Journal of Industrial Microbiology & Biotechnology (2001) 26, 2–8

www.nature.com/jim

In situ ^{1}H NMR study of the biodegradation of xenobiotics: Application to heterocyclic compounds

A-M Delort and B Combourieu

Laboratoire de Synthèse, Electrosynthèse et Etude de Systèmes à Intérêt Biologique, UMR 6504 Université Blaise Pascal, CNRS, Aubière Cedex 63177, France

***In vivo* or *in situ* nuclear magnetic resonance (NMR) offers a powerful tool to study the degradation of xenobiotics by microorganisms. Most studies reported are based on the use of heteronuclei, and experiments with xenobiotics have been limited because specifically labeled xenobiotics are not commercially available, with the exception of ^{19}F and ^{31}P. wn>^{1}H NMR is, thus, of great interest in this area. To avoid problems caused by the presence of water and intrinsic metabolite signals, some studies were performed using a deuterated medium or specific detection of protons linked to the ^{13}C–^{15}N enriched pattern. We report here the application of *in situ* ^{1}H NMR, performed directly on culture media, to study the metabolism of heterocyclic compounds. In this review, we show that a common pathway is involved in the biodegradation of morpholine, piperidine, and thiomorpholine by *Mycobacterium aurum* MO1 and *Mycobacterium* sp. RP1. In all cases, the first step is the cleavage of the C–N bond, which results in an amino acid. Thiomorpholine is first oxidized to sulfoxide before the opening of the ring. The second step is the deamination of the intermediate amino acid, which leads to the formation of a diacid. We have shown that the cleavage of the C–N bond and the oxidation of thiomorpholine are initiated by reactions involving a cytochrome *P*450.** *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 2–8.

Keywords: ^{1}H NMR; *Mycobacterium*; morpholine; degradation; cytochrome *P*450; xenobiotics

Introduction

Nuclear magnetic resonance (NMR) spectroscopy is a basic tool used to determine the molecular structure of organic compounds. It has been used in many studies to identify metabolites resulting from the degradation of xenobiotics by microorganisms. However, it was used mainly in a chemically conventional way, i.e., after purification of each intermediate and analysis by classical ^{1}H and ^{13}C NMR techniques (one- and two-dimensional experiments). This approach presents numerous drawbacks: large amounts of biomass (microorganisms), xenobiotics and metabolites (in the range of milligrams) are necessary. In addition, the metabolites must be purified.

Since the 1980s, a new NMR approach has been developed to study microbial metabolism: *in vivo* NMR, performed directly on living cells, or *in situ* NMR, performed on culture medium or cellular extracts [13, 17, 21, 31]. Metabolic NMR is usually based on the detection of heteronuclei such as ^{13}C, ^{31}P, ^{2}H, ^{19}F, and ^{15}N. Fluorinated and phosphorylated xenobiotics can be easily studied, but other nuclei must be enriched to be detected, because of their low sensitivity and low natural abundance. Specifically, ^{13}C-, ^{2}H-, or ^{15}N-labeled xenobiotics must be synthesized by specialized laboratories, as they are not commercially available. Consequently, there have been only a few studies concerning microbial degradation of xenobiotics using ^{13}C, ^{2}H, or ^{19}F NMR [2, 5, 6, 18, 24, 32].

^{1}H NMR has rarely been used, although the natural abundance of ^{1}H is almost 100%, and its sensitivity is excellent. Studies of biotransformation of xenobiotics using ^{1}H NMR are rare, due to the narrow range of chemical shifts (about 15 ppm), which induces overlapping of many signals and also because of the large amount of H_2O present (about 55 M).

To overcome these problems, some strategies were developed to eliminate ^{1}H signals from water and from intrinsic cellular metabolites.

Gaines *et al.* [15] studied the degradation of 100% ^{1}H benzoate and/or *p*-hydroxybenzoate by *Acinetobacter calcoaceticus* cells grown in a commercial ^{2}H-enriched medium (^{2}H IG medium). Under these conditions, only ^{1}H resonances from these aromatic compounds and their metabolites were detected. Nevertheless, this approach presents some drawbacks: *A. calcoaceticus* growth was reduced by half because of the well known isotopic effect of deuterium on many enzymatic reactions, and the large amount of deuterated medium needed in this type of experiment is very expensive.

Gard *et al.* [16] developed another concept based on the detection of ^{1}H coupled to specifically enriched heteroatoms (^{13}C–^{15}N). They studied metabolism of doubly labeled (^{13}C–^{15}N) glyphosate in two strains of *Ochobactrum antropi* using triple resonance edited NMR spectroscopy (TRIED). The combination of isotope editing and proton detection selectively cancels all but the signals arising from ^{13}C–^{15}N fragments, so the detected signals are specific to the xenobiotic and its metabolites. The limitation of this ^{1}H editing technique is the requirement for doubly labeled xenobiotics; in addition, the ^{13}C–^{15}N motif is not common among xenobiotics.

New techniques of high-performance liquid chromatography (HPLC) NMR have been intensively used in pharmacological studies [22], but there have been few studies which employ the technique on the degradation of xenobiotics by microorganisms

Correspondence: Dr. A-M Delort, Laboratoire de Synthèse, Electrosynthèse et Etude de Systèmes à Intérêt Biologique, UMR 6504 Université Blaise Pascal, CNRS, France
Received 27 December 1999; accepted 8 May 2000

[7]. These techniques allow the detection of lower concentrations of metabolites (a few micrograms) compared with conventional NMR analyses of isolated purified metabolites, but they require initial purification of the metabolites (on-line HPLC). One of the limitations of HPLC-NMR is the cost of the equipment.

In situ ^{1}H NMR, performed directly on biological fluids (e.g., plasma, urine, cerebrospinal fluid), is being increasingly used for clinical analyses [3]. This technique is simple and does not require any purification or specific labeling. It was also applied to study bacterial culture media and bacterial extracts [1,25,26], but no data have been reported about biodegradation of xenobiotics.

In this paper, we present a review of the results we obtained about the biodegradative pathway of heterocyclic compounds, such as morpholine, by two *Mycobacterium* strains, *Mycobacterium aurum* MO1 and *Mycobacterium* sp. RP1 [4,10–12,28], using *in situ* ^{1}H NMR. This new approach was particularly convenient, because the lack of chromophores and the high water solubility of these chemicals prevented easy purification. Only indirect tools for the detection of these xenobiotics and their metabolites are available, including measurements of chemical oxygen demand (COD), optical density (OD), or NH_3, growth on intermediates, and *in vitro* enzyme assays [8,14,19,27,33].

Degradative pathway of heterocyclic compounds

Several heterocyclic compounds including morpholine (X=O), thiomorpholine (X=S), piperidine (X=C) were incubated with the *Mycobacterium* strains and the kinetics of their degradation was monitored by *in situ* ^{1}H NMR using the experimental protocol described previously [12]. The results obtained allowed us to propose a general pathway of degradation (Figure 1).

Degradation of morpholine

An example of ^{1}H NMR spectra collected during the incubation of 10 mM morpholine with cells of *M. aurum* MO1 is presented in Figure 2.

In spectra taken at time zero, three main signals are visible: a singlet at 0 ppm that belongs to the methyl groups of $TSPd_4$ and two pseudotriplets at 2.88 and 3.72 ppm that correspond to the CH_2 of morpholine. After 10 h, the signals of morpholine had decreased while three new signals (Y) were present resonating, respectively, at 3.95 (singlet), 3.67 (pseudotriplet) and 3.05 ppm (pseudotriplet). These signals were assigned to the CH_2 of 2-(2-aminoethoxy)acetate (Figure 1). This assignment was confirmed by the addition of the authentic compound which we synthesized [12]. A singlet at 3.95 ppm increased. It was assigned to glycolate as evidenced by addition of the commercial compound to the sample. However, this chemical shift was also corresponding to the

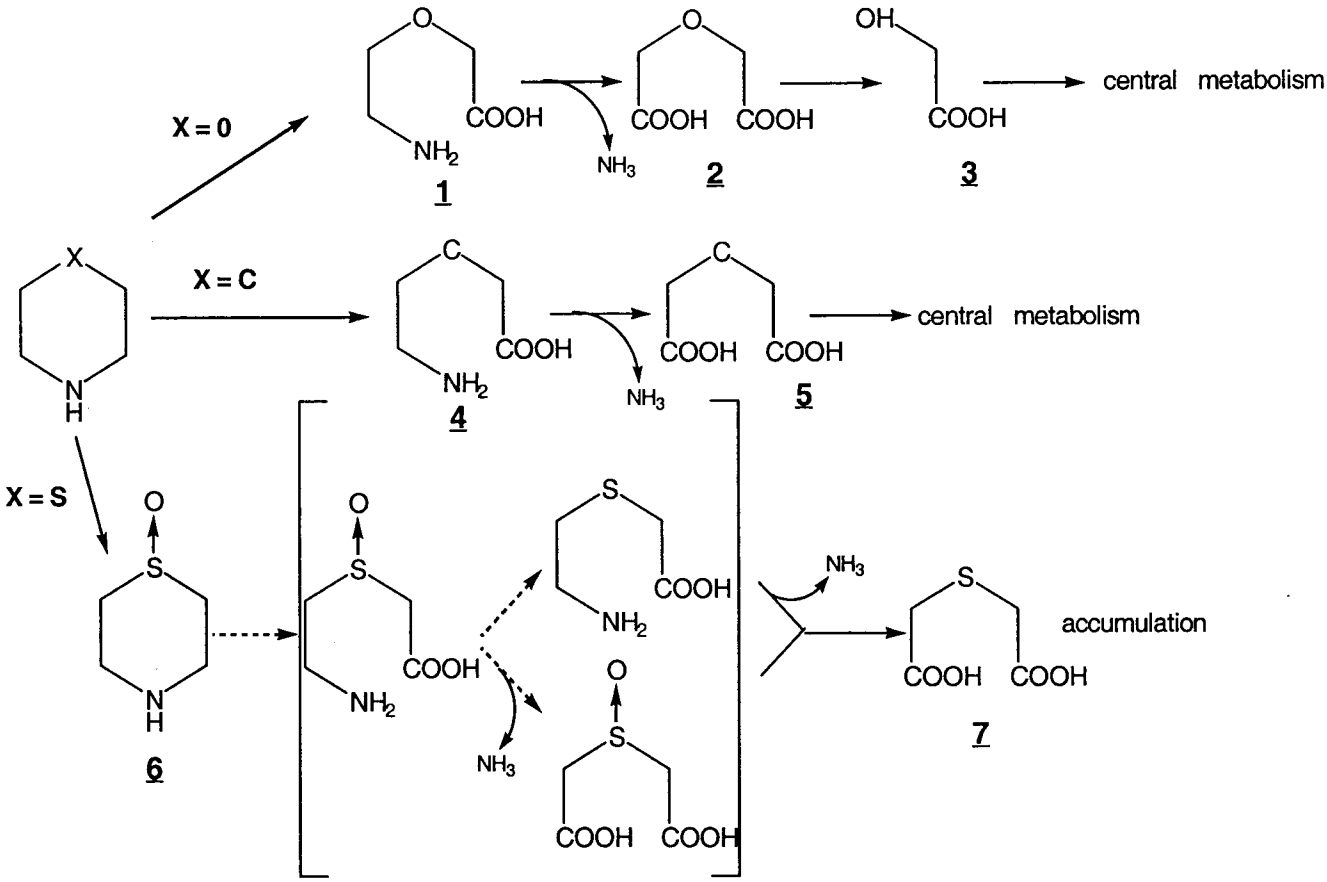

Figure 1 General pathway of degradation of heterocyclic compounds by *Mycobacterium aurum* MO1 and *Mycobacterium* sp. RP1. **1:** 2-(2-aminoethoxy) acetate, **2:** diglycolic acid, **3:** glycolic acid, **4:** 5-amino-valeric acid, **5:** glutaric acid, **6:** sulfoxide of thiomorpholine, **7:** thiodiglycolic acid.

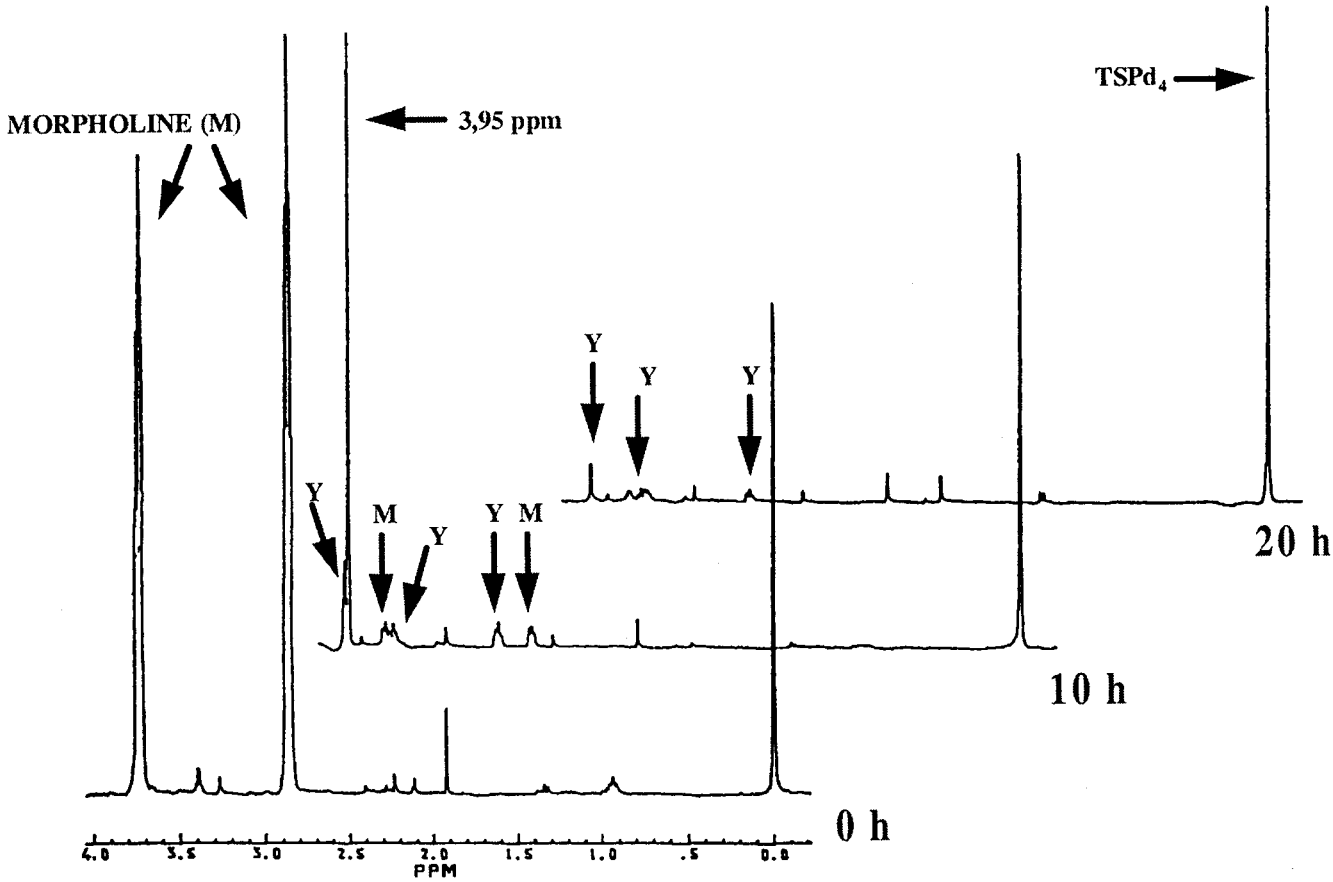

Figure 2 Kinetics of morpholine degradation by *Mycobacterium aurum* MO1. *In situ* ^{1}H NMR spectra were recorded at 300.13 MHz for 15 min on samples collected every hour from the incubation medium, centrifuged and adjusted to pH 10. $TSPd_4$ was used as chemical shift reference ($\delta = 0$ ppm) and for quantification. M: morpholine, Y: 2-(2-aminoethoxy)acetate.

resonance of diglycolic acid. Complementary experiments performed with *in situ* ion spray mass spectroscopy showed unambiguously the presence of diglycolic acid [10]. Both glycolate and diglycolate were degraded when incubated directly with the bacterial cells. Diglycolic acid is likely to be transformed to glycolic acid, which is further integrated in the central metabolism of bacteria via the glyoxylate pathway. After 20 h, morpholine was exhausted, and all intermediate compounds were decreasing and almost disappeared.

The time courses of the concentration of morpholine, 2-(2-aminoethoxy)acetate and the signal at 3.95 ppm are presented in Figure 3. These quantitative data were obtained by integrating the NMR signals in Figure 2, and the measured areas were compared with the integral of the $TSPd_4$ signal. The different concentrations were calculated as explained previously [12].

These experiments were repeated with *Mycobacterium* sp. RP1, and the same results were obtained [28].

Degradation of piperidine

An example of the ^{1}H NMR spectrum collected after 2 and 6 h of incubation of *M. aurum* MO1 with 10 mM piperidine is presented in Figure 4A. After 2 h, the signals belonging to the CH_2 of piperidine were still visible at 3.13 (triplet), 1.74 and 1.66 ppm (quintuplets). After 6 h, new signals resonating at 2.16 (triplet) and 1.76 ppm (quintuplet) were detected. They were assigned to the protons of glutarate, on carbons 2 and 3, respectively. This assignment was confirmed by adding commercial glutarate to the sample [10].

The quantitative analysis of the kinetics of degradation of piperidine was made by integrating the signals of the different metabolites in ^{1}H NMR spectra (Figure 4B). No glutarate was detected after 20 h. Consequently, it was degraded in the cells, presumably because it enters amino acid metabolism, especially lysine metabolism as observed for *Streptomyces* sp. [23] and *Candida tropicalis* [20].

We obtained similar results with the strain sp. RP1 (unpublished data).

Degradation of thiomorpholine

Figure 5A presents a spectrum collected after 10 h of incubation of *M. aurum* MO1 cells with 10 mM thiomorpholine. Four groups of multiplets resonating at 2.84, 2.98, 3.08 and 3.36 ppm were assigned to the four nonequivalent protons of the sulfoxide of thiomorpholine (Figure 1). To make this attribution, this sulfoxide was synthesized in our laboratory. Figure 5B shows the ^{1}H NMR spectrum of this synthetic sulfoxide (4 mM) in Knapp buffer, at pH 10; the signals perfectly overlap those of the sulfoxide obtained enzymatically.

Another signal (a singlet) is also present in Figure 5A at 3.25 ppm, which was assigned to the CH_2 of thiodiglycolic acid (see

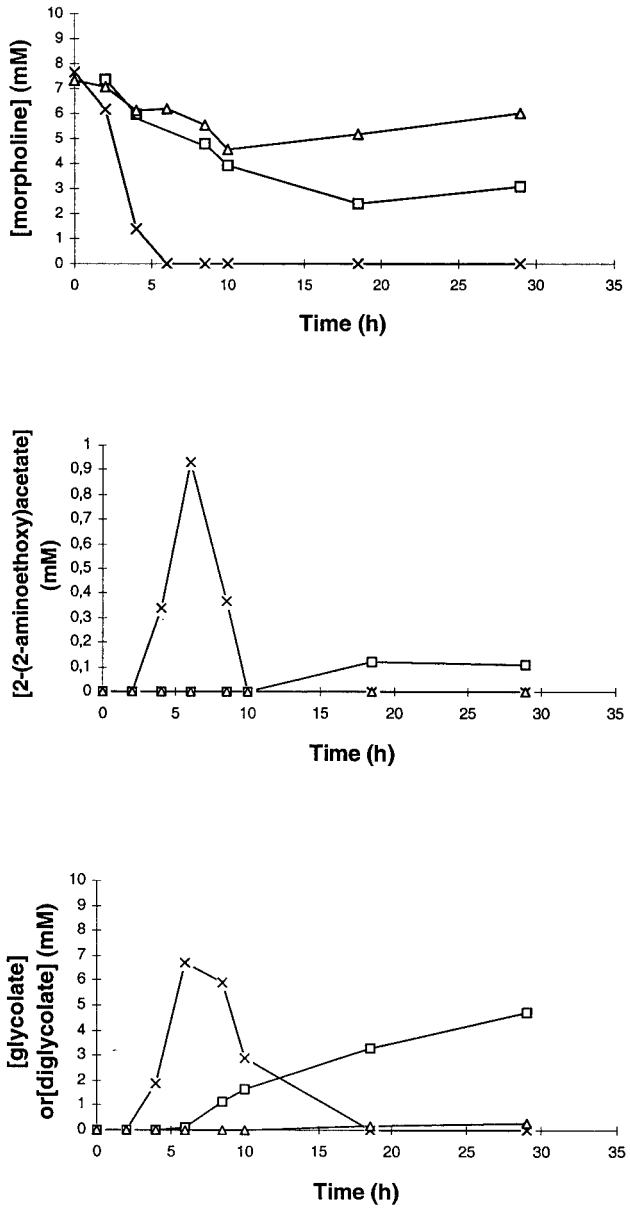

Figure 3 Morpholine degradation by *Mycobacterium aurum* MO1 in the absence (X) or in the presence of 5 mM (□) and 10 mM (△) metyrapone, a specific inhibitor of cytochrome *P*450. Concentrations were measured from metabolite integrals relative to $TSPd_4$ integrals in ^{1}H NMR spectra (Figure 2).

Figure 1). The same signal was obtained when the cells were incubated with the sulfoxide of thiomorpholine, showing that it is a downstream metabolite [11]. The assignment of this resonance was confirmed by addition of the commercial compound to this sample and complementary analysis by mass spectrometry. This metabolite accumulated in the cells, and the C–S bond was not cleaved further.

The time course of the concentrations of thiomorpholine sulfoxide and thiodiglycolic acid are presented in Figure 6.

The same metabolic pathway was observed when *Mycobacterium* sp. RP1 resting cells were incubated with thiomorpholine [4].

Conclusion

In all cases, the opening of the heterocyclic ring proceeds via the cleavage of the C–N bond. The absence of this C–N bond prevents any degradation of the heterocyclic compounds. This cleavage leads to an amino acid whose central atom varies (O, S or C) depending on the initial substrate.

The second step (Figure 1) is the deamination of the intermediate amino acid, which results in the formation of a diacid.

These results are in agreement with those of Swain *et al.* [33], who showed that pyrrolidine was metabolized via succinic acid in *Mycobacterium* sp. MORG.

Involvement of cytochrome *P*450 activity

Spectrophotometric evidence of the induction of a cytochrome P450

Mazure and Truffaut [27] have shown that morpholine degradation was associated with oxygen consumption, indicating that activity of a monooxygenase, namely a cytochrome *P*450, may be involved. To investigate the presence of a cytochrome *P*450 in *M. aurum* MO1, the carbon monoxide differential spectra of sodium dithionite-reduced soluble fractions of morpholine-induced and uninduced cells were measured [11,28]. The results are reported in Table 1. When *M. aurum* MO1 cells were grown on morpholine as a sole source of carbon, nitrogen, and energy, a peak at 449 nm was present in the spectrum, indicating the presence of a soluble cytochrome *P*450 [11]. As this peak was not observed in acetate-grown cells, the synthesis of this enzyme was induced by morpholine. The same results were obtained with *Mycobacterium* sp. RP1 [28].

Using the same spectrophotometric method, induction of cytochrome *P*450 by growth of each strain of mycobacteria on various heterocyclic compounds was tested [4,11,28] (Table 1). Induction in both strains of this soluble protein was observed in the case of morpholine, piperidine and thiomorpholine, which was consistent with the involvement of this enzyme in the cleavage of the heterocyclic ring. This cleavage probably occurs at the C–N bond, as shown by non-induction of a cytochrome *P*450 by tetrahydropyrane and tretrahydrofurane wherein this bond is absent.

Growth was usually correlated with induction of cytochrome *P*450, indicating the first step of degradation. When the degradation was not complete, as in the case of thiomorpholine, the bacteria could not grow on this substrate, despite its initial transformation.

The activity of *P*450 was also consistent with the oxidation of thiomorpholine into sulfoxide, this type of reaction being rather common.

Inhibition of morpholine and thiomorpholine transformation by metyrapone

To confirm the key role of a cytochrome *P*450 in the transformation of morpholine and thiomorpholine, a selective inhibitor of this enzyme was added to the incubation medium. Metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone) was chosen as a specific inhibitor of cytochrome *P*450 [34].

M. aurum MO1 cells (100 g/l) were incubated with different concentrations of metyrapone (5 and 10 mM) and with morpho-

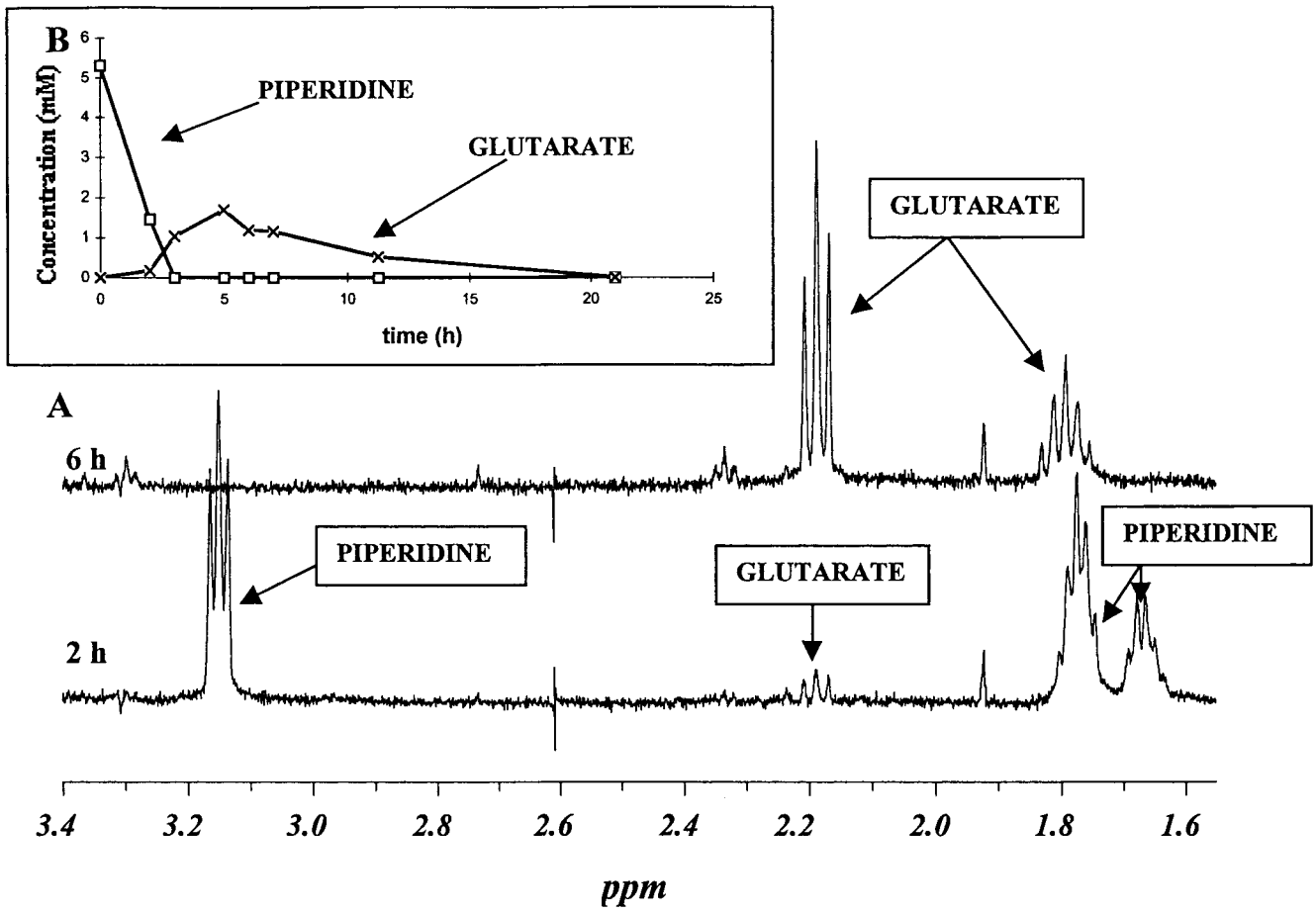

Figure 4 (A) *In situ* ^{1}H NMR spectra of a sample collected after 2 and 6 h of incubation of *Mycobacterium aurum* MO1 with 10 mM piperidine. Experimental conditions were as in Figure 2. (B) Time course of piperidine (□) and glutaric acid (×) concentrations during incubation of *Mycobacterium aurum* MO1 in the presence of 10 mM piperidine.

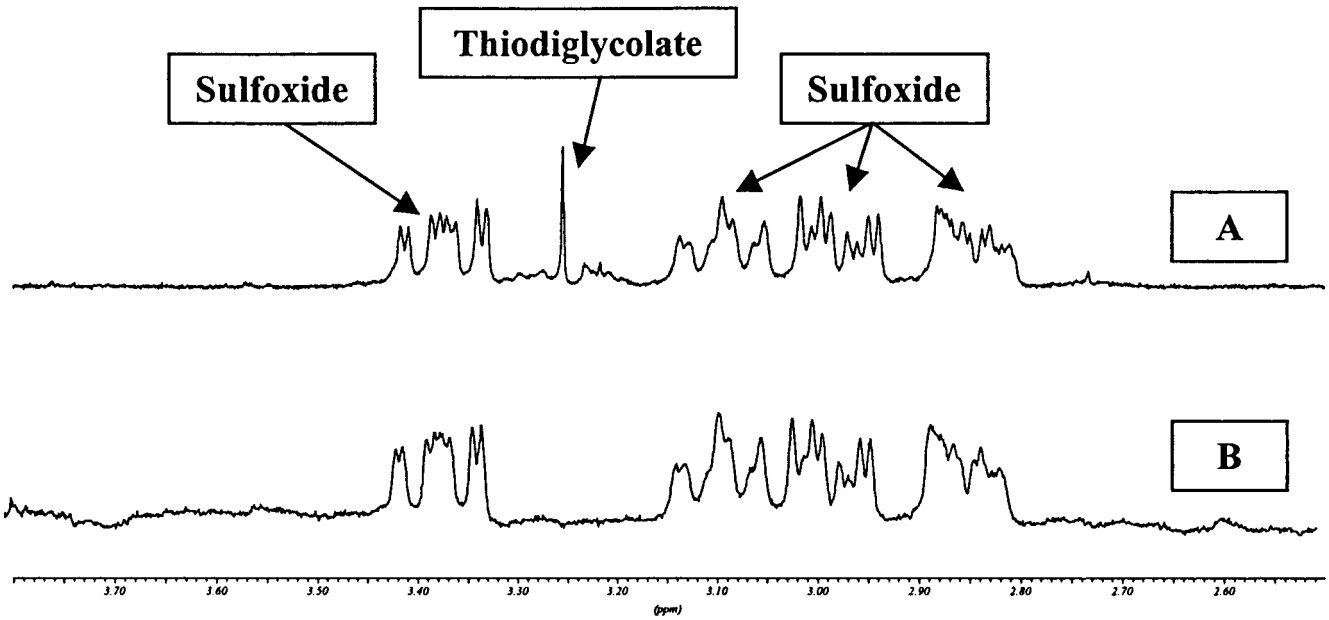

Figure 5 (A) ^{1}H NMR spectrum of a sample collected after 10 h of incubation of *Mycobacterium aurum* MO1 with 10 mM thiomorpholine, (B) ^{1}H NMR spectrum of synthetic sulfoxide of thiomorpholine in Knapp buffer.

line (10 mM) and analyzed by ^{1}H NMR. The kinetics of morpholine degradation is reported in Figure 3.

Addition of metyrapone resulted in inhibition of morpholine degradation. In the presence of metyrapone (5 mM), the rate of formation of 2-(2-aminoethoxyacetate) decreased and was completely inhibited at a concentration of 10 mM.

The direct effects of metyrapone on sulfoxide formation from thiomorpholine, the first intermediate in thiomorpholine biodegradation, showed that a cytochrome *P*450 was also involved in the first step of this degradation (Figure 6). The synthesis of thiodiglycolic acid was also decreased in the presence of metyrapone.

Similar results were obtained with *Mycobacterium* sp. RP1 [28].

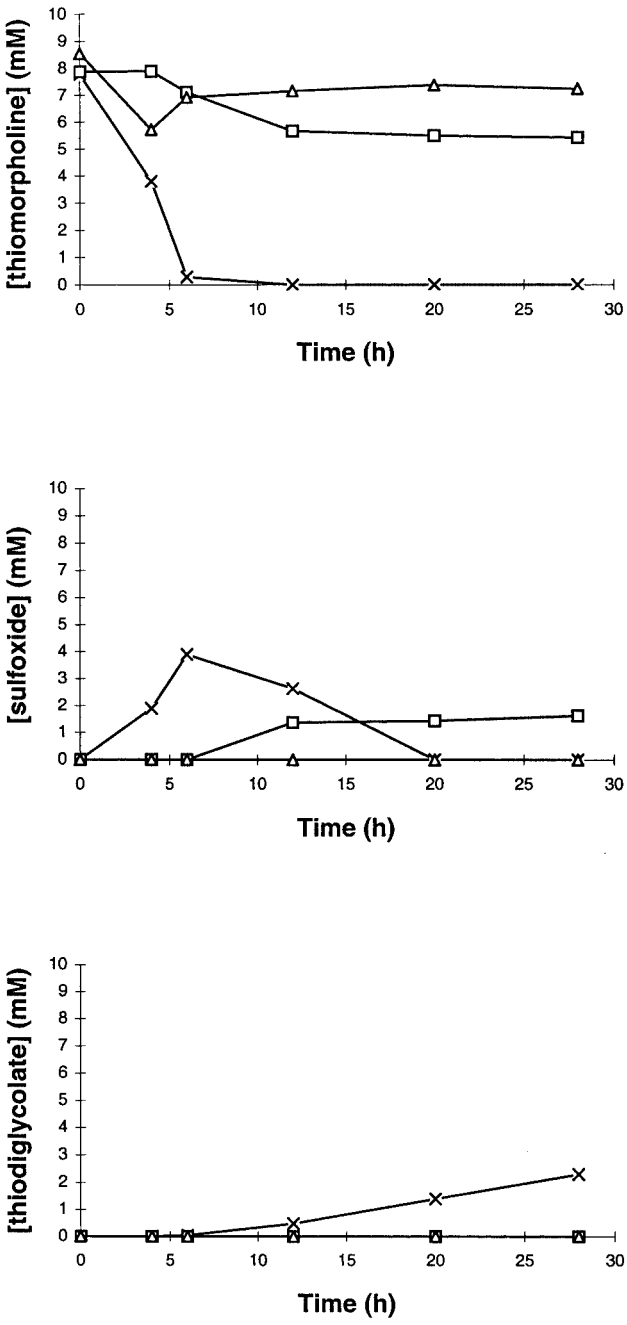

Figure 6 Thiomorpholine degradation by *Mycobacterium aurum* MO1 in the absence (×) or presence of 5 mM (□) or 10 mM (△) metyrapone, a specific inhibitor of cytochrome *P*450.

Table 1 Growth of *M. aurum* MO1 and *Mycobacterium* sp. RP1 on different substrates and induction of cytochrome *P*450 (From Refs. [20, 21, 28])

Substrate	Growth[a]		Induction of cytochrome *P* 450	
	MO1	RP1	MO1	RP1
Acetate	+	+	−	−
Succinate	+	+	−	−
Morpholine	+	+	+	+
Thiomorpholine	−	−	+	+
Pyrrolidine	+	+	+	+
Piperidine	+	+	+	+
Tetrahydrofurane	−	∓	−	−
Tetrahydropyrane	−	∓	−	−

[a](+) Growth; (∓) low growth; (−) no growth.

Conclusion

This was the first evidence of the presence of a cytochrome *P*450 in mycobacteria. Since then, Poupin et al. [29] showed induction of a soluble cytochrome *P*450 during degradation of morpholine in other strains of *Mycobacterium*. The genes encoding a cytochrome *P*450 involved in piperidine and pyrrolidine utilization and its regulatory protein were cloned and sequenced in *M. smegmatis* mc2155 [30], and 20 genes that potentially code for P450 proteins were found in the complete genome sequence of *M. tuberculosis* [9]. These examples show the key role of cytochrome *P*450 in mycobacteria, especially in biodegradation processes.

General conclusion

To date, only a few studies have been reported on the microbial degradation of xenobiotics using NMR. After the pioneering work carried out 10 years ago, new data have been published only recently. NMR is a high-performance tool to identify metabolic pathways involved in biodegradation of drugs and pollutants. The main limitation in this type of approach is the necessity of specifically (^{13}C, ^{15}N, ^{2}H) labeling non-commercial compounds to perform heteronucleus NMR experiments. However, the methods described in recent work using ^{1}H NMR in pure water, performed either by HPLC-NMR, or directly *in situ* in culture medium, are quite promising: natural abundance molecules can be studied under simple culture conditions. This strategy should be successfully developed in the future.

Acknowledgements

This paper is based on a presentation by A.M. Delort to a symposium on "NMR in Environmental Biotechnology" organized at the Wageningen NMR Center. This work was supported by an Environmental Program of the CNRS (ECOTECH).

References

1 Abel CB, JC Lindon, D Noble, BA Rudd, PJ Sidebottom and JK Nicholson. 1999. Characterization of metabolites in intact *Streptomyces citricolor* culture supernatants using high-resolution nuclear magnetic resonance and directly coupled high-pressure liquid chromatography nuclear magnetic resonance spectroscopy. *Anal Biochem* 270: 220–230.

2 Barber RD and TJ Donohue. 1998. Function of a glutathione-dependent formaldehyde dehydrogenase in *Rhodobacter sphaeroides* formaldehyde oxidation and assimilation. *Biochemistry* 37: 530–537.

3 Bell JD, JC Brown and PJ Sadler. 1989. NMR studies of body fluids NMR. *Biomedicine* 2: 246–256.

4 Besse P, B Combourieu, P Poupin, M Sancelme, N Truffaut, H Veschambre and AM Delort. 1998. Degradation of morpholine and thiomorpholine by an environmental *Mycobacterium* involves a cyt. *P*-450. Direct evidence of intermediates by *in situ* ^{1}H NMR. *J Mol Catal B Enzym* 5: 403–409.

5 Boersma MG, TY Dinarieva, WJ Middelhoven, WJH van Berkel, J Doran, J Vervoort and IMC. Rietjens. 1998. ^{19}F nuclear magnetic resonance as a tool to investigate microbial degradation of fluorophenols to fluorocatechols and fluoromuconates. *Appl Environ Microbiol* 64: 1256–1263.

6 Bondar VS, MG Boersma, EL Golovlev, J Vervoot, WJH van Berkel, ZI Finkelstein, IP Solyanikova, LA Golovleva and IMCM Rietjens. 1998. ^{19}F NMR study on the biodegradation of fluorophenols by various *Rhodococcus* species. *Biodegradation* 9: 475–486.

7 Cannell RJ, T Rashid, IM Ismail, PJ Sidebottom, AR Knaggs and P Marshall. 1997. Novel metabolites of warfarin produced by *Beauveria bassiana* and *Streptomyces rimosus*: a novel application of HPLC-NMR. *Xenobiotica* 27: 147–157.

8 Cech JS, P Hartman, M Slosarek and J Chudoba. 1988. Isolation and identification of a morpholine degrading bacterium. *Appl Environ Microbiol* 54: 619–621.

9 Cole ST, R Brosch, J Parkhill, T Garnier, C Churcher, D Harris, SV Gordon, K Eiglmeier, S Gas, CE Barry III, F Tekaia, K Badcock, D Basham, D Brown, T Chillingworth, R Connor, R Davies, K Devlin, T Feltwell, S Gentles, N Hamlin, S Holroyd, T Hornsby, K Jagels, A Krogh, J McLean, S Moule, L Murphy, S Oliver, J Osborne, MA Quail, MA Rajandream, J Rogers, S Rutter, K Seeger, S Skelton, R Squares, S Squares, JE Sulston, K Taylor, S Whitehead and BG Barrell. 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* 393: 537–544.

10 Combourieu B. 1999. Etude par RMN ^{1}H *in situ* de la dégradation d'hétérocycles azotés de type morpholine par des mycobactéries. Thèse de l'Université Blaise Pascal, Clermont-Ferrand, France.

11 Combourieu B, P Poupin, P Besse, M Sancelme, H Veschambre, N Truffaut and AM Delort. 1998a. Thiomorpholine and morpholine oxidation by a cytochrome *P*450 in *Mycobacterium aurum* MO1. Evidence of the intermediates by *in situ* ^{1}H NMR. *Biodegradation* 9: 433–442.

12 Combourieu B, P Besse, M Sancelme, H Veschambre, AM Delort, P Poupin and N Truffaut. 1998b. Morpholine degradative pathway of *Mycobacterium aurum* MO1. Direct evidence of intermediates by *in situ* ^{1}H NMR. *Appl Environ Microbiol* 64: 153–158.

13 Dale BE and RJ Gillies. 1991. Nuclear magnetic resonance spectroscopy of dense cell populations for metabolic studies and bioreactor engineering: a synergistic partnership. *Biotechnology* 17: 107–118.

14 Emtiazi G and JS Knapp. 1994. The biodegradation of piperazine and structurally related linear and cyclic amines. *Biodegradation* 5: 83–92.

15 Gaines III GL, L Smith and EL Neidle. 1996. Novel nuclear magnetic resonance spectroscopy methods demonstrate preferential carbon source utilization by *Acinetobacter calcoaceticus*. *J Bacteriol* 178: 6833–6841.

16 Gard JK, PCC Feng and WC Hutton. 1997. Nuclear magnetic resonance time course studies of glyphosate metabolism by microbial soil isolates. *Xenobiotica* 27: 633–644.

17 Grivet JP, M Durand and JL Tholozan. 1992. ^{13}C NMR studies of bacterial fermentations. *Biochimie* 74: 897–901.

18 Higgins TP, JE Hewlins and GF White. 1996. A ^{13}C NMR study of the bacterial metabolism of monomethyl sulfate. *Eur J Biochem* 236: 620–625.

19 Knapp JS, AG Callely and J Mainprize. 1982. The microbial degradation of morpholine. *J Appl Bacteriol* 52: 5–13.

20 Large PJ and A Robertson. 1991. The route of lysine breakdown in *Candida tropicalis*. *FEMS Microbiol Lett* 66 (2): 209–213.

21 Lens PNL and MA Hemminga. 1998. Nuclear magnetic resonance in environmental engineering: principles and applications. *Biodegradation* 9: 393–409.

22 Lindon JC, J Nicholson and ID Wilson. 1996. The development and application of coupled HPLC-NMR spectroscopy. *Adv Chromatogr* 36: 315–382.

23 Madduri K, C Stuttard and LC Vining. 1989. Lysine catabolism in *Streptomyces* sp. is through cadaverine: beta-lactam producers also make alpha-aminoadipate. *J Bacteriol* 171 (1): 299–302.

24 Mason RP and JK Sanders. 1989. *In vivo* enzymology: a deuterium NMR study of formaldehyde dismutase in *Pseudomonas putida* F61a and *Staphylococcus aureus*. *Biochemistry* 28: 2160–2168.

25 Matheron C, AM Delort, G Gaudet, E Forano and T Liptaj. 1998. ^{13}C and ^{1}H nuclear magnetic resonance study of glycogen futile cycling in strains of the genus *Fibrobacter*. *Appl Environ Microbiol* 64: 74–81.

26 Matheron C, AM Delort, G Gaudet, T Liptaj and E Forano. 1999. Interactions between carbon and nitrogen metabolism in *Fibrobacter succinogenes* S85. A ^{1}H and ^{13}C NMR and enzymatic study. *Appl Environ Microbiol* 65: 1941–1948.

27 Mazure N and N Truffaut. 1994. Degradation of morpholine by *Mycobacterium aurum* MO1. *Can J Microbiol* 40: 761–765.

28 Poupin P, N Truffaut, B Combourieu, P Besse, M Sancelme, H Veschambre and AM Delort. 1998. Degradation of morpholine by an environmental *Mycobacterium* strain involves a cytochrome *P*450. *Appl Environ Microbiol* 64: 159–165.

29 Poupin P, JJ Godon, E Zumqtein and N Truffaut. 1999a. Degradation of morpholine, piperidine and pyrrolidine by mycobacteria: evidences for the involvement of a cytochrome *P*450. *Can J Microbiol* 45: 209–216.

30 Poupin P, V Ducrocq, S Hallier-Soulier and N Truffaut. 1999b. Cloning and characterization of the genes encoding a cytochrome *P*450 (PipA) involved in piperidine and pyrrolidine utilization and its regulatory protein (PipR) in *Mycobacterium smegmatis* mc2155. *J Bacteriol* 181:3419–3426.

31 Santos H, P Fareleira, J Legall and AV Xavier. 1994. *In vivo* nuclear magnetic resonance in study of physiology of sulfate-reducing bacteria. *Methods Enzymol* 243: 543–558.

32 Selifonov SA, PJ Chapman, SB Akkerman, JE Gurst, JM Bortiatynski, M Nanny and PG Hatcher. 1998. Use of ^{13}C nuclear magnetic resonance to assess fossil fuel biodegradation: fate of [1-^{13}C]acenaphtene in creosote polycyclic aromatic compound mixtures degraded by bacteria. *Appl Environ Microbiol* 64: 1447–1453.

33 Swain A, KV Waterhouse, WA Venables, AG Callely and SE Lowe. 1991. Biochemical studies of morpholine catabolism by an environmental *Mycobacterium*. *Appl Microbiol Biotechnol* 35: 110–114.

34 Testa B and P Jenner. 1981. Inhibitors of cytochrome *P*450s and their mechanism of action. *Drug Metab Rev* 12: 1–117.