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GUAIACOL AND ISOVANILLIC ACID AS METABOLITES IN THE TRANSFORMATION OF METHOXYPHENOLIC ACIDS BY *NOCARDIA AUTOTROPHICA*

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Key Word Index—*Nocardia autotrophica*; bacteria; guaiacol; vanillic acid; transformation; metabolism.

Abstract—The transformation of methoxy derivatives of benzoic acid ^{14}C labelled in the ring or in the methoxyl or carboxyl groups were determined in the cultures of five selected strains of *Nocardia autotrophica*. It was shown that the transformation of vanillic acid to protocatechuic acid might proceed through guaiacol and isovanillic acid as intermediates. This metabolic conversion was found in three of the five bacterial strains examined.

INTRODUCTION

Nocardia, a genus of bacteria that transform organic substances in soil [1, 2], may be responsible for substantial modification of phenolic compounds present in the soil and the synthesis of new phenolic derivatives [3, 4]. Using isotopic and other modern techniques the pathways for transformations of phenolic compounds of some lignolytic *Nocardia* strains have been partly recognized [1, 5–7]. These strains perform an important role in the transformation of methoxyphenolic acids that are derived from the biological decomposition of lignin. *Nocardia*, which demethylate, decarboxylate or hydroxylate these substrates, produce substances which either undergo conversion to humus [8] or are dearomatized [9].

The processes mentioned above are crucial to soil quality and to the correct function of its microflora. Therefore the aim of the present work was to study in detail the metabolism of selected methoxyphenolic acids in five cultures of *Nocardia autotrophica* which have not yet been examined in that respect.

RESULTS AND DISCUSSION

All transformations described here were investigated in *Nocardia* cell culture media. Figure 1 shows the results of the transformation of methoxy acids labelled with ^{14}C in cultures of *N. autotrophica* DSM 43100. Of the four substrates examined vanillic acid was the first to undergo transformation, regardless of the localization of the carbon label (90% decrease of $O^{14}CH_3$ level and 80% $^{14}COOH$ within 3 days in comparison with 48 and 11% decrease of respective groups of benzylovanillic acid). Most substrates were degraded mainly in the first 2–4 days in the culture. Later, liberation of $^{14}CO_2$ decreased and quickly reached a plateau. Veratric acid labelled at the 3-methoxyl was a notable exception, since the release of $^{14}CO_2$ was at a lower level and only reached a plateau after 7 days incubation. Liberation of $^{14}CO_2$ from the aromatic ring of vanillic acid (about 77% after the plateau was reached) was rapid, proving that substrate dearomatization is due to the dioxygenases present in *Nocardia* [3, 5].

The metabolites that appeared during the incubation of aromatic substrates with *Nocardia* were identified by chromatography and autoradiography (Table 1). The decrease in vanillic acid levels was accompanied by the subsequent appearance and then disappearance of guaiacol and isovanillic acid. Protocatechuic acid was constantly present at a low concentration throughout the process. This is the first time that guaiacol and isovanillic acid have been detected as intermediates; in earlier work only protocatechuic acid was observed in *Nocardia* cultures [1, 3].

Oxygen uptake by *Nocardia* cells in the presence of phenolic substrates confirmed the above results (Fig. 2). On the first day of culture, the increase of oxygen uptake was observed towards vanillic acid and guaiacol only; in the case of isovanillic and protocatechuic acids the effect appeared later, i.e. on the second or third day, respectively. The appearance of guaiacol as an intermediate was confirmed in three of the five strains examined.

Labelled isovanillic acid failed to give rise to any labelled guaiacol (Table 2). It was confirmed, however, that isovanillic acid underwent slow transformation into protocatechuic acid but it remained in the culture unchanged for a long time. However, when isovanillic acid was produced from vanillic via guaiacol, it was metabolized quickly and it disappeared from the culture medium the next day (Table 1). Thus vanillic acid is a much more effective inducer of demethylase activity than isovanillic acid [10]. Hence the presence of the former in the medium is most probably conducive to the more rapid metabolism of the latter.

Guaiacol is not produced in the culture of *N. autotrophica* grown on veratric acid as sole carbon source. This acid is probably metabolized at a very early stage to a mixture of isovanillic and vanillic acids and the latter metabolite disappears more quickly from the culture than isovanillic acid [10].

The metabolic pathway of methoxyphenolic acids in

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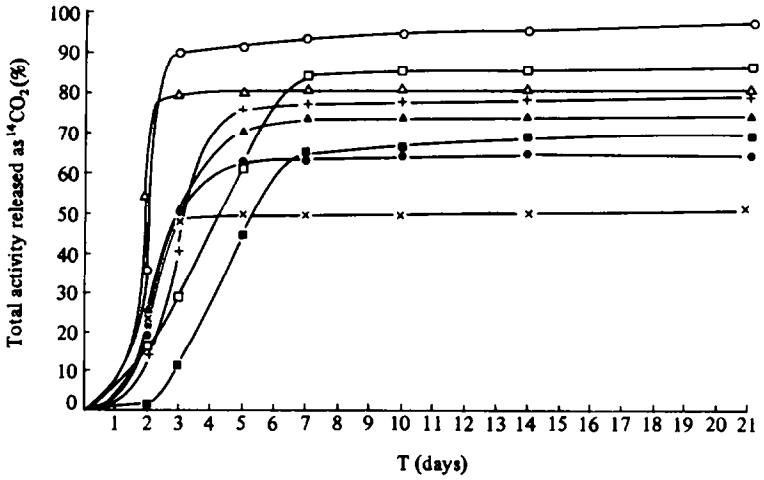

Fig. 1. Release of $^{14}CO_2$ by the *N. autotrophica* DSM 43100 during the growth on labelled methoxyphenolic derivatives of benzoic acid: (O) O^{14}CH$_3$-vanillic acid, (△) ^{14}COOH-vanillic acid, (+) ^{14}C-ring vanillic acid, (□) 3-O ^{14}CH$_3$-veratric acid, (▲) 4-O^{14}CH$_3$-veratric acid, (●) O^{14}CH$_3$-isovanillic acid, (×) O^{14}CH$_3$-benzylovanillic acid, (■) ^{14}COOH-benzylovanillic acid.

Table 1. The TLC data of products formed during the growth of *N. autotrophica* DSM 43100 on O^{14}CH$_3$- and ^{14}C-ring vanillic acids as the sole carbon source. All numbers represent average values of three chromatograms

Spot no.	Product identified	R_f	Radioactivity (Bq) Days of culture 1 –O^{14}CH$_3$	1 ^{14}C-ring	2 –O^{14}CH$_3$	2 ^{14}C-ring	3 –O^{14}CH$_3$	3 ^{14}C-ring	4 –O^{14}CH$_3$	4 ^{14}C-ring
1	Guaiacol	0.77	—	—	0.40	0.35	—	—	—	—
2	Veratric acid	0.66	0.25	0.12	0.27	0.10	0.25	0.10	0.10	—
3	Vanillic acid	0.48	69.3	73.0	20.0	21.22	—	—	—	—
4	Isovanillic acid	0.38	—	—	—	—	0.82	0.6	—	—
5	Protocatechuic acid	0.10	—	—	0.2	0.12	0.44	0.22	0.20	0.10

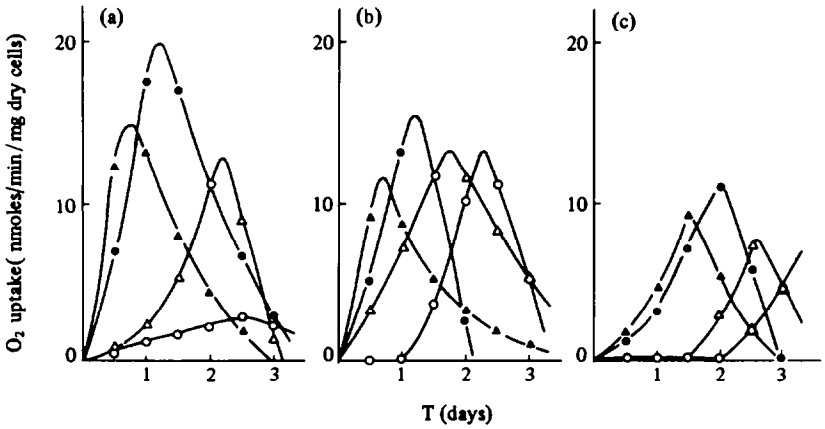

Fig. 2. The oxygen consumption of the strains of *N. autotrophica*: a—PCM 2186, b—DSM 43089, c—DSM 43100, towards methoxyphenolic acid. (O) Protocatechuic acid, (△) isovanillic acid, (●) guaiacol and (▲) vanillic acid. The strains were grown on vanillic acid as the sole carbon source.

Table 2. The TLC data of products formed during the growth of *N. autotrophica* DSM 43100 on $O^{14}CH_3$-isovanillic acid as the sole carbon source. All numbers represent average values of three chromatograms

Spot no.	Product identified	R_f	Radioactivity (Bq) Days of culture 1	2	3	4	5
1	Veratric acid	0.66	0.4	0.1	1.1	1.0	1.1
2	Anisic acid	0.64	1.1	0.8	—	—	—
3	Unidentified	0.58					
4	Unidentified	0.53				0.8	0.4
5	Unidentified	0.50					
6	Isovanillic acid	0.38	80.3	79.3	68.55	58.2	1.1
7	Protocatechuic acid	0.10	—	—	—	10.8	0.4

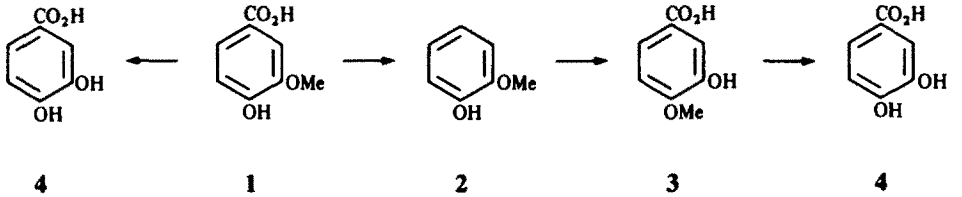

Fig. 3. A proposed pathway for the transformation of vanillic acid (1) to guaiacol (2), isovanillic acid (3) and protocatechuic acid (4) by five strains of *N. autotrophica*. Details in text.

cultures of *N. autotrophica* is indicated in Fig. 3. The identification of guaiacol by TLC was only possible in these strains of *Nocardia* because vanillic acid is transformed rather slowly. Earlier failures to detect this substance were probably due to its rapid metabolism in the growing cultures and its substantial volatility.

EXPERIMENTAL

Chemicals. ^{14}COOH-vanillic acid (20×10^3 Bq·mg^{-1}), $-O^{14}CH_3$-vanillic acid (38.7×10^3 Bq·mg^{-1}), ^{14}C ring-vanillic acid (99.7×10^3 Bq·mg^{-1}), $-^{14}$COOH-benzylvanillic acid (50×10^3 Bq·mg^{-1}), $-O^{14}CH_3$-benzylvanillic acid (23.3×10^3 Bq·mg^{-1}), 3-$O^{14}CH_3$-veratric acid (21×10^3 Bq·mg^{-1}) and 4-$O^{14}CH_3$-veratric acid (16.8×10^3 Bq·mg^{-1}) were obtained through the courtesy of Dr. K. Haider and Prof. J. Trojanowski. $-O^{14}CH_3$-isovanillic acid (92.4×10^3 Bq·mg^{-1}) came from the Institute of Nuclear Research, Swierk, Poland. All unlabelled methoxyphenolic acids were purchased from Fluka and protocatechuic acid was obtained from POCH (Gliwice, Poland).

Culture conditions. The four strains of *Nocardia autotrophica* came from the Collection of Microorganisms (DSM) at Göttingen, West Germany. Their respective numbers were: 43100, 43099, 43089, 43088. The strain PCM 2186 came from the Microbial Collection of the Institute of Immunology and Experimental Therapy, Polish Academy of Science, Wrocław. Strains were kept on 2% agar slants containing 0.4% glucose, 1.0% malt extract, 0.4% yeast extract [6, 7]. Vanillic, isovanillic, veratric or benzylvanillic acids at a final concentration of 3×10^{-6} mol·ml^{-1} each and the same acid but labelled with ^{14}C (*ca* 850 Bq or 50000 dpm each) were dissolved in 100 µl 10% KOH and added separately to the cultures as the sole carbon source in 100 ml media. The cultures were run on a rotary shaker at 25°. In some cultures only unlabelled methoxyphenolic acids were used.

$^{14}CO_2$ determination. The release of $^{14}CO_2$ from the cultures was counted daily after the absorption of KOH in glass cups according to ref. [5]. Uninoculated flasks containing separately each of the labelled compounds served as controls. The KOH in glass cups was changed each day by rinsing it with 2 cm^3 H_2O into counting flasks and 7 cm^3 of Dimilume Scintillator (Packard, Frankfurt, West Germany) were added. The radioactivity was counted in liquid scintillation counter (Isocap 300 Nuclear Chicago, U.S.A.). The percentage of liberated $^{14}CO_2$ was used as a criterion for the degradation of the labelled compounds.

TLC autoradiochromatography. 10 ml samples were taken from the cultures every 12 or 24 hr. The samples were acidified with 5% H_2SO_4 and extracted with Et_2O (2 × 10 ml). The extracts were evaporated to a small volume and analysed by TLC using silica gel F-254 plates. Each sample was simultaneously analysed × 3 on three separate plates. Chromatograms were developed in C_6H_6–MeOH–propionic acid (22:2:1) according to ref. [11] and two were sprayed with diazosulphanilamide (DASA) according to ref. [10]. In further experiments two chromatograms (sprayed with DASA and untreated) were deposited for 3 months in the dark attached to the Roentgen Photo-plates X5. The plates were then developed in order to obtain the visualization of the black spots which were then compared with the spots on chromatograms after DASA reaction and with the pattern of authentic markers. The metabolites were removed from the plates, extracted with acidified ether and their radioactivity counted as described in ref. [12].

Determination of oxygen uptake. Measurements were carried out using the Clark oxygen electrode under the same conditions as described in a previous paper [3]. *Nocardia* cells were progressively taken from the unlabelled culture twice a day and suspended in 0.1 M phosphate buffer to $E_{660} = 1$. The oxygen

uptake was determined as a quantity of O_2 nmol consumed during 1 min reaction by 1 mg of dry wt of *Nocardia* cells towards 10^{-6} ml of vanillic, isovanillic, protocatechuic acids or guaiacol [3].

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