NEW INSIGHT INTO THE ANAEROBIC BIODEGRADATION OF ALKYLBENZENES:
THE OXIDATION PATHWAY OF P-CYMENE IN A DENITRIFYING BACTERIUM

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Introduction

Various alkylbenzenes are common petroleum constituents and therefore occur widespread in natural environments due to oil seepages or oil spills. In addition, certain alkylbenzenes are formed biosynthetically. A prominent example of such natural products is the plant-derived monoterpene p-cymene (1). The aerobic biodegradation of alkylbenzenes is well-understood. In particular, various dioxygenases utilizing molecular oxygen as co-substrate are responsible both for converting alkylbenzenes to the corresponding benzene-1,2-diols as well as for ring cleavage of the latter yielding intermediates suitable for further degradation. In contrast, the full elucidation of biodegradation pathways of alkylbenzenes under anoxic conditions is currently extensively investigated by microbiologists and biogeochemists. Here, we report new insights into the pathways and mechanisms involved in anaerobic biodegradation of alkylbenzenes based on model studies with the denitrifying bacterium “Aromatoleum aromaticum” pCyN1 and p-cymene (1) as the substrate. “A. aromaticum” pCyN1 has been isolated from freshwater mud samples from ditches in Bremen, Germany, and is capable of utilizing toluene, p-ethyltoluene and p-cymene, but also various non-aromatic monoterpene hydrocarbons as sole sources of carbon and energy (Harms et al. 1999). We have previously shown that “A. aromaticum” pCyN1 initiates p-cymene degradation by oxidation to 4-isopropylbenzoyl-CoA (5) via 4-isopropylbenzyl alcohol (2), 4-isopropylbenzaldehyde (3) and 4-isopropyl-benzoate (4) (Fig. 1) (Strijkstra et al. 2014).

Results

Analysis of extracts from incubation experiments of “A. aromaticum” pCyN1 growing with p-cymene by gas chromatography-mass spectrometry revealed the presence of previously unknown metabolites. The constitutions and absolute configurations of these intermediates, in particular of (1S,2S,4S)-2-hydroxy-4-isopropylcyclohexane-1-carboxylate (8) and (5)-3-isopropylpimelate (10) were elucidated using reference standards for comparison. The reference standards were obtained by stereoselective syntheses via methyl 4-isopropyl-2-oxocyclohexane-1-carboxylate (9) as a key intermediate involving a rhodium-catalyzed asymmetric conjugate addition to establish the stereogenic center carrying the isopropyl group. The interpretations based on these metabolomic findings were supported by differential proteogenomic analysis. Overall, our study indicates that the degradation of p-cymene proceeds via (i) oxidation of the hydrocarbon 1 to 4-isopropylbenzoyl-CoA (5), (ii) a novel sub-type of class I benzoyl-CoA reductase (BCR) as known from the denitrifying betaproteobacterium Thauera aromaticum K172 and (iii) a modified β-oxidation pathway yielding 3-isopropylpimeloyl-CoA (10) analogously to benzoyl-CoA degradation in the phototrophic alphaproteobacterium Rhodopseudomonas palustris (Fig. 1). X-ray crystallography revealed that the thermodynamically most stable stereoisomer of 2-hydroxy-4-isopropylcyclohexane-1-carboxylate (8) is formed as the intermediate of the degradation...
pathway. Our findings imply that the reductive dearomatization of 4-isopropylbenzoyl-CoA (5) by the BCR employed by "A. aromaticum" pCyN1 stereospecifically forms (S)-4-isopropyl-1,5-cyclohexadiene-1-carbonyl-CoA (6). According to reasonable mechanistic models, the first protonation of the six-membered ring of 4-isopropylbenzoyl-CoA (5) takes place at the para-position forming the sp3-carbon center which later becomes the stereogenic center. However, its absolute configuration is defined by the second protonation step. In this presentation, the results for p-cymene degradation in "A. aromaticum" pCyN1 will be compared with those for other alkylbenzenes and akylbenzene-degrading anaerobic bacteria.

Conclusions

Our results for the first time allow suggesting a pathway for the complete oxidation of the plant-derived alkylbenzene p-cymene under strictly anoxic conditions. This pathway may be a blueprint for the degradation of other alkyl substituted toluenes and benzoates in natural habitats such as contaminated aquifers, marine oil seeps or petroleum reservoirs. The stereochemistry of the intriguing Birch-like reduction of the aromatic ring has been elucidated with implications for the mechanism of this key reaction in the anaerobic metabolism of a broad range of monoaromatic compounds including non-hydrocarbons.

![Oxidation pathway proposed for p-cymene in the denitrifying bacterium "Aromatoleum aromaticum" pCyN1. Structures shown in green represent metabolites identified by comparison with synthetic reference standards.](image_url)

**Figure 1** Oxidation pathway proposed for p-cymene in the denitrifying bacterium “Aromatoleum aromaticum” pCyN1. Structures shown in green represent metabolites identified by comparison with synthetic reference standards.

References
