Biosci. Biotechnol. Biochem., **64** (5), 1064–1066, 2000

Note

Substrate Specificity of Regiospecific Desaturation of Aliphatic Compounds by a Mutant *Rhodococcus* Strain

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Received November 9, 1999; Accepted January 4, 2000

Substrate specificity of *cis*-desaturation of alipahtic compounds by resting cells of a mutant, *Rhodococcus* sp. strain KSM-MT66, was examined. Among substrates tested, the rhodococcal cells were able to convert *n*-alkanes (C13–C19), 1-chloroalkanes (C16 and C18), ethyl fatty acids (C14–C17) and alkyl (C1–C4) esters of palmitic acid to their corresponding unsaturated products of *cis* configuration. The products from *n*-alkanes and 1-chloroalkanes had a double bond mainly at the 9th carbon from their terminal methyl groups, and the products from acyl fatty acids had a double bond mainly at the 6th carbon from their carbonyl carbons.

Key words: *Rhodococcus*; desaturation; regiospecificity; alkene; alkenoic acid

Recently, we demonstrated that a mutant strain designated KSM-B-3M of an alkane-utilizing *Rhodococcus* sp. had the ability to desaturate some aliphatic substrates at central positions of the chains and excrete large amounts of the unsaturated products.[1] For instance, the rhodococcal cells desaturated 1-chlorohexadecane and *n*-hexadecane to yield 1-chloro-*cis*-7-hexadecene and *cis*-7-hexadecene, respectively. We also found that a further improved mutant of the organism, designated KSM-MT66, was able to convert isopropyl hexadecanoate (palmitate) to isopropyl *cis*-6-hexadecenoate, and optimized its reaction conditions.[2] In this paper, we describe the substrate specificity of KSM-MT66 cells toward various aliphatic substrates, including alkanes and acyl fatty acids with different chain lengths.

Preparation of resting cells and the reaction conditions were described previously.[2] In brief, the reaction mixture was routinely composed of 5 ml of a substrate, 1 g (wet weight) of resting cells, and 15 ml of 0.25 M sodium phosphate buffer (pH 7.0) containing 1.0% sodium glutamate, 2 mM thiamine, and 2 mM $MgSO_4$, and the suspension was placed in a 500-ml flask. Products and residual substrates were extracted in 3 volumes of ethyl acetate from the spent media, and compounds in the solvent were measured by a gas chromatograph (GC). Fatty acids were methyl-esterified by treatment with a boron trifluoride methanol complex (BF_3/MeOH; Wako Pure Chemical) before GC analysis.[2] They were separated and purified in a HPLC system equipped with an Inertsil ODS-2 column (4.6 mm by 25 cm; Gas Chromatograph Industries) and a UV detector set at 210 nm. Double bond positions of products were identified on a gas chromatograph-mass spectrometer (GC-MS) by the alkylthiolation method using dimethyl disulfide (Wako Pure Chemical).[3,4] *Cis* olefinic hydrogens of unsaturated products were confirmed by the specific absorptions around 3,015, 2,935, and 2,865 cm^{-1} in infrared (IR) spectra and the proton signals around δ_H 5.56 ppm in proton nuclear magnetic resonance spectra.[1,2]

Acyl fatty acids were tested as substrates with different chain lengths of fatty acid and acyl moieties. Among ethyl esters of fatty acid (C10:0 through C20:0) tested, ethyl tetradecanoate, pentadecanoate, hexadecanoate, and heptadecanoate were *cis*-desaturated to form corresponding unsaturated fatty acids with yields of approximately 5, 7, 10, and 15 g/l after a 3-day incubation, respectively. The product from ethyl hexadecanoate was identified, for example, by the same method as described.[2] In a GC-MS spectrum, the M^+ ion of the product was detected at m/z 282, which corresponds to a mono-unsaturated compound of the substrate (M^+, m/z 284). A GC-MS spectrum of the alkylthiolated product showed the M^+ ion at m/z 376 and a single fragment pair of m/z 187 ($CH_3(CH_2)_8C^+HSCH_3$) and 189 ($CH_3CH_2COO(CH_2)_4C^+HSCH_3$). Thus, the structure of the product was deduced to be ethyl *cis*-6-hexadecenoate. GC-MS spectra of the alkylthiolated products from the other four etyl fatty acids showed that a double bond was also located exclusively at the 6th carbon from their carbonyl carbons: the base peak was observed at m/z 189 in every GC-MS spectra of the products.

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Abbreviations: GC, gas chromatograph; MS, mass spectrometer; IR, infrared

Methyl, propyl, isopropyl,[2] and isobutyl hexadecanoates were also *cis*-desaturated to yield corresponding unsaturated products at levels of 0.5, 20, 53, and 7 g/l after a 3-day incubation. The base peak was also observed at m/z 187 in every GC-MS spectra of the alkylthiolated products, indicating that desaturation occurs at the 6th carbon from the carbonyl carbons of the substrates. A GC-MS spectrum of the alkylthiolated isobutyl hexadecenoate (M^+, m/z 404) showed an additional fragment pair of m/z 201 and 203 (30%), as well as the main pair of m/z 187 and 217 (70%), indicating that a double bond was also introduced at the 5th carbon from the carbonyl carbon. Thus, the enzyme(s) responsible for the desaturation reaction appears to recognize mainly the 6th carbon from the carbonyl carbons, not from the methyl ends, of the acyl fatty acids. Ethyl esters and free acid forms of oleic acid (C18:1) and linoleic acid (C18:2), as well as unesterified octadecanoic, hexadecanoic, and tetradecanoic acids,[1] were inert in the reaction.

Among *n*-alkanes (C10 through C20) tested, KSM-MT66 cells *cis*-desaturated the alkanes with C13, C14, C15, C16, C17, C18, and C19, yielding corresponding unsaturated products with the yields of 3, 8, 25, 28, 18, 6, and 2 g/l after a 3-day incubation, respectively. Main products from these alkanes all had a double bond at the 9th carbon from the terminal methyl groups, as detected the base peak at m/z 173 of CH_3-$(CH_2)7$-C^+H-SCH_3 in every GC-MS spectra of the alkylthiolated products. In the case of *n*-tetradecane, a GC-MS spectrum of the alkylthiolated product (M^+, m/z 290) showed an additional fragment pair of m/z 131 and 159 (20%). This indicates the formation of a positional isomer with a double bond at the 8th carbon from the terminal methyl group, as in the *n*-hexedecane desaturation by the mutant KSM-B-3M cells.[1]

Conversion of 1-chlorohexadecane and 1-chlorooctadecane by KSM-MT66 cells yielded *cis*-unsaturated products at 28 and 32 g/l after a 3-day incubation, respectively. As in the case of 1-chlorohexadecane,[1] a double bond was introduced mainly at the 9th carbon from the terminal methyl group of 1-chlorooctadecane, since a main fragment pair of m/z 173 (base peak) and 207 (70%) was observed in a GC-MS spectrum of the alkylthiolated products (M^+, m/z 380). Two positional isomers having a double bond at the 7th (10%) and 8th carbons (20%) from the terminal methyl groups were also formed, as estimated by the relative abundance of the corresponding fragment pairs of m/z 145 and 235 and m/z 159 and 221, respectively.

In summary, the patterns of desaturation of aliphatic compounds by *Rhodococcus* sp. strain KSM-MT66 are illustrated in the Figure. Generally, alkanes are *cis*-desaturated mainly at the 9th carbon from their terminal methyl groups, and acyl fatty acids are *cis*-desaturated mainly at the 6th carbon from their carbonyl carbons. The difference in posi-

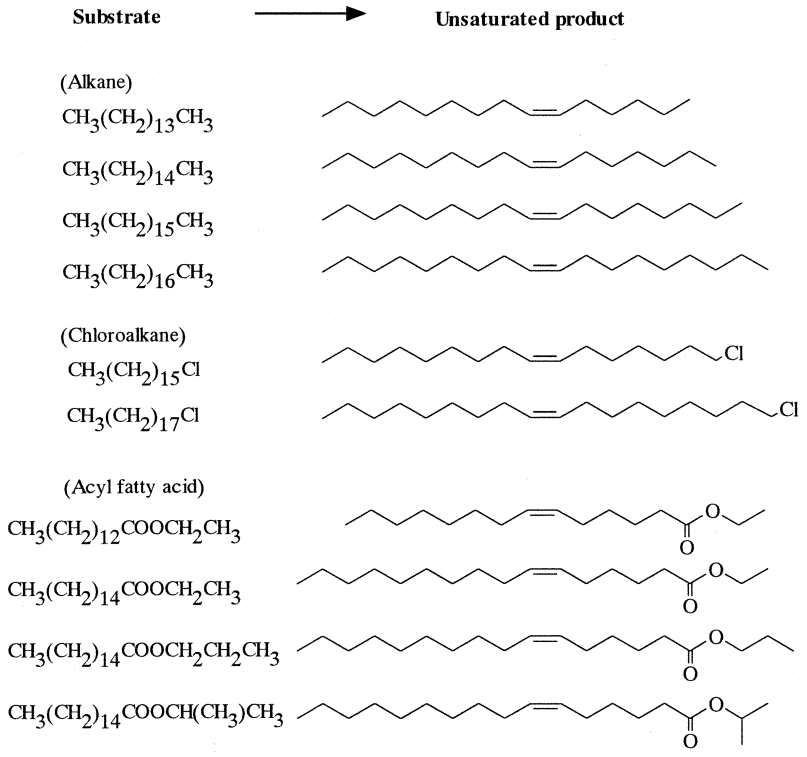

Figure. The Patterns of Regiospecific Desaturation of Aliphatic Substrates by *Rhodococcus* sp. Strain KSM-MT66 Cells.
The figure illustrates typical examples of the desaturation reactions catalyzed by the rhodococcal cells.

tional specificity toward alkanes and acyl fatty acids has not been clarified yet. These desaturation reactions by the rhodococcal mutant cells appear to be very unique and different from the well-known CoA-linked fatty acid desaturase reactions[5-8] and a subterminal oxidation system for *n*-alkanes.[9]

Acknowledgments

This study was financially supported by the Ministry of Trades and Industries of Japan, the New Energy and Industrial Technology Development Organization, and the Japan Association of Biotechnology.

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