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Isolation and characterization of efficient isoxaben-transforming *Microbacterium* sp. strains from four European soils

Suggestion for running title : **Efficient isoxaben-transforming *Microbacterium* sp. strains**

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Abstract

Nutrient-agar plates containing isoxaben (500 mg l^{-1}) were used to isolate isoxaben-metabolizing bacteria from four European soils incubated with the herbicide under laboratory conditions. In flask experiments, inoculation of a basal salts medium containing nitrogen and [*phenyl*-UL-^{14}C]isoxaben with an isolate (B2b) resulted in a 33% recovery of the initial radioactivity as $^{14}CO_2$ after two weeks. A major metabolite identified by GC-MS and NMR analysis as 5-amino-3-(1-ethyl-1-methyl-propyl)isoxazole accumulated both in basal salts and nutrient broth media. 2,6-dimethoxybenzoic acid, a suspected metabolite of isoxaben, was not detected in either liquid media. However, the capability of the B2b isolate to use 2,6-dimethoxybenzoic acid as a source of carbon was demonstrated. Soil inoculation with the B2b strain isolate resulted in an increase in the recovery of $^{14}C0_2$ from both *phenyl*-UL and 5

1 *isoxazolyl*-^{14}C-isoxaben. The previous metabolite identified as 5-amino-3-(1-ethyl-1-methyl-
2 propyl)isoxazole only accumulated if the soil was autoclaved before inoculation. This
3 metabolite was rapidly mineralized by the microflora of a natural soil without history of
4 isoxaben treatment. Homology patterns of sequenced 16S rDNA between isoxaben-
5 transforming isolates and reference strains show that the four isolates identified belong to the
6 genus *Microbacterium*.
7

8 ***Key words:*** actinomycetes; biodegradation; isoxaben; *Microbacterium*
9

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14 1 INTRODUCTION

15 Isoxaben (*N*-[3-(1-ethyl-1-methylpropyl)-5 isoxazolyl]-2,6-dimethoxybenzamide, compound
16 1, Figure 1), is a selective pre-emergence herbicide used to control annual broadleaf weeds in
17 winter cereals, ornamentals, turfgrass, trees and vines.[1,2] Isoxaben has a very low water
18 solubility (1-2 mg litre^{-1} at 25°C), is stable to hydrolysis at pH 5-9[3] and is susceptible to
19 photodegradation in aquatic systems.[4] Primary breakdown in soil is achieved through
20 microbial degradation. First report on soil degradation of isoxaben[5] refers to previous
21 unpublished data from D.P. Rayney and L.K. Graper 1984-85 (Lilly Research Laboratories).
22 These authors observed that *N*-[3-(1-hydroxy-1-methylpropyl)-5-isoxazolyl]-2,6-
23 dimethoxybenzamide, the major metabolite detected during their laboratory soil study,
24 reached levels of approximately 20% of the applied isoxaben. In their study, Rouchaud and
25 co-workers[6] identified demethoxy-isoxaben, 5-isoxazolone (3-[1-ethyl-1-

1 methylpropyl]isoxazolin-5-one) and 2,6-dimethoxybenzamide as the three main metabolites
2 of isoxaben in the soil of winter wheat crops treated with the herbicide. Three minor
3 compounds, 5-aminoisoxazole [5-amino-3-(1-ethyl-1-methylpropyl)isoxazole], 2-hydroxy-6-
4 methoxybenzamide and 2,6-dimethoxybenzoic acid were also detected. A relatively low
5 mineralization of ring-labeled ^{14}C-isoxaben in an acidic soil was also recently reported[7] but
6 metabolites were not definitively identified.

7 Application timing affects the dissipation of isoxaben from field soils and the duration
8 of weed control.[8-11] Herbicide persistence was less pronounced in wheat plots treated with
9 organic fertilisers.[8] In addition, repeated isoxaben treatments enhanced soil pesticide
10 biodegradation in several field experiments.[12]

11 The present work was carried out to further identify the key parameters governing
12 isoxaben degradation in fields. This paper presents preliminary investigations on the isolation
13 and characterization of isoxaben-transforming bacteria, as well as the identification of a major
14 transformation product of the pesticide.

15

16 **2 MATERIALS AND METHODS**

17 **2.1 Chemicals**

18 Technical grade isoxaben, [*isoxazole*-5-^{14}C]isoxaben (specific activity 3.71 MBq mmol^{-1}) and
19 [*phenyl*-UL-^{14}C]isoxaben (specific activity 17.80 MBq mmol^{-1}) were kindly provided by Dow
20 AgroSciences Ltd (Norfolk, England). Radioactive and unlabelled isoxaben were purified by
21 thin-layer chromatography (TLC) or crystallization by mixing a highly concentrated solution
22 of isoxaben in methanol with water.

23

24 **2.2 Enrichment, isolation and testing of isoxaben-degrading strains**

1 The soils were obtained from 4 field sites in Europe with known histories of isoxaben
2 treatments and from one site never previously treated with the herbicide (Table 1). Samples
3 were collected to a depth of 15 cm, sieved below 4 mm and stored at 4°C for 3-6 weeks prior
4 to use. For each soil, duplicates samples (50 g dry weight) were moistened to 90% of their
5 moisture-holding capacities and treated successively with 250 µl of methanolic solutions
6 containing isoxaben (0.5 and 5 mg per sample) at a one-month interval. The samples were
7 incubated at 20°C in a 1-litre-hermetic glass jar. Thirty days after the last treatment, soil (10 g)
8 was suspended in a sterile aqueous solution (100 ml) containing tetrasodium pyrophosphate
9 ($Na_4P_2O_7$; 01 g) and shaken for 10 min. Serial ten-fold dilutions were prepared and a fraction
10 of each dilution (0.1 ml µl) was spread onto nutrient-agar plates (Difco, Detroit, USA)
11 supplemented with isoxaben (500 mg litre^{-1}) in methanol (5 ml litre^{-1}). The agar plates were
12 cloudy as a result of particulate isoxaben in the medium, and visual inspection of the clearing
13 zone around isoxaben-degrading colonies was possible after a one week incubation period at
14 28°C. Isolates were maintained on slope tubes of nutrient agar supplemented with isoxaben
15 (250 mg litre^{-1}).

16 Primary testing of isoxaben-degradation by the selected isolates was carried out in 50
17 ml Erlenmayer flasks with nutrient broth (Difco) medium (10 ml) complemented with
18 [*isoxazole*-5-^{14}C]isoxaben (250 mg and 50 kBq litre^{-1}). The pesticide was added as filter-
19 sterilised solutions in methanol (0.1 ml per sample). The medium was inoculated with
20 approximately 10^8 bacteria (0.1 ml of each isolate suspension) and incubated on a rotary
21 shaker (150 rpm) in the dark at 28°C. Separate experiments with nutrient broth medium
22 complemented with 250 mg litre^{-1} of unlabelled isoxaben were also carried out in such a way
23 to produce metabolites useful for the identification tests.

24 Isoxaben-mineralization testing by the B2b isolate were carried out in 50 ml
25 Erlenmeyer flasks (four replicates) containing basal salts (BS) medium[13] with NO_3NH_4 (0.15

1 g.litre^{-1}) or nutrient broth medium (10 ml). Both media exhibited a pH of 6.9. The [2,6-
2 dimethoxybenzamide-*phenyl*-UL-^{14}C]isoxaben (50 mg and 50 kBq litre^{-1}) was added as filter-
3 sterilised solutions in methanol (0.1 ml per sample) in an empty-sterilised flask, plugged with
4 cotton whole, and the solvent was air-evaporated at 30°C in the dark for 24 hours before
5 adding sterile medium and inoculate to the different flasks as previously described. The flasks
6 were enclosed in 1-litre glass jars and incubated on a rotary shaker (150 rpm) in the dark at
7 28°C for two weeks. NaOH (5 ml of 1 M solution in vials) was used to trap the $^{14}CO_2$ released
8 in the incubation jars and the radioactivity was measured by liquid scintillation counting
9 (Tricarb TR 1900, Packard) after mixing with a scintillation cocktail (ACSII, Amersham
10 Pharmacia Biotech, Orsay, France).

11 The capability of B2b isolate to use 2,6-dimethoxybenzoïc acid as a source of carbon
12 was determined using the BS-nitrogen medium. Diluted sodium hydroxide was used to make
13 the acidic compound (2500 mg litre^{-1}) soluble and to maintain neutral pH of the medium.
14

15 **2.3 Soil incubation studies**

16 Soil incubation experiments were carried out using 25 g samples of natural or autoclaved (1 h,
17 120°C) soil from Dijon. Four of the eight replicates of each series were inoculated with a
18 suspension of 10^9 isoxaben-degrading bacteria in sterile water (isolate B2b). Control samples
19 received sterile water only. All soil samples were treated with a methanolic solution of
20 [*isoxazole*-5-^{14}C] or [2,6-dimethoxybenzamide-*phenyl*-UL-^{14}C]isoxaben (25 µl per sample) to
21 fortify the samples with the active ingredient (0.025 mg) and radioactive material (5 kBq).
22 Similar experiments were carried out with a chromatographically-purified radioactive
23 metabolite of isoxaben. In both cases, the soil moisture was adjusted to 90% of its water-
24 holding capacity. Sample-containing bottles were put into 1-litre glass jars and incubated in

1 the dark at 20°C for 61 days. Vials with NaOH (0.2 M; 5 ml) were used to trap the $^{14}CO_2$
2 released in the incubation jars and the radioactivity was measured as previously described.
3
4 **2.4 Extraction and analysis of pesticide residues from culture and soil experiments**
5 Axenic cultures in liquid or agar media were extracted twice by stirring with an equivalent
6 volume of dichloromethane for 60 min. The organic solution was dried on $MgSO_4$, filtered,
7 and concentrated to dryness under a N_2 stream. Soil samples were extracted under the same
8 conditions with methanol (200 ml per sample). For complementary analysis on the presence of
9 2,6-dimethoxybenzoic acid in liquid media, culture samples (10 ml) were introduced on 12 ml
10 SPE tubes containing reversed phase packing LC 18 (Supelco, Bellefonte, PA USA). After
11 elution with methanol (10 ml), the recovered eluate was concentrated to dryness as previously
12 described.
13 The different extracts were analyzed by TLC using silicagel 60F254 (20 x 20 cm, 0.25
14 mm thickness) plates with a concentration zone (Merck, Nogent-sur-Marne, France). The
15 following solvent systems were used: I, toluene:acetone (85+15 by volume; II,
16 toluene:dioxan:acetic acid (135+20+1 by volume). Radioactive spots were visualised with
17 Hyperfilm-max (Amersham, France).
18 Gas Chromatographic–Mass Spectrometric (GC-MS) analyses were carried out with a
19 5973 Mass Selective Detector coupled to a 6890 gas chromatograph (Agilent Technologies,
20 Palo Alto, CA) fitted with a split-splitless injector and a DB™FFAP fused silica capillary
21 column (30 m x 0.32 mm ID, 0.25 µm film thickness, J&W Scientific, Folsom, CA). The
22 column was held at 40°C for 2 min and then the oven temperature was programmed to
23 increase to 240°C at 10°C min^{-1}. The column was directly connected to the ion source of the
24 spectrometer through a heated transfer line maintained at 250°C.

1 Electron Impact (EI) mass spectra were obtained at 70 eV with the instrument
2 scanning from 29 to 350 amu and the source maintained at 230°C. Chemical Ionisation (CI)
3 mass spectra were generated at 63 eV using methane as a reactant gas with the instrument
4 scanning from 70 to 350 amu and the source temperature set at 250°C.
5 Fourier Transform Infrared (FTIR) data were collected with a Bio-Rad Digilab
6 FTS-60A spectrometer (Cambridge, MA). It was connected by a Digilab Tracer® direct
7 deposition interface to a Hewlett-Packard HP 5890 series II gas chromatograph (Palo Alto,
8 CA). The GC instrument was equipped with the column already described for GC-MS
9 analysis and operated in similar conditions. GC eluates were deposited onto a ZnSe window
10 cooled with liquid nitrogen. The spectral resolution was 8 cm^{-1}, and real-time spectra were
11 obtained by co-addition of four scans. A narrow-band (4000-700 cm^{-1}) mercury-cadmium-
12 telluride (MCT) detector was used.
13 NMR data (^{1}H, 300 MHz; ^{13}C, 75 MHz) were recorded on a Gemini 300 instrument
14 (Varian, Les Ulis, France). All NMR spectra were obtained in deuteriochloroform ($CDCl_3$).
15 Chemical shifts are reported in δ (parts per millions) relative to $CHCL_3$ ($CDCl_3$) as internal
16 reference: 7.27 ppm for ^{1}H (77.14 ppm for ^{13}C). Multiplicities are recorded as s (singlet), t
17 (triplet), m (multiplet) and br (broad).
18

19 **2.5 Molecular characterisation of isolates**

20 Bacteria for molecular analysis were grown for 2-3 days on isoxaben-agar slopes. The cells
21 were suspended in sterilised ultrapure water (Millipore-Q reagent) to obtain the optical density
22 of 1 U at 620 nm. The cells were then lysed by thermal shock (5 s in liquid nitrogen and 30 s
23 in boiling water, six times) and immediately used or stored at -20°C until PCR analysis.
24 ARDRA (Amplified Ribosomal DNA Restriction Analysis) of 16S rDNA and PCR-
25 RFLP (Restriction Fragment Length Polymorphism) analysis of the 16S-23S rDNA spacer

1 region (IGS) have previously been described in detail.[14] Only 4 restriction endonucleases were
2 used here: *Alu* I, *Dde* I, *Hae* III, and *Hinf* I.

3 Sequencing of 16S rDNA (1384 bp) was performed by Genome Express (Grenoble,
4 France). Sequences were compared by using the Infobiogen server (Villejuif, France) with
5 Fasta version 3.2t05 [15] and Align version 2.0u programs.[16] Partial 16S rDNA sequence of
6 isoxaben-degrading isolates from this study were deposited in Genbank under the following
7 accession number AY040877.

8

9 **2.6 Phenotypic testing**

10 Phenotypic tests were carried out with soil isolates and six reference strains of
11 *Microbacterium*: *M. dextranolyticum* DSM 8607, *M. flavescens* DSM 20643, *M.*
12 *ketoreductum* DSM 12510, *M.* sp. DSM 20028, *M. testaceum* DSM 20166 and *M.*
13 *trichotecenolyticum* DSM 8608. Nutrient agar plates or slope tubes supplemented with
14 isoxaben were inoculated with the test bacteria and incubated for one day at 28°C. The cells,
15 harvested with sterile water and suspended to an optical density of 1 U at 620 nm, were then
16 used as inoculum for the different tests. Biolog GP and GN (Biolog, Inc) microplates,[17,18]
17 were inoculated with cell suspension (0.1 ml per well) and incubated at 28°C for 3 days.
18 Colour development was automatically determined by means of a microplate reader
19 (Thermomax, Molecular Devices Corp., MenloPark, CA). Api Coryne microplates (Api
20 System S.A., La Balme les Grottes, France) were used according to the manufacturer's
21 recommendations. Gram reactions were recorded on 2- to 3-day-old colonies using a
22 fluorogenic substrate.[19]

23

24 **3 RESULTS AND DISCUSSION**

25 **3.1 Isolation of isoxaben-transforming strains**

1 All attempts to isolate isoxaben-degrading strains after soil enrichment and inoculation of the
2 liquid and agar-BS media failed, probably because the low solubility of isoxaben in the
3 medium. In contrast, on most of Nutrient-agar isoxaben plates, a few regular, brilliant, yellow,
4 colonies of bacteria (10^6 CFU g^{-1} of soil, 1-6% of total counts), grew in clearing zones. The
5 total colony count in soils I-IV incubated with isoxaben was approximately) Four to five
6 colonies forming clearing zones from each soil source were isolated to further characterise the
7 micro-organisms. No colony was observed in the soil from Dijon, suggesting that previous
8 field and/or laboratory treatments with isoxaben were necessary for bacterial proliferation.
9

10 **3.2 Isoxaben degradation in axenic cultures**

11 Four of the nineteen randomly selected isolates were used in this study: F2b from the Langon
12 French vineyard, A1j from the Serignac French orchard soil, Asp2 from the Bristol English
13 soil and B2b from the St Amand Belgian soil. Isoxaben degradation was never observed when
14 nitrogen was lacking in culture media suggesting that the pesticide cannot be used as sole
15 source of nitrogen by the isolates. Kinetics of mineralization of [2,6-dimethoxybenzamide-
16 *phenyl*-UL-^{14}C]isoxaben (50 mg litre^{-1}) inoculated by the B2b isolate are shown Figure 2.
17 After two weeks, 33%f the labelled isoxaben were recovered as $^{14}CO_2$. A $^{14}CO_2$ release was
18 also observed from flasks of nutrient broth medium with 250 mg litre^{-1} of pesticide. Under
19 such conditions, 36% of the initial radioactivity was trapped as $^{14}CO_2$ after two weeks (data
20 not shown). Less than 0.1% of the labelled isoxaben were recovered after two weeks both
21 from BS-nitrogen and nutrient broth uninoculated media.

22 TLC analysis of culture extracts (NB medium with 50 or 250 mg isoxaben litre^{-1})
23 showed a decrease in the residual ^{14}C-isoxaben and the accumulation of a slightly yellow,
24 non-radioactive spot with a 0.61 and 0.45 *rf* in solvent systems I and II, respectively
25 (metabolite I). In the same analysis control isoxaben spots presented a 0.71 and 0.63 *rf* in

solvent systems I and II, respectively. Only low traces of other labelled metabolites were generally observed.

In contrast to the previous results, release of $^{14}CO_2$ did not exceed 0.2% in two weeks when inoculated flasks of BS-nitrogen media were treated with [*isoxazole*-5-^{14}C]isoxaben (Figure 2) but as previously a major-radioactive metabolite with a TLC behaviour similar to metabolite I accumulated in the cultures. This metabolite was also present in the clearing zones extracts obtained from the culture of efficient isolates on the nutrient-agar plates with isoxaben. Similar results were observed in separate experiments with A1j, Asp2 and F2b isolates.

Labelled or unlabelled 2,6-dimethoxybenzoic acid (compound 2, Figure 1, 0.18 *rf* in solvent system II) neither accumulated in the different culture extracts although B2b rapidly grew on the BS-nitrogen medium supplemented with this compound as the sole source of carbon. This rapid growth could explain why this compound did not accumulate in the medium. However, as previously reported,[6] the presence of demethoxyisoxaben and of 2-hydroxy-6-methoxybenzamide in soil treated with isoxaben suggests a modification of the phenyl ring before isoxaben hydrolysis of the amide bond. This hypothesis is supported by the study of a chloridazon-degrading bacteria transforming metamitron to 2,-dihydroxy-metamitron which was then cleaved by *meta*-fission.[20] while a *Rhodococcus* sp. isolated from treated soil partly mineralized the phenyl ring of metamitron.[21]

3.3 Soil studies

Both [*isoxazole*-5-^{14}C] and [2,6-dimethoxybenzamide-*phenyl*-UL-^{14}C]isoxaben were poorly mineralized in natural and autoclaved samples of the soil from Dijon (Figures 3 and 4). Inoculation of natural samples with the suspension of the B2b isolate from soil IV greatly increased the mineralization rates of ^{14}C-isoxaben whatever the site of labelling.

1 Accumulation of the metabolite I was never observed in natural (non-autoclaved) soil. In
2 contrast, the radioactive metabolite I was present in methanol extracts from autoclaved soil
3 treated with [isoxazole-5-^{14}C]isoxaben] and inoculated with B2b. These results indicate that,
4 as in liquid system, the B2b isolate is capable of mineralizing the phenyl ring of isoxaben but
5 is unable to mineralize the isoxazole part of the pesticide. In separate experiments, addition of
6 the radioactive metabolite I in the non-inoculated natural soil resulted in a rapid
7 mineralization of the compound (Figure 5) while the compound was not mineralized in the
8 autoclaved soil. These results suggest a high degrading ability of the indigenous microflora for
9 metabolite I even if the soil has no previous history of isoxaben use. They also explain the
10 similarity of $^{14}CO_2$ kinetics observed with *phenyl* and *isoxazole*-^{14}C-isoxaben in the non-
11 autoclaved soil inoculated with B2b.

12

13 **3.4 Identification of the main metabolite formed from isoxaben**

14 The mass spectrum of metabolite I showed a base peak at m/z 139. The highest peak
15 observed, although with a very low abundance of ion, was at m/z 168. This ion could be
16 attributed to the molecular ion according to the results of chemical ionisation with methane as
17 reagent gas. In that case, the spectrum exhibited a base peak at m/z 169, and other peaks at
18 m/z 197 and m/z 209, due to the $[MH]^+$, $[M+C_2H_5]^+$ and $[M+C_3H_5]^+$ ions, respectively. The
19 isotopic contribution associated with these pseudomolecular ions suggested the formula of
20 $C_9H_{16}N_2O$ for metabolite I. The molecular weight of 168, even, was consistent with the
21 presence of two nitrogen atoms.

22 The presence of an amine group in the infrared spectrum of metabolite I was
23 confirmed by the two broad bands at 3327 and 3187 cm^{-1} (ν_{N-H} stretch). This low frequency
24 (approximately 100 cm^{-1}) was attributed to the solid phase of the compound deposited on the
25 zinc selenide window held at 77 K in the GC-FTIR interface.[22] The intense absorption band at

1665 cm^{-1} was attributed to the C-N stretching absorption of the imine function from the isoxazole cycle.

Taken together, these results, based on mass spectrometry, on calculation of isotopic contribution, and on IR analysis, suggested that the major metabolite of isoxaben observed in liquid or agar cultures and in inoculated soil was 5-amino-3-(1-ethyl-1-methyl-propyl)isoxazole (compound 3, Figure 1). A definitive proof was provided by NMR analysis. Chemical shifts δ measured by ^{1}H NMR were: 4.97 (s, 1 H, H-4), 4.36 (br s, 2 H, NH_2), 1.68-1.46 (m, 4 H, 2 CH_2), 1.19 (s, 3 H, CH_3), 0.80 (t, J = 7.4 Hz, 6 H, 2 CH_3), whereas these for ^{13}C NMR were: 171.8(C), 168.0 (C), 78.9 (CH), 39.4 (C), 33.3 (2 CH_2), 21.7 (CH_3), 8.7 (2 CH_3). They were consistent with the structure proposed for metabolite I.

In their previous study, Rouchaud and co-workers[6] also reported the presence of 5-amino-3-(1-ethyl-1-methyl-propyl)isoxazole in soil extract but the compound did not accumulate and they therefore considered the opportunity of an unusual mechanism for amide hydrolysis with 5-isoxazolone accumulation. Our results suggest that 5-amino-3-(1-ethyl-1-methyl-propyl)isoxazole could be an important intermediary product of the microbial degradation of isoxaben in soil. Alternative metabolic pathways correlated with specific microflora could therefore be proposed. Comparison with Camper's results[7] is more difficult because authors used a very acidic soil for their experiments. This could explain the relatively slow mineralization of labelled isoxaben. In contrast our results rather suggest the capability of certain microbial species to rapidly transform isoxaben and use part of the pesticide molecule as a source of carbon.

3.5 Characterization and identification of isolates

1 All the 19 isolates exhibited analogous RFLP profiles, whilst only one isolate from the French
2 orchard soil (A1j) exhibited a slightly different 16S-23S profile with a single restriction
3 endonuclease: *Dde I*. Only F2b, A1j, Asp2 and B2b isolates were retained for further
4 characterization: The 16S rDNA sequences analysed from the 4 selected isolates were
5 identical and consistent with the *Microbacterium* genus (98.3% identity with *Microbacterium*
6 *trichotecenolyticum* DSM 8608, 98.1% identity with *M. flavescens* DSM 20643, 97.6% with
7 *M. testaceum* DSM 20166, 97.6% with *M.* sp. DSM 20028, 97.3% with *M. dextranolyticum*
8 DSM 8607 and 97.4% with *M. ketoreductum* DSM 12510). All isolates and reference strains
9 exhibited a Gram positive reaction, as well as similar characteristics with Biolog and Api
10 Coryne tests

11 The *Microbacterium* genus belongs to the *Microbacteriaceae* family which is reported
12 to include *Microbacterium*, *Agrococcus*, *Agromyces*, *Aureobacterium*, *Clavibacter*,
13 *Curtobacterium, Rathayibacter, Leucobacter* and *Cryobacterium* genera.[23] The union of the
14 *Microbacterium* and *Aureobacterium* genera[24] and a reclassification of *Brevibacterium*
15 *oxydans* as *Microbacterium oxydans* have recently been proposed.[25]

16 Actinomycetes are phylogenetically and taxonomically related Gram positive bacteria
17 with a high G+C content in their DNA.[26] The degradation of pesticides by actinomycetes has
18 recently been reviewed.[27] Many pesticides, including phenoxyacetates, organochlorines, N-
19 methylcarbamates, *N*-phenylcarbamates, organophosphates or *s*-triazines can be degraded by
20 actinomycete species notably in the *Arthrobacter*, *Brevibacterium* and *Clavibacter* genera
21 which belong to the same suborder (*Micrococcineae*) as *Microbacterium.*

22 It can be surprising that all isolates from very different origins turn out to have the
23 same degradation intermediate and turn out to belong to the same genus and probably species.
24 Such presence of pesticide degrading strains with similar ribotypes in very different soils was
25 already observed with carbofuran[14]. However the high degree of genetic diversity within this

population possibly explained fortuitous similarities. Preliminary experiments did not suggest such high diversity within isoxaben degrading bacteria and did not allow locating isoxaben-catabolic genes in the *Microbacterium* sp. genome. Therefore, there is a need for further research on the genetic and enzymatic mechanisms involved in isoxaben transformation. Studies of actual involvement of microbial populations such as *Microbacterium* sp. strains on the field behaviour of isoxaben are in progress. Effects of repeated isoxaben treatments are considered at the present time by analogy with the microbial degradation of different amide herbicides such as napropamide, carbetamide or propyzamide.[28-30] In other respects there is an increasing environmental interest for natural strains such as *Microbacterium* sp isolates which could be used for bioremediation processes of pesticide wastes.

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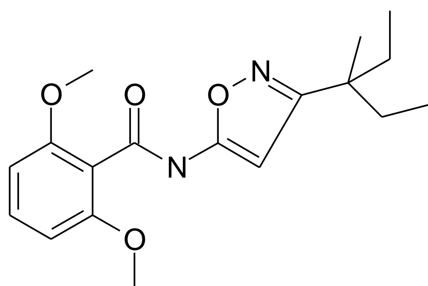
1

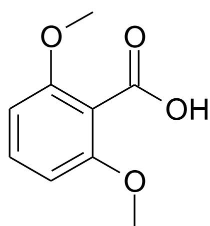
2 **Table 1.** Selected characteristics of soils used for this study

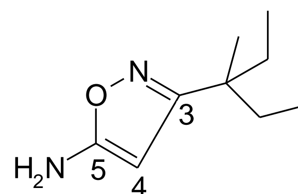
Soil	Origin	pH	W.H.C.	Organic matter	Sand	Silt	Clay	Crop and isoxaben history
			% dry weight	(g kg^{-1})		%		[treatment years]
I	Serignac, France	7.4	24.8	32	10	57	33	Orchard [4]
II	Langon, France	6.8	9.8	13	68	26	6	Vineyard [2]
III	Bristol, England	7.5	17.8	36	57	24	19	Turfgrass [2]
IV	Saint Amand, Belgium	7.4	24.8	24	12	72	16	Cereal [3]
V	Dijon, France	7.3	26.0	23	13	54	33	Turfgrass [0]

3

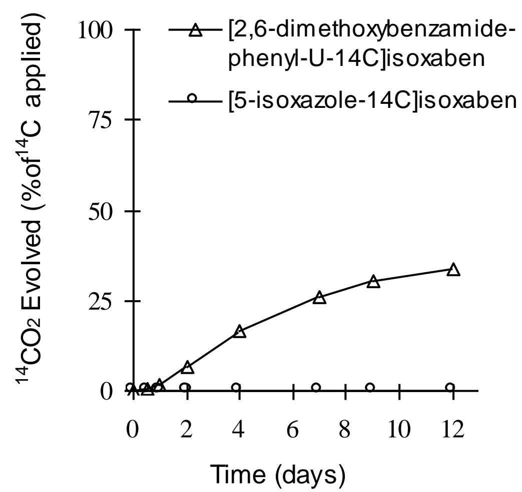
Figure 1. Structures of isoxaben (1), 2,6-dimethoxybenzoic acid (2) and 5-amino-3-(1-ethyl-1-methyl-propyl)isoxazole (metabolite I; 3).







1 **Figure 2**. Mineralization of labelled isoxaben in BS-nitrogen medium inoculated with the B2b isolate. Means of
2 4 replicates, S.D. below 5 %.
3



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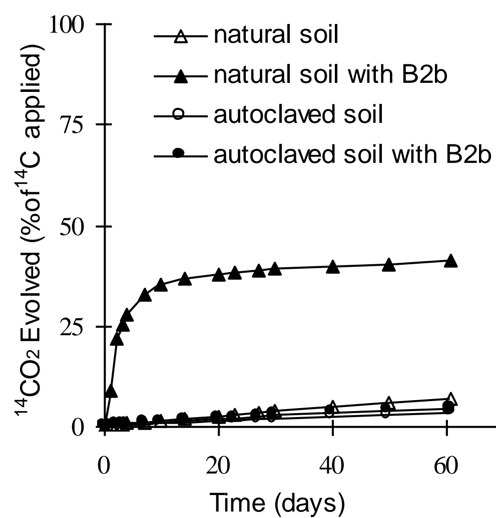
1 **Figure 3.** Mineralization of [*isoxazole*-5-^{14}C]isoxaben in natural and autoclaved soil from Dijon and effect of

2 inoculation with the B2b isolate. Means of 4 replicates, S.D. below 3 %.



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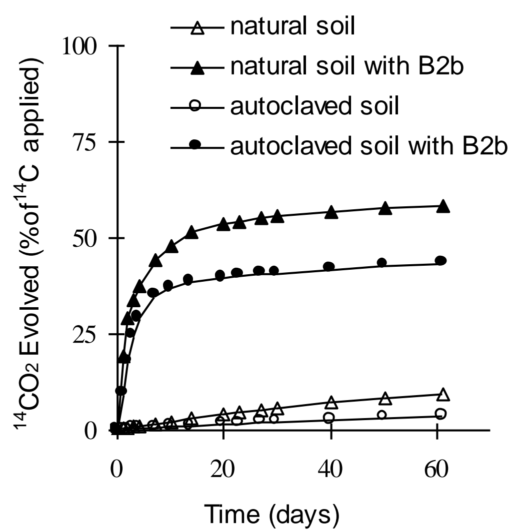
1 **Figure 4.** Mineralization of [2,6-dimethoxybenzamide-*phenyl*-UL-^{14}C]isoxaben in natural and autoclaved soil

2 from Dijon and effect of inoculation with the B2b isolate. Means of 4 replicates, S.D. below 4 %.



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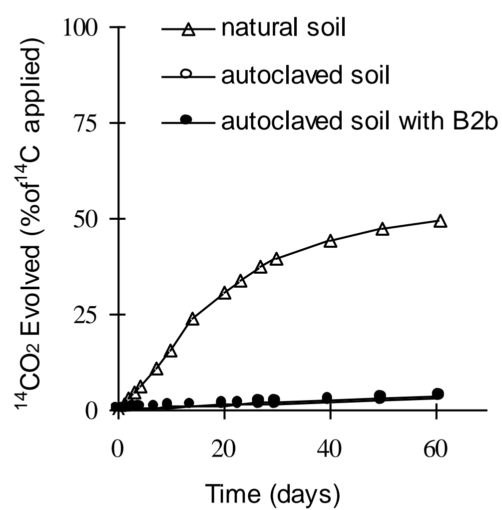
1 **Figure 5.** . Mineralization of 5-amino-3-(1-ethtyl-1-methylpropyl)isoxazole (metabolite I) in natural and
2 autoclaved Dijon soil and effect of inoculation with the B2b isolate. Means of 4 replicates, SD below 2 %. There
3 was no distinction between the curves for autoclaved soil with or without inoculum.
4



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