Conversion of methionine to phytotoxic 3-methylthiopropionic acid by *Xanthomonas campestris* pv. *manihotis*

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Incubation of the plant pathogenic bacterium *Xanthomonas campestris* pv. *manihotis* with either L-[3,4-¹⁴C]methionine or [3,4-¹⁴C]KMBA (2-keto-4-methylthiobutyric acid) led to the production of [¹⁴C]MTPA (3-methylthiopropionic acid) and [¹⁴C]MTAA (3-methylthioacrylic acid). When an excess of non-radioactive KMBA was present in the medium, formation of [¹⁴C]KMBA from L-[3,4-¹⁴C]methionine was observed. Conversely, [¹⁴C]methionine accumulated in a trapping pool of non-radioactive methionine as a result of biotransformation of [3,4-¹⁴C]KMBA. Aminooxyacetic acid, a transaminase inhibitor, suppressed totally the formation of [¹⁴C]MTPA from L-[3,4-¹⁴C]methionine but not from [3,4-¹⁴C]KMBA. Metabolism of L-[1-¹⁴C]methionine liberated ¹⁴CO₂ but did not produce [¹⁴C]MTPA. These results demonstrate that methionine is catabolized by *X. campestris* pv. *manihotis* into MTPA via transamination and subsequent decarboxylation of the intermediate α -keto acid KMBA.

Introduction

Cassava (*Manihot esculenta* Crantz) bacterial blight, caused by *Xanthomonas campestris* pv. *manihotis* (Berthet & Bondar, 1915; Young *et al.*, 1978) is characterized by water-soaked angular leaf spots which often develop rapidly into extensive blight areas (Maraite & Meyer, 1975). From such lesions, the blight-inducing toxin 3-methylthiopropionic acid (MTPA) was isolated by Perreux *et al.* (1986).

In broth media supplemented with methionine, MTPA is produced by *X. campestris* pv. *manihotis* (Perreux *et al.*, 1982) and several other pathovars of *X. campestris* (Noda *et al.*, 1980; Robeson & Cook, 1985). Although it is known that methionine undergoes biotransformation to MTPA (Arima *et al.*, 1970; Robeson & Cook, 1985), the precise reaction sequence is still unknown for the species of prokaryotes studied. In the case of mammalian liver cells, the catabolism of methionine has been described by Case & Benevenga (1976). In this system, the amino acid was first deaminated to produce 2-keto-4-methylthiobutyric acid (KMBA) which was subsequently decarboxylated into MTPA (Steele & Benevenga, 1978; Dixon & Benevenga,

1980). In this paper, our purpose is to establish the occurrence of such a catabolic sequence in a plant pathogenic bacterium such as *X. campestris*.

Methods

Chemicals. Glutamic acid, methionine, cysteine, all chemical salts used in the different broth culture and all solvents (analytical or HPLC grade) were purchased from Merck. Yeast extract was obtained from Difco; 2-keto-4-methylthiobutyric acid (KMBA), aminooxyacetic acid (AOAA), reduced glutathione, amino acid oxidase type I from *Crotalus adamanteus* and catalase were obtained from Sigma. 3-Methylthiopropionic acid (MTPA) was synthesized according to Holmberg (1945) and 3-methylthioacrylic acid (MTAA) was provided by Dr T. Noda (Laboratory of Plant Pathology, Joetsu, Niigata 943-01, Japan). Na-S HiPhi Diluent (0.2 M-sodium citrate buffer, pH 2.2 $\pm$ 0.01), used for sample preparation before amino acid analysis, was purchased from Dionex. Pic-Reagent A (tetrabutylammonium phosphate) for ion-pairing in HPLC and Sep-Pak C18 for sample preparation were from Waters. The radiolabelled compounds L-[3,4-¹⁴C]methionine [specific activity 56 mCi mmol⁻¹ (2.07 TBq mmol⁻¹)] and L-[1-¹⁴C]methionine [specific activity 52 mCi mmol⁻¹ (1.92 TBq mmol⁻¹)] were provided by CEA. Labelled [3,4-¹⁴C]KMBA was prepared and purified, using the procedure of Mitchell & Benevenga (1978), by incubating [3,4-¹⁴C]methionine with 1 unit of L-amino acid oxidase and 800 units of catalase. Purity was checked by HPLC and radioactivity measurements were made on collected fractions, as described below. Lumagel scintillation fluid (Lumac) was used for liquid scintillation counting.

Bacterial cultures. All experiments were done with strain HMB 23 (BM 12, ICPB XM 147) of *X. campestris* pv. *manihotis*. The strain was stored at room temperature on GYCA medium (Dye, 1962). For

Abbreviations: AOAA, aminooxyacetic acid; MTAA, 3-methylthioacrylic acid; MTPA, 3-methylthiopropionic acid; KMBA, 2-keto-4-methylthiobutyric acid.

inoculum production, bacteria were grown in GYS broth (per litre, 5.0 g glucose, 0.5 g $\text{NH}_4\text{H}_2\text{PO}_4$, 1.5 g K_2HPO_4 , 0.2 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 5.0 g NaCl, 5.0 g yeast extract). Culture samples (0.25 ml) harvested after 48 h at the end of the exponential growth phase were used to inoculate 50 ml of broth medium in 250 ml Erlenmeyer flasks. The basic culture medium used was a modified Watanabe broth (Watanabe *et al.*, 1967) [per litre, 1.0 g (6.80 mM) L-glutamic acid, 0.5 g (3.35 mM) L-methionine, 5.0 g sucrose, 3.0 g $(\text{NH}_4)_2\text{HPO}_4$, 2.0 g KH_2PO_4 , 0.2 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.01 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$]. For some cultures, the methionine in the basic medium was replaced by 0.40 g (3.30 mM) L-cysteine l^{-1} or 0.47 g (3.30 mM) sodium sulphate l^{-1} . Cultures were incubated for 84 h at 28 °C on a gyratory shaker operating at 150 r.p.m. (radius of motion 3 cm). Growth was estimated by optical density measurements at 600 nm. At regular intervals during growth, 2 ml samples were taken aseptically, bacteria were pelleted by centrifugation (10000 g for 15 min) and supernatants were assayed for organic acids and/or amino acids.

Preparation of bacterial suspension. Cells from 50 ml cultures in modified Watanabe broth were harvested after 48 h by centrifugation (10000 g for 15 min), washed twice with 50 ml 20 mM-potassium phosphate buffer, pH 7.2, and resuspended in an adequate volume of the same buffer to obtain approximately 2×10^{10} bacteria ml^{-1} , as determined by counting in a Petroff-Hausser cell. Protein was determined by the biuret procedure as modified by Stickland (1951); BSA was used as a standard. The bacterial suspension was used immediately in subsequent experiments involving incubation with labelled compounds.

Incubation of bacterial suspension. Bacterial suspension (0.5 ml) was incubated in 25 ml Erlenmeyer flasks containing 0.5 ml of an incubation mixture. Two different mixtures were used. The first contained 8 mM-L-[3,4- ^{14}C]methionine [2 μCi (74 kBq)], 8 mM-potassium phosphate buffer (pH 7.2) and 0 or 4 mM-KMBA. The second contained 5 mM-[3,4- ^{14}C]KMBA [0.5 μCi (18.5 kBq)], 8 mM-potassium phosphate buffer (pH 7.2) and 0 or 4 mM-L-methionine. The effect of AOAA (0.2 mM) was determined for both mixtures. After 2 h, incubations were stopped by addition of 0.5 ml 2 M- HClO_4 ; bacteria were pelleted by centrifugation (10000 g for 15 min) and radioactivity in the supernatant was determined by liquid scintillation counting of HPLC fractions.

Decarboxylation of methionine was measured by radiorespirometry, using the procedure described by Mayaudon (1971). The bacterial suspensions (0.5 ml) were mixed with 1 μCi (37 kBq) L-[1- ^{14}C]methionine, 1 μmol L-methionine and 4 μmol potassium phosphate buffer (pH 7.2) in a final volume of 1 ml, and incubated in a thermostatic 10 ml reactor connected by a closed loop to a Cary 401 β -counter (Varian). The atmosphere in the head-space of the reactor was forced, with a peristaltic pump, through the detector chamber and the

accumulation of the released $^{14}\text{CO}_2$ was measured for 1.5 h. Experiments were done at various temperatures between 10 and 30 °C.

Chromatographic procedures. Methionine and glutamic acid were assayed by ion-exchange chromatography and post-column derivatization with ninhydrin, using a Dionex Amino Acid Analyzer D300, according to the standard procedure of Spackman *et al.* (1958). Samples from broth culture (2 ml) were mixed with 8 ml methanol. The resulting polysaccharide precipitate was removed by filtration through a 0.45 μm (25 mm diameter) Schleicher and Schuell filter; 1 ml of the filtrate was dried thoroughly under vacuum and redissolved in 20 ml of Na-S HiPhi Diluent buffer before being analysed.

HPLC was used to quantify simultaneously MTPA, MTAA and KMBA released into the broth culture. The non-destructive UV detection also allowed further measurements by liquid scintillation counting on eluted fractions when ^{14}C -labelled compounds were used. Separation of carboxylic acids was done by the ion-pairing technique on a Rosil C18 reverse-phase column (length 250 mm, internal diam. 4.6 mm) obtained from Alltech. The HPLC system also comprised a Waters 510 pump, a Rheodyne 7125 injector valve and a Waters Lambda-Max 481 spectrophotometer detector. The elution solvent was water/acetonitrile (92:8, v/v). The ion-pairing agent (8.5 ml of Pic-Reagent A per litre of water) was dissolved in the water before addition of acetonitrile. Elution was isocratic with a flow rate of 1 ml min^{-1} ; detection was at 210 nm; chromatogram recording and peak integration were done with the software Baseline 810 (Waters). Samples were purified by liquid-solid extraction on a Sep-Pak C18 cartridge. The C18 solid phase was conditioned by successive washings with 5 ml methanol and 5 ml 10 mM- H_3PO_4 . After acidification to pH 2 with 2 M- H_3PO_4 the sample (2 ml) was passed through the Sep-Pak. The C18 adsorbent was then washed with 2 ml 10 mM- H_3PO_4 and carboxylic acids of interest were eluted with 2 ml 20 mM-potassium phosphate buffer (pH 7) and acetonitrile (80:20, v/v). When radioactivity measurements were to be done after HPLC separation, the elution solvent coming out of the detector was collected every 12 s in 3 ml glass tubes. Lumagel (2 ml) was added to each tube and radioactivity was measured by liquid scintillation counting on a Beckman LS 1701.

Results

Production of MTPA from methionine and KMBA

Addition of L-methionine to broth cultures of *X. campestris* pv. *manihotis* always resulted in an accumulation of MTPA and MTAA in the culture. After 48 h, concentrations were approximately 1.61 mM for MTPA and 0.28 mM for MTAA (Table 1). Equimolar replace-

Table 1. Conversion of various sulphur-containing compounds to MTPA and MTAA by cultures of *X. campestris* pv. *manihotis*

Data were obtained after 84 h, i.e. in the late stationary phase of growth. The values given are means of three replicates. ND, Not detected.

Potential precursor	Precursor concentration (mM)	Bacterial growth (OD_{600})	MTPA production (mM)	MTAA production (mM)
L-Methionine	3.35	3.06	1.70 ± 0.05	0.27 ± 0.03
KMBA	3.35	3.03	1.31 ± 0.03	0.13 ± 0.02
D-Methionine	3.35	2.30	1.01 ± 0.04	0.08 ± 0.02
L-Cysteine	3.30	2.80	ND	ND
Sodium sulphate	3.30	0.40	ND	ND

Fig. 1. Relationship between bacterial growth (□), assimilation of methionine (▲) and glutamic acid (■), and production of MTPA (△) and MTAA (○). Bacteria were grown for 84 h in 50 ml of a modified Watanabe broth at 28 °C on a gyratory shaker operating at 150 r.p.m.

ment of L-methionine by its D-enantiomer or its α -keto acid analogue (KMBA) also resulted in MTPA and MTAA production, though in smaller amounts (Table 1). None of these were detected in basal medium supplemented with L-cysteine or sodium sulphate.

In Watanabe broth containing L-methionine and L-glutamic acid, the assimilation curve of L-methionine showed a good temporal correlation with the production curve of MTPA (Fig. 1). Assimilation of methionine was delayed approximately 16 h with regard to bacterial growth and glutamic acid uptake (Fig. 1). No trace of KMBA was detected in the medium during any stage of bacterial growth. Methionine consumption and MTPA production were not affected by the absence of L-glutamic acid in the medium.

Demonstration of methionine conversion into MTPA

When a bacterial suspension was incubated for 2 h at 30 °C with 4 μ mol L-[3,4-¹⁴C]methionine, the radiochromatogram of fractions collected after HPLC showed three discernible peaks identified by comparing the retention time with standards (Fig. 2). The first peak, eluting between fractions 20 and 28 (retention time 4.35 min), was residual unmetabolized L-[3,4-¹⁴C]methionine. The second, at fractions 31 to 36, was MTPA (retention time 6.35 min). A third small peak corresponding to MTAA was discernible between fractions 42 to 46 (retention time 8.61 min). No radioactive KMBA was found when bacteria were incubated with L-[3,4-¹⁴C]methionine alone. In order to detect this presumed metabolic intermediate, a trapping pool of cold KMBA (2 μ mol) was added in conjunction with 4 μ mol L-[3,4-¹⁴C]methionine and incubated under standard conditions with bacteria. This complementation by KMBA induced a 2.35-fold decrease of radioactivity incorpo-

Fig. 2. HPLC elution profiles of radioactive compounds contained in supernatants from *X. campestris* pv. *manihotis* incubations. Bacterial suspensions (4.66 mg protein ml⁻¹) were incubated for 1 h with (●) or without 2 mM-KMBA (○) in 25 ml Erlenmeyer flasks containing 4 mM L-[3,4-¹⁴C]methionine (1 μ Ci) and 14 mM-potassium phosphate buffer (pH 7.2) in a final volume of 1 ml. For the control (△) 0.5 ml 2 M-HClO₄ was added at the start of the incubation.

Fig. 3. HPLC elution profiles of radioactive compounds contained in supernatants from *X. campestris* pv. *manihotis* incubations. Bacterial suspensions (5.96 mg protein ml⁻¹) were incubated for 1 h with (●) or without 2 mM-L-methionine (○) in 25 ml Erlenmeyer flasks containing 2.5 mM-[3,4-¹⁴C]KMBA (0.5 μ Ci) and 14 mM-potassium phosphate buffer (pH 7.2) in a final volume of 1 ml. For the control (△) 0.5 ml 2 M-HClO₄ was added at the start of the incubation.

ated in MTPA, a virtual disappearance of MTAA, and the appearance of a fourth radioactive peak eluting at fractions 54 to 61 (retention time 11.63 min) corresponding to the KMBA. Radioactive MTPA, MTAA and KMBA were never detected when bacteria were omitted from the mixture or initially killed by 2 M-HClO₄. On the basis of the recoveries of radioactivity and the methionine specific activity, the production rate of MTPA was estimated to be 3.1 nmol min⁻¹ (mg protein)⁻¹.

Incubation of bacteria with [3,4-¹⁴C]KMBA for 2 h under standard conditions led to the formation of radioactive MTPA and MTAA, but only traces of [¹⁴C]methionine were detected in the radiochromatogram of the mixture (Fig. 3). When the mixture

Table 2. *Effect of AOAA on the production recoveries of [¹⁴C]MTPA and [¹⁴C]MTAA*

L-[3,4-¹⁴C]Methionine (1.5 µCi, 2 mM) or [3,4-¹⁴C]KMBA (0.5 µCi, 2 mM) were incubated for 1 h in flasks containing bacterial suspension (5.42 mg protein ml⁻¹), 14 mM-potassium phosphate buffer (pH 7.2), with or without 0.2 mM-AOAA, in a final volume of 1 ml. Incubation mixtures were analysed by HPLC, fraction collection and subsequent liquid scintillation counting as described in Methods. Similar results were obtained in one repetition of this experiment.

Labelled substrate		Addition at the start of incubation	Total ¹⁴ C recovery (%)*	¹⁴ C recoveries (%) in HPLC fractions of:†			
Type	Amount (µCi)			Methionine (20–28)	KMBA (54–61)	MTPA (31–36)	MTAA (42–46)
L-[3,4- ¹⁴ C]Methionine	1.5	H ₃ PO ₄ (2 M)	89.1	82.0	0.0	0.0	0.0
	1.5	None	92.5	44.7	0.0	19.4	3.2
	1.5	AOAA (0.2 mM)	94.0	72.8	0.0	0.6	0.0
[3,4- ¹⁴ C]KMBA	0.5	H ₃ PO ₄ (2 M)	71.5	7.1‡	61.5	4.0‡	0.0
	0.5	None	80.6	15.0	11.7	52.4	6.2
	0.5	AOAA (0.2 mM)	77.6	11.3	20.2	45.6	6.1

* Percentage of radioactivity recovered in the sample before HPLC analysis.

† Numbers in parentheses are the fractions in which the compound was recovered.

‡ Traces of [¹⁴C]methionine and [¹⁴C]MTPA contaminants in the radioactive [3,4-¹⁴C]KMBA prepared according to the method developed by Mitchell & Benevenga (1978).

contained both [3,4-¹⁴C]KMBA and cold L-methionine (2 µmol) there was a 1.52-fold decrease in the recovery of radioactivity into MTPA, while 25% of the total radioactivity was incorporated into the methionine pool (Fig. 3). The production of [¹⁴C]MTPA and/or [¹⁴C]methionine from [3,4-¹⁴C]KMBA was, as above, strictly associated with bacterial metabolism.

AOAA at a concentration of 0.2 mM totally inhibited production of [¹⁴C]MTPA by a bacterial suspension incubated with L-[3,4-¹⁴C]methionine (Table 2). On the other hand, incubation of the bacteria with [3,4-¹⁴C]KMBA and AOAA still led to the accumulation of radiolabelled MTPA in the mixture. In this latter case, production of [¹⁴C]MTPA was reduced by only 8% compared to bacteria incubated in absence of AOAA. At the same time, the consumption of KMBA was reduced by about the same amount (8.5%). Similar relationships were observed for the MTPA-associated production of MTAA.

A release of ¹⁴CO₂ was recorded by radiorespirometry when bacterial suspensions were incubated with L-[1-¹⁴C]methionine for 1.5 h, but no radioactive MTPA was detected in the mixture at the end of the run. The rate of ¹⁴CO₂ production increased with temperature, from 10 to 30 °C, according to the Arrhenius relationship; an activation energy of 55.6 kJ mol⁻¹ was calculated for the microbiological system involved in the decarboxylation. At 30 °C, with a mixture containing 1 µmol L-methionine, the decarboxylation rate of the amino acid was 2.25 nmol min⁻¹ (mg protein)⁻¹.

Discussion

This work establishes for plant pathogenic bacteria the

catabolic sequence by which methionine is converted into MTPA, a reportedly phytotoxic organic acid. As in the experiment done by Robeson & Cook (1985), the absolute requirement of methionine for MTPA production and the incorporation of radioactivity from L-[3,4-¹⁴C]methionine into MTPA demonstrate the occurrence of a metabolic link between these two compounds. The release of ¹⁴CO₂ and the absence of incorporation of radioactivity into MTPA from L-[1-¹⁴C]methionine indicate that one step of the pathway leading to the production of MTPA is decarboxylation of methionine. Moreover, the fact that KMBA undergoes biotransformation to MTPA, the difficulties in detecting its accumulation *in vitro* and the need to use a trapping pool of cold KMBA to observe incorporation of radioactivity from L-[3,4-¹⁴C]methionine into KMBA suggest that KMBA is an intermediate in the overall biotransformation process. Incubation of bacteria with either L-[3,4-¹⁴C]methionine or [3,4-¹⁴C]KMBA demonstrate the reality and reversibility of a bioconversion between these two compounds, suggesting a transaminase activity. From these results, transamination of methionine into KMBA and subsequent decarboxylation into MTPA is proposed as the catabolic sequence for production of the phytotoxin. Evidence for this was provided by adding the transaminase inhibitor AOAA (Rognstad & Clark, 1974) to the incubation mixture. Production of [¹⁴C]MTPA from L-[3,4-¹⁴C]methionine was totally inhibited by AOAA, whereas production of [¹⁴C]MTPA from [3,4-¹⁴C]KMBA was unaffected. These results accord with the catabolic pathway described by Steele & Benevenga (1978) for eukaryotes (rat or monkey liver cells). Extension of this catabolic sequence to some other amino acids (leucine, isoleucine, phenylalanine and

tryptophan) is in accordance with the production of low molecular mass carboxylic acids observed for *X. campestris* pv. *campestris* (Robeson & Cook, 1985) and pv. *manihotis* (Ewbank & Maraite, 1990).

Ince & Knowles (1985, 1986) described the formation of ethylene from methionine by cultures or cell-free extracts of *Escherichia coli*. They demonstrated that the first step of the process implied the production of KMBA by transamination of methionine (Ince & Knowles, 1986). On the other hand, many plant pathogenic bacteria belonging to the genera *Erwinia*, *Pseudomonas* and *Xanthomonas* show increased production of ethylene in the presence of methionine (Swanson *et al.*, 1979). Methionine could therefore be involved via its conversion to KMBA in a dual metabolic role, leading to the production of phytotoxins (MTPA and MTAA) and to excessive production of the phytohormone ethylene. Further investigation could determine more precisely the basis of pathogenicity of *Xanthomonas* spp.

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