Note

Carotenogenesis Pathway of Novel Carotenoid Glucoside Mycolic Acid Esters in *Rhodococcus rhodochrous* Using Carotenogenesis Mutants and Inhibitors

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Received May 26, 1999; Accepted July 2, 1999

Carotenogenesis in Nocardioform actinomycete *Rhodococcus rhodochrous* was investigated using carotenogenesis mutants and inhibitors, and a postulated carotenogenesis pathway was proposed. At the end of the synthesis, fatty acid or mycolic acid was esterified by different esterases to the same C-6 hydroxyl group of β-δ-glucoside.

**Key words:** carotenogenesis mutant; carotenogenesis pathway; carotenoid glucoside mycolic acid ester; *Rhodococcus rhodochrous*

*Rhodococcus*, a Gram-positive and aerobic bacterium, belongs to Nocardioform actinomycetes, and forms rods to extensively branched substrate mycelium. *Rhodococcus rhodochrous* forms red to orange colonies. Its color is due to carotenoid pigments, which, including novel carotenoid glucoside mycolic acid esters, have already been identified (Fig. 1); a monocyclic carotenoid with a tertiary hydroxyl group at C-1′ (carotenoid B), a keto derivative of carotenoid B (carotenoid K), their β-δ-glucosides at C-1′ (carotenoids B-G and K-G), their fatty acid monoesters at a C-6 hydroxyl group of the β-δ-glucoside moiety (carotenoids B-G-FA and K-G-FA), and their mycolic acid monoesters at the same position of the glucoside moiety (carotenoids B-G-Myc and K-G-Myc). The fatty acid composition of carotenoid glucoside fatty acid esters was different from that of the total cellular lipids and was influenced by growth temperature, while the mycolic acid composition of carotenoid glucoside mycolic acid esters was similar to that of the total cellular mycolic acids.

All of the carotenogenesis genes including zeaxanthin glucosylase have been cloned from *Erwinia*, and the characteristics of their products have been investigated. UDP-glucose is the origin of glucoside. Little is known, however, about the pathway of the synthesis of carotenoid glycoside esters even now. In *Myxococcus* sp. MY-18, the formation of monocyclic carotenoid glucoside fatty acid esters has been proposed that glucose is bound to carotenoid and then fatty acid is esterified to glucoside, but the pathway was different from that of *Myxococcus fulvus* Mx2. From *Myxococcus xanthus*, a cluster of several carotenogenesis genes for carotenoid glucoside fatty acid esters have been cloned, but the functions of their products have not been identified yet. In this study, we investigated both the carotenoid compositions of some carotenogenesis mutants and the effects of carotenogenesis inhibitors. From these results, a postulated carotenogenesis pathway in *R. rhodochrous* was proposed.

*Rhodococcus rhodochrous* RNMS1 (IAM 13988, IFO 14894, JCM 7553) was used as a wild-type strain. Carotenogenesis mutants were obtained by a treatment of the wild-type strain with N-methyl-N-nitro-N-nitrosoguanidine. Colonies with different colors were selected. The cells were grown in a nutrient broth with shaking at 30°C for 72 h. To inhibit carotenogenesis, 18 μM diphenylamine (DPA) or 6.2 mM nicotine was added to the medium. The cells were harvested with a GF/F glass microfiber filter (Whatman, U.S.A.), washed with 0.9% NaCl, and stored at −30°C. Carotenoids were extracted from the harvested cells with chloroform-methanol (1:2, v/v) several times. The combined extract was washed with 30 mM Tris-HCl (pH 8.0) buffer containing 2% NaCl. After centrifugation, the chloroform fraction was collected and evaporated. The pigments were dissolved in chloroform-methanol (3:1, v/v) and were analyzed with HPLC equipped with a μBondapak C18 column (Waters, U.S.A.) and a photodiode array detector. Methanol was an eluent for 6 min, followed by a linear gradient of chloroform to 25%, and 28.5 min an isocratic chloroform-methanol (1:3, v/v) at a constant flow rate of 2.0 ml/min. The molar absorption coefficient at 458 nm of carotenoids in the eluent of HPLC was assumed to be the same.

The wild-type strain produced eight kinds of carotenoids, the chemical structures of which have been identified previously and are shown in Fig. 1. HPLC elution profile has been shown in Fig. 1 of ref. 3. Major components were carotenoid glucoside fatty acid esters, and novel carotenoid glucoside mycolic acid esters were also found (Table 1). From the results described below, a predicted carotenogenesis pathway is proposed in Fig. 2. This pathway and the presence of the carotenogenesis enzymes were further confirmed using the carotenogenesis mutants and the inhibitors.

A white mutant P-1 accumulated only phytoene, and might be a phytoene desaturase mutant. DPA is known.
Carotenogenesis in *Rhodococcus rhodochrous*

![Chemical Structure](image)

<table>
<thead>
<tr>
<th>Carotenoid</th>
<th>X</th>
<th>Y</th>
<th>Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>K</td>
<td>O</td>
<td>H</td>
<td></td>
</tr>
<tr>
<td>B-G</td>
<td>H</td>
<td>G</td>
<td>H</td>
</tr>
<tr>
<td>K-G</td>
<td>O</td>
<td>G</td>
<td>H</td>
</tr>
<tr>
<td>B-G-FA</td>
<td>H</td>
<td>G</td>
<td>FA</td>
</tr>
<tr>
<td>K-G-FA</td>
<td>O</td>
<td>G</td>
<td>FA</td>
</tr>
<tr>
<td>B-G-Myc</td>
<td>H</td>
<td>G</td>
<td>Myc</td>
</tr>
<tr>
<td>K-G-Myc</td>
<td>O</td>
<td>G</td>
<td>Myc</td>
</tr>
</tbody>
</table>

*Fig. 1. Structures of Carotenoids from *Rhodococcus rhodochrous*. G, glucoside; FA, fatty acid; Myc, mycolic acid.*

![Chemical Reactions](image)

**Table 1. Carotenoid Compositions (% of Total Carotenoids) of Wild-type and Mutant Strains of *Rhodococcus rhodochrous***

<table>
<thead>
<tr>
<th>Carotenoid</th>
<th>Strain</th>
<th>Wild</th>
<th>GM-1a</th>
<th>F-1</th>
<th>F-2</th>
<th>K-1</th>
<th>K-2</th>
</tr>
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<tbody>
<tr>
<td>B</td>
<td></td>
<td>2</td>
<td>31</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>K</td>
<td></td>
<td>1</td>
<td>6</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B-G</td>
<td></td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>13</td>
<td>4</td>
</tr>
<tr>
<td>K-G</td>
<td></td>
<td>7</td>
<td>4</td>
<td>26</td>
<td>61</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B-G-FA</td>
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<td>25</td>
<td>19</td>
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<td>0</td>
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<td>91</td>
</tr>
<tr>
<td>K-G-FA</td>
<td></td>
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<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B-G-Myc</td>
<td></td>
<td>3</td>
<td>0</td>
<td>11</td>
<td>5</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>K-G-Myc</td>
<td></td>
<td>13</td>
<td>0</td>
<td>55</td>
<td>31</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>

*Fig. 2. Postulated Carotenogenesis Pathway in *Rhodococcus rhodochrous*. Parentheses indicate mutants and inhibitors. In this figure, enzymes except for ketolase act at the vertical arrows in both sides, and ketolase acts at all of the horizontal arrows.*

\[ \text{Phytoene} \downarrow \quad \text{Phytoene desaturase (P-1, DPA)} \]
\[ \quad \downarrow \quad \text{Lycopene cyclase or } \gamma-\text{-carotene synthase (C-1)} \]
\[ \gamma-\text{-Carotene} \quad \rightarrow \quad 4\text{-Keto-}\gamma-\text{-Carotene} \]
\[ \quad \downarrow \quad \text{Hydratase (H-1, Nicotine)} \]
\[ \text{Carotenoid B} \quad \rightarrow \quad \text{Carotenoid K} \]
\[ \quad \downarrow \quad \text{Glucosyl transferase (GM-1)} \]
\[ \text{Carotenoid B-G} \quad \rightarrow \quad \text{Carotenoid K-G} \]
\[ \quad \downarrow \quad \text{Fatty acid esterase (F-1, F-2)} \]
\[ \text{Carotenoid B-G-FA} \quad \rightarrow \quad \text{Carotenoid K-G-FA} \]
\[ \text{Mycolic acid esterase (GM-1)} \]
\[ \quad \rightarrow \quad \text{Carotenoid B-G-Myc} \quad \rightarrow \quad \text{Carotenoid K-G-Myc} \quad \rightarrow \quad \text{Ketolase (K-1, K-2)} \]

An amount of 33% can be obtained from 16% wild-type strain or 84% mutant.

*Fig. 2. Postulated Carotenogenesis Pathway in *Rhodococcus rhodochrous*. Parentheses indicate mutants and inhibitors. In this figure, enzymes except for ketolase act at the vertical arrows in both sides, and ketolase acts at all of the horizontal arrows.*

A mutant C-1 accumulated two carotenoids, which were not found in the wild-type strain. From absorption spectra and the retention times on HPLC (data not shown), they seemed to be dihydroxyxycopene diglucoside and dihydroxyxycopene di-(glucoside ester). Small amounts of other carotenoids found in the wild type were also produced. This mutant had low activity to form \( \gamma \)-carotene from lycopene, and without this step, further synthesis occurred. Since one of the \( \psi \) end groups of lycopene was crystallized to the \( \beta \) end group to form \( \gamma \)-carotene in the wild-type strain, this enzyme was lycopene cyclase or \( \gamma \)-carotene synthase (Fig. 2).

A mutant H-1 produced only two carotenoids, 16% \( \gamma \)-carotene and 84% 4-keto-\( \gamma \)-carotene of total carotenoids. These carotenoids were identified with absorption spectra, the retention times on HPLC, and the relative molecular weights after purification (data not shown). Although nicotine is known to inhibit hydroylation to the C-1, 2 double bond of the \( \psi \) end group by H2O and cyclization to form the \( \beta \) end group, both inhibitions are depending on the species. When the wild-type strain was cultured with nicotine, 50% \( \gamma \)-carotene, 46% 4-keto-\( \gamma \)-carotene, 2% carotenoid B and 2% carotenoid K of total carotenoids were found. This mutant H-1 was a hydratase mutant and nicotine inhibited only hydroylation not cyclase (Fig. 2).

A mutant GM-1 accumulated 33% \( \gamma \)-carotene, 31% carotenoid B and 6% carotenoid K of total carotenoids (Table 1). This mutant had low activity of glucosyl transferase (Fig. 2).

Red mutants F-1 (KA-2, previous name in ref. 3) and F-2 accumulated carotenoid glucoside mycolic acid esters but not carotenoid glucoside fatty acid esters (Table 1). HPLC elution profile of F-1 has also been shown in Fig. 1 of ref. 3. These mutants inhibited fatty acid esterase for glucoside. On the other hand, the mutant GM-1 could not produce carotenoid glucoside mycolic acid esters, and this might be a double mutant. Since there were two types of the mutants, F-type and GM-1, carotenoid B-G might be esterified to form its fatty acid esters by fatty acid esterase or to form its mycolic acid esters by mycolic acid esterase. Fatty acid esterase might be specific enzyme for this reaction, since the fatty acid composition of carotenoid glucoside fatty acid esters was very different from that of total cellular fatty acids. Since the esterified position of fatty acid and mycolic acid is the same C-6 hydroxyl group of glucoside, these esterases must act competitively at the end of carotenogenesis (Fig. 2). Furthermore, attachment of
esterified glucose to carotenoid B or K was excluded in this bacterium.

Since these four mutants, that is, hydratase, glucosyl transferase, fatty acid esterase and mycolic acid esterase mutants, accumulated corresponding both B and K-type carotenoids (Table 1), the end groups, the β (B-type) and the 4-keto-β (K-type) end groups, were not responsible to the activity of these enzymes.

Yellow mutants K-1 and K-2 accumulated B-type carotenoids (carotenoids B, B-G, B-G-FA and B-G-Myc) but not K-type carotenoids (carotenoids K, K-G, K-G-FA and K-G-Myc) (Table 1). These mutants were the ketolase mutants. Although all of B-type carotenoids and γ-carotene could have a keto group, it was not sure that the enzyme could oxidize all of B-type carotenoids or one was the suitable substrate in the cells (Fig. 2). In the case of ketolase from Agrobacterium aurantiacum, wide substrate specificities have been reported.54 When the mutant K-1 was cultured with nicotine, only γ-carotene was accumulated as expected.

In conclusion, the postulated carotenogenesis pathway of R. rhodochrous is proposed in Fig. 2 using carotenogenesis mutants and inhibitors. At the end of the synthesis, fatty acid or mycolic acid was esterified by different esterases to the same C-6 hydroxyl group of β-δ-glucoside. In this paper, we described only typical mutants, but we obtained many similar mutants. These carotenogenesis mutants might be useful for analysis of the carotenogenesis genes, and functions of carotenoids and carotenoid derivatives, such as protection of highlight and/or UV-light, and scavenger activity for active oxygens.

References