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Comparative Analysis of β-Carotene Hydroxylase Genes for Astaxanthin Biosynthesis

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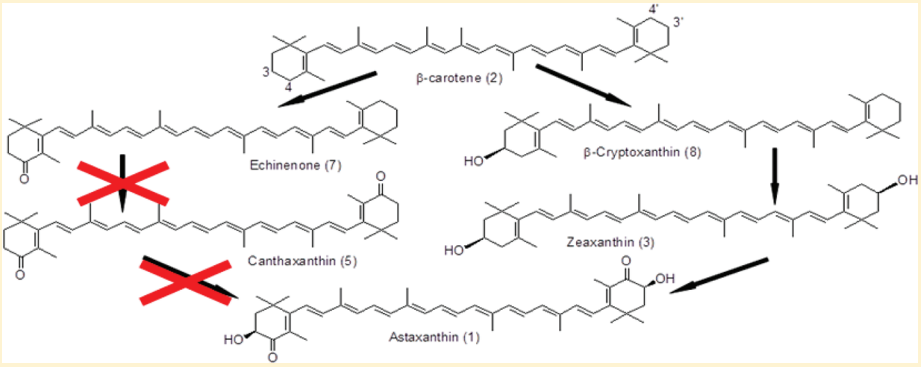
Supporting Information



ABSTRACT: Astaxanthin (3,3′-dihydroxy-4,4′-diketo-β-carotene) (**1**) is a carotenoid of significant commercial value due to its superior antioxidant potential, application as a component of animal feeds, and ongoing research that links its application to the treatment and prevention of human pathologies. The high commercial cost of **1** is also based upon its complex synthesis. Chemical synthesis has been demonstrated, but produces a mixture of stereoisomers with limited applications. Production from biological sources is limited to natural producers with complex culture requirements. The biosynthetic pathway for **1** is well studied; however, questions remain that prevent optimized production in heterologous systems. Presented is a direct comparison of 12 β-carotene (**2**) hydroxylases derived from archaea, bacteria, cyanobacteria, and plants. Expression in *Escherichia coli* enables a comparison of catalytic activity with respect to zeaxanthin (**3**) and **1** biosynthesis. The most suitable β-carotene hydroxylases were subsequently expressed from an efficient dual expression vector, enabling **1** biosynthesis at levels up to 84% of total carotenoids. This supports efficient **1** biosynthesis by balanced expression of β-carotene ketolase and β-carotene hydroxylase genes. Moreover, our work suggests that the most efficient route for astaxanthin biosynthesis proceeds by hydroxylation of β-carotene to zeaxanthin, followed by ketolation.

Carotenoids compose a vast, diverse and commercially important group of pigmented natural products that are produced ubiquitously by photosynthetic organisms. Colors vary from yellow through orange, to red. This chromophore results from the absorption of specific wavelengths of light by the variable system of conjugated double bonds at the center of carotenoid compounds.

Carotenoid biosynthesis proceeds from abundant C_5 isoprenoid precursors, isopentenyl diphosphate and dimethylallyl diphosphate, through to β-carotene (**2**). The subsequent biosynthesis of astaxanthin (**1**) from **2** is less well understood. Due to this, the biosynthetic pathway is commonly represented not as a linear pathway, but as a metabolic web (Figure 1). The complexity associated with the synthesis of **1** represents a significant challenge to metabolic engineers developing biological production systems for **1**.

The most common route for **1** biosynthesis requires two proteins, β-carotene ketolase (β-CK), to add keto functional groups to carbons 4 and 4′ of **2**, and β-carotene hydroxylase (β-CH), to add hydroxy functional groups to carbons 3 and 3′ of **2** (Figure 1). The complexity of **1** biosynthesis comes from a required cooperative function of these two proteins. Thus, optimal **1** biosynthesis requires either careful control of carbon flux along a specific reaction route, employing a single bifunctional protein with respect to substrate specificity, or the application of two bifunctional proteins.[1−3] This complexity is evident in the literature and further compounded by the lack of genetic characterization, or novel nature, of natural astaxanthin-producing organisms.[4]

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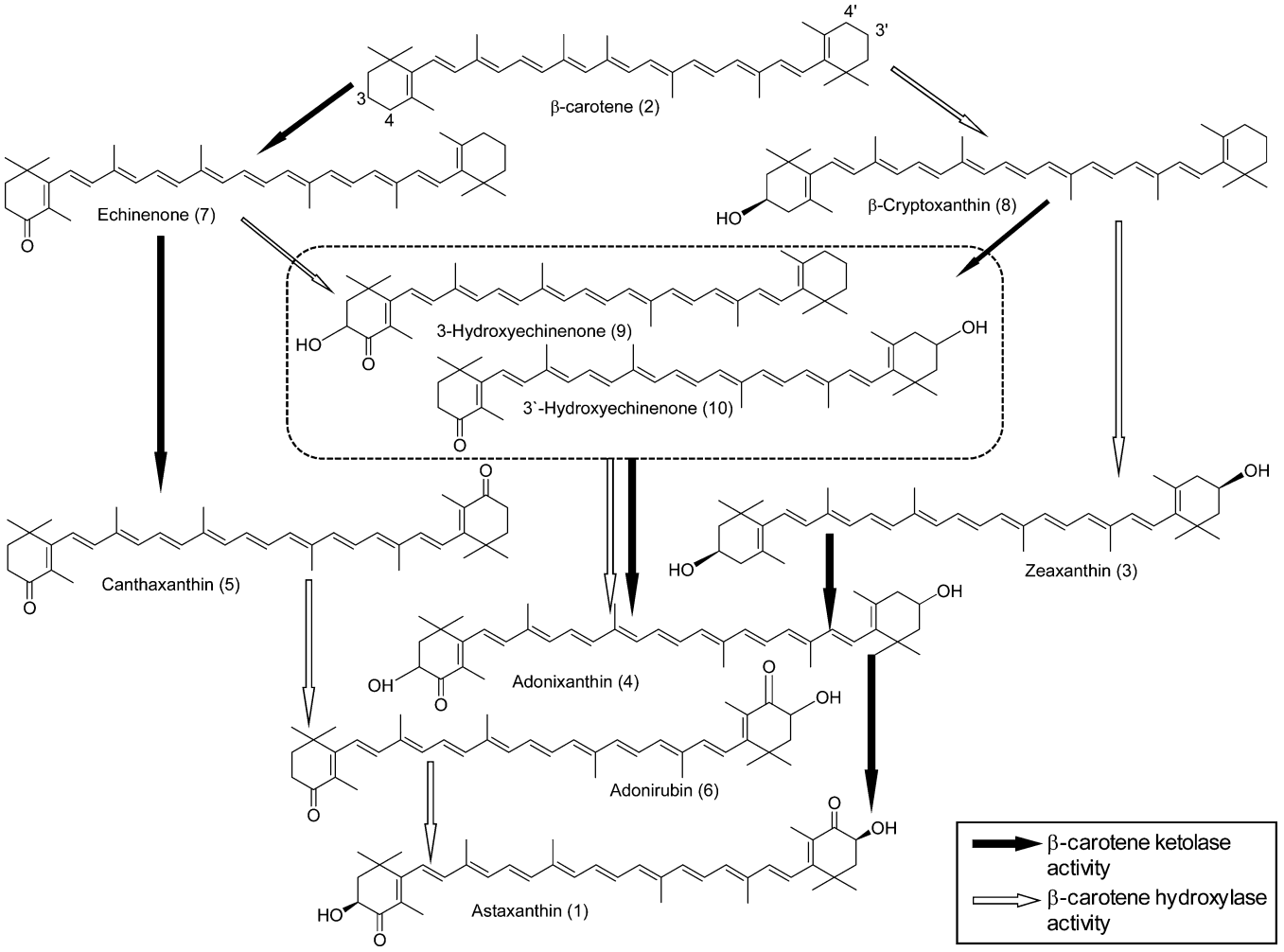

Figure 1. Proposed routes for the synthesis of astaxanthin (**1**) from β-carotene (**2**). The hashed box demonstrated that both echinenone (**7**) and β-cryptoxanthin (**8**) could function as precursor to both 3 and 3-hydroxyechinenone (**9** and **10**).

It has been hypothesized that **1** biosynthesis proceeds in a universal manner along all potential routes depicted in Figure 1.[2,3] Both reports also state that adonixanthin (**4**), a monoketolated product of zeaxanthin (**3**), was accumulated as the dominant intermediate.[2,3] This was hypothesized to be because the capacity of β-CK to use hydroxylated substrates is rate limiting.[5] The presence of canthaxanthin (**5**), or hydroxy derivatives thereof, was not reported, supporting a biosynthetic route progressing from **2** to **3** and then **1**. We recently demonstrated that efficient **1** biosynthesis does not proceed in a universal manner; rather, tight regulation of the relative expression levels of β-CK and β-CH genes is required, pushing carbon flux along the same route.[6] In doing this, the most efficient engineered **1** biosynthesis system reported to date was developed. More recently, it was demonstrated that reduced expression of β-CK with respect to β-CH increased **1** biosynthesis efficiency.[7] It has also been shown that β-CK activity on zeaxanthin (**3**) is the limiting step to **1** biosynthesis in bacterial and plant systems.[8] Importantly, each of these studies achieved >90% **1** within the final carotenoid extract.[6−8] This exceeds the concentration of **1** in *Haematococcus pluvialis*, a freshwater unicellular green microalga and the most prolific natural producer of **1**.[9,10] One measure of successful metabolic engineering toward a given goal is the production of the desired product at levels greater than or equal to those of a natural producer (with respect to yield and purity), at reduced cost. Thus, at lab scale, these studies hold potential to succeed in the development of commercially viable astaxanthin production processes.

The activity and function of β-CK proteins, with respect to **1** biosynthesis, has been studied extensively, while the function of β-CH proteins has been largely overlooked. Our work compared 12 β-CH proteins, including several novel candidates, within *Escherichia coli*, with respect to the hydroxylation of β-carotene (**2**) and canthaxanthin (**5**) as substrates. Subsequently, we employed the most suitable candidate β-CH proteins to generate a series of dual expression vectors for comparison of activity in **1** biosynthesis. This was done with the intention of identifying a superior β-CH for **1** biosynthesis, highlighting the limiting steps of **1** biosynthesis and demonstrating the importance of β-CH selection.

RESULTS AND DISCUSSION

Identification of Target β-Carotene Hydroxylase Genes. Analysis of known and putative β-CH genes in the public domain revealed significant diversity within this class of proteins. Of the putative and characterized β-CH proteins, 28 were selected on the basis of published literature describing capacity to function in **1** biosynthesis or novel nature. These were subjected to phylogenetic analysis using MEGA4[11]

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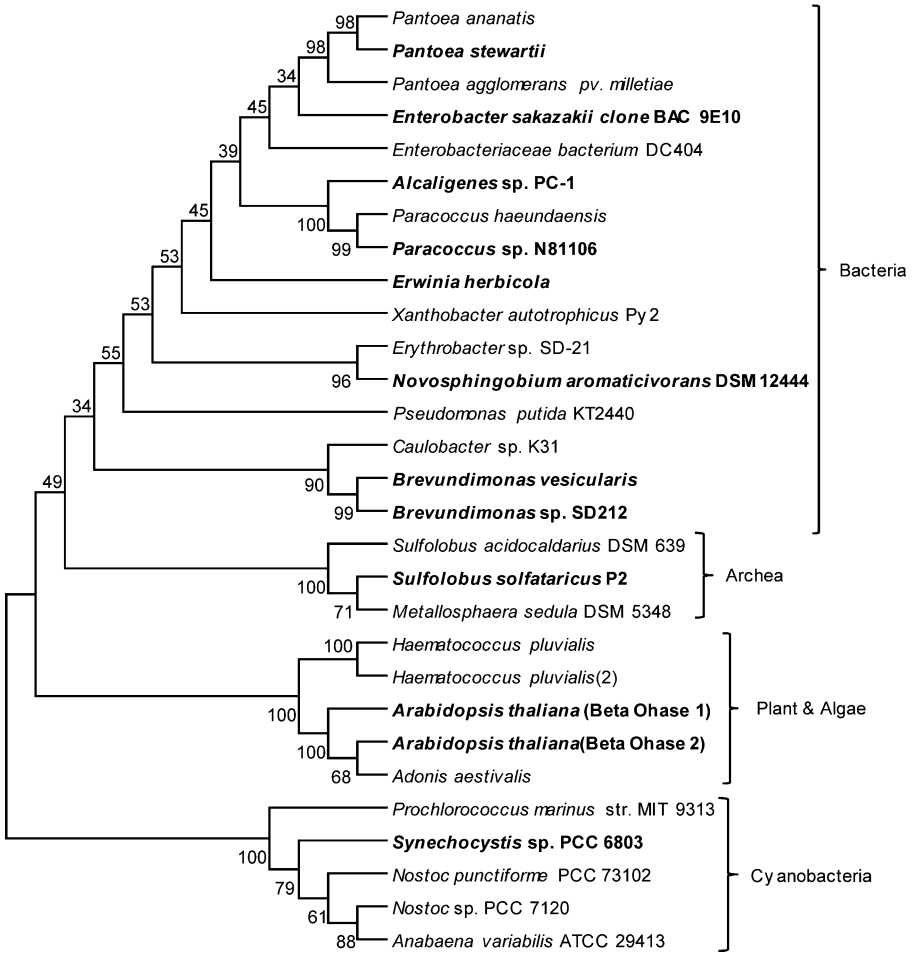

Figure 2. Phylogenetic analysis of β-hydroxylase genes, based on translated sequences. Target β-hydroxylase genes are highlighted in bold. Phylogenetic analyses was conducted in MEGA4[10] and inferred by the neighbor-joining method. Bootstrap consensus was inferred from 1000 replicates. Evolutionary distances were computed using the Poisson correction method (number of amino acid substitutions per site).

(Figure 2, selected β-CH genes are in bold text). This analysis demonstrated that in spite of significant diversity within the β-CH gene set, specific clustering of genes, based on taxonomic grouping, was observed. It has been previously reported that nonheme di-iron monooxygenase β-CHs can be categorized based on primary structure.[12]

As a group, plant and green algal β-CHs are highly conserved, sharing <30% protein identity with other β-CHs. Cyanobacterial β-CHs form a second distinct group, and nonphotosynthetic bacteria a third. *Sulfolobus solfataricus* P2, a thermoacidophilic archaeon, represents a fourth distinct class of β-CH. The extensive diversity within the β-CH protein class, homology to both β-CK and fatty acid desaturases, and a conserved functional mechanism[12,13] suggest that in spite of this diversity in primary structure, the catalytic mechanism is both robust and flexible.

Functional Characterization of Target β-Carotene Hydroxylase Genes. Twelve β-CH genes were expressed and directly compared in *E. coli*. For this, target genes were cloned into plasmid pBAD24 and expressed in *E. coli* strain TOP10 previously transformed with plasmid pAC-Beta,[14] using the stringent, arabinose inducible pBAD promoter.[15] *E. coli* strain TOP10 constitutively expressing carotenogenic genes from plasmid pAC-Beta accumulates **2** at 85 μg/g.[6] To account for variations in codon usage and protein primary sequence, potentially impacting protein expression level and solubility, coexpression of target β-CH genes was initially assessed in a screen fashion. Cultures were set up, and protein expression was induced with 0%, 0.02%, or 0.2% L-arabinose (final concentration in culture medium) (Table 1). As expected, application of L-arabinose impacted both total carotenoid biosynthesis and the carotenoid profile. Results of this screen were used for subsequent accurate quantification using the most suitable culture conditions derived from the screen, in triplicate (Figure 3 and Table 2).

Pantoea agglomerans-derived β-CH was considered the control due to its established function in **1** biosynthesis.[6] Thus, β-CH derived from *Pantoea stewartii*, *Alcaligenes* sp. PC-1, *Brevundimonas* sp. SD212, *Enterobacter sakazakii* BAC 9E10, *Novosphingobium aromaticivorans* DSM 1244, *Brevundimonas vesicularis*, and *S. solfataricus* P2 exhibited comparable or increased catalytic activity under used experimental conditions, when compared to the control. Conversely, the *Paracoccus* sp. N81106- and *Synechocystis* sp. PCC 6803-derived β-CH failed to produce significant hydroxy carotenoid products under all tested conditions. Function of these enzymes was demonstrated previously, suggesting that the employed expression conditions may be suboptimal for these proteins.[5,6,16,17]

Differences in culture conditions influence protein expression, solubility, and therefore activity. The purpose of this study was not to achieve optimal expression of each protein, and no true expression quantification was performed. Our goal was to

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Table 1. Quantification of Carotenoids (μg/g) Due to a β-Carotene Hydroxylase Expression Screen in *E. coli* Engineered to Accumulate β-Carotene (2)

sample	arabinose (%)	astaxanthin (1)	zeaxanthin (3)	canthaxanthin (5)	β-cryptoxanthin (8)	β-carotene (2)
P. stewartii	0	0.0	472	593	0.0	0.0
	0.02	37.1	34.3	0.0	0.0	0.0
	0.2	32.9	27.9	0.0	0.0	0.0
Alcaligenes sp. PC-1	0	0.0	0.0	0.0	0.0	904
	0.02	4.6	204	213	19.5	725
	0.2	3.7	169	234	12.0	727
Paracoccus sp. N81106	0	0.0	4.3	0.0	265	848
	0.02	0.0	38.9	63.9	0.0	732
	0.2	0.0	34.4	58.5	0.0	648
Brevundimonas sp. SD212	0	0.0	15.7	0.0	180	1022
	0.02	3.2	34.1	55.4	0.0	735
	0.2	0.0	30.5	66.9	0.0	753
P. agglomerans	0	0.0	155	311	20.1	7.9
	0.02	6.0	195	172	38.4	17.2
	0.2	2.8	120	94.0	5.6	2.2
N. aromaticivorans DSM 1244	0	5.0	0.0	0.0	145	802
	0.02	5.2	269	404	12.4	16.8
	0.2	9.3	320	389	15.5	13.6
E. sakazakii clone BAC 9E10	0	0.0	308	462	21.5	10.1
	0.02	5.8	407	504	25.6	12.1
	0.2	4.7	25.1	24.3	0.0	14.4
B. vesicularis	0	0.0	3.1	0.0	134	803
	0.02	6.0	290	423	15.1	17.0
	0.2	10.1	298	396	18.4	13.8
A. thaliana (Ohase 1)	0	0.0	0.0	0.0	140	1198
	0.02	0.0	137	180	1655	98.8
	0.2	0.0	161	224	1804	75.2
A. thaliana (Ohase 2)	0	0.0	0.0	0.0	25.4	1018
	0.02	0.0	53.4	0.0	1070	122
	0.2	0.0	45.1	0.0	1321	179
S. solfataricus P2	0	4.4	352	760	40.5	27.4
	0.02	0.0	14.5	0.0	0.0	697
	0.2	0.0	11.0	0.0	0.0	696
Synechocystis sp. PCC 6803	0	0.0	0.0	0.0	0.0	1103
	0.02	0.0	0.0	0.0	0.0	830
	0.2	0.0	0.0	0.0	0.0	829

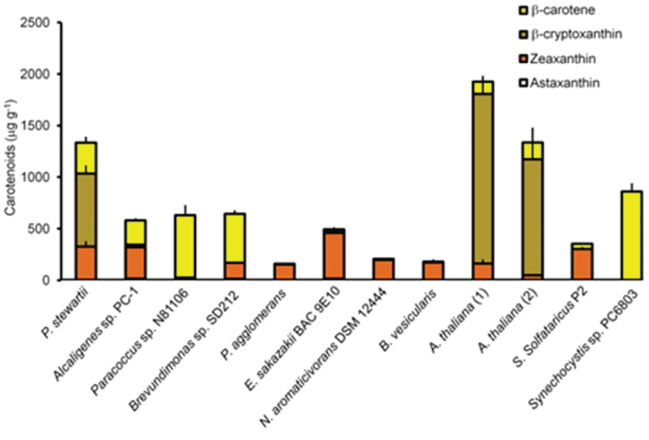

Figure 3. Accurate assessment of carotenoid profiles due to heterologous β-CH expression in *E. coli* accumulating β-carotene (**2**), under most suitable conditions. Data presented are the average of three biological replicates (n = 3) ± SD (standard deviation).

assess the function of each protein under standard conditions, thereby elucidating the most suitable for subsequent investigation.

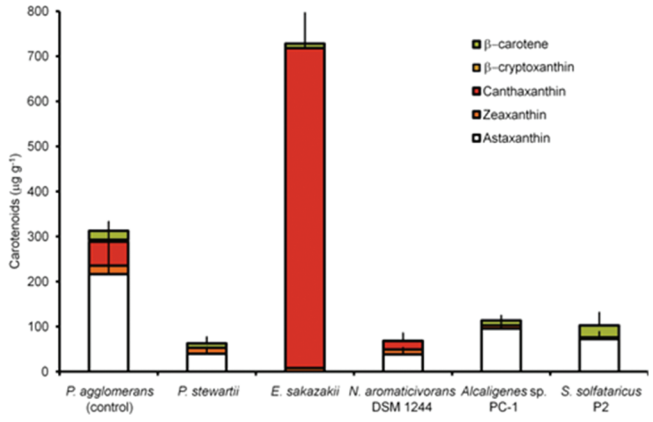

Figure 4. Comparison of astaxanthin biosynthesis due to different β-hydroxylase use (pBADAv.2W-β-CH) (n = 3) ± SD.

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Table 2. Quantification of Carotenoids (μg/g) (±SD) Due to Expression of β-Carotene Hydroxylase Proteins in *E. coli* Accumulating β-Carotene (2) (n = 3)

sample	astaxanthin (1)	zeaxanthin (3)	β-cryptoxanthin (8)	β-carotene (2)
P. stewartii	0.0 ± 0.0	327 ± 52.2	708 ± 79.8	299 ± 58.8
Alcaligene sp. PC-1[a]	15.7 ± 2.3	305 ± 27.7	25.2 ± 10.8	234 ± 22.2
Paracoccus sp. N81106	0.0 ± 0.0	27 ± 3.1	0.0 ± 0.0	605 ± 96.4
Brevundimonas sp. SD212[a]	13.3 ± 7.2	157 ± 5.2	0.0 ± 0.0	472 ± 36.7
P. agglomerans	10.1 ± 1.1	154 ± 6.2	4.1 ± 3.6	2.4 ± 0.5
E. sakazakii BAC 9E10[a]	15.1 ± 3.8	445 ± 39.7	26.6 ± 27.6	6 ± 10.4
N. aromaticivorans DSM 12444[a]	21.6 ± 0.2	197 ± 22.3	0.0 ± 0.0	9.8 ± 1.3
B. vesicularis[a]	3.1 ± 0.5	170 ± 29.8	0.0 ± 0.0	8.1 ± 2.3
A. thaliana (Ohase1)	0.0 ± 0.0	162 ± 37.8	1645 ± 176	118 ± 19.7
A. thaliana (Ohase2)	0.0 ± 0.0	48.3 ± 11.1	1125 ± 306	161 ± 57.8
S. solfataricus P2[a]	2.4 ± 0.2	303 ± 21.1	118 ± 16.8	50.7 ± 6.4
Synechocystis sp. PCC6803	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	860 ± 82.0

[a] β-CHs suitable for further investigation.

Table 3. Quantification of Carotenoids (μg/g) Due to Expression of β-Carotene Hydroxylase Proteins in *E. Coli* Engineered to Accumulate Canthaxanthin (5)

sample	arabinose (%)	astaxanthin (1)	zeaxanthin (3)	canthaxanthin (5)	β-cryptoxanthin (8)	echinenone (7)	β-carotene (2)
P. stewartii	0	34.7	61.2	724	80.9	19.4	319
	0.02	25.5	21.0	120	1.9	0.4	2.6
	0.2	26.4	22.3	114	0.0	0.0	3.3
Alcaligenes sp. PC-1	0	0.0	9.0	1073	15.9	23.8	316
	0.02	93.0	152	496	0.0	4.9	39.0
	0.2	116	159	439	0.0	4.7	39.5
Paracoccus sp. N81106	0	42.5	86.9	557	20.3	25.6	325
	0.02	31.7	48.7	504	20.5	5.6	107
	0.2	25.0	47.5	505	21.2	7.0	126
Brevundimonas sp. SD212	0	68.6	58.3	661	37.2	23.4	337
	0.02	39.1	158	269	0.0	1.9	10.8
	0.2	37.8	173	275	0.0	0.0	10.2
P. agglomerans	0	213	40.5	399	279	11.1	258
	0.02	26.2	14.1	731	18.5	7.5	99.8
	0.2	27.1	16.7	699	17.1	6.3	81.8
N. aromaticivorans DSM 1244	0	253	172	248	62.4	3.3	35.2
	0.02	9.4	6.4	969	20.2	7.7	125
	0.2	3.7	7.7	966	16.7	7.3	118
E. sakazakii clone BAC 9E10	0	0.0	6.4	1001	16.3	25.2	298
	0.02	55.7	85.8	310	0.0	3.5	26.4
	0.2	38.9	106	299	0.0	3.9	31.0
B. vesicularis	0	11.6	25.7	927	16.4	26.8	315
	0.02	58.0	107	538	0.0	5.6	57.8
	0.2	68.6	142	515	0.0	5.1	50.3
A. thaliana (Ohase 1)	0	0.0	16.6	1317	19.2	35.2	381
	0.02	66.0	45.2	524	205	7.7	156
	0.2	107	42.5	530	256	7.9	148
A. thaliana (Ohase 2)	0	0.0	10.5	1125	18.3	25.5	321
	0.02	66.1	14.0	1692	185	13.6	205
	0.2	53.7	13.1	1789	124	16.3	180
S. solfataricus P2	0	0.0	4.7	906	13.6	25.7	289
	0.02	213	79.4	306	44.0	3.0	30.3
	0.2	313	66.2	251	44.3	2.4	25.3
Synechocystis sp. PCC 6803	0	0.0	0.0	1095	15.8	28.2	342
	0.02	0.0	0.0	1245	22.6	21.7	224
	0.2	0.0	0.0	1144	21.9	16.5	167

Arabidopsis thaliana-derived β-CH proteins, Ohase 1 and Ohase 2, produced significant quantities of hydroxy carotenoids. However, of the total carotenoids produced, 81% and 82%, respectively, were composed of the monohydroxylated carotenoid β-cryptoxanthin (**8**). This high-level production of **8** was unique to the *A. thaliana*-derived β-CH protein, although the *P. stewartii*-derived protein also produced significant levels of **8** at 50% of total carotenoids.

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For *A. thaliana*-derived β-CH proteins, asymmetrical activity may be explained by the typical plant carotenoid profile, which is predominantly composed of lutein. Lutein, a metabolite of α-carotene, like **3** contains two hydroxylated rings. However, unlike **3**, lutein contains one β-ring and one ε-ring.[14] Thus, from an evolutionary perspective, plant-derived β-CH proteins have evolved to predominantly catalyze a monohydroxylation reaction and may not be suitable for application in **1** biosynthesis. The function of the *A. thaliana*-derived β−-CH has been reported previously,[13] as has its propensity to produce **8**. However, this was previously observed only when a 5′ *BglI* truncated gene was expressed in *E. coli*.[14]

Our work represents the first functional characterization of the *E. sakazakii* BAC 9E10-, *B. vesicularis*-, and *S. solfataricus* P2-derived β-CH gene. *S. solfataricus* P2 is an important model organism for the archaea. In spite of the unique biology and environmental niche of *S. solfataricus* P2, the derived protein exhibits significant activity in *E. coli*. Heterologous expression resulted in the synthesis of 354 μg/g total carotenoids, composed of 50.7 μg/g **2** and 303 μg/g **3**, with no **8** detected.

Expression of target β-CH genes within this system resulted in the synthesis of at least one unknown compound (≠ in Figure S1, Supporting Information). Although this compound eluted at the same time point as the first peak of the **5** standard (Figure S1, panel A), this lacks the characteristic second smaller peak of **5**. This compound is produced in varying quantities by all β-CH proteins investigated. On the basis of peak area, the *S. solfataricus* P2-derived protein is the most prolific producer of this compound.

Of the β-CH proteins studied, *Brevundimonas* sp. SD212, *Alcaligenes* sp. PC-1, and *Paracoccus* sp. N81106 have been compared previously via heterologous expression,[5] concluding that the *Brevundimonas* sp. SD212 β-CH was best suited for **1** production. However, no comparison was made between these proteins with respect to the hydroxylation of **2**. The *Brevundimonas* sp. SD212-derived β-CH, combined with a β-CK isolated from the same marine bacterium, was employed in the efficient biosynthesis of **1** in transgenic tobacco plants, producing **1** at 0.5% dry cell weight and 70% of total carotenoids.[18] For the first time we present the comparison of 12 β-CH proteins within a heterologous production host, revealing that the *A. thaliana*-derived β-CH Ohase 1 resulted in the greatest production of carotenoids within *E. coli*, at 1926 μg/g, although only 162 μg/g was **3**.

Additionally, the *E. sakazakii* BAC 9E10-derived protein produced the most **3**, at 445 μg/g, closely followed by *P. stewartii*, *Alcaligene* sp. PC-1, and *S. solfataricus*, producing 327, 305, and 303 μg/g **3**, respectively. This work therefore identified eight β-CH proteins as more active than the control, of which six were deemed suitable for further investigation with respect to **1** biosynthesis, based on **3** biosynthesis (indicated in Table 2). Improved use of **2** as a substrate may not directly correlate with increased capacity to function in **1** biosynthesis; thus, the capacity of the six selected β-CH genes to function in this manner was assessed via coexpression in *E. coli* TOP10 engineered to accumulate **5**.

Capacity to Function within Astaxanthin (1) Biosynthetic Pathways. Progressing from **2**, a level of indecision surrounds the route of **1** biosynthesis. Thus, β-CH proteins may, or may not, use keto-carotenoid substrates in efficient **1** biosynthesis, and this may be a gene-specific property rather than a universal rule. To investigate this, *E. coli* was modified to accumulate high levels of canthaxanthin (**5**) via transformation with plasmid pAC-Canth. pAC-Canth expresses a highly active β-CK derived from *A. variabilis* via the constitutive pTrc promoter.[6] **5** accumulating *E. coli* was transformed with target β-CH genes. Application of pAC-Canth resulted in the biosynthesis of total carotenoids at 1.49 mg/g, 89% **5**.[19] This efficiency was hypothesized to limit **2** availability within the cell due to its efficient conversion into **5**, facilitating an investigation into the capacity of β-CH proteins to hydroxylate **5**.

As described previously, the assay was completed in a screen format. Table 3 presents accurate quantification of carotenoids for which standards were available in our laboratory (Table S1). Completion of this assay resulted in the biosynthesis of several unknown compounds (Figure S2). This work did not demonstrate a correlation between unknown metabolites (based on presence/absence or peak area variance) and **1** biosynthesis. Therefore, for the purpose of this assay, it was not deemed necessary to identify or quantify these compounds.

Despite production of several unknown compounds, all target β-CH proteins analyzed (with the exception of that from *Synechocystis* sp. PCC 6803) participated in the biosynthesis of **1** (as a minor product). To the best of our knowledge, this is the first report of such activity for β-CH proteins isolated from *E. sakazakii* BAC 9E10, *N. aromaticivorans* DSM 1244, *B. vesicularis*, *A. thaliana* Ohase 1 and 2, and *S. solfataricus* P2. Under employed assay conditions, **5** biosynthesis was greater than its metabolism (based on accumulation within the cell), forming the dominant substrate for potential hydroxylation by the expressed β-CH proteins. However, biosynthesis of **1** remained low in all instances, suggesting that **5** represents a poor substrate for all employed β-CH proteins.

The presence of hydroxylated products of **2**, **3**, and **8** within the extract demonstrated that ketolase activity was not completely sufficient to prevent use of **2** by expressed β-CH proteins, but their levels remain significantly reduced. Thus, the route of **1** biosynthesis cannot be deduced from these data. Results demonstrated that efficient biosynthesis of **1** is not achieved by the high-level expression of β-CK, accompanied by various levels of expression of β-CH genes. Moreover, efficient **1** biosynthesis is not achieved when **5** is the dominant precursor. These facts were recently demonstrated elsewhere.[6,7] Therefore, this investigation supports the hypothesis that efficient **1** biosynthesis proceeds via hydroxylation of **2**, to **3**, and subsequently ketolation.

Dual Expression Vectors to Make Astaxanthin (1). Having demonstrated that efficient **1** was not achieved by a simple coexpression system, and to allow accurate assessment of select β-CH proteins, an efficient **1** biosynthesis method was used. This enabled a direct and accurate assessment of the β-CH proteins with respect to **1** biosynthesis. Dual expression vectors enable expression of different β-CH genes with the β-CK gene from *A. variabilis*. Following creations, triplicate single colonies transformed with the target dual expression vector and pAC-Beta were cultured and inducted at 0%, 0.002%, 0.02%, 0.2%, or 2% L-arabinose (final concentration in culture media). In all instances, 2% L-arabinose resulted in the highest percentage composition of **1** within the carotenoid extract, although actual **1** was not always the highest under these conditions (Figures S3 and 4).

This work demonstrated that β-CH proteins derived from *P. stewartii*, *N. aromaticivorans* DSM 1244, *Alcaligenes* sp. PC-1, and *S. solfataricus* P2 hold potential in the biosynthesis of **1**, as determined by **1** production at 63%, 57%, 84%, and 71% of total carotenoids, compared to 69% of the *P. agglomerans*-

derived control β-CH. However, on a μg/g basis, **1** biosynthesis remained significantly elevated for *P. agglomerans* β-CH.

Despite initial activity, the *E. sakazakii* BAC 9E10-derived β-CH failed to produce **1** within this system. Regardless, these results suggest that the creation of pBADAv.2W-β-CH dual expression vectors represents an efficient mechanism for the analysis of β-CH genes with respect to **1** biosynthesis. Moreover, presented data demonstrate that although several β-CH proteins function in **1** biosynthesis, final yield varies significantly and cannot be predicted on the basis of conversion of **2** to **3**.

CONCLUSIONS

β-CH derived from *S. solfataricus* P2 produced **1** at elevated levels with respect to total carotenoids. Comparison of actual productivity revealed that this, and all other candidate β-CH proteins, produced 56−82% less total **1** when compared to the control. Thus, we demonstrated that β-CH selection can significantly impact **1** yield and that β-CH derived from *P. agglomerans* is the most suitable for application in the development of an efficient **1** biosynthesis system. Our work also supports the hypothesis that efficient biosynthesis of astaxanthin requires tight control of β-CK and β-CH expression to drive carbon flux from β-carotene to zeaxanthin and on to astaxanthin, suggesting that relative β-CK and β-CH expression levels may be a critical factor.

EXPERIMENTAL SECTION

Bioinformatics: Identification of Target β-Carotene Hydroxylase Proteins. Target β-CH were identified initially by review of published literature, focusing on targets with proven capacity to function in the biosynthesis of **1**. Subsequently, a basic local alignment search tool (BLAST) was used to identify additional targets with the GenBank public database. This used *P. agglomerans*-derived β-CH as the query and so was specific for CrtZ-type (bacterial β-CH) nonheme di-iron mono-oxygenases. Identified sequences were subjected to a brief bioinformatic investigation to ensure suitable diversity within the investigated β-CH genes. Specifically, phylogenetic analysis and bootstrap values were generated for 28 β-CH genes belonging to different taxonomic groups, using MEGA4[11] (Figure 2, target β-CH genes are highlighted).

***E. coli* Strains and Plasmids.** *E. coli* strain TOP10 (1×10^9) (Invitrogen) was used as the host for carotenoid biosynthesis and general molecular biology work. Plasmid pAC-Beta was employed for **2** biosynthesis in coexpression experiments, while pAC-Zeax was used as a source of *P. agglomerans* β-CH.[14,20] Creation of pTrc-Av.2W and pAC-Canth has been described previously.[6,19]

Creation of pBAD-CrtZ Expression Vectors. β-CH genes derived from *P. stewartii*, *E. sakazakii* clone BAC 9E10, *Alcaligenes* sp. PC-1, *Paracoccus* sp. N81106, *Brevundimonas* sp. SD212, and *B. vesicularis* (see Table S2 for GenBank ID) were synthesized by Geneart, with the additional 5′ sequence GGTACCGAATTCAGG upstream of the start codon, and subsequently cloned into the unique *Eco*RI (underlined) and *Hin*dIII sites of plasmid pBAD24.[21] The *N. aromaticivorans* DSM 1244 β-CH was synthesized with an additional 5′ extension of CGCCC (converting the start codon into an *NcoI* (CCATGG) recognition sequence) and cloned into the unique *NcoI* and *SphI* sites of pBAD24.

A. thaliana-, *Synechocystis* sp. PCC 6803-, *P. agglomerans*-, and *S. solfataricus* P2-derived β-CH genes were amplified and cloned into pBAD24 as follows: *A. thaliana* hydroxylase Ohase 1 and Ohase 2 genes (see Table S2 for GenBank ID) were amplified via PCR from plasmids U09533 and U18630 (The *Arabidopsis* Information Resource (TAIR)), respectively, using primers detailed in Table S3 (Supporting Information). The *Synechocystis* sp. PCC 6803-derived β-CH (see Table S2 for GenBank ID) was isolated from plasmid pTrcSynR[6] (Table S2), while the *P. agglomerans* (previously *Erwinia herbicola*)-derived target was created previously.[6]

The β-CH derived from *S. solfataricus* P2 (see Table S2 for GenBank accession number) was isolated from genomic DNA via PCR. Primers for the amplification and creation of new pBAD-β-CH plasmids are detailed in Table S3. PCR was completed with Bio-x-act short DNA polymerase and reaction buffers (Cedarlane), as per the manufacturer's instructions. Amplification was confirmed via TAE agarose gel electrophoresis.

Plasmid DNA was removed via digestion with *DpnI* (New England Biolabs), when required. A 5.5 μL amount of 10× NEB buffer 4 and 1 μL of *DpnI* (10U/μL) were combined directly with the PCR sample and incubated at 37 °C for 4 to 16 h. *DpnI* was inactivated by incubation at 80 °C for 20 min. Subsequently, the PCR product was desalted and concentrated via precipitation with SureClean reagent (Cedarlane), as per the manufacturer's instructions, and used in a PCR-mediated cloning reaction designed to insert the DNA fragment in a site-specific manner into plasmid pBAD24.[15] The PCR-mediated cloning reaction is described below.

PCR-Mediated Cloning. PCR-mediated cloning was completed as described previously,[21] using the Bio-x-act long DNA polymerase system (Cedarlane). PCR products were desalted and concentrated via application of YM-100 commercially available centrifugal filter devices (Millipore), and 2 μL of the DNA eluent (approximately 10 μL) was transformed into commercially available TOP10 competent cells (Invitrogen), according to the manufacturer's instructions.

Creation of pTrc-β-CH Vectors. Target β-CH genes were cloned into the expression vector pTrcHis2 (Invitrogen). Oligo nucleotide primers described in Table S4 allowed amplification of target β-CH genes from the corresponding pBAD-β-CH plasmid (Table S2). The Bio-x-act short DNA polymerase system plus corresponding reaction buffers (Cedarlane) were used, and amplification, template (plasmid) DNA removal, and DNA precipitation were completed as described previously. Of the resultant PCR product, 100 ng was employed in a PCR-mediated cloning reaction designed to directly replace the β-CK gene present in plasmid pTrcAv.2W,[6] creating the pTrcHis2-based expression constructs for β-CH genes.

A PCR-mediated cloning reaction was completed, as described previously. The resultant library was screened by colony screen PCR using primers TrcSeqF and TrcSeqR (Table S4). Size differentiation, determined by agarose gel electrophoresis, was used to identify positive clones. Plasmid DNA was subsequently isolated by standard protocols, and samples were sequenced to confirm β-CH gene presence and fidelity.

Dual Expression Vectors. Dual expression vectors were created as described previously.[6] Briefly, β-CH genes, along with vector encoded promoter (pTrc), were amplified via PCR using Bio-x-act short DNA polymerase and primers detailed in Table S5, as per the manufacturer's instructions. Amplification was confirmed via TAE agarose gel electrophoresis. Template (plasmid) DNA was removed from the PCR via *DpnI* digestion and target DNA precipitated, as described previously. Of the resultant PCR product, 100 ng was employed in a PCR-mediated cloning reaction that inserted target β-CH genes, plus promoter (pTrc), into *Sal*I-digested pBADAv.2W, as described previously. Electrocompetent cells were prepared and transformed according to standard protocols.[22] Selection on LB agar media was performed with suitable antibiotics (Table S2). Colonies derived from cloning reactions were screened via PCR to confirm incorporation of target genes, prior to sequence analysis, via modification of a previously described method.[23] Briefly, 5 μL of nuclease-free water in a sterile 200 μL PCR tube was inoculated with a single colony using a sterile pipet tip. The colony was then replicated by streaking onto an LB agar plate. To this was added 15 μL of *Taq* PCR master mix (New England Biolabs) per reaction. PCR was performed as per the manufacturer's instructions with an initial 10 min denaturing step at 94 °C.

Carotenoid Biosynthesis and HPLC Analysis. Carotenoid biosynthesis was characterized via cultivation of distinct clones, combined with extraction and accurate quantification via HPLC. For accurate assessment of carotenoid biosynthesis, three distinct clones

were selected to account for potential variations between colonies in plasmid copy number and overall expression level. Generated data are presented as means ± SD (standard deviation) in figures and tables.

Single colonies were selected from an LB agar plate containing appropriate antibiotics, and used to inoculate 5 mL of LB broth containing appropriate antibiotics, in 15 mL Falcon tubes, and incubated for 16 h at 37 °C, 200 rpm. Of this culture 3 mL was transferred to 50 mL of LB broth, containing appropriate antibiotics, within a 500 mL conical flask. Expression cultures were incubated at 37 °C to an OD_{600} 0.6, at which point incubation temperature was reduced to 30 °C, and chemical inductants were added to the culture media. Carotenoid biosynthesis proceeded for an additional 24 h. Biomass was harvested via centrifugation, 3000*g*, at 4 °C for 10 min. Supernatant was decanted to waste, and the cell pellet frozen at −80 °C and lyophilized for 16 h. Extraction and HPLC analysis of carotenoids were performed as described previously.[6,24]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional information regarding compounds produced in coexpression experiments, accurate quantification of extracted carotenoids, in the form of standard curves, and primer sequences used throughout this study are provided. This information is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Ukibe, K.; Hashida, K.; Yoshida, N.; Takagi, H. *Appl. Environ. Microbiol.* **2009**, *75*, 7205−7211.
(2) Tao, L.; Wilczek, J.; Odom, J. M.; Cheng, Q. *Metab. Eng.* **2006**, *8*, 523−531.
(3) Ye, R. W.; Stead, K. J.; Yao, H.; He, H. *Appl. Environ. Microbiol.* **2006**, *72*, 5829−5837.
(4) Cunningham, F. X.; Gantt, E. *Plant Cell.* **2011**, *23*, 3055−3069.
(5) Choi, S. K.; Matsuda, S.; Hoshino, T.; Peng, X.; Misawa, N. *Appl. Microbiol. Biotechnol.* **2006**, *72*, 1238−1246.
(6) Scaife, M. A.; Burja, A. M.; Wright, P. C. *Biotechnol. Bioeng.* **2009**, *103*, 944−955.
(7) Lemuth, K.; Steuer, K.; Albermann, C. *Microb. Cell Fact.* **2011**, *10*, no. 29.
(8) Zhong, Y. J.; Huang, J. C.; Liu, J.; Li, Y.; Jiang, Y.; Xu, Z. F.; Sandmann, G.; Chen, F. *J. Exp. Bot.* **2011**, *62*, 3659−3669.
(9) Grung, M.; D'Souza, F.; Borowitzka, M.; Liaaen-Jensen, S. *J. Appl. Phycol.* **1992**, *4*, 165−171.
(10) Renstrom, B.; Borch, G.; Skulberg, O. M.; Liaaen-Jensen, S. *Phytochemistry.* **1981**, *20*, 2561−2564.
(11) Tamura, K.; Dudley, J.; Nei, M.; Kumar, S. *Mol. Biol. Evol.* **2007**, *24*, 1596−1599.
(12) Tian, L.; DellaPenna, D. *Arch. Biochem. Biophys.* **2004**, *430*, 22−29.
(13) Bouvier, F.; Keller, Y.; d'Harlingue, A.; Camara, B. *Biochim. Biophys. Acta, Lipids Lipid Metabol.* **1998**, *1391*, 320−328.
(14) Cunningham, F. X., Jr.; Pogson, B.; Sun, Z.; McDonald, K. A.; DellaPenna, D.; Gantt, E. *Plant Cell* **1996**, *8*, 1613−1626.
(15) Guzman, L. M.; Belin, D.; Carson, M. J.; Beckwith, J. *J. Bacteriol.* **1995**, *177*, 4121−4130.
(16) Fraser, P. D.; Miura, Y.; Misawa, N. *J. Biol. Chem.* **1997**, *272*, 6128−6135.
(17) Masamoto, K.; Misawa, N.; Kaneko, T.; Kikuno, R.; Toh, H. *Plant Cell Physiol.* **1998**, *39*, 560−564.
(18) Hasunuma, T.; Miyazawa, S.; Yoshimura, S.; Shinzaki, Y.; Tomizawa, K.; Shindo, K.; Choi, S.; Misawa, N.; Miyake, C. *Plant J.* **2008**, *55*, 857−868.
(19) Scaife, M. A.; Ma, C. A.; Norman, A.; Armenta, R. E. *Process Biochem.* **2012**, submitted.
(20) Sun, Z.; Gantt, E.; Cunningham, F. X., Jr. *J. Biol. Chem.* **1996**, *271*, 24349−24352.
(21) Bryksin, A. V.; Matsumur, I. *BioTechniques* **2010**, *48*, 463−465.
(22) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*, 2nd ed.; Cold Spring Harbor: New York, 1981.
(23) Güssow, D.; Clackson, T. *Nucleic Acids Res.* **1989**, *17*, 4000.
(24) Armenta, R. E.; Burja, A.; Radianingtyas, H.; Barrow, C. J. *J. Agric. Food Chem.* **2006**, *54*, 9752−9758.