

Overexpression of Carotenoid Pathway Genes *dxs*, *crtE*, *crtI*, and *crtB* in *Rhodococcus erythropolis* AN12

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Abstract

The carotenoid pathway has not been studied in great depth in *Rhodococcus*. We studied the carotenoid pathway in *Rhodococcus erythropolis* AN12 by attempting to overexpress several genes of the carotenoid pathway. We cloned the genes and introduced them into expression vectors. The expression vectors were introduced into wildtype *R. erythropolis* AN12 and their effect on carotenoid accumulation was measured. Expression vectors containing *dxs*, which encodes 1-deoxy-D-xylulose-5-phosphate (DOXP) synthase, or *crtE*, which encodes geranylgeranyl pyrophosphate synthase, increased the carotenoid accumulation approximately two fold when compared to the controls. In contrast, overexpression of *crtB* and *crtI* had no obvious effect on carotenoid accumulation in *R. erythropolis* AN12. This study presents the first analysis of carotenoid biosynthetic pathway in *Rhodococcus*.

Introduction

The carotenoid pathway in *Rhodococcus*, and most bacteria, is largely understudied. A better understanding of carotenoid synthesis in bacteria such as *Rhodococcus* has important commercial as well as scientific implications (Oh et al., 2000). Carotenoids function as antioxidants because they reduce free radical concentrations and consume O₂ (Nelson et al., 2003). In nature, carotenoids serve as precursors to vitamins, including vitamin A, and phytohormones, and function as accessory pigments in photosynthetic membranes. Carotenoids are also animal feed additives, as they are necessary to the health of animals but found in minimal levels in the starchy feed. Carotenoids are used as pigments for salmon and other food products, and represent a large portion of the salmon farming cost (Lorenz and Cysewski, 2000).

Carotenoid biosynthesis in bacteria starts with the dxp (non-mevolanate) pathway, which forms isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) (Kim and Keasling, 2001). A multi-step pathway converts IPP and DMAPP to geranylgeranyl pyrophosphate (GGPP), the precursor molecule to carotenoids. Enzymes encoded by the *crt* genes consume GGPP to form specific carotenoids (Figure 1) (Misawa et al, 1995).

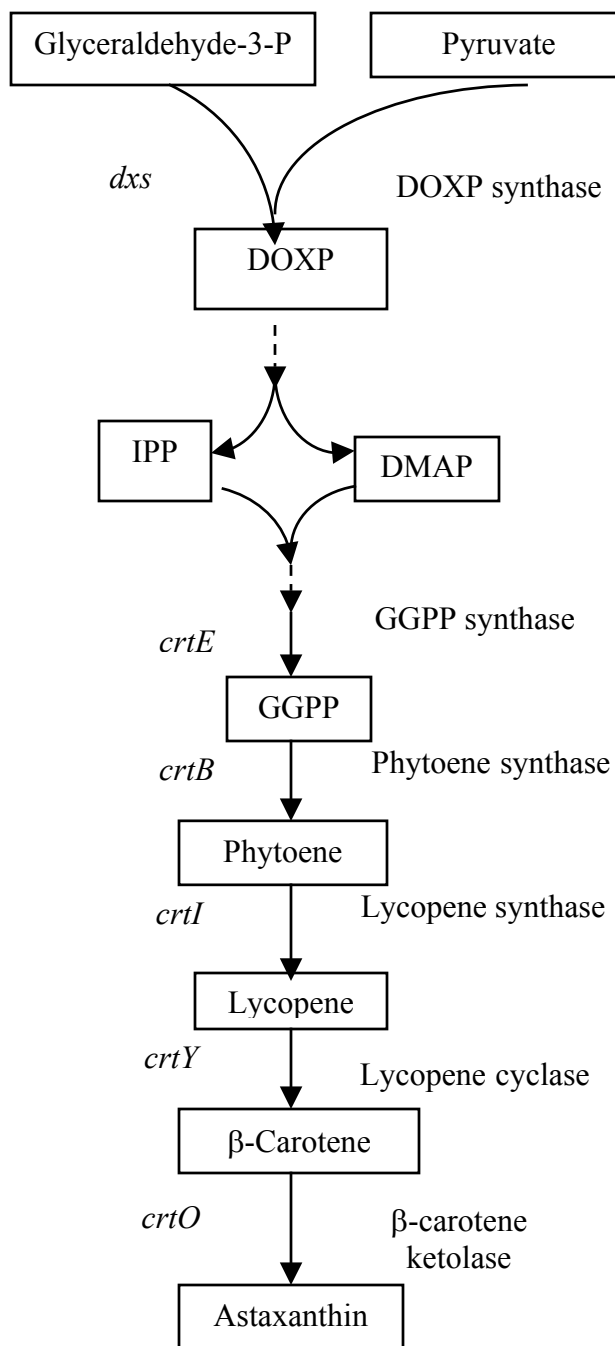


Figure 1. Carotenoid biosynthetic pathway. Metabolite names are boxed. Gene names are indicated to the left of the arrows, while the names of the enzymes they encode are listed to the right. Dashed lines indicate multi-step processes (individual genes and enzymes not shown). DOXP stands for 1-deoxy-D-xylulose-5-phosphate; GGPP stands for geranylgeranyl pyrophosphate

Carotenoid gene expression has been manipulated in many organisms, including yeast, *Escherichia coli*, *Rhodococcus*, and plants (Kostichka et al., 2003; Mann et al., 2000; Verdoes et al., 2003; Matthews and Wurtzel, 2000). Overexpressing *crtI* and *crtYB* in the yeast *Xanthophyllomyces dendrorhous* caused a significant increase in total accumulated carotenoids. In *X. dendrorhous*, the *crtYB* gene serves the same function as the separate bacterial *crtY* and *crtB* genes. *E. coli*, which does not naturally produce carotenoids, accumulated beta-carotene when *crtE*, *crtI*, *crtB*, and *dxs* were expressed from a plasmid (Verdoes et al, 2003).

Unlike *E. coli*, *Rhodococcus* naturally produce carotenoids, such as beta-carotene. Some strains of *Rhodococcus* accumulate high levels of carotenoids. In addition to being able to make these compounds, *Rhodococcus erythropolis* AN12 is also relatively easy to transform with expression vectors. While these features are attractive, the AN12 strain does not accumulate carotenoids as effectively as other *Rhodococcus* strains.

In this study, we overexpressed four carotenoid genes - *crtB*, *crtE*, *crtI*, and *dxs*- individually in *R. erythropolis* AN12. This method allowed us to assess whether the amount of enzyme available is a significant controlling factor for the carotenoid pathway in *Rhodococcus*.

Materials and Methods

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. All DNA-modifying enzymes and associated buffers were purchased from New England Biolabs (Beverly, MA), unless otherwise noted.

Bacterial Strains and Plasmids

All bacterial strains and plasmids used in this study are listed in Table 1. All cell cultures were grown in LB broth (5 g/L yeast extract, 5 g/L sodium chloride, 10 g/L tryptone, pH 7.5) with appropriate antibiotics, unless otherwise noted. *E. coli* cultures were grown at 37°C. *Rhodococcus* cultures were grown at 30°C. Kanamycin (Kan) was added at a concentration of 100 mg/L to cultures that contained pCR2.1-TOPO plasmids or its derivatives. Spectinomycin (Spec) was added at a concentration of 100 mg/L to cultures containing pJP10 or its derivatives. Frozen stocks were made of each bacterial strain by adding 0.8 mL 40% glycerol to 0.8 mL of cell culture and storing at -80°C.

Polymerase Chain Reaction (PCR)

PCR was performed using the primers shown in Table 2. They were all purchased from Integrated DNA Technologies, Inc. (Coralville, IA). The PCR reaction consisted of 10 µL 10x PCR buffer, 20 µL Q-solution, 2 µL dNTP mix (10 mM each dNTP), 1 µL *R. erythropolis* AN12 genomic DNA, 1 µL 5' primer (100 mM), 1 µL 3' primer (100 mM), 0.5 µL Hot Star Taq (2.5 units), and 64.5 µL distilled water. PCR buffer, Q-solution, dNTP mix, and Hot Star Taq were all purchased from Qiagen Inc. (Valencia, CA). The PCR reaction was heated to 95°C for 15 minutes to activate the Hot Star Taq. The reaction was then put through 35 cycles of 95°C for 30 seconds, 52°C for 30 seconds, and 72°C for 2 minutes and 30 seconds. After completion of the cycles, the reaction stayed at 72°C for an additional 10 minutes. The amplified reaction was stored at 4°C until use.

Agarose Gel Electrophoresis and Gel Purification

All agarose gels were run in 1% agarose (ISC Bioexpress; Kaysville, UT) in 1x TAE buffer (10x TAE was purchased from Invitrogen (Carlsbad, CA)). The gel was stained with 0.5 µg/mL ethidium bromide. Loading dye (15% Ficoll, 0.1% Bromphenol Blue, 0.1% Xylene Cyanol) was added to the samples at a concentration of 0.3 µL per 1 µL of sample. The samples were run at 100 V in 1x TAE buffer. The size standard used was the 1 kb Ladder from New England Biolabs. The DNA was extracted and purified from the gels using the GeneClean II Kit (Bio101; La Jolla, CA), following the manufacturer's instructions.

Table 1. Bacterial strains and plasmids used in this study.

Abbreviations: SpecR indicates spectinomycin resistance; AmpR indicates ampicillin resistance; KanR indicates kanamycin

Bacterial Strains	Description	Reference/Source
<i>Escherichia coli</i> One Shot Top10	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) Φ80 <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>recA1</i> <i>araD139</i> Δ(<i>ara-leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> (Str ^R) <i>endA1</i> <i>nupG</i>	Invitrogen
<i>Escherichia coli</i> XL1-Blue	<i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>hsdR17</i> <i>supE44</i> <i>relA1</i> <i>lac</i> [F' <i>proAB</i> <i>lacI</i> ^q ZDM15 Tn10 (Tet ^r)]	Stratagene (La Jolla, CA)
<i>Rhodococcus erythropolis</i> AN12	Transformable; Produces carotenoids	Kostichka et al. (2003)

Plasmids	Description	Reference/Source
pCR2.1-TOPO	KanR, AmpR, <i>ColE1</i> origin	Invitrogen
pJP10	<i>Trc</i> promoter, SpecR, KanR, NG2 origin, inducible <i>lac</i> promoter	P. Lessard, MIT Biology Dept.
pTOPO-B	pCR2.1-TOPO derivative with <i>crtB</i> PCR product insertion	This work
pTOPO-E	pCR2.1-TOPO derivative with with <i>crtE</i> PCR product insertion	This work
pTOPO-I	pCR2.1-TOPO derivative with <i>crtI</i> PCR product insertion	This work
pTOPO-dxs	pCR2.1-TOPO derivative with <i>dxs</i> PCR product insertion	This work
pJP10-B	pJP10 derivative with <i>crtB</i> inserted downstream of <i>trc</i> promoter	This work
pJP10-E	pJP10 derivative with <i>crtE</i> inserted downstream of <i>trc</i> promoter	This work
pJP10-I	pJP10 derivative with <i>crtI</i> inserted downstream of <i>trc</i> promoter	This work
pJP10-dxs	pJP10 derivative with <i>dxs</i> inserted downstream of <i>trc</i> promoter	This work

resistance; NG2 ori is an origin of replication that is able to replicate in *R. erythropolis* AN12; *trc* is an IPTG-inducible promoter; *ColE1* origin is an origin of replication capable of replicating in *E. coli*.

Table 2. Primers used in this study.

Primer Name	Sequence (5' to 3')	Use(s)	Flanking Restriction Enzyme Sites	Expected PCR Product Size
<i>crtB</i> -down	GCATGCGCGATCACAAGATCATCGCA	PCR; sequencing	<i>SphI</i>	1338 bp
<i>crtB</i> -up	GCTGCAGGGAAGATCCACTCAGACTTCGT	PCR; sequencing	<i>PstI</i>	
<i>crtB</i> -middown	CGTCGCGGATGAAGTTGGT	sequencing		
<i>crtB</i> -midup	CCAACTTCATCCGCGACGT	sequencing		1571 bp
<i>crtE</i> -down	GCTGCAGGGATCGAGCTTGTGCACAACGA	PCR; sequencing	<i>PstI</i>	
<i>crtE</i> -up	GGATCCGGAATCTCAGGATCACGCA	PCR; sequencing	<i>BamHI</i>	
<i>crtE</i> -middown	GCTGATGTCGAGGAATTGGCCA	sequencing		2170 bp
<i>crtE</i> -midup	GGCCAATTCCTCGACATCAGCA	sequencing		
<i>crtI</i> -down	GCACTAGTGGATGAATCCTCGGTTACGA	PCR; sequencing	<i>SpeI</i>	
<i>crtI</i> -up	GCATGCCAATTCCTCGACATCAGCA	PCR; sequencing	<i>SphI</i>	2192 bp
<i>crtI</i> -middown	GCTCAGATCCTTGACTGCT	sequencing		
<i>crtI</i> -midup	CGTGAAGCAGTCAAGGA	sequencing		
<i>dxs</i> -down	GCACTAGTCGAGATTATCGTGAGACGA	PCR; sequencing	<i>SpeI</i>	2192 bp
<i>dxs</i> -up	GCGGCCGCCCATTTGTCTGTGATTGCGA	PCR; sequencing	<i>NotI</i>	
<i>dxs</i> -middown	GGTCATCGTTCATGCCGT	sequencing		
<i>dxs</i> -midup	GCATGAACGATGACCGGT	sequencing		

TOPO Cloning

PCR products were inserted into the pCR2.1-TOPO vector and transformed into *E. coli* One Shot Top10 chemically competent cells using reagents and protocols provided by Invitrogen.

Plasmid Purification

Plasmids from *E. coli* cultures of less than 10 mLs were purified using Perfectprep Plasmid Mini Kits (Eppendorf; Westbury, NY), following the manufacturer's instructions. Plasmids from larger *E. coli* cell cultures (200 mL) were purified using the Wizard Plus Maxiprep from Promega (Madison, WI), according to accompanying instructions.

Sequence Verification

Sequencing reactions were carried out by the MIT Biopolymers Lab, using ABI Prism Big Dye v. 3.0 on an AB 3700 Capillary Automated Sequencer (both from Applied Biosystems; Foster City, CA). The sequencing reactions were prepared according to MIT Biopolymers Lab specifications. Analysis was performed using BLAST software (Altschul et al, 1990).

Ligation and Transformation of Plasmids

Ligations were performed using T4 ligase and the instructions provided by New England Biolabs. Transformations into *E. coli* XL1-Blue electrocompetent cells were performed by adding 2 μ L ligated DNA to 40 μ L thawed cells. The transformations sat on ice for 5 minutes before being electroporated at 2.5 kV, 25 μ F and 200 Ω using a Bio-Rad Gene Pulser (Bio-Rad; Hercules, CA). They were then recovered in 300 μ L SOC (Invitrogen) and placed at 37°C for 1 hour. All transformations were then plated on LB Kan agar plates and returned to 37°C. Transformations into *R. erythropolis* AN12 were performed by adding 3 μ L plasmid DNA to 70 μ L thawed cells. The transformations sat on ice for 5 minutes. They were then electroporated at 2.5 kV, 25 μ F and 400 Ω . They were recovered with 300 μ L NBYE (8 g/L nutrient broth (Difco; Detroit, MI), 5 g/L Bacto yeast extract (Difco)) and placed at 30°C for 2 hours. The transformations were then plated on LB Spec agar plates and returned to 30°C.

Electrocompetent *R. erythropolis* AN12 cells

A 10 mL culture of *R. erythropolis* AN12 was grown in LB at 30°C. 100 μ L of this culture was used to inoculate 200 mL NBYE with 0.05% Tween-80. This culture was grown at 30°C until the OD(600nm) was between 0.8 and 1.0. The cells were pelleted by spinning them at 6000RPM for 5 minutes. The pellet was resuspended in 30 mL ice-cold water and pelleted again as before. The pellet was again resuspended in ice-cold water and pelleted. The pellet was washed with 10 mL ice-cold 10% glycerol and centrifuged at 8000RPM for 5 minutes. The final cell pellet was resuspended in 1 mL of ice-cold 10% glycerol. The cells were aliquoted and stored at -80°C.

Genomic DNA Preparation

A 10 mL overnight culture of cells was grown in LB. The cells were pelleted by spinning at 6000 RPM for 5 minutes. The pellet was frozen at -20°C for one hour. The

pellet was resuspended in 250 μ L lysozyme (10 mg/mL) (Sigma-Aldrich) in TE (10 mM TrisHCl, 1 mM EDTA, pH 8.0). 20 μ L mutanolysin (1 mg/mL; Sigma-Aldrich) was added, and the reaction was shaken at 37°C for one hour. 50 μ L 0.5M EDTA, 50 μ L 10% SDS, and 50 μ L 5M sodium chloride were added, and the mixture was gently shaken to mix. 10 μ L proteinase K (20 mg/mL) was added, and the reaction was then incubated at 37°C for 1 hour. 50 μ L sodium perchlorate solution (1g/mL) was added to the cell slurry, which was then mixed gently. The DNA was extracted with one volume of phenol:chloroform:isoamyl alcohol (25:24:1). The slurry was then mixed well and centrifuged for 5 minutes in a microcentrifuge. The aqueous phase was extracted with 0.5 volumes chloroform:isoamyl alcohol (24:1). The mixture was then spun down in the microcentrifuge for 5 minutes. To the aqueous phase, 1 volume of isopropanol was added. This mixture was then centrifuged for 15 minutes in the microcentrifuge. The DNA was washed with 70% ethanol. The pellet was then dried and resuspended in 200 μ L TE. The DNA was stored at 4°C.

Carotenoid Extraction

200 mL cultures of *R. erythropolis* AN12 were grown in medium-rare defined medium (1.4 g/L (NH₄)₂SO₄, 1.0 g/L MgSO₄-7H₂O, 0.015 g/L CaCl₂-2H₂O, 1 g/L MOPS, 0.5 mg/L FeSO₄-7H₂O, 0.4 mg/L ZnSO₄-7H₂O, 0.02 mg/L MnSO₄-H₂O, 0.015 mg/L H₃BO₃, 0.01 mg/L NiCl₂-6H₂O, 0.25 mg/L EDTA, 0.05 mg/L CoCl₂-6H₂O, 0.005 mg/L CuCl₂-2H₂O, 2.0 mg/L NaMoO₄-2H₂O, 5.0 mg/L FeNa-EDTA, 3.98 g/L K₂HPO₄ and 1.65 g/L KH₂PO₄) with 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) and appropriate antibiotics. 10 mLs of the culture was spun down at 6500 RPM for 5 minutes. The cell pellet was resuspended in 1 mL of 1 M phosphate buffer (113 g/L K₂HPO₄, 47 g/L KH₂PO₄), transferred to an eppendorf tube, and spun down in a microcentrifuge for 5 minutes at 14000 RPM. The supernatant was discarded, and the wet weight of the pellet was determined. The pellet was resuspended in 5 mL/g methanol and spun down for 5 minutes at 14000 RPM. The supernatant was removed and stored in foil. The methanol extraction was performed 4 more times, saving the supernatants in separate tubes. The A(462 nm) was measured of each sample. The fractions were then pooled, filter-sterilized, and the A(462 nm) was measured of the pool. The volume of the pool was measured, and the milligrams of carotenoids were determined by the following formula:

$$\text{mg carotenoids} = \text{Volume}_{\text{pool}} * A(462 \text{ nm})_{\text{pool}} * 1000/25000$$

Results

Four expression vectors were created that individually expressed the *dxs*, *crtE*, *crtI*, and *crtB* genes. The expression vectors were then transformed into *R. erythropolis* AN12 and induced to express their respective genes. Carotenoid extractions were then carried out on the transformants.

Generation of PCR Products

Primers were designed to flank the sequences of the targeted genes' open reading frames. The open reading frames were provided by Phil Lessard (MIT Biology

Department). Each primer to be used for PCR had an added restriction enzyme digest site (the flanking restriction enzyme site) on the 5' end of the primer (Table 2). PCR was then performed using the appropriate primer pairs. The template for the PCR reactions was *R. erythropolis* AN12 genomic DNA, which was extracted by a genomic preparation. The PCR products were then run on an agarose gel and expected band sizes were viewed (Figure 2).

Generation and Verification of pTOPO Plasmids

The PCR products were purified from the agarose gel on which they had been run. A TOPO-cloning was then performed on each separate PCR product. The TOPO-cloning reactions were transformed into chemically-competent TOP10 cells and plated onto LB Kan plates with 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal). White colonies, which had interrupted *lacZ* genes because of an insertion, were picked and minipreparations were performed. Three restriction enzymes digests were performed to verify that the expected PCR products had been incorporated into the pCR2.1-TOPO vector. The *EcoRI* digest was performed on all plasmids, in order to check the size of the insert, as *EcoRI* sites flank the insert site on pCR2.1-TOPO (Figures 3 and 4). To further evaluate these plasmids, two additional restriction enzymes digests were performed in each case, in which the restriction enzymes cut the expected insert. Maxipreparations were then performed on all samples that appeared correct after the three verification digests. These samples were again verified by three appropriate restriction enzyme digests. Maxipreparation samples that appeared correct were then sequenced and verified by comparing them to the provided sequence (data not shown).

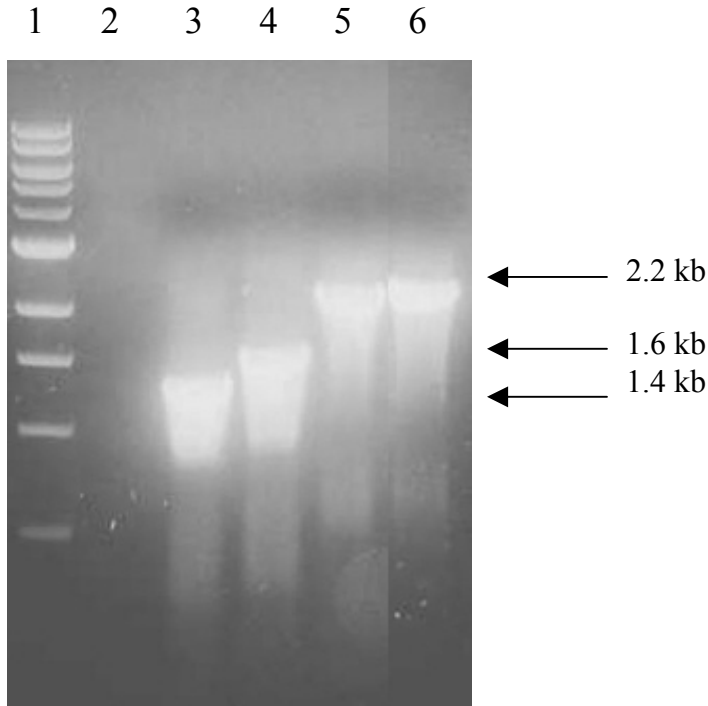


Figure 2. PCR products. Lane 1 contains the 1 kb ladder. Lane 2 is empty. Lane 3 contains the *crtB* PCR product that was created using primers *crtB*-down and *crtB*-up. Lane 4 contains the *crtE* PCR product that was created using primers *crtE*-down and *crtE* up. Lane 5 contains the *crtI* PCR product that was created using primers *crtI*-down and *crtI*-up. Lane 6 contains the *dxs* PCR product that was created using primers *dxs*-down and *dxs*-up.

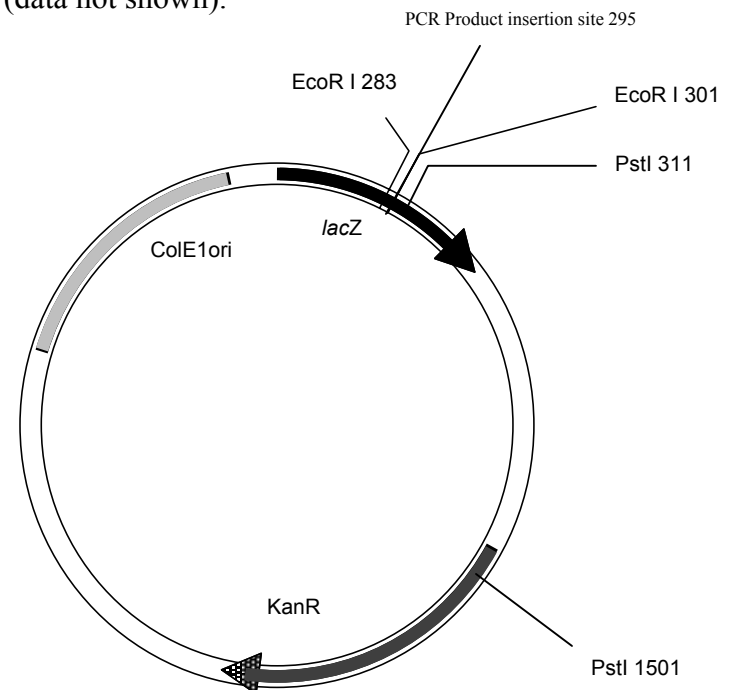


Figure 3. pCR2.1-TOPO vector. The ColE1 ori is the origin of replication. KanR indicates kanamycin resistance. *lacZ* is a gene that allows metabolism of X-gal. PCR product insertion site at 295 bp is where the TOPO-cloning reaction inserts PCR products that have been created by HotStarTaq. The pCR2.1-TOPO vector is 3908 base pairs long.

Generation and Verification of Expression Vectors

In order to create the expression vectors to be transformed into *R. erythropolis* AN12, the purified pTOPO plasmids, which were obtained by maxipreparation, were digested with the two flanking restriction enzymes except in the case of *dxs*. pTOPO-*dxs* was digested with *SpeI* and *PstI*. The maxipreparation protocol was performed on *E. coli* strain JM109 (pJP10) to purify the expression vector, pJP10. A sample of pJP10 was digested with the corresponding restriction enzymes that had been used to cut the pTOPO-derivative plasmids. The digests were run on an agarose gel and appropriate bands were excised (data not shown). The purified fragments were then ligated.

The ligated plasmids were transformed into *E. coli* XL1-Blue cells and grown on LB Spec plates to select for spectinomycin resistance. Colonies that grew were then picked and minipreparations were performed. The plasmids were verified by three restriction enzyme digests, including digestion with both of the restriction enzymes used to create the plasmids (this digest will be referred to as the dropout digest from this point on). Plasmids that produced the expected fragments in all of the minipreparation verification digests were then isolated on a larger scale via the maxipreparation protocol. Three restriction enzymes digests, including the dropout digest (Figure 5), verified the composition of plasmids recovered from the maxipreparations (Figure 6).

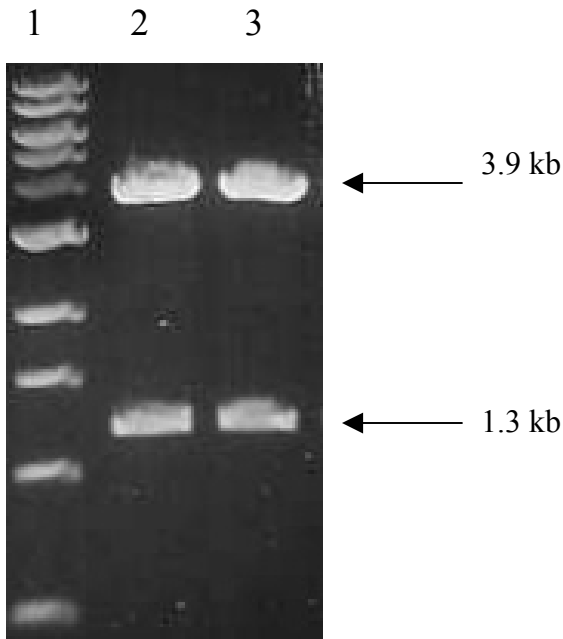


Figure 4. EcoRI digest of pTOPO-B. Lane 1 contains the 1 kb ladder. Lane 2 and Lane 3 contain two samples of suspected pTOPO-B minipreparation samples. Both were found to have correct insertions.

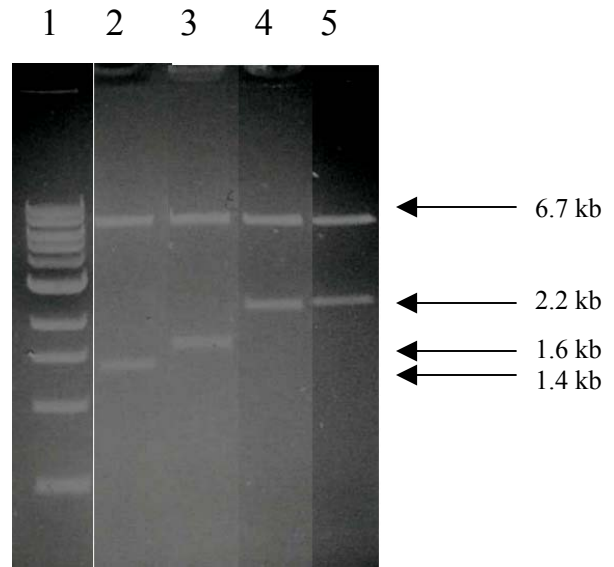


Figure 5. Test digests of pJP10-derivatives. Lane 1 contains the 1 kb ladder. Lane 2 contains the pJP10-B dropout digest products. Lane 3 contains the pJP10-E dropout digest products. Lane 4 contains the pJP10-I dropout digest products. Lane 5 contains the pJP10-*dxs* dropout digest products. All products were correct.

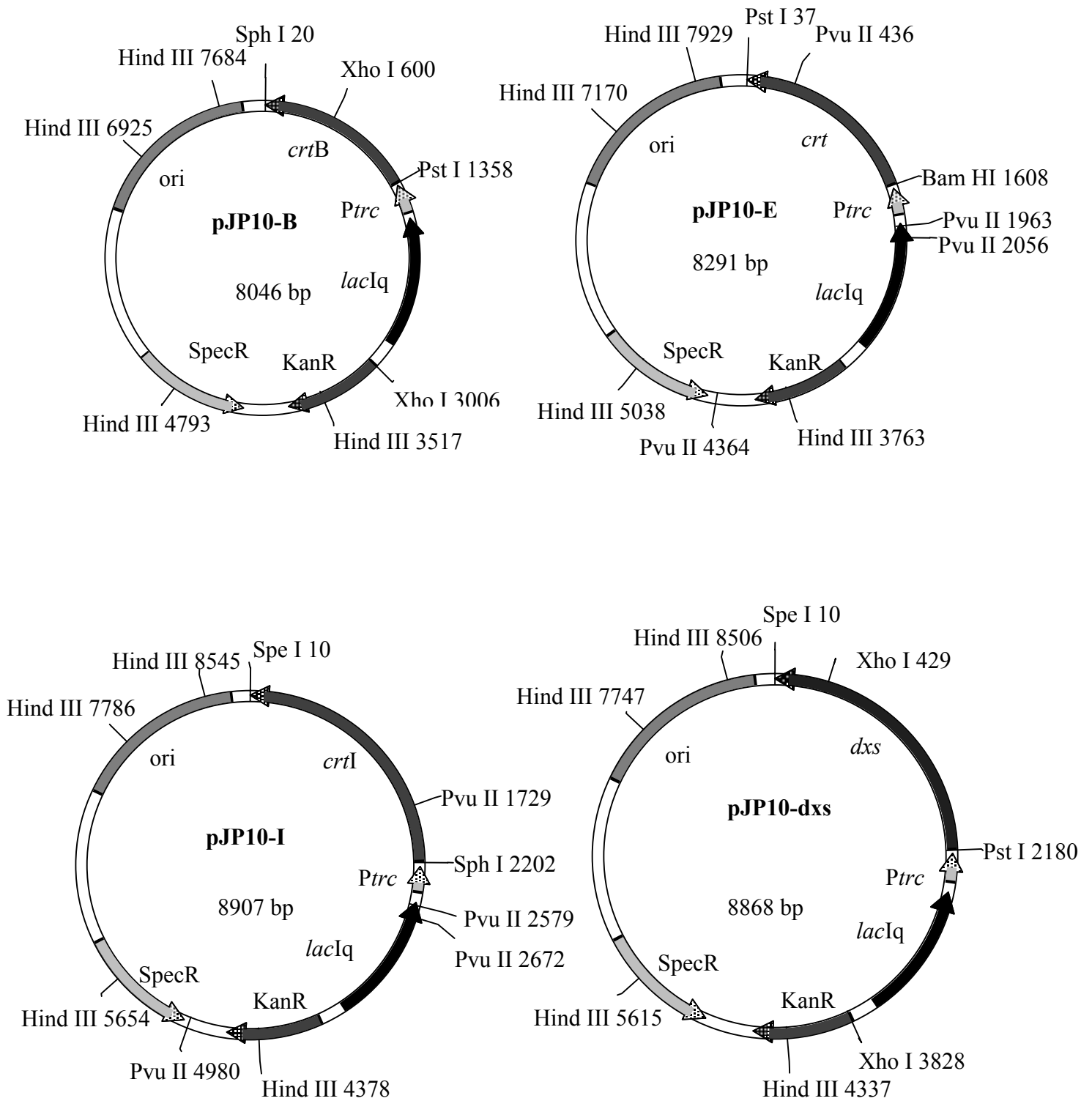


Figure 6. pJP10 derivative plasmids. *Ptrc* is the IPTG-inducible promoter that expresses the inserted genes. *Ori* is the origin of replication, which is capable of replicating in AN12. *SpecR* indicates spectinomycin resistance. *KanR* indicates kanamycin resistance. *lacIq* is the *lac* repressor gene. Restriction enzyme sites are provided for use in verification digests.

Expression in *R. erythropolis* AN12

Electrocompetent *R. erythropolis* AN12 cells were transformed with the pJP10-derivative plasmids. Transformants were selected using the spectinomycin resistance of the pJP10-derivative plasmids. We then performed the genomic preparation procedure on selected colonies. Verification restriction digests were performed on the genomic DNA, along with genomic DNA from untransformed *R. erythropolis* AN12 DNA (Figure 7).

Genomic preparation samples were also verified by plasmid rescue, which consisted of transforming XL1-Blue competent cells with samples of genomic DNA isolated from the candidate *R. erythropolis* AN12 transformants. The cells were plated on LB Spec plates, to select for spectinomycin resistance, and plasmids were recovered via the miniprep protocol. The plasmids were digested with three restriction enzymes and verified by comparing the resulting bands on an agarose gel to those of similarly-digested reference plasmids with which the *R. erythropolis* AN12 cells had originally been transformed (data not shown).

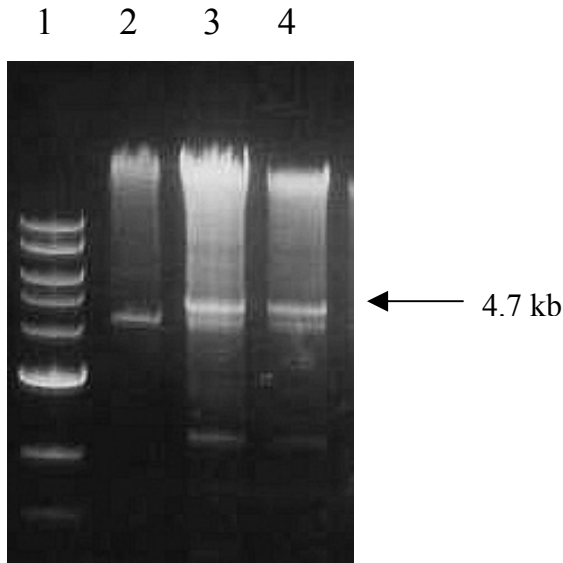


Figure 7. HindIII digest of wildtype and AN12 and AN12 with pJP10-I. Lane 1 contains the 1 kb ladder. Lane 2 contains wildtype *R. erythropolis* AN12 genomic DNA. Lanes 3 and 4 contain two samples of genomic preparation samples of *R. erythropolis* AN12 with pJP10-I transformants. Note that whereas the wildtype genomic DNA produces a band at approximately 4 kb, the transformed strains carry additional bands a approximately 4.7 kb and approximately 2.1 kb, as expected from pJP10-I.

Carotenoid Extraction of *R. erythropolis* AN12 Transformants

Carotenoid extractions were performed on three 120-hour cultures of each successful *R. erythropolis* AN12 transformants carrying either of pJP10-I, pJP10-E or pJP10-dxs. One carotenoid extraction was performed each on an unaltered pJP10 transformant in *R. erythropolis* AN12 and a pJP10-B transformant (Figure 8). The pJP10-dxs transformants had a marked increase of accumulated carotenoids when compared to the control [AN12(pJP10)]. Similarly, the pJP10-E transformants appeared to have increased carotenoid accumulation, when evaluated against that of the pJP10 transformant. Neither the pJP10-B transformant nor the pJP10-I transformants showed a convincing difference in accumulated carotenoids from the control.

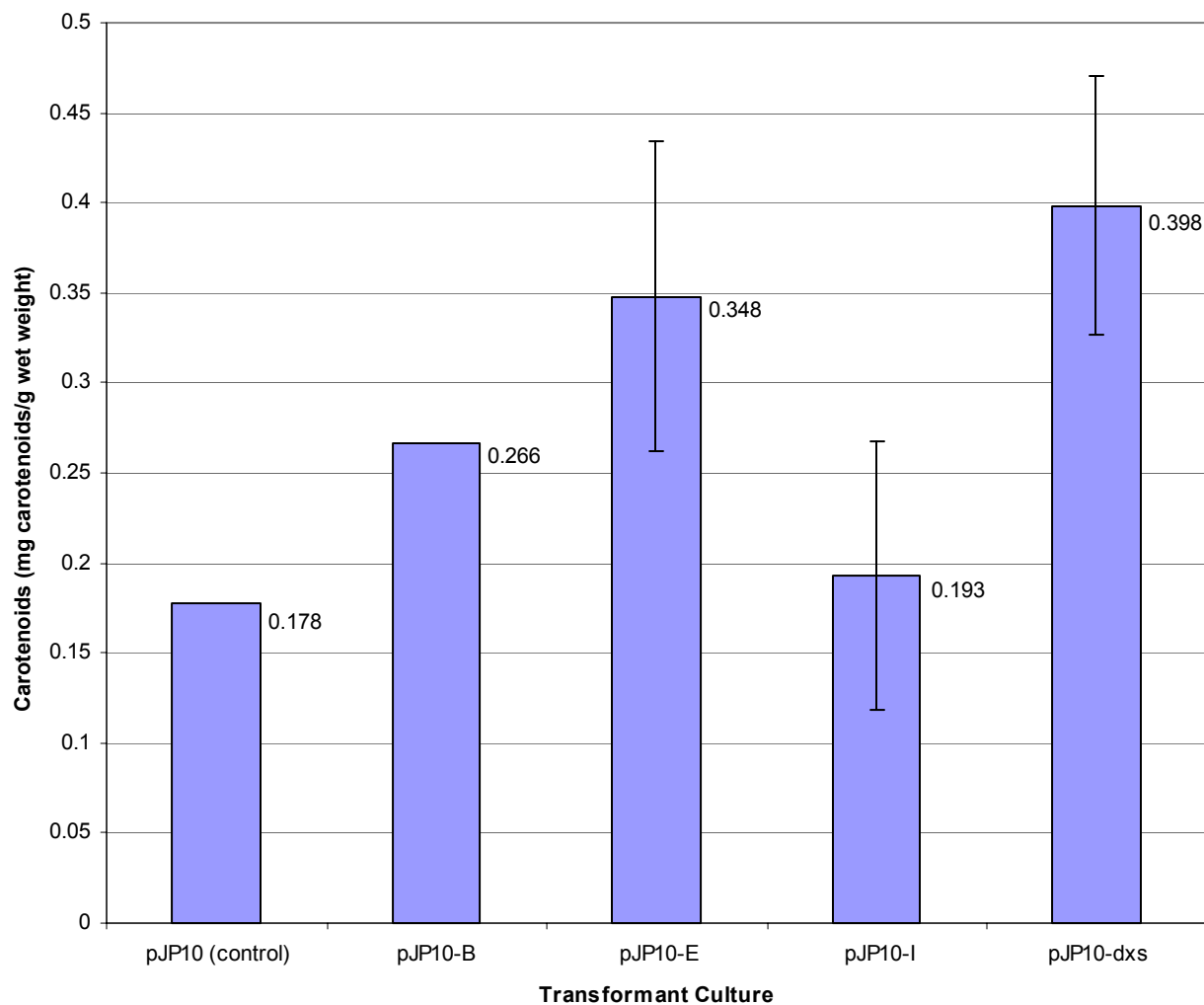


Figure 8. Carotenoid accumulation. This graph shows the carotenoid accumulation of the transformants after 120 hours. The Y-axis represents the amount of carotenoids accumulated, in mg carotenoids per gram wet weight. The X-axis represents the culture. The top of the bar indicates the mean carotenoid amount, with the numerical value written to the right of the bar. The error bars represent the standard deviation of the data.

Discussion

Very little is known about the carotenoid biosynthetic pathway in *Rhodococcus*. While we know that *Rhodococcus* has all the genes of the pathway, practically nothing else is known about the regulatory mechanisms that control carotenoid accumulation. We are trying to study these regulatory mechanisms by overexpressing the *dxs*, *crtE*, *crtB*, and *crtI* genes and gauging the contribution each corresponding enzyme has on the total carotenoid accumulation. From our results, we found that overexpression of *crtE* and overexpression of *dxs* had strong effects on carotenoid accumulation.

When *dxs* was overexpressed, we saw a greater than two fold increase in accumulated carotenoids. As the gateway gene to the isoprenoid pathway, *dxs* is suspected to have the greatest effect on carotenoid production out of the four genes in this study, because more carotenoids could potentially be formed by increasing the precursor molecules. As observed by Kostichka et al (2003), overexpression of the *R. erythropolis*

AN12 *dxs* has been performed in *R. erythropolis* CW23 and overall carotenoid production was doubled. Our results coincide quite accurately with these results, which shows that the results can be repeated, even under rather different circumstances, as separate carotenoid extraction methods and strains were used. Matthews and Wurtzel (2000) saw a greater than 10 fold increase of total carotenoids when *dxs* was overexpressed with *crtE*, *crtI*, and *crtB* in *E. coli*, when compared to just *crtE*, *crtI*, and *crtB*. Similarly, Lois et al. (2000) observed a strong correlation between *dxs* overexpression and carotenoid synthesis in plants. All of these sources, along with our own data, support that carotenoid synthesis regulation is, to some extent, controlled by the amount of enzyme present in the cell, as seen in plants and bacteria.

The *crtE* transformant increased the carotenoids approximately two fold. The increase caused by *crtE* is somewhat surprising. In bacteria, *crtE* is not the committed step in the carotenoid pathway, as GGPP is also used for diterpenes and geranylgeranylated proteins (Math et al., 1992). This concept, however, shows similarity to *dxs*, as DOXP synthase does not catalyze a committed step to carotenoid production, but a marked increase in carotenoid accumulation was still observed in many cases (Lois et al., 2000; Matthews and Wurtzel, 2000; Kostichka et al., 2003). By expressing *crtE* and a construct containing *crtI*, *crtB*, *crtY*, *crtZ* and *crtW*, Wang et al (1999) saw increased carotenoid expression over the 5-gene constructs and proposed that *crtE* was the first rate-controlling step for carotenoid production in *E. coli*. This finding would suggest that overexpressing *crtE* would have a greater effect than overexpressing the genes farther down the pathway, which coincides with our observations.

In contrast to *dxs* and *crtE*, neither *crtI* nor *crtB* appeared to have an evident effect, at least not within our conditions and our sample size. The committed step to the carotenoid pathway is phytoene synthase, *crtB*, so we would expect a noticeable increase in carotenoids produced. In this respect, our result was unexpected, in that there was no marked increase in carotenoid accumulation, when compared to the control. By overexpressing the endogenous phytoene synthase gene, *crtB*, Lindgren et al (2003) observed a significant increase in the total carotenoid content in *Arabidopsis*. Like *R. erythropolis*, *Arabidopsis* naturally produces carotenoids, so it is possible that this result should carry over to *Rhodococcus*, but our result does not seem to coincide with this idea. However, carotenoid extraction was only performed once on the pJP10-B transformant, so this may be reflect an artifact of the small sample size. Also, the product of phytoene synthase, phytoene, is not pigmented (Armstrong, 1994), so the transformant may be accumulating phytoene, but we were just unable to quantify it by our spectrophotometric method. Although other carotenoid genes, such as *crtI*, *crtO* and *crtY*, are farther down the carotenoid pathway, they are still likely candidates for increasing carotenoid production. The phytoene desaturase gene, *crtI*, which is also known as lycopene synthase, has been overexpressed in *Xanthophyllomyces dendrorhous* (Verdoes et al, 2003). In this case, a decrease in total carotenoids was observed. It is important to mention that a major shift in the distribution of individual carotenoid production was noted in this system, indicating that overexpressing the *crtI* gene may still act as a limiting factor for the latter part of the pathway. The distribution shift viewed by Verdoes et al. (2003) could be occurring in AN12(pJP10-I), as we did not distinguish the individual carotenoids that were formed.

Based on our results, the carotenoid pathway in *Rhodococcus* seems homologous to many of the pathways studied. This similarity suggests that the regulatory mechanism for the carotenoid pathway is controlled, in part, by the concentration of enzymes in the cell, and that the pathway can be manipulated by overexpressing the carotenoid genes. In order to have a more complete study of the enzymatic regulation of the carotenoid pathway in *R. erythropolis* AN12, overexpressing other genes of the carotenoid pathway, such as *crtY* and *crtO*, in the same manner, would be useful. Similarly, overexpressing multiple genes in one cell, especially *crtE* and *dxs*, would provide us with a more thorough understanding of the carotenoid pathway genes. This process could be performed by using multiple expression vectors, with different selectable markers, or by inserting multiple genes into a single expression vector. Ravello et al. (2003) saw an increase in carotenoid expression in when a three-gene construct containing *crtB*, *crtI* and *crtY* was overexpressed in *Brassica napus*. This increase was markedly greater than solely overexpressing *crtB*. This observation would suggest that overexpressing multiple carotenoid genes would permit greater carotenoid accumulation in *Rhodococcus* as well.

Despite these observations, it is clear that our research should be continued using more samples, so that statistical observations could be made on the carotenoids accumulated by transformants. In addition, the IPTG-responsiveness of the *trc* promoter in *R. erythropolis* AN12 was never determined in this study. While our data may suggest that overexpressing certain carotenoid genes increases overall carotenoid production, the level of overexpression of the encoded enzymes has not been directly determined. Thus, perhaps another expression vector would have allowed for a greater increase of carotenoids. Furthermore, the plasmids we have created can be used as tools to express multiple genes at a time in *R. erythropolis* AN12. The flanking restriction sites have been designed so that two genes can fit into the expression vector at a time.

Most bacterial carotenoid expression studies have been performed in *E. coli* (Wang et al., 1999; Matthews and Wurtzel, 2000), which has no natural carotenoid pathway (Gallagher et al., 2003). Thus, in order to study carotenoid accumulation in *E. coli*, the entire carotenoid pathway must be introduced. By looking at *Rhodococcus* instead of *E. coli*, we can explore the native carotenoid pathway, studying how the regulatory mechanisms interact with carotenoid accumulation in the cell. Given that the AN12 strain of *R. erythropolis* possesses the genes required for carotenoid synthesis and that this strain accumulates less carotenoids than many of its relatives, it seems reasonable that further genetic manipulation of this strain might unlock its biosynthetic potential. Continuation of this work may even allow *Rhodococcus* to accumulate higher levels of carotenoids than can more familiar genetically-modified hosts, such as *E. coli*.

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