobenil mineralization was slower and occurred to a lower extent within a comparable concentration range. The highest dichlobenil mineralization of 50.9 ± 0.4\% was observed with a concentration of 58.1 µM within the 10 day experiment. The highest dichlobenil concentration of 290.7 µM was mineralized to the lowest extent during the 10 day experiment (Fig. 2B), and it took
20 additional days of incubation before this dichlobenil concentration was mineralized to approximately 50% $^{14}$CO$_2$ (data not shown). This might reflect an inhibition of the degradative activity by strain MSH1 with high dichlobenil concentrations. Minor evaporation of $^{14}$C-dichlobenil into the base trap was measured during the 10 day experiment with a maximum of 2.5 ± 1.0% detected in the flask initiated with 5.8 µM dichlobenil. Aminobacter sp. ASI1 was tested for mineralization with the three lowest concentrations of BAM and dichlobenil (Fig. 2C and 2D). ASI1 mineralized BAM to a higher extent than MSH1, with 43.5 – 66.6% of the added BAM mineralized to $^{14}$CO$_2$ within 10 days (Fig. 2C), and ASI1 in contrast to strain MSH1 had no degradative activity towards dichlobenil (Fig. 2D and Table 1). Dichlobenil evaporation into the base trap was quantified to a maximum of 2.4% (± 0.1%) of the initially added $^{14}$C-dichlobenil during the 18 day experiment with ASI1.

**Growth and yield studies with strains MSH1 and ASI1.** Strain MSH1 used BAM as well as dichlobenil as sources of carbon, nitrogen and energy for growth and the cell density after mineralization of different BAM concentrations estimated by plating unto R2A is presented in Figure 3. Initial cell densities were 2 – 4 × 10$^6$ ml$^{-1}$ for both strains and in all experiments and after mineralization of 263.1 µM BAM, the highest tested concentration, 9.6 × 10$^{10}$ ± 0.4 × 10$^{10}$ cells of MSH1 per liter culture media was measured. In comparison, 3.4 × 10$^9$ ± 0.6 × 10$^9$ cells of MSH1 per liter culture media was measured with the lowest tested BAM concentration (7.9 nM). Strain ASI1 showed minor growth with cell densities of 1.8 × 10$^8$ ± 0.4 × 10$^8$, 2.4 × 10$^8$ ± 1.2 × 10$^8$ and 3.1 × 10$^8$ ± 0.8 × 10$^8$ ASI cells per liter culture medium measured following the mineralization of 7.9 nM, 534.1 nM and 5.3 µM BAM, respectively. This growth was not clearly distinct from the background growth in MS medium. It is however possible to produce dense and flocculated ASI1 cells cultures with higher BAM concentrations and extended periods of incubation (data not shown).
Residual BAM concentrations following BAM and dichlobenil mineralization by strains MSH1 and ASI1. A quantitative ELISA assay was used to measure residual BAM concentrations following the mineralization experiments with strains MSH1 and ASI1 presented in Figures 2A and 2C. Residue concentrations of 0.22 – 18.05 nM BAM were measured following mineralization of initial BAM concentrations from 7.9 nM to 263.1 µM by MSH1 and ASI1 corresponding to a depletion of BAM in the range of 95.6% to 99.9% relative to the initial concentration (Table 2). With a 99.9% BAM depletion the most efficient degradation was apparent with strain MSH1 and initial BAM concentrations from 5.3 µM BAM and above. At the BAM concentrations below 5.3 µM the two strains were highly similar in their BAM efficiency with remaining residues in concentrations of 0.35 – 0.41 nM BAM. This is in contrast to the different extent of 14C-BAM mineralization observed for the two strains by measuring 14CO2 production (Fig 2A and 2C).

Following the mineralization of dichlobenil by MSH1 (Fig. 2B) sub-samples of the liquid medium were analyzed using the ELISA-approach. No BAM was detected below an initial dichlobenil concentration of 5.9 µM (Table 3). Above this initial dichlobenil concentration only 0.004 – 0.006% of the initially added herbicide was detected as BAM, corresponding to detections of 0.22 to 3.34 nM of BAM after the 10 day mineralization assay.

Aminobacter spp. substrate range. The results of testing our BAM-mineralizing Aminobacter isolates and the closely related Aminobacter type strains for degradative capacity towards dichlobenil, BAM and several of their potential metabolites and structural analogs is summarized in Table 1. Strain MSH1 is the most versatile amongst the tested strains and degrades all tested compounds. In contrast, strain ASI1 only degrades BAM, 2,6-dichlorobenzoic acid, ortho-chlorobenzamide, benzamide and benzoic acid (Table 1). Surprisingly, however, A. aminovorans DSM 7048T, A. aganoensis DSM 7051T and A. niigataensis DSM 7050T have a native ability to degrade benzonitrile (Table 1) with a corresponding accumulation of a compound tentatively
identified as benzamide by comparing its HPLC retention time with that of an authentic standard (data not shown). The only other metabolite detected within the screening experiment summarized in Table 1 was a compound produced by degradation of ortho-chlorobenzamide by strain ASI1 with an HPLC retention time similar to ortho-chlorobenzoic acid. Both *A. aganoensis* DSM 7051^T^ and *A. niigataensis* DSM 7050^T^ degraded benzoic acid separating them from *A. aminovorans* DSM 7048^T^ that had no degradative activity towards this compound (Table 1).

**DISCUSSION**

Following a screening of samples obtained from different dichlobenil-treated locations in Denmark we have obtained a collection of soils with indigenous microorganisms capable of mineralizing the groundwater contaminant BAM. This metabolite was previously considered persistent in the environment. Within this screening we also located one apparently unique soil, sampled from the courtyard of a former plant nursery, able to perform a rapid and extensive mineralization of dichlobenil itself. This soil served as the basis for the BAM-grown enrichment culture revived and used to isolate *Aminobacter* sp. MSH1 within the current study. This isolate reflects the degradation capacity of the original soil and mineralizes both BAM and the mother compound dichlobenil. Similarly, the recently isolated *Aminobacter* sp. strain ASI1 mirrors the degradation capacity of its native soil performing only BAM mineralization and has no capacity to degrade dichlobenil. However, the ability to degrade dichlobenil to BAM seems to be widespread, as even bacteria without any previous contact with the herbicide are capable of performing this initial degradation step (9) possibly explaining the much more frequent detection of BAM as a water contaminant in comparison to dichlobenil. Furthermore, dichlobenil is moderately to strongly sorbed to soils, opposite BAM that has a much lower soil sorption and higher water solubility (5),
which suggests that dichlobenil could act as a soil-sorbed reservoir slowly releasing BAM to the soil water. The fact that our previous screening only located one soil performing extensive dichlobenil mineralization, in contrast to several soils performing rapid BAM mineralization, likewise suggests that the high soil sorption of dichlobenil restricts its availability to the soil microorganisms as shown for other pesticide residues (e.g. 11), whereby the microbial populations within a dichlobenil-treated area are more exposed to BAM than to dichlobenil itself. Microbial adaptation to biodegradation of various persistent pesticides as a consequence of prolonged exposure has been described before (e.g. 7,36), and the same phenomenon seems like a plausible explanation for the occurrence of BAM-mineralizing bacteria in dichlobenil-treated soils.

Besides the difference in the metabolic versatility of our two *Aminobacter* isolates they seem to be highly taxonomically similar. The α-proteobacteria genus *Aminobacter* was proposed by Urakami *et al.* (38) and originally consisted of three species, *A. aminovorans*, *A. aganoensis* and *A. niigataensis*, and only recently have two new species, *A. lissarensis* and *A. ciceronei* been included (15). Members of this genus appear to be widely distributed in terrestrial environments (15,21,32,33), and several isolates have been enriched and cultivated based on their ability to use different xenobiotics as growth substrates including the pesticides atrazine and carbofuran (15,21,32,33). Other *Aminobacter* strains have been isolated based on the ability to utilize methylhalides as carbon and energy sources (15) or nitrilo-acetate as carbon, nitrogen and energy source (12), suggesting that the members of this genus are very metabolically versatile. Besides being closely related to each other our two BAM-mineralizing strains are also highly similar to all five *Aminobacter* species (Fig. 1). 16S rRNA gene sequencing and alignment suggests that strains ASI1 and MSH1 belong to either *A. aganoensis* or *A. niigataensis*. However, neither the fatty acid analysis nor the phenotypic characterizations are sufficient to assign the isolates to one of these species with certainty.
The fact that such highly similar *Aminobacter* strains can be enriched and isolated from geographically different soils when providing BAM as sole source of carbon, nitrogen and energy seems remarkable. Interestingly, similar results have been presented by Topp *et al.* (32) in a study where 14 indistinguishable atrazine-degraders were isolated from different French and Canadian agricultural soils. These strains were originally reported to be members of the genus *Pseudaminobacter* (32) but one representative for the 14 isolates, strain C147, has recently been assigned to the genus *Aminobacter* as the new species *A. ciceronei* (15). Topp *et al.* (32) suggested that enrichment of *Aminobacter* strains from the different atrazine-treated soils may be due to a native ability of their isolates to utilize alkyl-amines, which allowed for the usage of atrazine as a source of nitrogen and carbon when the *atzABC* genes are acquired. Three *Aminobacter* type strains closely related to our BAM-mineralizing isolates, *A. aminovorans*, *A. aganoensis* and *A. niigataensis*, all appeared to harbor nitrile hydratase activity degrading benzonitrile to benzamide.

Additionally, *A. aganoensis* and *A. niigataensis* were both capable of degrading benzoic acid (Table 1). These compounds are non-chlorinated structural analogs to dichlobenil and the dichlobenil- and BAM-metabolite 2,6-dichlorobenzoic acid which indicates that these *Aminobacter* strains naturally may develop the entire degradation pathway upon acquiring or evolving genes encoding enzymes involved in the initial steps of the mineralization pathway. One defining feature of members of the genus *Aminobacter* is that they are facultative methylotrophs capable of utilizing methyl-amine as growth substrates (38). According to this utilization of the side-chain of BAM may also be part of the explanation for the proliferation of the BAM-mineralizing *Aminobacter* strains in our study.

Besides using the nitrogen and possibly also the carbon from the side-chain of BAM, both strains ASII and MSH1 were capable of mineralizing the ring structure with a maximum of 60 – 70% $^{14}$C-ring-carbon metabolized to $^{14}$CO$_2$. The remaining $^{14}$C may serve as a carbon source whereby it is incorporated into the biomass as known from other pesticide-mineralizing isolates (27,28). The
occurrence of $^{14}$C-labeled metabolites not measured with our HPLC method can however not be excluded.

Many studies with environmental samples (e.g. 22,30,34) and isolated microorganisms (22,39) have reported threshold concentrations for biodegradation of xenobiotics below which no extensive degradative activity has been detected. The phenomenon is often explained by lack of microbial growth below a critical substrate concentration being in the micro- to nano-molar range (e.g. 23,34,35). Such thresholds therefore appear as an obvious explanation for the persistence of biodegradable pesticide residues at trace concentrations in water resources. Some observations however suggest that bacteria adapted to processing low concentrations of xenobiotic compounds can be isolated (20,35). Both *Aminobacter* sp. strain MSH1 and strain ASI1 appeared to be very efficient at degrading and mineralizing ecologically relevant concentrations of BAM. The EC threshold limit of 0.1 µg l$^{-1}$ for pesticide residues in drinking water corresponds to 0.53 nM BAM and both isolates were capable of removing BAM to concentrations below this threshold limit when provided with initial BAM concentrations of 7.9 nM and 534.1 nM (Fig. 2 and Table 2). The presence of metabolites from partial degradation of BAM can however not be excluded as the ELISA-assay exclusively targets BAM (4). There might be a threshold for BAM degradation at about 0.2 – 0.4 nM, as both isolates appear to have BAM residues in this concentration range in the liquid media following the mineralization experiments (Table 2). Further studies are however needed to address the possibility of degradation to even lower residual concentrations given prolonged incubation periods and following additional optimization of the cultivation process. Measurements of the BAM concentrations following the dichlobenil mineralization experiment with strain MSH1 revealed none or only minor amounts of BAM (Table 3), which could suggest that the strain is very efficient in degrading dichlobenil. Based on the specificity of the ELISA-approach it
can however not be excluded that other metabolites as well as dichlobenil itself still are present in the liquid media.

In conclusion, this is the first time that a dichlobenil-mineralizing bacterium has been isolated and characterized. There is a considerable interest in developing efficient remediation methods for BAM contaminated water resources and our results demonstrate that this new isolate, *Aminobacter* sp. MSH1 is a promising candidate for use in bioremediation processes aimed at cleaning natural waters polluted with low concentrations of BAM or dichlobenil. The high degree of similarity to the recently isolated BAM-mineralizing *Aminobacter* ASI1, and the fact that closely related *Aminobacter* type strains are capable of degrading non-chlorinated structural analogs, adds to the knowledge on the metabolic capacities of this genus, and suggests that this closely related group of *Aminobacter* spp. could have a selective advantage in BAM-contaminated environments.

**ACKNOWLEDGEMENT**

This work was supported by the Danish Technical Research Council, talent grant 26-04-0051 (funding for S.R.S.) and the PESTICON Project, grant 274-05-0399, financed by the Danish Research Council for Technology and Production Sciences (funding for M.S.H.). We thank Pia B. Jacobsen (GEUS) and Spire M. Kiersgaard (GEUS) for skillful technical assistance and Patricia Simpson for excellent help during the writing of the manuscript. Additionally, we appreciate the help from Dr. C. Spröer (DSMZ) and Dr. S. Chen (The Questor Centre and School of Biology and Biochemistry, The Queen's University of Belfast, United Kingdom) in the taxonomic analysis of strains MSH1 and ASI1.

**REFERENCES**


40. Verloop, A. 1972. Fate of the herbicide dichlobenil in plants and soil in relation to its biological activity. Residue Reviews 43:55-103


FIGURE LEGENDS

FIG. 1. Phylogenetic affiliations of 16S rRNA gene sequences derived from strains ASI1 and MSH1 and closely related α-proteobacteria type strains. The phylogenetic tree was constructed using a neighbor-joining algorithm with Jukes-Cantor distance correction. The root of the tree was determined by including the 16S rRNA gene sequence of *Mesorhizobium loti* into the analysis. Accession number for each sequence is given in parentheses. The scale bar indicates 3 nucleotide substitutions per 100 nucleotides.

FIG. 2. Mineralization of $^{14}$C-labeled 2,6-dichlorobenzamide ($^{14}$C-BAM) to $^{14}$CO$_2$ by *Aminobacter* sp. MSH1 (A) or *Aminobacter* sp. ASI1 (C) and the mineralization of $^{14}$C-labeled dichlobenil ($^{14}$C-DCB) to $^{14}$CO$_2$ by *Aminobacter* sp. MSH1 (B) or *Aminobacter* sp. ASI1 (D). Initial cell density was $2 \times 10^6$ ml$^{-1}$. The data is mean values ($n = 3$). The bars indicate the standard deviation and some are smaller than the symbols.

FIG. 3. Cell density of *Aminobacter* sp. MSH1 after mineralization of different concentrations of 2,6-dichlorobenzamide (BAM) provided as sole source of carbon, nitrogen and energy (7.9 nM, 534.1 nM, 5.3 µM, 52.6 µM and 263.1 µM; see Fig. 2A) measured as CFU following plating on R2A. The data are mean values ($n = 3$). The bars indicate the standard deviation. Some bars are smaller than the symbols.
Sørensen et al. fig 2

A. 

\[ ^{14}C\text{-DCB mineralized to } ^{14} \text{CO}_2 \] (in % of added \(^{14}C\))

- 7.9 nM
- 590.1 nM
- 58.1 µM
- 290.7 µM

B. 

\[ ^{14}C\text{-BAM mineralized to } ^{14} \text{CO}_2 \] (in % of added \(^{14}C\))

- 7.9 nM
- 534.1 nM
- 52.6 µM
- 263.1 µM

C. 

\[ ^{14}C\text{-DCB mineralized to } ^{14} \text{CO}_2 \] (in % of added \(^{14}C\))

- 7.9 nM
- 534.1 nM
- 52.6 µM

D. 

\[ ^{14}C\text{-BAM mineralized to } ^{14} \text{CO}_2 \] (in % of added \(^{14}C\))

- 82.7 nM
- 590.1 nM
- 5.8 µM

<table>
<thead>
<tr>
<th>Compound</th>
<th>DCB</th>
<th>BAM</th>
<th>2,6-DCBA</th>
<th>OBN</th>
<th>OBAM</th>
<th>OBA</th>
<th>BN</th>
<th>BAD</th>
<th>BA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain MSH1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Strain ASII</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Strain DSM7048T</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Strain DSM7051T</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Strain DSM7050T</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

The abbreviations are BAM: 2,6-dichlorobenzamide, 2,6-DCBA: 2,6-dichlorobenzoic acid, OBN: ortho-chlorobenzonitrile, OBAM: ortho-chlorobenzamide, OBA: ortho-chlorobenzoic acid, BN: benzonitrile, BAD: benzamide and BA: benzoic acid. All compounds were added in concentrations of 50 μM. * Degradation (+), no degradation (−).
TABLE 2. Residual 2,6-dichlorobenzamide (BAM) concentrations determined by a quantitative enzyme-linked immunosorbent analysis following BAM mineralization experiments with *Aminobacter* sp. MSH1 or *Aminobacter* sp. ASI1.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Initial concentration:</th>
<th>BAM conc.</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.0</td>
<td>7.9 nM</td>
<td>534.1 nM</td>
<td>52.6 nM</td>
<td>263.1 nM</td>
</tr>
<tr>
<td>MSH1</td>
<td>Residual concentration (nM) (a)</td>
<td>0.00</td>
<td>0.35 (±0.03)</td>
<td>0.35 (±0.11)</td>
<td>0.70 (±0.08)</td>
<td>7.54 (±0.61)</td>
</tr>
<tr>
<td></td>
<td>BAM depletion (%)</td>
<td>95.60 (±0.40)</td>
<td>99.93 (±0.02)</td>
<td>99.99 (±0.00)</td>
<td>99.99 (±0.00)</td>
<td>99.99 (±0.00)</td>
</tr>
<tr>
<td>ASI1</td>
<td>Residual concentration (nM)</td>
<td>0.00</td>
<td>0.22 (±0.04)</td>
<td>0.41 (±0.27)</td>
<td>3.47 (±0.60)</td>
<td>ND (b)</td>
</tr>
<tr>
<td></td>
<td>BAM depletion (%)</td>
<td>97.27 (±0.50)</td>
<td>99.92 (±0.05)</td>
<td>99.93 (±0.01)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(a\) Means and standard deviations (given in parentheses) of residual BAM concentrations in triplicate flasks following the 10 day (strain MSH1) or 18 day (strain ASI1) BAM mineralization presented in figures 2. Initial cell density was \(2 – 4 \times 10^6\) ml\(^{-1}\).  
\(b\) Not determined.
TABLE 3. 2,6-dichlorobenzamide (BAM) concentrations determined by a quantitative enzyme-linked immunoassay analysis following dichlobenil (DCB) mineralization experiments with *Aminobacter* sp. MSH1

<table>
<thead>
<tr>
<th>Initial DCB concentration:</th>
<th>0.0</th>
<th>8.7 nM</th>
<th>590.1 nM</th>
<th>58 μM</th>
<th>58.1 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAM concentration (nM) <em>a</em></td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.22 (±0.06)</td>
<td>3.34 (±0.60)</td>
</tr>
<tr>
<td>DCB accumulated as BAM</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.004 (±0.001)</td>
<td>0.006 (±0.001)</td>
</tr>
</tbody>
</table>

(in % of added DCB)

*a* Means and standard deviations (given in parentheses) of BAM concentrations in triplicate flasks after the 10 days DCB mineralization presented in figures 2B. Initial cell density was $4 \times 10^6$ ml$^{-1}$. 