Functional Expression and Extension of Staphylococcal Staphyloxanthin Biosynthetic Pathway in *Escherichia coli*<sup>**S**</sup>

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**Background:** The biosynthetic pathway for staphyloxanthin has previously been proposed to consist of five enzymes. Here, we report a missing sixth enzyme, 4,4′-diaponeurosporen-4-aldehyde dehydrogenase (AldH), in the staphyloxanthin biosynthetic pathway and describe the functional expression of the complete staphyloxanthin biosynthetic pathway in *Escherichia coli*. When we expressed the five known pathway enzymes through artificial synthetic operons and the wild-type operon (crtOPQMN) in *E. coli*, carotenoid aldehyde intermediates such as 4,4′-diaponeurosporen-4-al accumulated without being converted into staphyloxanthin or other intermediates. We identified an *aldH* gene located 670 kilobase pairs from the known staphyloxanthin gene cluster in the *S. aureus* genome and an *aldH* gene in the non-staphyloxanthin-producing *Staphylococcus carnosus* genome. These two putative enzymes catalyzed the missing oxidation reaction to convert 4,4′-diaponeurosporen-4-al into 4,4′-diaponeurosporenoic acid in *E. coli*. Deletion of the *aldH* gene in *S. aureus* abolished staphyloxanthin biosynthesis and caused accumulation of 4,4′-diaponeurosporen-4-al, confirming the role of AldH in staphyloxanthin biosynthesis. When the complete staphyloxanthin biosynthetic pathway was expressed using an artificial synthetic operon in *E. coli*, staphyloxanthin-like compounds, which contained altered fatty acid acyl chains, and novel carotenoid compounds were produced, indicating functional expression and coordination of the six staphyloxanthin pathway enzymes.

**Results:** A sixth pathway enzyme, 4,4′-diaponeurosporen-4-aldehyde dehydrogenase, was identified using a synthetic module approach.

**Conclusion:** The complete staphyloxanthin biosynthetic pathway consists of six enzymes in *Staphylococcus aureus*.

**Significance:** This is the first report demonstrating the complete staphyloxanthin pathway.

The biosynthetic pathway for staphyloxanthin, a C<sub>30</sub> carotenoid biosynthesized by *Staphylococcus aureus*, has previously been proposed to consist of five enzymes (CrtO, CrtP, CrtQ, CrtM, and CrtN). Here, we report a missing sixth enzyme, 4,4′-diaponeurosporen-aldehyde dehydrogenase (AldH), in the staphyloxanthin biosynthetic pathway and describe the functional expression of the complete staphyloxanthin biosynthetic pathway in *Escherichia coli*. When we expressed the five known pathway enzymes through artificial synthetic operons and the wild-type operon (crtOPQMN) in *E. coli*, carotenoid aldehyde intermediates such as 4,4′-diaponeurosporen-4-al accumulated without being converted into staphyloxanthin or other intermediates. We identified an *aldH* gene located 670 kilobase pairs from the known staphyloxanthin gene cluster in the *S. aureus* genome and an *aldH* gene in the non-staphyloxanthin-producing *Staphylococcus carnosus* genome. These two putative enzymes catalyzed the missing oxidation reaction to convert 4,4′-diaponeurosporen-4-al into 4,4′-diaponeurosporenoic acid in *E. coli*. Deletion of the *aldH* gene in *S. aureus* abolished staphyloxanthin biosynthesis and caused accumulation of 4,4′-diaponeurosporen-4-al, confirming the role of AldH in staphyloxanthin biosynthesis. When the complete staphyloxanthin biosynthetic pathway was expressed using an artificial synthetic operon in *E. coli*, staphyloxanthin-like compounds, which contained altered fatty acid acyl chains, and novel carotenoid compounds were produced, indicating functional expression and coordination of the six staphyloxanthin pathway enzymes.

Synthetic biological approaches have been widely used for the redesign and reconstruction of biosynthetic pathways in heterologous hosts (1, 2). The functional expression of redesigned pathway enzymes in a modular manner can improve the productivity and yield of target molecules and can demonstrate previously undefined functions of pathway enzymes (3–6). Moreover, the modular expression of a pathway enzyme can be used as a tool for diversifying the structures of molecules such as carotenoids (7) using combinatorial biosynthesis and directed evolution in heterologous hosts (8–10).

Carotenoids are compounds that belong to a class of isoprenoid derivatives found in many organisms, including photosynthetic and non-photosynthetic bacteria. The carotenoids found in bacteria and yeast have antioxidative functions and play roles in light harvesting, energy transfer, and the regulation of membrane fluidity (11–13). The protective functions of carotenoids against oxidative stress, singlet oxygen, and peroxy radicals promote the survival of pathogenic microbes during host immune responses (14, 15).

The pathogen *Staphylococcus aureus* is a Gram-positive, gold-colored bacterium that is resistant to methicillin and all available β-lactam antibiotics (16). The antibiotic resistance of *S. aureus* has necessitated the development of new types of anti-infective drugs, such as virulence factor-specific drugs (14, 17). The gold color of this pathogen is derived from the yellow-orange carotenoid staphyloxanthin, a virulence factor. The chemical characterization of staphyloxanthin, combined with analysis of *S. aureus* mutants, enabled the elucidation of the staphyloxanthin biosynthetic pathway (18), which was thought to consist of five enzymes: 4,4′-diapophytoene synthase (CrtM), 4,4′-diapophytoene desaturase (CrtN), 4,4′-diaponeurosporenoic acid oxidase (CrtP), glycosyltransferase (CrtQ), and acyltransferase (CrtO) (see Fig. 1A) (19). CrtP introduces a terminal oxygen molecule into 4,4′-diaponeurosporenoic acid, which results from sequential activities of CrtM and CrtN, to form a carboxylic acid intermediate (4,4′-diaponeurosporenoic acid) via an aldehyde intermediate (20). Staphyloxanthin is finally synthesized by further modification of 4,4′-diaponeurosporenoic acid by glycosylation (CrtQ) and acylation (CrtO) at a terminal carboxyl group. Interestingly, CrtP was reported to function as both an oxidase and an aldehyde dehydrogenase (20). Here, we report the identification of a sixth enzyme, 4,4′-diaponeurosporen-aldehyde dehydrogenase (AldH), in the *S. aureus* staphyloxanthin biosynthetic pathway. With a complete and redesigned staphyloxanthin pathway, staphyloxanthin-like
compounds and novel carotenoids were successfully produced in *Escherichia coli* for the first time.

**EXPERIMENTAL PROCEDURES**

Cloning and Synthetic Module Construction—All cloning and carotenoid expression experiments were performed in the *E. coli* SURE strain except for using pBBR1MCS-2-derived vectors. *E. coli* XL1-Blue was used for cloning and expressing pBBR1MCS-2-derived vectors (21). Genes encoding CrtM, CrtN, CrtP, CrtQ, and CrtO from *S. aureus* ssp. *aureus* (KCTC 1928) were cloned into the constitutive expression vector pUCM (22). Four genes, encoding AldH (*aldH*), glycine betaine aldehyde dehydrogenase (*gbsA*), aldehyde dehydrogenase homolog (*aldA*), and aldehyde dehydrogenase family protein, were amplified from the genomic DNA of *S. aureus* strain Newman (AP009351) and cloned into the pUCM vector. The *aldH* gene from *S. aureus* KCTC 1928 was cloned into pBBR1MCS-2, a vector that is compatible with pUCM and pACYC184 in *E. coli*. A putative *aldH* gene was amplified from the genomic DNA of *Staphylococcus carnosus* ssp. *carnosus* KCTC 3580 and cloned into pUCM. To reconstruct the staphyloxanthin biosynthetic pathway in *E. coli*, a synthetic module containing *crtM*, *crtN*, *crtP*, or *crtQ* was sequentially assembled into the pACYC184 vector to generate pACM-M_{SA}-N_{SA}-P_{SA}, pACM-M_{SA}-N_{SA}-P_{SA}, and pACM-M_{SA}-N_{SA}-P_{SA}-Q_{SA}. Briefly, a gene was subcloned from pUCM-X_{SA} (where X is a pathway gene) into pACYC184.

![Diagram of carotenoid biosynthetic pathway.](image-url)

**FIGURE 1.** Complete staphyloxanthin biosynthetic pathway, gene cluster organization, and reconstructed staphyloxanthin pathway in heterologous host *E. coli*. A, structures of carotenoids produced in recombinant *E. coli* but not in *S. aureus* are shown in blue. B, the C_{30} carotenogenic gene cluster found in *Methylomonas* sp. strain 16a, the staphyloxanthin gene cluster, and the *aldH* gene encoding 4,4'-diaponeurosporen-aldehyde dehydrogenase in *S. aureus*. IPP, isopentenyl diphosphate; FPP, farnesyl diphosphate.

**FIGURE 2.** HPLC analysis of cell extracts of recombinant *E. coli* expressing pACM-M_{SA}-N_{SA}-P_{SA} (A), pACM-M_{SA}-N_{SA}-P_{SA}-Q_{SA} (B), and pUC19-OQMN_{SA} (C). The following carotenoids were identified: 4,4'-diaponeurosporen-4-al (peak 1) and 4,4'-diapolyycopene-dial (peak 2). The inset shows the recorded UV-visible absorption spectra for individual peaks (see Fig. 1A and Table 2 for details).
by amplifying the gene together with a modified constitutive lac promoter. The staphyloxanthin gene cluster (crtOPQMN) from *S. aureus* was amplified with a forward PCR primer for *crtO* and a reverse PCR primer for *crtN* containing an XbaI restriction enzyme site at its 5′-end (supplemental Table S1). The PCR product was then digested with XbaI and cloned into the corresponding site in the pUC19 vector to facilitate controlled expression of the genes from a lac promoter. All plasmids and strains used in this study are listed in supplemental Table S2.

**Culture Growth for Carotenoid Production**—For carotenoid production, *S. aureus* strains (KCTC 1928, RN4220, and an *aldH* deletion mutant of RN4220) were cultivated for 24 h in the dark at 30 °C in B-medium. For carotenoid production, recombinant *E. coli* SURE or XL1-Blue was cultivated for 36 h in the dark at 30 °C with shaking at 250 rpm in Terrific broth medium (50 ml of medium in a 300-ml flask or 200 ml of medium in a 1-liter flask) supplemented with the appropriate antibiotics: 50 μg/ml chloramphenicol, 100 μg/ml ampicillin, and/or 30 μg/ml kanamycin.

**Isolation of Carotenoids**—Carotenoids were extracted from cell pellets using 15 or 30 ml of acetone or methanol until all visible pigments were removed. When performing saponification, carotenoids were extracted using 15 ml of methanol containing 6% KOH and incubated for 14 h at 4 °C. Colored supernatants were pooled after centrifugation (4 °C and 4000 rpm) and concentrated into a small volume using an EZ-2 Plus centrifugal evaporator (Genevac Inc., Gardiner, NY). Five milliliters of EtOAc was added to the concentrated solution and re-extracted after the addition of 5 ml of NaCl (5 N) solution for salting out. The upper organic phase containing carotenoids was collected, washed with distilled water, and completely dried using the EZ-2 Plus centrifugal evaporator. Dried samples were resuspended in 300 μl of EtOAc, run through a silica column, and eluted stepwise with increasing amounts of acetone in a 9:1 hexane/EtOAc solvent. A 20-μl aliquot of each fraction was subjected to TLC to confirm the purity of the compound in the fractions. The fractions were collected, concentrated using the EZ-2 Plus centrifugal evaporator, and subjected to a Varian 1200L LC/MS system.

**Allelic Replacement**—Allelic replacement of the *aldH* gene in *S. aureus* strain RN4220 (modification-positive, restriction-negative) was performed according to the protocol described by Wyatt et al. (23). The erythromycin gene was amplified from pSAKON and fused with the flanking region of the *aldH* gene at both ends by overlapping PCR. The PCR product was purified and ligated into the pGEM-T Easy vector (Promega). Following sequencing, the erythromycin cassette was treated with Ncol and PsI and subcloned into the corresponding sites in pCL522, a temperature-sensitive *E. coli*/S. aureus shuttle vector. The resulting pCL-ALDKO plasmid was electrotansformed into *S. aureus* RN4220 (24) and incubated at 43 °C on B-medium-agar plates containing 10 μg/ml erythromycin (BM-agar Erm10 plates) for 2 days. Colonies were restreaked onto BM-agar Erm10 plates and incubated at 43 °C for 2 days. A single colony was inoculated into 30 ml of B-medium without antibiotics and sequentially diluted (1:1000) into 30 ml of B-medium each day at 30 °C for 5 days. After 5 days, 1×10^6 diluted cells were spread onto BM-agar Erm10 plates, and colonies were visually screened. Deletion mutants that developed an orange color were confirmed by PCR and carotenoid profiling.

**Analysis of Carotenoids**—The initial TLC analysis was performed using a 9:1:1 hexane/EtOAc/acetone solvent system. A 9:1:3 hexane/EtOAc/acetone solvent was used for analyzing glycosylated C30 carotenoids. A 10–20-μl aliquot of the crude extract or the collected polar fraction was applied to a ZORBAX Eclipse XDB-C18 column (4.6 × 150 mm, 5.0 μm; Agilent Technologies, Inc., Santa Clara, CA) and eluted under isocratic conditions with a solvent system (80:15:5 acetonitrile/methanol/isopropyl alcohol) at a flow rate of 1 ml/min using an Agilent 1200 HPLC system equipped with a photodiode array
detector. For structural elucidation, carotenoids were identified using a combination of HPLC retention times, UV-visible absorption spectra, and mass fragmentation spectra. Mass fragmentation spectra were monitored using both negative and positive ion modes in a mass range of \( m/z \) 200–900 on the Varian 1200L LC/MS system equipped with an atmospheric pressure chemical ionization interface.

RESULTS

Reconstruction of Partial Staphyloxanthin Biosynthetic Pathway in E. coli—To establish the biosynthetic pathway of staphylococcal staphyloxanthin in E. coli, \( \text{crtM, \text{crtN, or \text{crtP}} \) expression cassettes in pUCM-CrtM, pUCM-CrtN, or pUCM-CrtP vectors, containing constitutive lac promoters (25) that controlled the individual expression of \( \text{crtM, \text{crtN, or \text{crtP}} \), were sequentially assembled to generate pACM-MSA-NSA-PSA. Reddish recombinant E. coli expressing pACM-MSA-NSA-PSA produced 4,4\(^{'\prime}\)-diaponeurosporen-4-al (structure 1 in Fig. 1A) and 4,4\(^{'\prime}\)-diapolycope-dial (structure 2 in Fig. 1A), as shown in Fig. 2A. No further oxidized carboxylic acid intermediates were detected. The dominant formation of 4,4\(^{'\prime}\)-diaponeurosporen-4-al and 4,4\(^{'\prime}\)-diapolycope-dial without 4,4\(^{'\prime}\)-diaponeurosporene-dial and 4,4\(^{'\prime}\)-diapolycope-4-al (or 4,4\(^{'\prime}\)-diapolycope-4-al) suggests that \( \text{CrtP} \) recognizes one end region containing a conjugated double bond and preferably introduces the aldehyde function group to this conjugated double bond-containing region, unlike the \( \text{CrtP-type carotenoid oxidase} \) expressed in \( \text{Methylomonas sp. strain 16a} \) (6).

For further extension of the staphyloxanthin pathway in E. coli, the fourth gene, \( \text{crtQ} \), was expressed by (i) a two-plasmid system (pUC19-CrtQ/pACM-MSA-NSA-PSA or pUCM-CrtQ/pACM-MSA-NSA-PSA) and (ii) a one-plasmid system (pACM-MSA-NSA-PSA-QSA). In both cases, carotenoid aldehyde intermediates (4,4\(^{'\prime}\)-diaponeurosporen-4-al and 4,4\(^{'\prime}\)-diapolycope-dial) accumulated without being converted into the corresponding glycosylated carotenoid structures (Fig. 2B). The accumulation of carotenoid aldehyde intermediates was also observed in recombinant E. coli expressing the wild-type gene cluster (\( \text{crtOPQMN} \)) (Fig. 2C). These results indicate that non-functionality of \( \text{CrtQ} \) or the absence of the sixth pathway enzyme (AldH) may be attributable to the accumulation of...
carotenoid aldehyde intermediates. Therefore, we focused on the discovery of the missing AldH because, even though CrtP was reported to have dual functions as both an oxidase and AldH, no further oxidized carotenoid carboxylic acid intermediates such as 4,4'-diaponeurosporenoic acid were detected in recombinant E. coli expressing pACM-MSA-NSA-PSA.

Identification of Sixth Enzyme in Staphyloxanthin Biosynthetic Pathway of S. aureus—AldH from the carotenogenic Methylomonas sp. strain 16a was previously reported to catalyze the oxidation of aldehyde groups of 4,4'-diapolyoctopyrene- dial to carboxyl groups (6). Although the five pathway enzymes were reported to be sufficient for staphyloxanthin biosynthesis in S. aureus, we decided to search for a sixth enzyme with AldH functionality that could catalyze the oxidation of carotenoid aldehyde intermediates to carotenoid carboxylic acid intermediates. An NCBI BLAST search (tblastx) with the aldH gene sequence of Methylomonas sp. strain 16a returned seven aldehyde dehydrogenase genes in S. aureus strain Newman with a high similarity (Table 1). Among these seven genes, we selected four, encoding AldH (aldH), glycine betaine aldehyde dehydro-
Functional Expression of Staphyloxanthin Pathway

Functional Verification of 4,4'-Diaponeurosporen-aldehyde Dehydrogenase (AldH) in S. aureus Using Allelic Replacement—To confirm the function of AldH in the original host S. aureus, we replaced the aldH gene with an erythromycin resistance cassette (23). To do this, the modification-positive S. aureus strain RN4220 was visually screened, and deletion of the aldH gene was verified by PCR (Fig. 5A). The aldH dele-

two-plasmid system (pUCM-aldHSA/pUCM-gbsASA/pUCM-aldASA/pUCM-2026SA + pACM-MSA-NSA-PSA) in E. coli. Surprisingly, 4,4'-diaponeurosporen-aldehyde (structure 3 in Fig. 1A) and 4,4'-diapolyocopen-4'-4'-dioic acid (structure 4 in Fig. 1A) were detected in E. coli expressing pUCM-aldHSA + pACM-MSA-NSA-PSA (Fig. 3A). TLC (Fig. 3B) and LC/MS (Fig. 4, A and B) analysis also supported the presence of the carboxylic acid group in the carotenoid structures 3 and 4. Furthermore, the novel structures 4,4'-diapolyocopen-4'-al-4-oic acid (or 4,4'-diapolyocopen-4-al-4'-oic acid) (structure 5 in Fig. 1A) and 4,4'-diapolyocopen-aldehyde (structure 6 in Fig. 1A) were also identified by LC/MS analysis (Fig. 4, C and D). This confirms that AldH has a broad substrate specificity like other carotenoid enzymes (7, 10, 26).

Identification of Enzyme Catalyzing Oxidation of Carotenoid Aldehyde to Carboxylic Acid in S. carnosus—To date, staphyloxanthin biosynthesis had been studied both in S. aureus and in the non-carotenogenic bacterium S. carnosus (19). Staphyloxanthin was heterologously produced in recombinant S. carnosus transformed with the S. aureus gene cluster encoding the five pathway enzymes (19). This indicates that wild-type S. carnosus expresses a carotenoid AldH, similar to the AldH of S. aureus, that can catalyze the oxidation of carotenoid aldehyde (4,4'-diaponeurosporen-4-al) to a carboxylic acid intermediate (4,4'-diaponeurosporen-aldehyde). Therefore, we searched for AldH candidates in S. carnosus using the amino acid sequence of the S. aureus AldH and found a putative AldH enzyme in S. carnosus that shared 78% amino acid identity with the AldH of S. aureus. As expected, when the S. carnosus AldH was expressed in recombinant E. coli also expressing the pACM-MSA-NSA-PSA vector, 4,4'-diaponeurosporen-4-al was successfully converted into 4,4'-diaponeurosporen-aldehyde (Fig. 3C), as observed when the S. aureus AldH was expressed in recombinant E. coli expressing pACM-MSA-NSA-PSA. The AldH-mediated oxidation of 4,4'-diaponeurosporen-4-al into 4,4'-diaponeurosporen-aldehyde suggests that the AldH enzymes from S. carnosus and S. aureus have similar functions. This result explains how recombinant S. carnosus expressing only five staphyloxanthin enzymes can still produce staphyloxanthin without requiring the exogenous expression of the sixth S. aureus AldH. It is not clear why the genome of non-pigmented S. carnosus contains the aldH gene. The presence of aldH in the S. carnosus genome may be a clue to an evolutionary event and suggests that S. carnosus may lose a carotenoid biosynthetic pathway. Future studies investigating the regulation of AldH expression and its physiological function in S. carnosus are necessary.

Functional Verification of 4,4'-Diaponeurosporen-aldehyde Dehydrogenase (AldH) in S. aureus Using Allelic Replacement—To confirm the function of AldH in the original host (S. aureus), we replaced the aldH gene with an erythromycin resistance cassette (23). To do this, the modification-positive and restriction-negative S. aureus strain RN4220 was used instead of the multidrug-resistant S. aureus strain KCTC 1928. The carotenoid profile of S. aureus RN4220 was confirmed to be the same as that of S. aureus KCTC 1928. An aldH deletion mutant of S. aureus RN4220 was visually screened, and deletion of the aldH gene was verified by PCR (Fig. 5A). The aldH dele-

FIGURE 7. LC/MS analysis of staphyloxanthin and staphyloxanthin-like compounds. Tetradecanoyl-glucosyl-4,4'-diaponeurosporen-aldehyde (A), hexadecanoyl-glucosyl-4,4'-diaponeurosporen-aldehyde (B), and staphyloxanthin (C) were isolated and analyzed. The structures, fragments obtained after ionization, and corresponding masses are indicated in the insets.

genase (gbsA), aldehyde dehydrogenase homolog (aldA), and aldehyde dehydrogenase family protein (NWMN_2026). These genes were then cloned from S. aureus and expressed by the
tion mutant accumulated 4,4′-diaponeurosporen-4-al as a major product but did not produce 4,4′-diaponeurosporenoic acid or staphyloxanthin, as shown in the HPLC chromatogram (Fig. 5, B and D) and TLC plate (Fig. 5C). This accumulation of 4,4′-diaponeurosporen-4-al was consistent with that seen in recombinant E. coli expressing pACM-M<sub>SA</sub>-N<sub>SA</sub>-P<sub>SA</sub>-Q<sub>SA</sub> or pUC19-OPQMNSA (Fig. 2, B and C). Therefore, this mutational study confirms that AldH catalyzes the oxidation of 4,4′-diaponeurosporen-4-al to 4,4′-diaponeurosporenoic acid in the staphyloxanthin biosynthetic pathway in S. aureus.

Taken together, our data demonstrated that AldH is essential for staphyloxanthin formation and is the sixth enzyme in the staphyloxanthin biosynthetic pathway of S. aureus. Interestingly, unlike the aldH gene of Methylophilus sp. strain 16a, which is located between crtP and crtN in a gene cluster, the aldH gene of S. aureus is located 670 kilobase pairs from the staphyloxanthin biosynthetic gene cluster (Fig. 1B).

Expression of Complete Staphyloxanthin Pathway in E. coli and Characterization of Staphyloxanthin-like Compounds Produced in Engineered E. coli—After we had identified 4,4′-diaponeurosporen-aldehyde dehydrogenase as the sixth enzyme in the staphyloxanthin biosynthetic pathway, the complete pathway was then modularly reconstructed and expressed in E. coli using three compatible plasmids. Recombinant E. coli expressing pACM-M<sub>SA</sub>-N<sub>SA</sub>-P<sub>SA</sub>-Q<sub>SA</sub> or pUC19-OPQMNSA (Fig. 2, B and C). Therefore, this mutational study confirms that AldH catalyzes the oxidation of 4,4′-diaponeurosporen-4-al to 4,4′-diaponeurosporenoic acid in the staphyloxanthin biosynthetic pathway in S. aureus.

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### TABLE 2

| Major carotenoids (structures in Fig. 1) | Mass analysis (m/z) (M) |
|-----------------------------------------|------------------------|
| **Absorption**                           |                        |
| **Clones Expressed genes**               | **Major carotenoids**   |
| **Wild-type**                            |                        |
| pACM-M<sub>sa</sub>-N<sub>sa</sub>-Q<sub>sa</sub> |                        |
| ctfN, ctfP                                | 4,4'-Diaponeurosporenoic acid (3) |
| ctfN, ctfP, crtO                           | 4,4'-Diaponeurosporenoic acid (4) |
| crtN, ctfN, ctfP, aldH                      | 4,4'-Diaponeurosporenoic acid (5) |
| crtN, ctfN, aldH                            | 4,4'-Diaponeurosporenoic acid (6) |
| crtN, ctfN, ctfP, aldH, crtM                | 4,4'-Diaponeurosporenoic acid (7) |
| crtN, ctfN, ctfP, aldH, crtM, crtO, crtQ, crtM | 4,4'-Diaponeurosporenoic acid (8) |
| crtN, ctfN, ctfP, aldH, crtM, crtO, crtQ, crtO, crtM | 4,4'-Diaponeurosporenoic acid (9) |
| crtN, ctfN, ctfP, aldH, crtM, crtO, crtQ, crtO, crtO, crtM | 4,4'-Diaponeurosporenoic acid (10) |

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To date, the biosynthesis of staphyloxanthin had been exclusively studied in *S. aureus* and *S. carnosus*. The alternative *S. carnosus* system had been successfully used for the identification of the five pathway enzymes (CrtO, CrtP, CrtQ, CrtM, and CrtN); however, it failed to elucidate the missing sixth pathway enzyme, AldH from *S. aureus*. Although the aldH gene is not located near the pathway gene cluster in *S. aureus*, we identified its function by coexpressing the aldH gene with synthetic staphyloxanthin pathway modules in *E. coli*. An aldH deletion mutant abolished staphyloxanthin formation and caused accumulation of an aldehyde intermediate, confirming the role of AldH in the oxidation of aldehyde intermediates (4,4'-diaponeurosporenoic acid) to carboxylic acid intermediates (4,4'-diaponeurosporenoic acid) in the complete staphyloxanthin biosynthetic pathway in *S. aureus*. For the first time, we succeeded in reconstructing the complete staphyloxanthin biosynthetic pathway in *E. coli*. The six staphyloxanthin pathway enzymes were functionally active and coordinated in heterologous *E. coli*. Staphyloxanthin-like structures were produced instead of staphyloxanthin due to differences in the types of fatty acids available for staphyloxanthin synthesis in *E. coli* versus *S. aureus*. Furthermore, the redesigned staphyloxanthin pathway produced novel carotenoids, which were not detected in *S. aureus*, proving that synthetic modules of biosynthesis are a powerful methodology for generating structural diversity of biochemicals. Finally, engineered *E. coli* cells expressing the synthetic staphyloxanthin pathway could supply pathway intermediates (Table 2) for biological studies and represent a good model system for investigating the role of staphyloxanthin or staphyloxanthin-like compounds in the virulence of *S. aureus*.

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