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^{19}F NMR study on the biodegradation of fluorophenols by various *Rhodococcus* species

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Abstract

Of all NMR observable isotopes ^{19}F is the one perhaps most convenient for studies on biodegradation of environmental pollutants. The reasons underlying this potential of ^{19}F NMR are discussed and illustrated on the basis of a study on the biodegradation of fluorophenols by four *Rhodococcus* strains. The results indicate marked differences between the biodegradation pathways of fluorophenols among the various *Rhodococcus* species. This holds not only for the level and nature of the fluorinated biodegradation pathway intermediates that accumulate, but also for the regioselectivity of the initial hydroxylation step. Several of the *Rhodococcus* species contain a phenol hydroxylase that catalyses the oxidative defluorination of ortho-fluorinated di- and trifluorophenols. Furthermore, it is illustrated how the ^{19}F NMR technique can be used as a tool in the process of identification of an accumulated unknown metabolite, in this case most likely 5-fluoromaleylacetate. Altogether, the ^{19}F NMR technique proved valid to obtain detailed information on the microbial biodegradation pathways of fluorinated organics, but also to provide information on the specificity of enzymes generally considered unstable and, for this reason, not much studied so far.

Abbreviation: NMR – Nuclear Magnetic Resonance

Introduction

The number of fluorine containing xenobiotics and environmental contaminants has strongly increased during the past decades (Key et al. 1997). A number of fluorine containing drugs are currently in clinical use, a large number of fluorinated compounds are intermediates or end products in the synthesis of industrial and agrochemicals, and many fluorine containing biodegradation products result from the chemical and/or microbial degradation of these fluorinated chemicals (Banks & Lowe 1994; Banks 1995; Walker 1990; Banks & Tatlow 1994; Edwards 1994). The objective of the present study is to illustrate the use of ^{19}F NMR as a technique to study the microbial degradation of fluorinated environmental pollutants.

Of all NMR observable isotopes ^{19}F is the one perhaps most convenient for studies on biodegradation of environmental pollutants (Malet-Martino & Martino 1989; Rietjens et al. 1993). This originates from several advantages of the ^{19}F isotope as compared to other nuclei. First, the intrinsic sensitivity of the ^{19}F nucleus is high and almost comparable to that of the ^{1}H nucleus. Sensitivity is an important issue, because xenobiotics and their metabolites are usually present

in relatively low concentrations. Second, for fluorine the sensitivity is further increased due to the absence of background signals, because biological systems do not contain ^{19}F NMR visible fluorinated endogenous compounds. This implies that all resonances observed can be unambiguously ascribed to the xenobiotic compound and its biodegradation products. Third, the ^{19}F nucleus is known to have a broad chemical shift range of about 500 ppm. This is large compared to the chemical shift range of for example ^{1}H (15 ppm) and that of ^{13}C (250 ppm). The chemical shift of a ^{19}F nucleus is highly sensitive to its molecular surroundings resulting in widespread changes in chemical shifts upon biotransformation of fluorinated organic compounds, thereby reducing the chances of peak overlap.

In the framework of a project devoted to the biodegradation of halophenols by gram-positive bacteria, the objective of the present study was to use the ^{19}F NMR technique to investigate the biodegradation pathways of fluorophenols in several *Rhodococcus* strains. Special emphasis was on the first step in the catabolic pathways, namely the conversion of fluorophenols to fluorocatechols. Generally, in *Rhodococcus* species these studies are limited by the instability and/or poor activity of the phenol hydroxylase(s) involved (Straube 1987; Janke et al. 1988). However, the ^{19}F NMR technique provides the unique opportunity to study the activity of the phenol hydroxylases *in vivo*.

Thus, the conversions of a series of ortho fluorinated di- and trifluorophenols by four different strains of *Rhodococcus* were investigated. These strains were originally isolated in the framework of a project searching for bacteria able to degrade crude oil, but showed also capable of growing on phenols. All fluorophenol derivatives studied contain a fluorinated C2 position and a non-fluorinated C6 position. This provides the possibility to detect unambiguously the regioselectivity of the initial hydroxylation step catalysed by the phenol hydroxylase.

Materials and methods

Chemicals

Phenol was purchased from Merck (Darmstadt, Germany). 2,4-Difluoro- and 2,5-difluorophenol were purchased from Aldrich (Steinheim, Germany). 2,3-Difluoro-, 2,3,4-trifluoro-, 2,3,5-trifluoro- and 2,4,5-trifluorophenol were obtained from Fluorochem (Derbyshire, UK). Fluorocatechols were prepared from the corresponding fluorophenols using purified phenol hydroxylase from *Trichosporon cutaneum* (Peelen et al. 1995). Fluoromuconates were prepared and identified as described previously (Boersma et al., 1998) by incubating the fluorocatechols with catechol 1,2-dioxygenase from *Pseudomonas arvilla* C-1.

Isolation and growth of strains

The conversions of a series of ortho-fluorinated di- and trifluorophenols by four different strains of *Rhodococcus* were investigated. These strains were originally isolated while searching for bacteria able to degrade crude oil, but showed also capable of growing on phenols.

The strain *Rhodococcus opacus* 1G was isolated by selective enrichment from the soil of oil fields in the Volga river valley near Samara. Chemostat cultivation with liquid paraffins as carbon source was used for isolation of the strain. The cells were grown at 28 °C on an orbital shaker (250 rpm) in a synthetic medium containing per liter: NH_4NO_3, 1g; K_2HPO_4, 1g; KH_2PO_4, 1g; $MgSO_4{*}7H_2O$, 0.2 g; $CaCl_2$, 0.02 g; $FeCl_3$, 2 drops of a saturated solution; pH 7.2. Phenol, used as the sole carbon and energy source, was added several times at 1.0 mM (final concentration) over a period of 24 h. The strain was maintained at 4 °C on agar slants of the same medium containing phenol (0.2 g/l). *Rhodococcus corallinus* 135 was isolated from soil contaminated with diesel oil from an abandoned military base. The strain *Rhodococcus strain* 89 was isolated from sand soil of pine forest in the Volga river valley near Nignyi Novgorod. For isolation of both strains a continuous enrichment culture technique was applied using n-alkanes as a sole source of carbon. The strains were grown at 28 °C on a synthetic medium containing per liter: NH_4NO_3, 1g; K_2HPO_4, 1g; KH_2PO_4, 1g; $MgSO_4{*}7H_2O$, 0.2 g; $CaCl_2$, 0.02 g; $FeCl_3$, 2 drops of a saturated solution; pH 7.2. Phenol, used as the substrate, was added several times up to 3.2 mM (total concentration) over a period of 24 h. The strain was maintained at 4 °C on agar slants of the same medium containing phenol (0.2 g/l).

Rhodococcus erythropolis 1CP was isolated as described previously (Gorlatov et al. 1989). The strain was grown at 28 °C on a synthetic medium containing per liter: NH_4NO_3, 1g; K_2HPO_4, 1g; KH_2PO_4, 1g; $MgSO_4{*}7H_2O$, 0.2 g; $CaCl_2$, 0.02 g; $FeCl_3$, 2 drops of a saturated solution; pH 7.2. For growth, 4-chlorophenol, proven to be the best substrate for *Rh. erythropolis* 1CP, was used as the sole carbon and en-

ergy source. The substrate was added several times up to 3.2 mM (total concentration) over a period of 24 h. The strain was maintained at 4 °C on agar slants of the same medium containing 4-chlorophenol (0.2 g/l).

Incubations with whole cells

Cells were harvested by centrifugation (6500 g) and washed three times in medium without phenol. To test biodegradation of the fluorophenol derivatives, cells were resuspended in medium without phenol containing per liter: peptone, 10 g; yeast extract, 5 g; NaCl, 8 g; pH 7.4. The optical density at 600 nm of this cell suspension was 1.8-2.1. To 80 ml of this culture the fluorophenol to be tested was added to a final concentration of 0.7 mM and the cultures were incubated at 28 °C on an orbital shaker. Although the detection limit of the ^{19}F NMR measurements is 1 μm, the substrate was added in much higher concentration in order to provide optimal possibilities for detection of intermediates. To prevent autoxidation of the fluorocatechols formed, 2 mM ascorbic acid (final concentration) was added at the start of the incubation followed by 1 mM every next hour. To monitor the rate and characteristics of the conversion of the fluorophenols, samples were taken at one hour time intervals for 6 hours. Before freezing the samples into liquid nitrogen, another 10 mM of ascorbic acid was added. Samples were stored at -20 °C until analysed. Before ^{19}F NMR analysis, samples were thawed and centrifuged (5 min 13,000 g at 0 °C).

Incubations with purified enzymes

Phenol hydroxylase from *Trichosporon cutaneum* was purified essentially as described by Sejlitz and Neujahr (1987). Catechol 1,2-dioxygenase from *Pseudomonas arvilla* C-1 was purified as described by Nakai et al. (1988). Chloromuconate cycloisomerase from *Rhodococcus erythropolis* 1CP was purified essentially as reported elsewhere (Solyanikova et al. 1995). Incubation of 2,3-difluorophenol with phenol hydroxylase and catechol 1,2-dioxygenase was performed at 30 °C in closed reaction vessels to prevent evaporation of the phenolic substrate. Incubations contained (final concentrations) 0.1 M potassium phosphate pH 7.6, 0.7 mM of 2,3-difluorophenol, added as 1% (v/v) of a 70 mM stock solution in dimethylsulphoxide, 10 μM FAD, 1 mM ascorbic acid and 1 mM NADPH. The incubations were started by the addition of catalytic amounts of the purified enzymes (5.3×10^{-3} U/ml with phenol as final concentration of phenol hydroxylase and 7.2×10^{-3} U/ml with catechol as final concentration of catechol 1,2-dioxygenase). The sample thus obtained was analysed by ^{19}F NMR, showing significant formation of 2,3-difluoromuconate, and then used for another incubation with purified chloromuconate cycloisomerase. Upon addition of a catalytic amount of chloromuconate cycloisomerase (9.6×10^{-3} U/ml with 2-chloromuconate as final concentration) the conversion of 2,3-difluoromuconate was followed in time by recording ^{19}F NMR spectra while incubating the sample in the NMR spectrometer at 30 °C.

^{19}F NMR measurements

^{19}F NMR measurements were performed on a Bruker AMX 300 and a Bruker DPX 400 NMR spectrometer as described previously (Vervoort et al. 1990: Boersma et al. 1998). The temperature was 7 °C unless indicated otherwise. A dedicated 10 mM ^{19}F NMR probehead was used at both instruments. The spectral width for the ^{19}F NMR measurements was between 20,000 and 50,000 Hz depending on the sample of interest. The number of datapoints used for data acquisition was 65536. Pulse angles of 30° were used. Between 2,000 and 60,000 scans were recorded, depending on the concentrations of the fluorine-containing compounds and the signal to noise ratio required. The detection limit of an overnight run (60,000 scans) is 1 μM. The sample volume was 1.71 ml, containing 1.4 ml incubation mixture, 200 μl 0.8 M potassium phosphate pH 7.6, 100 μl of ^2H_2O, used as deuterium lock and 10 μl of 8.4 mM 4-fluorobenzoate added as an internal standard. Concentrations of the various metabolites were calculated by comparison of the integrals of the ^{19}F NMR resonances of the metabolites to the integral of the 4-fluorobenzoate resonance. Chemical shifts are reported relative to $CFCl_3$. ^{19}F NMR chemical shift values of the various fluorine containing compounds were identified using authentic reference compounds for fluoride anions and all fluorophenols. The resonances of the different fluorocatechol and fluoromuconate metabolites have been identified and reported previously (Peelen et al. 1995; Boersma et al. 1998). ^{1}H decoupling was achieved with a Waltz16 decoupling sequence.

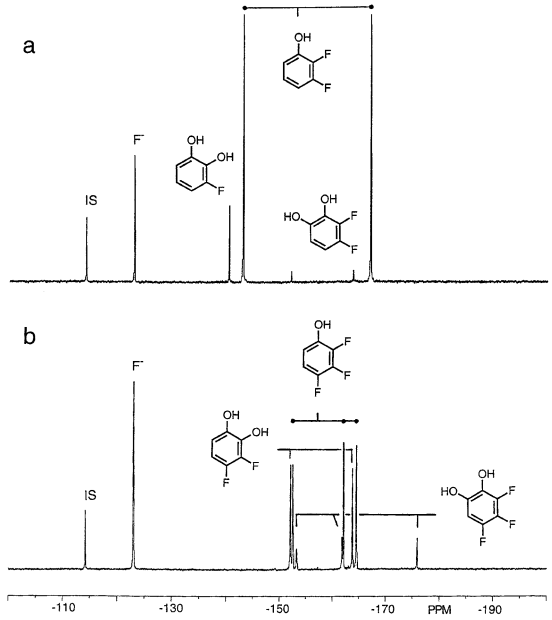

Figure 1. Representative ^{19}F NMR spectra showing metabolite profiles of a) 2,3-difluorophenol and b) 2,3,4-trifluorophenol, by *Rh. opacus* 1G. Spectra presented were those obtained at t = 2 h. Resonances were identified as previously described (Peelen et al. 1995; Boersma et al. 1998). The resonance marked IS is from the internal standard 4-fluorobenzoate. The resonances marked with an asteriks indicate unidentified metabolite signals.

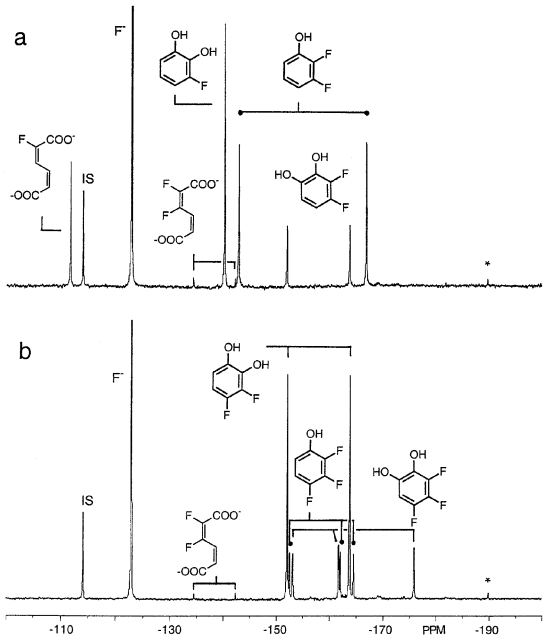

Figure 2. Representative ^{19}F NMR spectra showing metabolite profiles of a) 2,3-difluorophenol and b) 2,3,4-trifluorophenol, by *Rh. corallinus* 135. Spectra presented were those obtained at t = 2 h. Resonances were identified as described in the legend of Figure 1.

Results

Fluorophenol biodegradation and fluoride anion formation as determined by ^{19}F NMR

Figures 1–4 present some representative ^{19}F NMR spectra showing the metabolite patterns of the ortho-fluorinated di- and trifluorophenols by different *Rhodococcus* strains. First of all, these spectra illustrate that the ^{19}F NMR analysis provides a way to characterise and quantify metabolite profiles. The positions of the peaks indicate which metabolites are present, the peak areas reflect the amount of the various metabolites, and the number of fluorine signals belonging to one metabolite indicate the number of different type of fluorine substituents present in the molecule. From these and additional spectra the metabolite profiles of the fluorophenols in time by the different *Rhodococcus* species could be quantified. Table 1 presents the percentage of fluorinated substrate left, the percentage of fluorine recovered as fluoride anions, and the percentage of fluorinated metabolic intermediates observed, all at t = 2 h of incubation of the different *Rhodococcus* species with the various fluorophenols.

All *Rhodococcus* species appeared able to convert the fluorophenols, although at a different rate. Based on the results presented (Table 1), the overall extent of fluorophenol conversion decreased for the four species in the order *Rh. corallinus* 135 > *Rh. opacus* 1G > *Rh. erythropolis* 1CP > *Rh. strain 89*.

Furthermore, there is no consistent order in the preference of the *Rhococcus* species for the different fluorophenols. For *Rh. opacus* 1G, the order in the rate of conversion of the fluorophenols is 2,5-difluoro- > 2,3,4-trifluoro- > 2,4-difluoro- > 2,3-difluoro- > 2,3,5-trifluoro- = 2,4,5-trifluorophenol (Table 1). *Rh. corallinus* 135 converts all fluorophenols extremely efficient, resulting in full elimination of 5 out of 6 fluorophenols upon 2 to 6 h of incubation (^{19}F NMR spectra not shown). From the ^{19}F NMR spectra obtained at 2 h (Table 1) the following order for the rate of degradation of the various fluorophenols by *Rh. corallinus* 135 can be derived: 2,5-difluoro- > 2,3,4-trifluoro- > 2,4,5-trifluoro- > 2,3-difluoro- > 2,4-difluoro- > 2,3,5-trifluorophenol. In addition, the data presented in Table 1 indicate that for *Rh. strain 89* the fluorophenols are degraded with rates decreas-

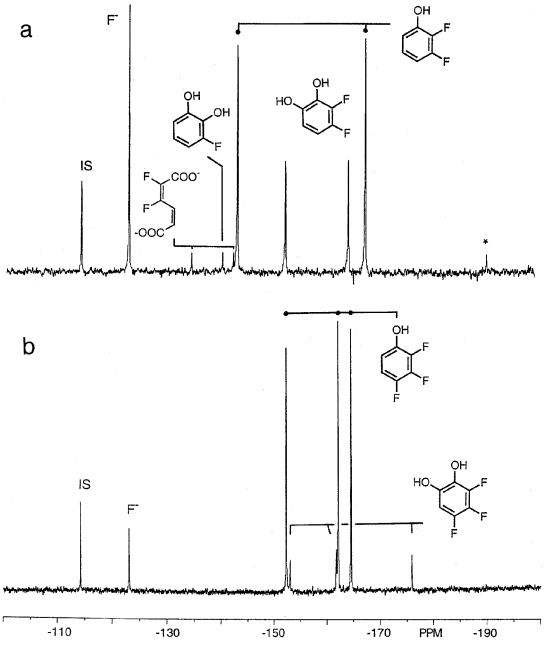

Figure 3. Representative ^{19}F NMR spectra showing metabolite profiles of a) 2,3-difluorophenol and b) 2,3,4-trifluorophenol, by *Rh. strain 89*. Spectra presented were those obtained at t = 6 h. Resonances were identified as described in the legend of Figure 1.

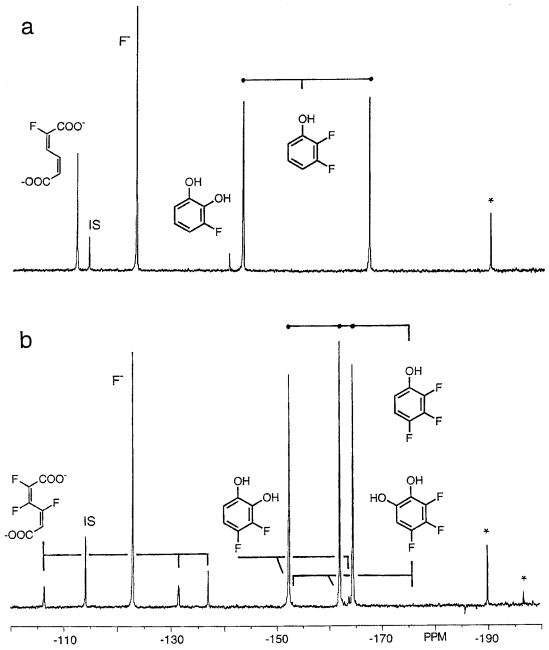

Figure 4. Representative ^{19}F NMR spectra showing metabolite profiles of a) 2,3-difluorophenol and b) 2,3,4-trifluorophenol, by *Rh. erythropolis* 1CP. Spectra presented were those obtained at t = 4h. Resonances were identified as described in the legend of Figure 1.

ing in the order 2,3-difluoro- = 2,4-difluoro- > 2,5-difluoro- > 2,3,4-trifluoro- > 2,3,5-trifluoro- = 2,4,5-trifluorophenol. For *Rh. erythropolis* 1CP the rates decrease in the order 2,5-difluoro- > 2,3,5-trifluoro- = 2,4-difluoro > 2,3-difluoro- > 2,4,5-trifluoro- > 2,3,4-trifluorophenol. Thus, generally there is a tendency to convert the difluorophenols with rates higher or about equal to the rates for conversion of the trifluorophenols. This is in line with the expected effect of fluorine substituents, decreasing the nucleophilic reactivity of the π-electrons in the fluorophenol, a characteristic that is of importance for their rate of conversion by phenol hydroxylase from *Trichosporon cutaneum* (Peelen et al. 1995).

Fluorophenol biodegradation patterns as determined by ^{19}F NMR

Table 1 and Figures 1–4 also illustrate that the ^{19}F NMR spectra provide insight in the fluorinated biodegradation pathway intermediates formed upon the conversion of the different fluorophenols. During the first hours of the incubations and as long as a residual amount of the parent fluorophenol is present, the relative amount of fluorinated intermediates is constant. Only when all fluorophenol is converted, the amount of accumulated intermediates starts to decrease, accompanied by, as expected, a further increase in fluoride anions.

Thus, the difference between the amount of fluorophenol degraded and the amount of fluoride anions formed (Table 1) results from the accumulation of fluorinated intermediates in the biodegradation pathways. From the ^{19}F NMR spectra presented in Figures 1–4 it can be derived that the fluorinated biodegradation metabolites which accumulate are mainly fluorocatechols and fluoromuconates. These metabolites result from ortho-hydroxylation of the fluorophenols by a phenol hydroxylase, followed by intradiol ring cleavage of the fluorocatechols to fluoromuconates by a catechol 1,2-dioxygenase. Other fluorinated intermediate metabolites generally do not accumulate to a significant extent, except from the conversion of fluorophenols by *Rh. erythropolis* 1CP (Figure 4). Table 2 presents the relative contribution of the different fluorinated catechols, muconates and unidentified metabolites, to the total amount of fluorine containing intermediates observed. From these data the ratio between the two possible fluorocatechol metabolites, resulting from hydroxylation of the fluorophenols at respectively their fluorinated C2 and their non-fluorinated C6

Table 1. Relative fluorophenol loss, fluoride anion release and accumulation of fluorinated intermediates in incubations of *Rhodococcus* species with different ortho-fluorinated di- and trifluorophenols. The incubation time was t = 2 h. The standard deviation was less than 10%. In abiotic controls no reaction took place

fluorinated substrate	*Rhodococcus opacus* 1G %	*Rhodococcus corallinus* 135 %	*Rhodococcus strain 89* %	*Rhodococcus erythropolis* 1CP %
2,3-difluorophenol				
Phenol left	55	26	74	74
F^- release	26	38	9	16
F in intermediates	19	36	17	10
2,4-difluorophenol				
Phenol left	49	46	75	66
F^- release	42	53	11	32
F in intermediates	9	1	14	2
2,5-difluorophenol				
Phenol left	30	0	82	61
F^- release	57	90	3	35
F in intermediates	13	10	15	4
2,3,4-trifluorophenol				
Phenol left	43	12	90	80
F^- release	31	35	3	13
F in intermediates	26	53	7	7
2,3,5-trifluorophenol				
Phenol left	63	59	94	66
F^- release	23	38	4	34
F in intermediates	14	3	2	0
2,4,5-trifluorophenol				
Phenol left	65	19	94	79
F^- release	28	79	5	21
F in intermediates	7	2	1	0

position, can be derived, as well as the ratio between the two corresponding muconate metabolites. The hydroxylation at the fluorinated C2 position of the substrates represents a so called oxidative or oxygenolytic dehalogenation in which the insertion of an oxygen atom derived from molecular oxygen is accompanied by defluorination.

The results obtained and quantified in Table 2 indicate marked differences between the biodegradation pathways of the fluorophenols by the different *Rhodococcus* species. This holds not only for the extent of accumulation of fluorinated biodegradation pathway intermediates, but also for the nature of these intermediates.

For *Rh. opacus* 1G, incubations with fluorophenols result in accumulation of especially the corresponding fluorocatechols. Of all fluorinated intermediate metabolites observed, the major part appear to be fluorocatechols (Figure 1, Table 2). This points at a rate limiting role for the catechol 1,2-dioxygenase of *Rh. opacus* 1G in the biodegradation of the fluorophenols. The ratio of C2: C6 hydroxylation of the various fluorophenols suggests that the phenol hydroxylase of *Rh. opacus* 1G favours formation of the defluorinated catechol metabolite, i.e. prefers to catalyse oxidative dehalogenation at the fluorinated C2 of the fluorophenol instead of hydroxylation at the non-fluorinated C6 position.

For *Rh. corallinus* 135 there is no consistency in the nature and level of the intermediates that tend to accumulate. For 2,3-difluorophenol, catechols as well as muconates accumulate, for 2,3,4-trifluoro- and 2,3,5-trifluorophenol mainly fluorocatechols accumulate, for 2,5-difluorophenol accumulation of especially

Table 2. Relative composition of the metabolite profiles of the various *Rhodococcus* species incubated with ortho-fluorinated di- and trifluorophenols as detected by ^{19}F NMR. The total amount of fluorinated products other than the parent phenol and fluoride anions was taken as 100%. Values presented are the mean of the data obtained at t = 1, 2, 4 and 6 h (n = 4), except for the conversion of 2,3-difluoro-, 2,5-difluoro- and 2,3,4-trifluorophenol by *Rh. corallinus* 135, and of 2,3-difluoro-, 2,4-difluoro-, 2,5-difluoro- and 2,3,4-trifluorophenol by *R. opacus* 1G, for which the mean of t = 1 and 2 h is presented (n = 2) because the amount of phenol was fully degraded at 4 and 6 h. For incubations where the amount of intermediates was < 2% of the total amount of fluorinated metabolites (Table 1), the metabolite profile is not specified

Fluorinated substrate metabolite	*Rhodococcus opacus* 1G %	*Rhodococcus corallinus* 135 %	*Rhodococcus strain* 89 %	*Rhodococcus erythropolis* 1CP %
2,3-difluorophenol				
3-fluorocatechol	81 ± 2	50 ± 3	5 ± 3	11 ± 3
3,4-difluorocatechol	16 ± 1	23 ± 5	87 ± 6	0
2-fluoromuconate	1 ± 1	24 ± 2	0	58 ± 4
2,3-difluoromuconate	0	1 ± 1	5 ± 3	0
unknown	2 ± 1	2 ± 1	3 ± 2	31 ± 2*
2,4-difluorophenol				
4-fluorocatechol	47 ± 30	–	5 ± 3	–
3,5-difluorocatechol	16 ± 8	–	94 ± 3	–
3-fluoromuconate	0	–	0	–
2,4-difluoromuconate	0	–	1 ± 1	–
Unknown	37 ± 37*	–	0	–
2,5-difluorophenol				
4-fluorocatechol	64 ± 9	9 ± 9	0	1 ± 1
3,6-difluorocatechol	0	12 ± 12	47 ± 5	2 ± 1
3-fluoromuconate	0	0	0	0
2,5-difluoromuconate	0	74 ± 17	53 ± 5	17 ± 6
Unknown	36 ± 9*	5 ± 5	0	80 ± 7*
2,3,4-trifluorophenol				
3,4-difluorocatechol	81 ± 1	79 ± 1	0	7 ± 4
3,4,5-trifluorocatechol	19 ± 1	21 ± 2	100 ± 0	3 ± 2
2,3-difluoromuconate	0	1 ± 1	0	0
2,3,4-trifluoromuconate	0	0	0	39 ± 8
Unknown	0	0	0	51 ± 2*
2,3,5-trifluorophenol				
3,5-difluorocatechol	98 ± 1	78 ± 5	–	–
3,4,6-trifluorocatechol	0	2 ± 2	–	–
2,4-difluoromuconate	0	1 ± 1	–	–
2,3,5-trifluoromuconate	0	5 ± 5	–	–
Unknown	2 ± 1	14 ± 9	–	–
2,4,5-trifluorophenol				
4,5-difluorocatechol	95 ± 2	–	–	–
3,4,6-trifluorocatechol	0	–	–	–
3,4-difluoromuconate	0	–	–	–
2,3,5-trifluoromuconate	0	–	–	–
Unknown	5 ± 2	–	–	–

∗ For 2,3-difluorophenol and 2,3,4-trifluorophenol these were a major metabolite at −189.9 ppm and a minor one at −196.6 ppm (see Figure 4), for 2,4-difluorophenol and 2,5-difluorophenol these were a major metabolite at −90.3 ppm, and minor metabolites with resonances at −100.3 and −113.2 ppm (for 2,4-difluorophenol) and −126.1 and −113.2 ppm (for 2,5-difluorophenol)(^{19}F NMR spectra not shown).

the fluoromuconates is observed, whereas for 2,4-difluoro- and 2,4,5-trifluorophenol no significant accumulation of fluorinated intermediates is observed (Figure 2, Table 1 and 2). This variation in the nature and level of the accumulated metabolites does not result from different rates of conversion of the parent fluorophenol. This follows for example from the observation that 2,3,4-trifluorophenol is converted relatively rapidly, leaving no parent fluorophenol upon 4 h of incubation (^{19}F NMR data not shown), whereas even at t = 6 h the fluorinated catechols still make up 73% of all intermediates observed (^{19}F NMR data not shown). Furthermore, the nature of the accumulated metabolites is not influenced primarily by the type of fluorocatechol formed, since from 2,4-difluoro- and 2,3,5-trifluorophenol both 3,5-difluorocatechol is formed, whereas only for 2,3,5-trifluorophenol preferential accumulation of this 3,5-difluorocatechol is observed. Most likely the phenomenon is dependent on the as yet not characterised effect of the fluorine substituent pattern on both the activity of phenol hydroxylase as well as on the activity of the catechol 1,2-dioxygenase from *Rh. corallinus* 135. For *Rh. corallinus* 135 also the preference of its phenol hydroxylase for C2 as compared to C6 hydroxylation varies with the fluorine substituent pattern of the fluorophenol, although in most cases, as for the phenol hydroxylase from *Rh. opacus* 1G, oxidative dehalogenation at C2 is favoured over hydroxylation at C6 (Table 2). There is some tendency that when oxidative defluorination in the first step is favoured over hydroxylation at C6, such as in the case of 2,3,4-trifluoro-, 2,3,5-trifluorophenol and, to a lesser extent, 2,3-difluorophenol, accumulation of the catechols is observed. When hydroxylation at C6 of the fluorophenol is preferred, such as for 2,5-difluorophenol, accumulation of the muconates and not of the catechols is observed. Together these data suggest that the relative activities of phenol hydroxylase and catechol 1,2-dioxygenase of *Rh. corallinus* 135 are affected by the nature of the fluorine substituent pattern in their substrates. The data also suggest that the *in situ* activity of phenol hydroxylase and catechol 1,2-dioxygenase of *Rh. corallinus* 135 are in the same order of magnitude, resulting in the possibility for an influence of the fluorine substituent pattern on the nature of the metabolites that accumulate. From the fact that fluorocatechols and fluoromuconates are the only intermediates that accumulate, it is concluded that the rate limiting step in the biodegradation pathway of the fluorophenols by *Rh. corallinus* 135 is related to the enzyme catalysing the conversion of the fluoromuconates.

For *Rh. strain 89*, in all cases where significant metabolite accumulation was observed, except for 2,5-difluorophenol, preferential accumulation of the fluorocatechols is observed, pointing at a rate limiting role in the biodegradation pathway for the catechol 1,2-dioxygenase. The regioselectivity of the phenol hydroxylase from *Rh. strain 89* is such that it catalyses preferential hydroxylation at C6 instead of catalysing oxidative defluorination at C2 (Figure 3, Table 2).

The metabolite profiles observed for *Rh. erythropolis* 1CP, differ from those of the other *Rhodococcus* species, especially because accumulation of as yet not identified fluorinated intermediates is observed to a relatively high extent (Figure 4, Table 2). Incubations with 2,3-difluorophenol and 2,3,4-trifluorophenol give rise to a major unidentified metabolite with a resonance peak at −189.9 ppm (Figure 4). In incubations with 2,4-difluorophenol and 2,5-difluorophenol accumulation of a major unidentified metabolite with its resonance at −90.3 ppm is observed (Table 2, ^{19}F NMR spectra not shown). Furthermore, in the incubations with *Rh. erythropolis* 1CP, fluoromuconates accumulate to a higher extent than the corresponding fluorocatechols. All together the metabolite profiles obtained for *Rh. erythropolis* 1CP indicate that the rate limiting step in the fluorophenol degradation occurs after the steps catalysed by phenol hydroxylase, and catechol 1,2-dioxygenase. The regioselectivity of the reaction catalysed by phenol hydroxylase from *Rh. erythropolis* 1CP is dependent on the substituent pattern in the fluorophenol. In analogy with the phenol hydroxylase from *Rh. corallinus* 135 and that of *Rh. opacus* 1G, the enzyme from *Rh. erythropolis* 1CP is able to catalyse oxidative defluorination, for example of 2,3-difluorophenol (Table 2).

Use of ^{19}F NMR in elucidation of the nature of unidentified metabolites

The ^{19}F NMR spectra of especially *Rh. erythropolis* 1CP incubated with either 2,3-difluorophenol or 2,3,4-trifluorophenol showed accumulation of an as yet unidentified major intermediate metabolite with its resonance at −189.9 ppm (Figure 4). Close inspection of the ^{19}F NMR spectra of incubations of the other *Rhodococcus* species of the present study reveals the presence of a very small amount of this metabolite also in incubations of *Rh. corallinus* 135 (Figure 2) and *Rh. strain 89* (Figure 3) with 2,3-

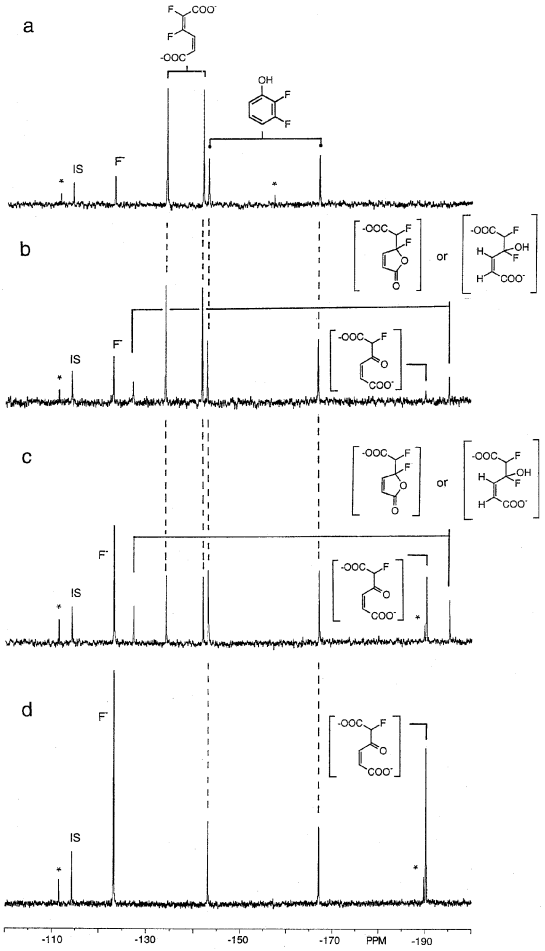

Figure 5. Time-dependent ^{19}F NMR spectra of the incubation of 2,3-difluoromuconate with chloromuconate cycloisomerase from *Rh. erythropolis* 1CP. Spectra were obtained at a) t = 0, b) t = 30 min, c) t = 1.5h and d) t = 6 h. Metabolites were assigned on the basis of arguments presented in the text. The resonance marked IS is from the internal standard 4-fluorobenzoate. The resonances marked with an asteriks indicate unidentified signals mostly already present at t = 0.

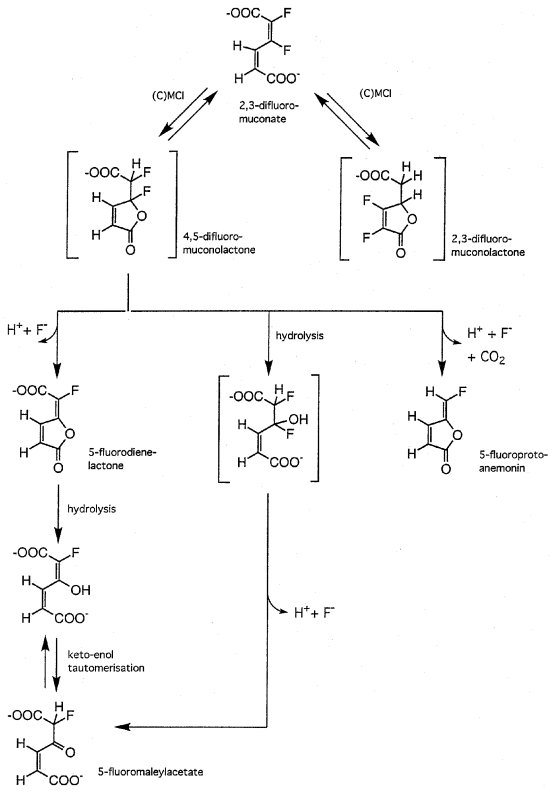

Figure 6. Schematic presentation of the possible conversion of 2,3-difluoromuconate by chloromuconate cycloisomerase, based on the conversion of 2-chloromuconate and 3-chloromuconate by muconate cycloisomerase and chloromuconate cycloisomerase.

difluoro- and 2,3,4-trifluorophenol. In order to further illustrate the potential of the ^{19}F NMR technique in studies on biodegradation, additional ^{19}F NMR experiments were performed to obtain information on the nature of this major metabolite.

Figure 5a shows the ^{19}F NMR spectrum of 2,3-difluoromuconate prepared by incubation of 2,3-difluorophenol with purified phenol hydroxylase and catechol 1,2-dioxygenase. Upon addition to this sample of chloromuconate cycloisomerase, purified from *Rh. erythropolis* 1CP, the ^{19}F NMR spectra presented in Figure 5b–d were obtained in time. Figure 5b–d show that the metabolite with its resonance at −189.9 ppm, also observed in the incubations with whole cells (Figure 4), is readily formed upon the addition of chloromuconate cycloisomerase to 2,3-difluoromuconate. The fact that the metabolite shows only one peak in the ^{19}F NMR spectrum indicates that this compound contains only one (type of) fluorine substituent.

Furthermore, the spectra in Figure 5b and 5c show the formation of another metabolite upon the incubation of 2,3-difluoromuconate with chloromuconate cycloisomerase. This metabolite contains two fluorine substituents as reflected by two peaks of identical intensity at −127.2 and −195.3 ppm. This metabolite seems to be formed initially to a larger extent than the metabolite at −189.9 ppm, but disappears in time, giving rise to an increase in the metabolite with its resonance at −189.9 ppm and significant fluoride anion formation. Based on literature data on the conversion of 2-chloro-, 3-chloro- and 3-fluoromuconate by (chloro)muconate cycloisomerases (Harper & Blakley 1971; Schmidt & Knackmuss 1980; Schreiber

et al. 1980; Schlömann et al. 1990b; Vollmer et al. 1994; Vollmer and Schlömann 1995; Prucha et al. 1996; Blasco et al. 1995; Kaschabek & Reineke 1995), possible pathways for the biodehalogenation of a 2,3-dihalomuconate by chloromuconate cycloisomerase can be put forward. Figure 6 schematically presents these possible pathways. Cyclisation of 2,3-difluoromuconate to 2,3-difluoromuconolactone can not lead to dehalogenation. The other possible product resulting from cyclisation of 2,3-difluoromuconate would be 4,5-difluoromuconolactone. Defluorination of 4,5-difluoromuconolactone can result in formation of 5-fluorodienelactone (Figure 6). This reaction would be analogous to the dechlorination of 3-chloromuconate proceeding by its initial conversion to 4-chloromuconolactone catalysed by chloromuconate cycloisomerase, followed by chloride elimination resulting in the dienelactone. The 5-fluorodienelactone formed may be sensitive to chemical hydrolysis leading to formation of 5-fluoromaleylacetate in either its enol or keto form (Kaschabek & Reineke 1995) (Figure 6). Alternatively, hydrolysis of the 4,5-difluoromuconolactone may occur prior to fluoride elimination in a pathway also leading to formation of 5-fluoromaleylacetate (Figure 6). This pathway would be analogous to the one reported for defluorination of 4-fluoromuconolactone (Harper and Blakley 1971; Schlömann et al. 1990b). Elimination of the C5-fluorine substituent from 4,5-difluoromuconolactone, analogous to the dechlorination of 2-chloromuconate through 5-chloromuconolactone, is hampered by the fluorine substituent at C4. Finally, elimination of a fluoride anion with concomitant decarboxylation of 4,5-difluoromuconolactone, to give 5-fluoroprotoanemonin, can be proposed (Figure 6), analogous to the conversion of 3-chloromuconate through 4-chloromuconolactone to protoanemonin (Blasco et al. 1995). This route, however, seems less likely, since the second step in this reaction pathway has been reported to be catalysed by muconate cycloisomerase, not chloromuconate cycloisomerase (Blasco et al. 1995).

Next, a proton coupled ^{19}F NMR spectrum of the metabolite with its ^{19}F NMR resonance at -189.9 ppm was measured to further elucidate its possible nature. The ^{1}H coupled ^{19}F NMR pattern of the resonance showed one J_{FH} coupling of 50.4 Hz. This value can be compared to coupling constants reported in the literature, where $^{4}J_{FH}$ values, for coupling over one double and three single bonds, vary between 1–8 Hz and $^{2}J_{FH}$ values are between 45 and 65 Hz when the hydrogen and fluorine atoms are bound to an sp3 type carbon, but between 70–90 Hz when bound to an sp2 type carbon (Wray 1983). This implies that the J_{FH} coupling constant of 50.4 Hz, combined with the various options for the monofluorinated reaction product formed from 2,3-difluoromuconate by chloromuconate cycloisomerase (Figure 6), strongly argue in favour of identification of the metabolite with the resonance at −189.9 ppm as the keto form of 5-fluoromaleylacetate.

The metabolite in Figure 5b–d with ^{19}F NMR resonances at −127.2 and −195.3 ppm both showing a J_{FF} of 16.6 Hz, could then be ascribed to either one of the two possible difluorinated intermediates depicted in Figure 6. This difluorinated intermediate is not observed in the incubations with whole cells (Figures 1–4) and was therefore not identified further at this stage.

Discussion

In the present study, the biodegradation of ortho-fluorinated di- and trifluorophenols by different strains of *Rhodococcus* was analysed by ^{19}F NMR. The results obtained illustrate that ^{19}F NMR is an efficient technique to gain insight in the biodegradation of fluorinated environmental pollutants. Marked differences between the various *Rhodococcus* strains were observed especially with respect to: 1) their ability and rates for biodegradation of the fluorophenols, 2) the nature of the rate limiting step in the biodegradation pathways, and, as a result, 3) the nature of the fluorinated metabolites that accumulate, 4) the effect of the fluorine substituent pattern on the biodegradation pathway and the enzymes involved and 5) the regioselectivity of the first step in the degradation pathway catalysed by phenol hydroxylases which, in several cases, appear to favour the catalysis of oxidative defluorination over hydroxylation at a nonfluorinated ortho position.

Phenol hydroxylase from *Rh. opacus* 1G favours oxidative defluorination of all fluorophenols tested, whereas the regioselectivity of the phenol hydroxylase from *Rh. strain 89* is such that it catalyses preferential hydroxylation at C6 instead of catalysing oxidative defluorination at C2. The activity of the catechol 1,2-dioxygenase, catalysing the subsequent reaction step, appears to be an important rate limiting factor in the biodegradation of the fluorophenols by *Rh. opacus* 1G and *Rh. strain* 89, possibly due to the sensitivity

of the enzyme to suicide inhibition by fluorocatechol metabolites. This impairment of ring cleavage by the presence of halogen substituents *ortho* with respect to the hydroxyl moieties of a ring cleavage metabolite is similar to the inhibition of protocatechuate dioxygenase by *ortho*-halogenated protocatechuates (Walsh and Ballou 1983) and of catechol 2,3-dioxygenase by *ortho*-halogenated catechols (Bartels et al. 1984). The phenomenon was reported to originate from formation of an abortive complex between the *ortho*-halogenated catechol and the dioxygenase (Walsh et al. 1983). *Rh. corallinus* 135 appeared very efficient in fluorophenol biodegradation, being able to catalyse oxidative defluorination, and containing a catechol 1,2-dioxygenase with an unusual broad substrate specificity. Especially in incubations of *Rh. erythropolis* 1CP, accumulation of fluorinated metabolites different from the fluorophenols, fluorocatechols or fluoromuconates, was observed. From additional experiments with purified chloromuconate cycloisomerase from *Rh. erythropolis* 1CP, evidence was provided suggesting that one of these metabolites is the keto form of 5-fluoromaleylacetate (Figure 6). The experimental data presented for the conversion of the 2,3-difluoromuconate by chloromuconate cycloisomerase (Figure 5) are in line with literature data on the conversion of 2-chloro-, 3-chloro- and 3-fluoromuconate by chloromuconate cycloisomerases (Harper & Blakley 1971; Schmidt and Knackmuss 1980; Schreiber et al. 1980; Schlömann et al. 1990b; Solyanikova et al. 1995; Vollmer et al. 1994; Vollmer and Schlömann 1995; Prucha et al. 1996; Blasco et al. 1995; Kaschabek & Reineke 1995). The data presented here provide a first indication on the nature of the unidentified accumulated metabolites, and are included to illustrate the use of ^{19}F NMR in studies on biodegradation. However, the exact nature of all possible fluoromuconolactone, fluorodienelactone and fluoromaleylacetate metabolites remains to be elucidated.

Previous studies have reported on the biodegradation of fluorobenzoic acids and fluorocatechols by many different microorganisms (Goldman et al. 1967; Clarke et al. 1975; Schreiber et al. 1980; Ivoilov et al. 1987; Engesser et al. 1990; Schlömann et al. 1990a; 1990b), but in only a limited number of studies, ^{19}F NMR was applied to identify the metabolites and/or quantify the metabolite profiles (Van Berkel et al. 1994; Boersma et al. 1998). The metabolic profiles reported for the degradation of mono-fluorocatechols, formed from monofluorobenzoates in an initial oxidative decarboxylation step, are similar to the ones reported here for fluorocatechols, i.e. ring cleavage by an intradiol dioxygenase followed by defluorination of the fluoromuconates thus formed in subsequent reaction steps (Goldman et al. 1967; Harper & Blakley 1971; Clarke et al. 1975; Schreiber et al. 1980; Ivoilov et al. 1987; Engesser et al. 1990; Schlömann et al. 1990a).

Altogether, the present paper illustrates the possibilities and advantages of the use of ^{19}F NMR in studies on the biodegradation of fluorinated compounds. Clearly, the main advantages, as compared to other techniques, such as for example HPLC or GC-MS, are that the ^{19}F NMR data can be easily quantified, that no disturbing background signals are present, that no metabolite extraction or separation procedures are required and that the amount of fluoride anion formation, i.e. the extent of dehalogenation, can be quantified. The method is restricted to compounds containing fluorine substituents. However, with the expected future increase in the number of fluorinated chemicals in both industry and agriculture (Schreiber et al. 1980; Oltmanns et al. 1989; Engesser et al. 1990), the ^{19}F NMR technique may develop as an important tool in this field of environmental microbiology.

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