Degradation and Mineralization of Nanomolar Concentrations of the Herbicide Dichlobenil and Its Persistent Metabolite 2,6-Dichlorobenzamide by *Aminobacter* spp. Isolated from Dichlobenil-Treated Soils[▽]

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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 strain MSH1) was cultivated and identified by 16S rRNA gene sequencing and fatty acid analysis as being closely related to members of the genus *Aminobacter*, including the only cultured BAM degrader, *Aminobacter* sp. strain ASI1. Strain MSH1 mineralized 15 to 64% of the added [*ring*-U-^{14}C]BAM to ^{14}CO$_2$ with BAM at initial concentrations in the range of 7.9 nM to 263.1 μM provided as the sole carbon, nitrogen, and energy source. A quantitative enzyme-linked immunoassay analysis with antibodies against BAM revealed residue concentrations of 0.35 to 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. strain 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 nonchlorinated structural analogs. Combined, these results suggest that closely related *Aminobacter* strains may have a selective advantage in BAM-contaminated environments, since they are able to use this metabolite or structurally related compounds as a carbon and nitrogen source.

Each year, millions of tons of xenobiotic compounds are applied globally as pesticides in agricultural production and in 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 more than 99% of the drinking water originates from groundwater, the detection of pesticide residues above the European Commission (EC) threshold limit of 0.1 μg liter^{-1} has resulted in the costly closure of numerous groundwater abstraction wells (29). Not only are the pesticides themselves monitored, but 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 nonagricultural 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 liter^{-1} for drinking water (3). Similar results were 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 to 2,079 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 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 6 soils that have a potential for mineralization of [*ring*-U-^{14}C]BAM to ^{14}CO$_2$, out of a total of 79 samples screened for mineralization activity, obtained from 39 different

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Danish locations previously exposed to dichlobenil. One of the soils with a potential for BAM mineralization but with no evident dichlobenil mineralization was recently used to enrich and isolate a BAM-mineralizing bacterium identified as an *Aminobacter* isolate, 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 [*ring*-U-^{14}C]dichlobenil to $^{14}CO_2$ (25), and we later succeeded in obtaining stable BAM-mineralizing enrichment cultures (10), 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. strain (designated strain MSH1). This strain is closely related to our BAM-mineralizing *Aminobacter* sp. strain ASI1, and these two strains along with selected *Aminobacter* type strains were compared with regard to their taxonomy, degradative capacity toward dichlobenil and related compounds, and ability to degrade 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 liter^{-1} water solubility at 20°C), BAM (CAS RN 002008-58-4) (95.5% purity; 2,730 mg liter^{-1} water solubility at 23°C), 2,6-dichlorobenzoic acid (CAS RN 000050-30-6) (99.5% purity; 14,100 mg liter^{-1} water solubility at 20°C), benzonitrile (CAS RN 000100-47-0) (99.5% purity; water solubility, 2,000 mg liter^{-1} at 25°C), and benzoic acid (CAS RN 000065-85-0) (99.5% purity; water solubility, 3,400 mg liter^{-1} at 25°C) were all purchased from 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), and *ortho*-chlorobenzoic acid (CAS RN 000118-91-2) (98% purity; water solubility, 2,090 mg liter^{-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). [*ring*-U-^{14}C]dichlobenil (28.3 mCi mmol^{-1}) ([^{14}C]dichlobenil) and [*ring*-U-^{14}C]BAM (25.2 mCi mmol^{-1}) ([^{14}C]BAM) were purchased from International Izotop (Budapest, Hungary). Radiochemical purities better than 95% were verified for the [^{14}C]dichlobenil and [^{14}C]BAM in our laboratory by a thin-layer-chromatography-based assay. Both these ^{14}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}C tracer is recovered as $^{14}CO_2$.

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 liter^{-1} stock solution in high-performance liquid chromatography (HPLC)-grade dimethyl sulfoxide (Merck, Darmstadt, Germany) directly to the medium, providing 263.12 μM BAM (50 mg liter^{-1}) as the 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, a 10% volume was transferred to fresh BAM-containing MS medium to a final volume of 25.0 ml. After 10 subculturings 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 1 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. strain 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 (1,463

bp sequenced) and ASI1 (1,485 bp sequenced) within the current study. Genomic DNA extraction, PCR amplification of the 16S rRNA gene, and purification of the PCR products were performed as described previously (19). 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, rifampin, nalidixin, and tetracycline in 1/10-strength Luria-Bertani medium (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^T, *A. aganoensis* DSM 7051^T, and *A. niigataensis* DSM 7050^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 liter^{-1} proteose peptone no. 3 (Difco Laboratories) and 3.0 g liter^{-1} yeast extract (Difco Laboratories) (MS-C) prior to the degradation experiments. The strains MSH1 and ASI1 were either pregrown in MS with 25 to 50 mg liter^{-1} BAM (132.5 to 263.1 μM) or washed from R2A plates prior to the degradation and mineralization experiments.

Degradation and mineralization experiments. Mineralization of [^{14}C]BAM in MS was followed by insertion of a 10-ml glass tube containing 2.0 ml 0.5 M NaOH to trap $^{14}CO_2$ into the 100-ml culture flasks following inoculation. The final volume of liquid medium 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 [^{14}C]dichlobenil into the NaOH trap was necessary due to its high vapor pressure (31) potentially leading to an overestimation of the extent of mineralization. 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 ^{14}C trapped in the NaOH trap by scintillation counting and another 1.0 ml used for estimating the amount of [^{14}C]dichlobenil evaporated into the trap. The trapped [^{14}C]dichlobenil was quantified by acidifying the 1.0 ml base solution with 1.5 ml 1 M HCl, allowing the $^{14}CO_2$ to evaporate during incubation for 1 h followed by scintillation counting as described above. The dichlobenil mineralization was calculated by subtracting the evaporated [^{14}C]dichlobenil from the total ^{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 ^{14}C and unlabeled compound in dimethyl sulfoxide. 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 level of 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. MS medium (4.9 ml) was inoculated with 0.1 ml of a washed cell suspension having an optical density at 600 nm of 0.5, and subsamples were analyzed by the 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, United Kingdom) as described by Holtze et al. (8). Prior to the HPLC analysis, 750-μl subsamples of the medium were filtered through a 0.2-μm polytetrafluoroethylene membrane 17-mm syringe filter (Titan Filtration Systems; Sun SRI, Wilmington, NC), and the last 250 μl was collected for analysis. The HPLC detection limits for the nine compounds were <1 μM (0.035 to 0.159 mg liter^{-1}) by direct HPLC analysis of the filtered liquid medium (8). The medium samples for HPLC measurements were stored at −15°C for up to 2 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 subsamples of the culture medium following the mineralization experiments. This ELISA assay has a detection limit of 0.02 μg liter^{-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). Medium samples for ELISA were stored at −15°C for up to 2 weeks before analysis.

Nucleotide sequence accession numbers. The 16S rRNA sequences for *Aminobacter* sp. strain MSH1 and *Aminobacter* sp. strain ASI1 have been deposited in the GenBank database under accession numbers DQ401867 and DQ401866, respectively.
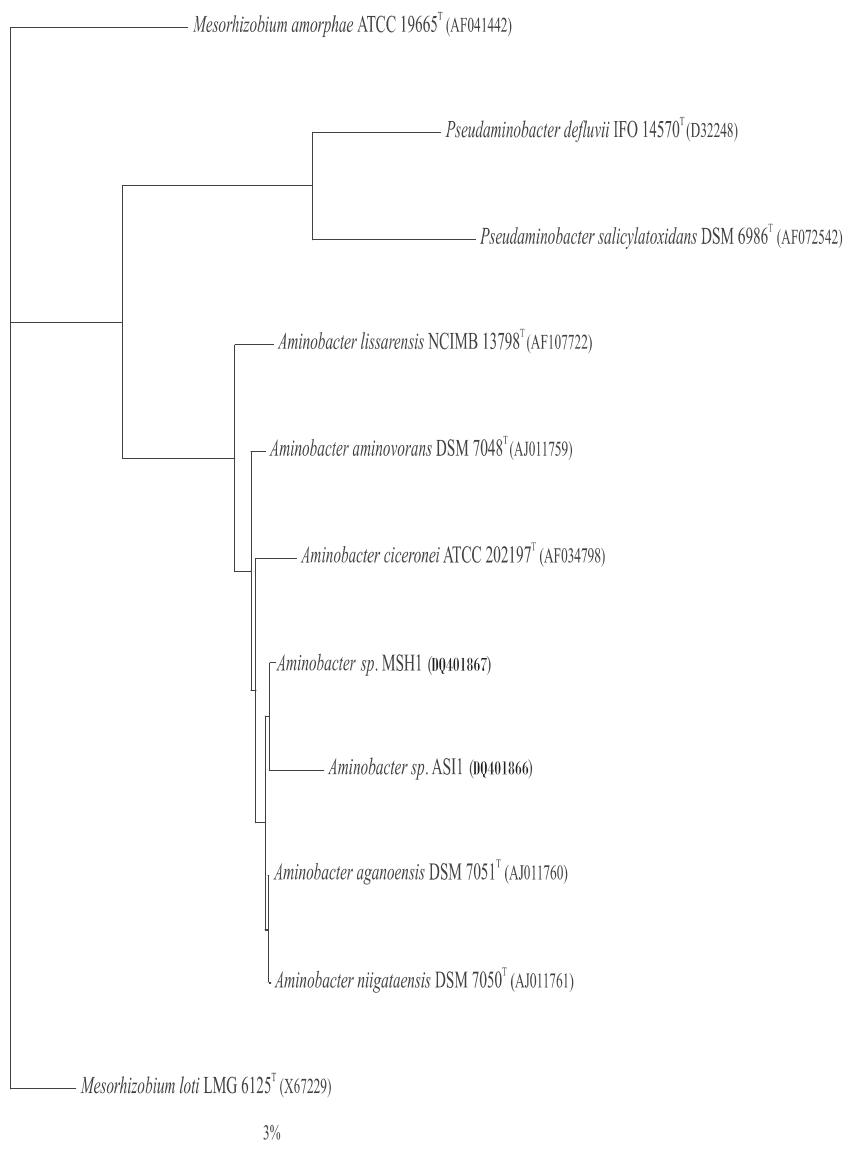
FIG. 1. Phylogenetic affiliations of 16S rRNA gene sequences derived from strains ASI1 and MSH1 and closely related α-proteobacteria type strains. The phylogenetic tree was constructed using a neighbor-joining algorithm with Jukes-Cantor distance correction. The root of the tree was determined by including the 16S rRNA gene sequence of *Mesorhizobium loti* in the analysis. The accession number for each sequence is given in parentheses. The scale bar indicates 3 nucleotide substitutions per 100 nucleotides.

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 to 0.8 μm and a length of 2.0 to 3.5 μm after growth in nutrient-rich broth. It is oxidase, catalase, and urease positive. MSH1 did not hydrolyze esculin, gelatin, Tween 80, DNA, or starch. It gives negative results in tests for nitrate reduction and production of H_2S. When grown on R2A agar, its colony morphology is similar to that of *Aminobacter* sp. strain ASI1, with white colonies having a characteristic reddish-brown center appearing after 4 to 5 days at 20°C.

MSH1 used glucose, D-arabinose, mannose, maltose, *N*-acetylglucosamine, acetate, D-arabitol, D-fructose, fumarate, pyruvate, L-rhamnose, and D-sucrose as growth substrates. In contrast, no growth was detectable with L-arabinose, malate, adipate, citrate, caprate, gluconate, 2-OH-valerate, lactose, and lactulose. Both MSH1 and ASI1 were insensitive to the antibiotic streptomycin (50 mg liter^{-1}) but sensitive to ampicillin, chloramphenicol, kanamycin, penicillin G, rifampin, nalidixin, and tetracycline. It was not possible to distinguish MSH1 from *Aminobacter* sp. strain 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. strain ASI1 (25). For both strains, the dominant fatty acid was 18:
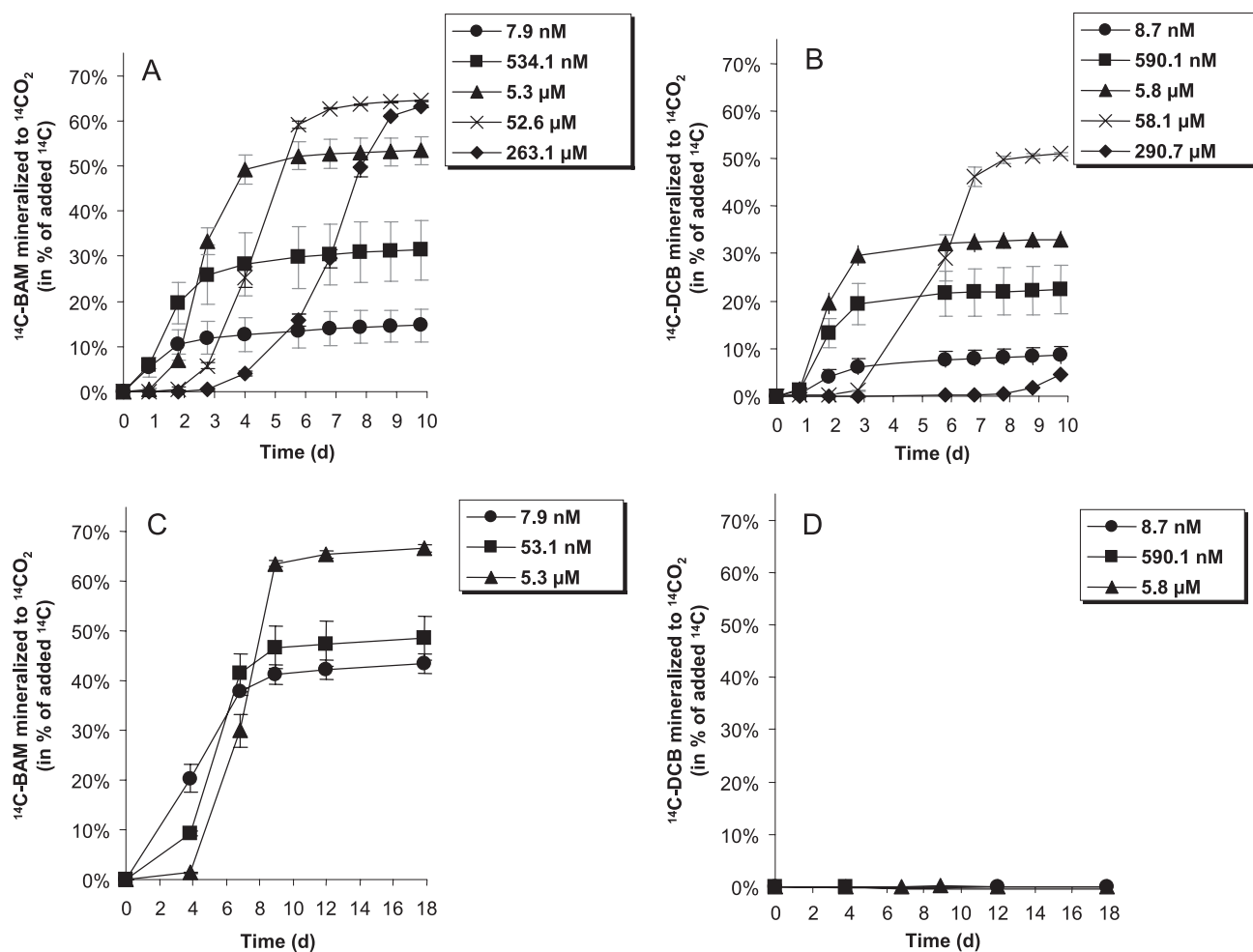
FIG. 2. Mineralization of [^{14}C]BAM to $^{14}CO_2$ by *Aminobacter* sp. strain MSH1 (A) or *Aminobacter* sp. strain ASI1 (C) and mineralization of [^{14}C]DCB to $^{14}CO_2$ by *Aminobacter* sp. strain MSH1 (B) or *Aminobacter* sp. strain ASI1 (D). Initial cell density was 2×10^6 to 4×10^6 ml^{-1}. The data are mean values ($n = 3$). The bars indicate standard deviations, and some are smaller than the symbols.
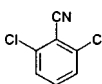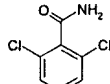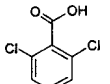
$1\omega7c$, with 75.0% in MSH1 and 67.8% in ASI1. The second-most-dominant fatty acid in MSH was 16:0, with 8.3% in comparison to 7.2% in ASI1. In contrast, 11-methyl-18:1$\omega7c$ was the second-most-dominating fatty acid in ASI1 with 10.6%, while MSH1 had only 1.2% of this fatty acid.

The 1,463-bp 16S rRNA gene sequence obtained from isolate MSH1 is identical to the 16S rRNA gene sequences from *A. aganoensis* DSM 7051^T and *A. niigataensis* DSM 7050^T. It has 99.5 to 99.8% similarity to the three remaining members of the genus *Aminobacter*: *A. aminovorans*, *A. lissarensis*, and *A. ciceronei*. Similarities of 96.8 to 97.8% are apparent with different type strains from the genera *Pseudaminobacter* and *Mesorhizobium*. A 99.6% similarity to the 1,485-bp 16S rRNA gene sequence obtained from the recently described BAM-mineralizing *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 Fig. 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

[^{14}C]BAM was mineralized to $^{14}CO_2$ within 10 days. Increasing the BAM concentration to 534.1 nM gave a greater extent of mineralization, with 31.4% ± 6.6% measured as $^{14}CO_2$ 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 to 263.1 μM, and at the end of the experiment, 53.4% to 64.4% was mineralized (Fig. 2A). Generally, a similar pattern was observed with the mineralization of dichlobenil, with the lowest concentrations having the smallest extent of mineralization (Fig. 2B). However, compared to the mineralization of BAM, dichlobenil mineralization was slow and occurred to a small 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. Dichlobenil at the highest concentration of 290.7 μM was mineralized to the smallest 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 concen-

TABLE 1. Degradation of dichlobenil and eight potential metabolites by *Aminobacter* strains[a]

Organism or control[b]	Degradation of compound[b]								
	DCB	BAM	2,6-DCBA	OBN	OBAM	OBA	BN	BAD	BA
Strain MSH1	+	+	+	+	+	+	+	+	+
Strain ASI1	−	+	+	−	+	−	−	+	+
Strain DSM7048[T]	−	−	−	−	−	−	+	−	−
Strain DSM7051[T]	−	−	−	−	−	−	+	−	+
Strain DSM7050[T]	−	−	−	−	−	−	+	−	+
Abiotic control	−	−	−	−	−	−	−	−	−

[a] *Aminobacter* sp. strain MSH1, *Aminobacter* sp. strain ASI1, *Aminobacter aminovorans* DSM 7048[T], *Aminobacter aganoensis* DSM 7051[T], and *Aminobacter niigataensis* DSM 7050[T] were used. Degradation was determined by high-performance liquid chromatography analysis of the liquid medium following 14 days of incubation.

[b] Abbreviations: DCB, dichlobenil; 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; BA, benzoic acid. All compounds were added in concentrations of 50 μM. +, degradation; −, no degradation.

trations. 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. strain ASI1 was tested for mineralization with the three lowest concentrations of BAM and dichlobenil (Fig. 2C and D). ASI1 mineralized BAM to a greater extent than MSH1, with 43.5 to 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; Table 1). Evaporation of dichlobenil 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 onto R2A is presented in Fig. 3. Initial cell densities were 2×10^6 to 4×10^6 ml^{-1} for both strains and in all experiments, and after mineralization of 263.1 μM BAM,

the highest tested concentration, $9.6 \times 10^{10} \pm 0.4 \times 10^{10}$ cells of MSH1 per liter culture medium, was measured. In comparison, $3.4 \times 10^9 \pm 0.6 \times 10^9$ cells of MSH1 per liter culture medium was measured with the lowest tested BAM concentration (7.9 nM) possibly representing smaller cell sizes (17). Strain ASI1 showed minor growth, with cell densities of $1.8 \times 10^8 \pm 0.4 \times 10^8$, $2.4 \times 10^8 \pm 1.2 \times 10^8$, and $3.1 \times 10^8 \pm 0.8 \times 10^8$ ASI cells per liter culture medium measured following the mineralization of 7.9 nM, 534.1 nM, or 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 cell 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 concen-
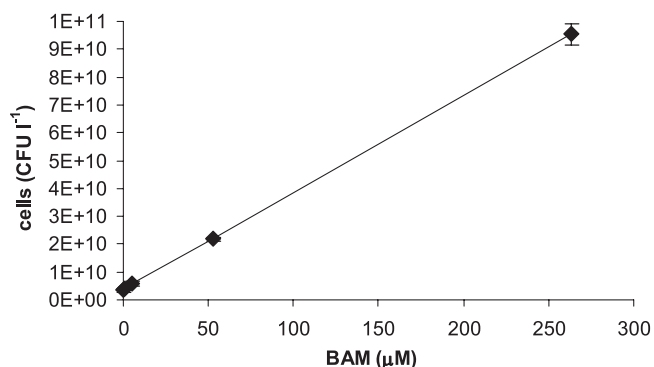

FIG. 3. Cell densities of *Aminobacter* sp. strain MSH1 after mineralization of different concentrations of BAM provided as the sole source of carbon, nitrogen, and energy (7.9 nM, 534.1 nM, 5.3 μM, 52.6 μM, or 263.1 μM; see Fig. 2A) measured as CFU following plating on R2A. The data are mean values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

TABLE 2. Residual BAM concentrations determined by a quantitative ELISA analysis following BAM mineralization experiments with *Aminobacter* sp. strain MSH1 or *Aminobacter* sp. strain ASI1

Strain	Initial concn of BAM	Residual concn of BAM (nM)[a]	BAM depletion (%)
MSH1	0.0	0.0	
	7.9 nM	0.35 (±0.03)	95.60 (±0.40)
	534.1 nM	0.35 (±0.11)	99.93 (±0.02)
	5.3 μM	0.70 (±0.08)	99.99 (±0.00)
	52.6 μM	7.54 (±0.61)	99.99 (±0.00)
	263.1 μM	18.05 (±0.82)	99.99 (±0.00)
ASI1	0.0	0.0	
	7.9 nM	0.22 (±0.04)	97.27 (±0.50)
	534.1 nM	0.41 (±0.27)	99.92 (±0.05)
	5.3 μM	3.47 (±0.60)	99.93 (±0.01)
	52.6 μM	ND[b]	ND
	263.1 μM	ND	ND

[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 Fig. 2. Initial cell density was (2 to 4) × 10^6 ml^{-1}.

[b] ND, Not determined.

TABLE 3. BAM concentrations determined by a quantitative
ELISA analysis following dichlobenil mineralization
experiments with *Aminobacter* sp. strain MSH1

Initial concn of DCB	Concn of BAM (nM)[a]	Amt of DCB accumulated as BAM (%[b])
0.0	0.00	0.000
8.7 nM	0.00	0.000
590.1 nM	0.00	0.000
5.8 μM	0.22 (±0.06)	0.004 (±0.001)
58.1 μM	3.34 (±0.60)	0.006 (±0.001)

[a] Means and standard deviations (given in parentheses) of BAM concentrations in triplicate flasks after the 10-day DCB mineralization presented in Fig. 2B. Initial cell density was 4×10^6 ml^{-1}.
[b] Expressed as a percentage of added DCB.

trations following the mineralization experiments with strains MSH1 and ASI1 presented in Fig. 2A and C. Residue concentrations of 0.22 to 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 degradation efficiencies, with remaining residues in concentrations of 0.35 to 0.41 nM BAM. This is in contrast to the different extents of [14C]BAM mineralization observed for the two strains by measurement of $^{14}CO_2$ production (Fig. 2A and C).

Following the mineralization of dichlobenil by MSH1 (Fig. 2B), subsamples 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 to 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.

Substrate range for *Aminobacter* spp. The results of testing of 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 are summarized in Table 1. Strain MSH1 is the most versatile among the tested strains and degrades all tested compounds. In contrast, strain ASI1 degrades only BAM, 2,6-dichlorobenzoic acid, *ortho*-chlorobenzamide, benzamide, and benzoic acid (Table 1). Surprisingly, however, *A. aminovorans* DSM 7048[T], *A. aganoensis* DSM 7051[T], and *A. niigataensis* DSM 7050[T] 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 that of *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], which 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. strain 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, since 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, which 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 located only 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., see reference 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., see references 7 and 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 methyl halides as carbon and energy sources (15) or nitriloacetate as a 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 suggest 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 BAM is provided as the 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 1 representative for the 14 isolates, strain C147, was recently 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 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 nonchlorinated structural analogs of 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 amines 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 ASI1 and MSH1 were capable of mineralizing the ring structure with a maximum of 60 to 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 for other pesticide-mineralizing isolates (27, 28). The occurrence of ^{14}C-labeled metabolites not measured with our HPLC method cannot be excluded, however.

Many studies with environmental samples (e.g., see references 22, 30, and 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 nanomolar range (e.g., see references 23, 34, and 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 liter^{-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; Table 2). The presence of metabolites from partial degradation of BAM cannot be excluded, however, since the ELISA assay exclusively targets BAM (4). There might be a threshold for BAM degradation at about 0.2 to 0.4 nM, since both isolates appear to have BAM residues in this concentration range in the liquid medium following the mineralization experiments (Table 2). Further studies are needed, however, 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 no BAM 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, however, it cannot be excluded that other metabolites, as well as dichlobenil itself, still are present in the liquid medium.

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. strain 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* strain ASI1 and the fact that closely related *Aminobacter* type strains are capable of degrading nonchlorinated structural analogs add to knowledge of the metabolic capacities of this genus and suggest that this closely related group of *Aminobacter* spp. could have a selective advantage in BAM-contaminated environments.

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