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Degradation of *o*-methoxybenzoate by a two-member consortium made up of a gram-positive *Arthrobacter* strain and a gram-negative *Pantotea* strain

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Abstract

Aromatic carboxylic acids substituted with methoxylated groups are among the most abundant products in "alpechin", the wastes resulting from pressing olives to obtain olive oil. Degradation of *o*-methoxybenzoate by an stable consortium made of a gram positive bacterium, *Arthrobacter oxydans*, and gram negative one, *Pantotea agglomerans*, was shown to mineralize this compound efficiently. The concerted action of both microorganisms was needed for the two first steps in the process, namely, the conversion of *o*-methoxybenzoate into salycilate, and the hydroxylation of the latter to gentisate. Gentisate was further degraded by the *Arthrobacter* strain.

Introduction

The wastes resulting from the pressing of olives for obtaining olive oil are defined under the generic name of "alpechin". Several methods are used that vary in the amount of water used in the process, but in all cases the wastes exhibit a high oxygen demand, and represent the main environmental pollution problem in the Mediterranean basin (Hamdi 1993; Paredes et al. 1987).

We and others have carried out sequential extractions of products from alpechin by using solvents of increasing polarity. These fractions were analysed by ^{1}H-NMR, ^{13}C-NMR and gas chromatography-mass spectrometry. These analyses revealed that more than 200 different chemical products are present in this waste. Among the most abundant products in "alpechin" are aromatic compounds substituted with methoxylated and carboxylic acid groups on the aromatic ring. Other substituents found often on the aromatic ring are short aliphatic chains, hydroxy, and alkyl groups (Akasbi et al. 1993; Bianchi et al. 1994; Bianco et al. 1993; Brenes-Balbuena et al. 1992; Fleming et al. 1969; Gil et al. 1997; Gil et al. 1998; Gil 1999; Martínez-Nieto et al. 1992; Rodríguez et al. 1988). Among the unequivocally identified products in "alpechin" was *o*-methoxybenzoate and because of the presence of carboxyl and methoxyl groups on the aromatic ring, we chose this compound as a model to study degradation of methoxylated aromatics in alpechin.

Materials and methods

Culture media and growth conditions

Bacterial strains were grown routinely on M9 minimal medium with 5 mM *o*-methoxybenzoate, 5 mM salicylate or 5 mM 2,5-dihydroxybenzoate as a C-source or on LB medium (Abril et al. 1989). All flasks were incubated at 30 °C and shaken on an orbital platform operating at 200 to 250 strokes per min. Growth was measured as the increase in turbidity at 660 nm and by determining colony forming units per ml on solid medium.

Isolation of metabolites

Culture supernatants were extracted thrice with ethyl acetate at pH 7. The resulting aqueous solution was acidified to pH 2 with HCl and extracted again twice

with ethyl acetate. The extracts were dried over anhydrous sodium sulfate, and the excess solvent was removed by evaporation under reduced pressure at 35 °C.

Analytical methods

Products were analyzed by high-performance liquid chromatography (HPLC) on a Hewlett-Packard model 1050 chromatograph equipped with a diode array detector and a 5 μm C18RP column (Hypersil-C18-ODS 10 cm $\times$ 4.6 mm). The column was first washed with a mixture of acetonitrile (ACM) and a solution of 13.5 mM trifluoroacetic acid (TFA) in water for 2 min. Then a linear gradient was applied to reach 60% (vol/vol) ACM over 6 min, the column was finally washed for 4 mm with this mixture. The flow was kept constant at 1 ml/min, and the detector was set at 230 and 254 nm to detect aromatic compounds.

Gas chromatography-mass spectrometry (GC-MS) analyses were carried out with an HP6890 GC-MS apparatus. The GC was equipped with a capillary HP-1 methyl silicone column (25 m $\times$ 0.025 mm).

^{1}H nuclear magnetic resonance (^{1}H MMR) was performed with a Bruker AM300 (300 MHz) spectrometer on solutions prepared in deuterated acetone. Tetramethylsilane was used as an internal standard.

Results and discussion

Enrichment for microorganisms that use o-methoxybenzoate

A soil sample from an olive tree field taken in Villacarrillo (Jaen, Spain) was suspended in a modified M9 minimal medium with *o*-methoxybenzoate as the sole C-source (Abril et al. 1989). To enrich the soil suspension for fast growing microorganisms, the culture suspension was 100-fold diluted every 24–30 h in the same culture medium. After 10-dilution rounds the suspension was spread on Luria-Bertani (LB) medium plates, where an apparent single type of colony was evident. However, when these colonies were streaked separately on M9 minimal medium plates with 5 mM *o*-methoxybenzoate as the sole C-source no growth was observed. In contrast, when the last-dilution round suspension was directly plated on plates made of M9 minimal medium with 5 mM *o*-methoxybenzoate as the sole C-source, two types of colonies were distinguished, one larger than the other and yellow coloured, and another which was colourless (both colonies were colourless on LB medium and had a very similar appearance). None of these two types of colonies grew when streaked separately on plates made of M9 minimal medium with 5 mM *o*-methoxybenzoate. We then tested to see if the two isolates could grow alone with other C-sources. It was found that both strains could grow on M9 minimal medium with glucose and succinate as the sole C-source. This suggests that the need of the consortium to degrade *o*-methoxybenzoate was not due to auxotrophies of the strains that could be "supplemented" by each other, but that growth on *o*-methoxybenzoate required some cooperative action between both microbes to allow for the successful metabolism of the methoxylated aromatic compound.

Subsequently, the two microorganisms were microbiologically characterized. The colourless colony was found to be a gram-positive bacterium that based on API20 tests was classified as a member of the *Arthrobacter* genus. Phospholipid analysis identified it as *Arthrobacter oxydans*. The yellow colony was identified as a gram-negative bacteria and based on API20 tests was ascribed to the genus *Pantotea*. Phospholipid analysis assigned it to the species *Pantotea agglomerans*. (The phospholipid analysis were done by Microbiol Identification Co., VT, USA.)

Stability of the consortium and demonstration that initiation of the metabolism of o-methoxybenzoate requires the concerted action of the two members of the consortium

We tested the stability of the consortium with time by determining the relative ratio of the two members on plates of M9 minimal medium with *o*-methoxybenzoate as the sole C-source (we counted the yellow and the colourless colonies). This also allowed us to determine the generation time of the two bacteria. In five independent assays we found that the generation time of the two strains was in the order of 14 h, and that regardless of the growth state of the cultures, the relative ratio of *Arthrobacter oxydans* to *Pantotea agglomerans* was in the range of 1.02 to 1.30. This indicates that the consortium was stable and that none of the strains was predominant.

To determine which strain could initiate the metabolism of *o*-methoxybenzoate each strain was grown on M9 minimal medium with glucose (0.5% wt/vol) or 10 mM succinate, and the cultures were supplemented with 5 mM *o*-methoxybenzoate. We tested whether *o*-methoxybenzoate was consumed with time by each of the strains and if putative metabolites resulting from

Table 1. ^{1}H-NMR data of dicamba and 2-hydroxy-3,6-dichlorobenzoic acid

Dicamba		2-Hydroxy-3, 6-Dichlorobenzoate	
H-4	7.39d (8.7 Hz)	H-4	7.46d (8.6 Hz)
H-5	7.15d (8.7 Hz)	H-5	6.98d (8.6 Hz)
$-OCH_3$	3.97 s		

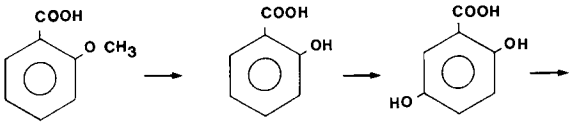

Figure 1. Catabolic pathway proposed for the mineralization of *o*-methoxybenzoate by a consortium made of *Arthrobacter oxydans* and *Pantotea agglomerans*.

the biotransformation appeared in the culture medium. We found that the amount of *o*-methoxybenzoate did not decrease with time, and that no metabolites derived from *o*-methoxybenzoate accumulated in the medium so that it could be detected. In contrast the consortium consumed 100% of *o*-methoxybenzoate in 24 h (not shown).

Dicamba (3,6-dichloro-*o*-methoxybenzoate) has been used as an analog of *o*-methoxybenzoate to study the initial step in the degradation of this methoxylated aromatic carboxylic acid (Krueger et al. 1989; Wang et al. 1997; Yang et al. 1994). We supplied glucose-growing cultures of each of the strains or a mixture of the two strains in the consortium with 1 mM dicamba. We found that while the pure cultures did not transform dicamba, the consortium converted dicamba into another compound. This compound was extracted from the culture medium and analyzed by GC-MS and H-NMR. The compound was unequivocally identified as 2-hydroxy-3,6-dichlorobenzoic acid (Table 1). This suggests that the initial attack on *o*-methoxybenzoate requires the concerted action of the two microbes and that the initial step likely leads to the production of salycilate (Figure 1). This finding was unexpected since *o*-demethylases are intracellular enzymes and at present we cannot explain how the concerted action occurs at the molecular level.

The *o*-demethylation of methoxylated aromatic compounds is a common step in the metabolism of this type of compound and it has been described before for degradation of vanillic acid, veratric acid and syringic acid by several *Pseudomonas* sp. and *Sphingomonas* sp. strains (Berhhardt et al. 1975; Enoki et al. 1981; Mali et al. 1985; Nishikawa et al. 1998; Priefert et al. 1997; Ribbons 1971; Vicuña 1988).

Metabolism of hydroxylated aromatic compounds by the consortium and each of its members

Based on the above results we tested to see if each of the consortium members, and the consortium itself would be able to use monohydroxy- and dihydroxybenzoates as the sole C-source. We found that none of the pure cultures could use salycilate, *m*-, or *p*-hydroxybenzoate as the sole C-source; however, the consortium was able to thrive with salycilate as the sole C-source (turbidity of the culture at 660 nm > 1), but neither *m*- nor *p*-hydroxybenzoate supported the growth of the consortium (turbidity of the cultures at 660 nm $\leq$ 0.1). This again suggests that a concerted action of the two microbes is needed for the second step in the process. Culture supernatants of the consortium growing on salycilate accumulated a compound that absorbed at 254 nm, and that by GC-MS analysis was unequivocally identified as 2,5-dihydroxybenzoate (Figure 1).

Consistent with the degradation of salycilate through 2,5-dihydroxybenzoate (gentisate) was that the consortium was able to grow on M9 minimal medium with this dihydroxylated aromatic acid, while 2,3-, 2,4-, 3,4- and 2,6-dihydroxybenzoate were not used by the consortium. We found that *Arthrobacter oxydans* but not *Pantotea agglomerans* was able to grow at the expense of 2,5-dihydroxybenzoate as the sole C-source. This suggests that the last steps of the degradation of the aromatic compound are likely to be only mediated by the *Arthrobacter* strain without the need for further assistance by *Pantotea*. However, it should be noted that the consortium can be kept when gentisic acid is the sole C-source and that upon repetitive growth on this C-source the ratio of *Arthrobacter oxydans* to *Pantotea agglomerans* was kept in the range between 1.07 and 1.30. It may be possible that the role of *Pantotea* in the consortium be that of supplying an inducer of the *Arthrobacter* strain, and that in turn the latter excretes enough carbon to support the growth of the former. This hypothesis needs experimental support.

Although not studied in detail here, the degradation of gentisate by *Arthrobacter*sp. is likely to occur via “1-2” fission yielding maleylpyruvic acid, which should be further converted to pyruvic acid and maleic

acid, which are therefore channeled into the Krebs cycle, in accordance with the well established pathway for the degradation of gentisate in gram positive and gram negative bacteria (Bayly and Barbour 1984; Feng et al. 1999; Fuenmayor et al. 1998; Harpel and Lipscomb 1990; Romine et al. 1999; Werwath et al. 1998).

Conclusion

In summary the two-members consortium that degrade *o*-methoxybenzoate and salycilate is highly stable in time as the ratio of the consortium members is relatively constant. This is further supported by the fact that both members of the consortium had a similar generation time when growing on M9 minimal medium with *o*-methoxybenzoate or salycilate. Therefore, the two strains in the consortium should keep an efficient exchange of nutrients so that mutual benefit and carbon is derived from a single compound to allow the balanced growth of the two consortium members. The nature of the exchanges between the two members of the consortium is unknown, but they do not represent a simple supplementation of a nutrient to bypass an auxotrophy, since both members of the consortium were able to grow on minimal medium with glucose as the sole C-source.

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