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Complete genome sequence of *Planococcus faecalis* AJ003^T, the type species of the genus *Planococcus* and a microbial C30 carotenoid producer

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Highlights

- - The first complete genome sequence of the type-strains *Planococcus faecalis* AJ003^T is reported.
- - A single circular genome of *P. faecalis* AJ003^T consists of 3,495,892 bp with 3511 coding DNA sequences and 99 RNAs.
- - Seven genes associated with the MEP pathway and five genes involved in the carotenoid pathway were identified.

ABSTRACT

A novel type strain, *Planococcus faecalis* AJ003^T, isolated from the feces of Antarctic penguins, synthesizes a rare C30 carotenoid, glycosyl-4,4'-diaponeurosporen-4'-ol-4-oic acid. The complete genome of *P. faecalis* AJ003^T comprises a single circular chromosome (3,495,892 bp; 40.9% G+C content). Annotation analysis has revealed 3511 coding DNA sequences and 99 RNAs; seven genes associated with the MEP pathway and five genes involved in the carotenoid pathway have been identified. The functionality and complementation of 4,4'-diapophytoene synthase (CrtM) and two copies of heterologous 4,4'-diapophytoene desaturase (CrtN) involved in carotenoid biosynthesis were analyzed in *Escherichia coli*.

Abbreviations: IPP, isopentenyl diphosphate; DMAPP, dimethylallyl pyrophosphate; FPP, farnesyl diphosphate. (B) Diagram representing a whole gene cluster consisting of five carotenoid pathway genes showing the translational direction of the genes.

Keywords: *Planococcus faecalis*; Carotenoids; Gene cluster; Complete genome sequence

Carotenoids have diverse biological functions in nature, including coloration, photoprotection, and light harvesting; they are also the precursors for many hormones (Holt et al., 2005; Johnson and Schmidt-Dannert, 2008). In the past, carotenoids have mainly been used as food colorants, antioxidants, and animal feed supplements (Nishino et al., 2009).

Carotenoids are now also used in other applications such as nutraceuticals, cosmetics, and pharmaceuticals. Recently, we described the biological functions of C30 carotenoids, which have a backbone of 30 carbons; such functions include stem cell proliferation and antioxidative activity (Kim et al., 2016). Compared with C40 carotenoids—including lycopene and β -carotene—C30 carotenoid is relatively rare in nature, and there is little information regarding the biosynthetic organization and regulation of its genes (Kim and Lee, 2012). Consequently, the fermentative production of bioactive C30 carotenoids in wild-type strains and heterologous hosts has not been researched extensively, and its use in biotechnological applications has been limited.

We recently isolated and characterized a type strain, *Planococcus faecalis* AJ003^T, which is an orange, aerobic, gram-positive coccus bacterium that belongs to the family Planococcaceae (Kim et al., 2015). Notably, *P. faecalis* AJ003^T produces a rare C30 carotenoid, glycosyl-4,4'-diaponeurosporen-4'-ol-4-oic acid, which is distinguishable from the other carotenoids produced by some *Planococcus* strains, such as methyl glucosyl-3,4-dehydro-apo-8'-lycopenoate (Shindo et al., 2008; Ganapathy et al., 2016). To date, the genomes of only five type strains have been completely sequenced, i.e., those of *P. donghaensis* (See-Too et al., 2017b), *P. kocurii* (See-Too et al., 2016b), *P. massiliensis* (Hegedus et al., 2017), *P. rifietoensis* (See-Too et al., 2016a), and *P. versutus* (See-Too et al., 2017a). Because the novel type strain *P. faecalis* AJ003^T is a potential C30 carotenoid producer, it is worth investigating in detail. Such an investigation should include the

determination of its whole-genome sequence. The genome sequence could provide the basis for the development of a recombinant *Planococcus* strain that is suitable for the large-scale fermentation of biotechnologically important C30 carotenoids, and for the elucidation of C30 carotenoid production mechanisms.

Herein, we report the complete genome sequence of *P. faecalis* AJ003^T, and propose a mechanism by which it produces C30 carotenoids based on genome annotation analysis and functional expression in *Escherichia coli*.

Genomic DNA was extracted using a Genomic DNA Kit (Macrogen, Korea). The genome of *P. faecalis* AJ003^T was sequenced using PacBio RS II single-molecule real-time sequencing technology (Pacific Biosciences, CA, USA). After sub-read filtering of the raw data from the PacBio RS II sequencer, 70,249 long reads and 832,715,567 base pairs, with a 238-fold genome coverage, were generated and assembled *de novo* using a Canu v1.3 assembler (Koren et al., 2017). The overlapping regions at both ends of one contig were identified and trimmed to generate a unique stretch at both ends using Circlator (Hunt et al., 2015). The open reading frames (ORFs) were predicted using the online RAST server (Aziz et al., 2008), Prodigal version 2.6.3 (Hyatt et al., 2010), and Glimmer 3.2 (Delcher et al., 1999). Transfer RNA (tRNA) and ribosomal RNA (rRNA) were predicted using tRNAscan-SE v1.21 (Lowe and Eddy, 1997) and RNAmmer v1.2 (Lagesen et al., 2007), respectively. Function predictions were based on RPS-BLAST searches (E-value < 10⁻³) against the non-redundant GenBank protein database (www.ncbi.nlm.nih.gov/protein), the clusters of orthologous groups (COG) database (www.ncbi.nlm.nih.gov/COG), and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (www.genome.ad.jp/kegg). The graphical circular map of the genome was constructed and visualized using Circos v0.67 (Krzywinski et al., 2009). Putative carotenoid genes were amplified by polymerase chain reaction (PCR) with

gene-specific primers (Table S1), and cloned into pUC19 or pUCM plasmids (**Table 1**).

Functional expression and complementation of the cloned pathway genes were investigated in *E. coli* strain XL1-blue. The *E. coli* strains were grown at 30°C in 250 mL LB medium supplemented with 100 µg/mL ampicillin and/or 50 µg/mL chloramphenicol, whereas *P. faecalis* AJ003^T was grown in 250 mL MR medium (Kim et al., 2015). The carotenoids were repeatedly extracted from wet pellets (approximately 5 g) of *P. faecalis* AJ003^T and recombinant *E. coli* strains using 30 mL of acetone or methanol until all visible pigments had been removed from the pellets. After concentration, two-phase extraction, drying, and dissolving in methanol according to our previous paper (Kim et al., 2016), the carotenoids were analyzed using an Agilent 1200 high-performance liquid chromatography (HPLC) system equipped with a photodiode array detector, according to the process described in a previous study (Kim et al., 2012).

The genome of *P. faecalis* AJ003^T comprises a 3,495,892 bp circular chromosome with a G+C content of 40.9% (**Fig. 1A**), and is devoid of any extrachromosomal plasmids. A total of 3511 coding DNA sequences (CDSs) were predicted, with 27 rRNA and 72 tRNA genes (**Fig. 1B**), constituting a gene density of 1033 genes/megabase. The 2757 identified genes were classified in functional categories based on the COG designation (Tatusov et al., 2000). The classified genes are represented in the circular format shown in **Figure 1A**.

In nature, the carotenoid precursor isopentenyl diphosphate (IPP) is synthesized via two distinct pathways, namely the mevalonate (MVA) and 2-C-methyl-D-erythritol-4-phosphate (MEP) pathways (Lee and Schmidt-Dannert, 2002). Genome annotation analysis revealed that *P. faecalis* AJ003^T has a complete MEP pathway for synthesizing IPP, similar to the other strains of the *Planococcus* family. Seven MEP pathway genes were identified. They encode the following enzymes : 1-deoxy-D-xylulose-5-phosphate synthase (*dxs*;

AJGP001_07970), 1-deoxy-D-xylulose-5-phosphate reductoisomerase (*ispC*;
 AJGP001_11285), 2-C-methyl-D-erythritol 4-phosphate cytidyltransferase (*ispD*;
 AJGP001_00505), 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (*ispE*;
 AJGP001_00240), 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (*ispF*;
 AJGP001_00510), (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate synthase (*ispG*;
 AJGP001_07750), and 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (*ispH*;
 AJGP001_07715) (**Fig. 2A**).

Based on the chemical structure of glycosyl-4,4'-diaponeurosporen-4'-ol-4-oic acid, as described in our previous study (Kim et al., 2015), six genes encoding the glycosyl-4,4'-diaponeurosporen-4'-ol-4-oic acid pathway enzymes were expected in the genome of *P. faecalis* AJ003^T. However, genome annotation analysis revealed that *P. faecalis* AJ003^T has a carotenoid gene cluster comprising five genes encoding four C30 carotenoid pathway enzymes (**Fig. 2B**): two heterologous copies of the *crtN* gene, namely *crtN1* (AJGP001_15430) and *crtN2* (AJGP001_15440), encoding 4,4'-diapophytone desaturase (CrtN); *crtM*, encoding 4, 4'-diapophytone synthase (CrtM; AJGP001_15435); *crtP*, encoding 4, 4'-diaponeurosporene oxidase (CrtP; AJGP001_15445); and *crtQ*, encoding glycosyltransferase (CrtQ; AJGP001_15425). As indicated by the two question marks in **Fig. 2B**, two genes encoding AldH-like aldehyde dehydrogenase (Kim and Lee, 2012) and CrtA-like monooxygenase (Lee et al., 2010) were not computationally identified near the carotenoid gene cluster. Notably, this is not unique to *P. faecalis* AJ003^T because it is often reported that one or two genes are located far away from a carotenoid gene cluster, for instance in the C30 carotenoid pathway gene organization in *Staphylococcus aureus* (Kim and Lee, 2012) and *Methylomonas* sp. (Tao et al., 2005). Therefore, the two essential carotenoid genes are thought to be located away from the carotenoid gene cluster in the

genome of *P. faecalis* AJ003^T. Although these two genes have not yet been computationally identified, the C30 carotenoid glycosyl-4, 4'-diaponeurosporen-4'-ol-4-oic acid pathway of *P. faecalis* AJ003^T has been proposed, as shown in **Fig 2B**, based on the information gathered from the structure of glycosyl-4, 4'-diaponeurosporen-4'-ol-4-oic acid, the predicted carotenoid gene cluster, and the MEP pathway genes.

We investigated the function of the proposed carotenoid pathway genes in *P. faecalis* AJ003^T by heterologously expressing the whole gene cluster comprising *crtN1*, *crtN2*, *crtM*, *crtP*, and *crtQ* in *E. coli* under the control of one constitutive modified *lac* promoter, even though the translational direction of the *crtP* and *crtQ* genes is opposite to that of the others. Notably, we detected 4,4'-diapolycopene (peak 1 in **Fig. 3A**), which is not present in the C30 carotenoid pathway of *P. faecalis* AJ003^T, as a major carotenoid, with a small amount of 4, 4'-diaponeurosporene (peak 2 in **Fig. 3A**), which is a pathway precursor of glycosyl-4,4'-diaponeurosporen-4'-ol-4-oic acid. We suspected that the transcription of the oppositely located *crtP* and *crtQ* genes might affect the expression of the *crtN1*, *crtN2*, and *crtM* genes, and the resulting unbalanced ratio of substrate to expressed enzyme might influence the unexpected formation of 4,4'-diapolycopene. It is well documented that substrate/enzyme dynamic ratios significantly affect the substrate channeling of multienzyme complexes, and influence product profiles in heterologous hosts (Song et al., 2013; Lee and Schmidt-Dannert, 2002; Sacchettini and Poulter, 1997). Therefore, to understand the formation of 4,4'-diapolycopene, we constructed a subcluster, *crtN1-crtN2-crtM*, and expressed it under the control of a single promoter in *E. coli*. Unlike the detection of 4,4'-diapolycopene in the heterologous expression of the whole gene cluster, 4,4'-diaponeurosporene (peak 2 in **Fig. 3B**) was detected as a sole carotenoid.

Because two genes (*crtN1* and *crtN2*) encoding CrtN were present in the cluster, we investigated the function of the *crtN1* and *crtN2* genes by coexpressing CrtN1 and CrtM (**Fig. 3C**), and CrtN2 and CrtM (**Fig. 3D**), respectively. In both combinations, 4,4'-diaplycopenes (peak 1 in **Fig. 3C** and **3D**) and 4,4'-diaponeurosporene (peak 2 in **Fig. 3C** and **3D**) were detected as the main carotenoids. Both 4,4'-diaplycopenes and 4,4'-diaponeurosporene were expressed in considerable amounts, in contrast to 4,4'-diaponeurosporene obtained when expressing the subcluster *crtN1-crtN2-crtM*. An explanation for this observation requires further investigation. However, we suspect that, unlike the finely controlled expression in native *P. faecalis* AJ003^T, the overexpression of CrtN1, CrtN2, and CrtM under the control of a constitutive promoter in a heterologous *E. coli* host might change substrate channeling or the coordination of carotenoid enzyme complexes, which might subsequently affect the carotenoid profiles, as reported in other studies (Lee et al., 2010; Kim et al., 2012). Based on the carotenoid profile, the *crtN2* gene encodes the main CrtN and the *crtN1* gene encodes an auxiliary CrtN.

We next coexpressed the subcluster *crtN1-crtN2-crtM* and the other subcluster *crtQ-crtP* in *E. coli* to investigate the functions of the *crtP* and *crtQ* genes. As shown in **Fig. 3E**, the expected 4,4'-diaponeurosporene-4-al was not detected as 4,4'-diaponeurosporene and 4,4'-diaplycopenes accumulated. This suggests that the CrtP-based reaction that converts 4,4'-diaponeurosporene to 4,4'-diaponeurosporene-4-al did not occur. We suspect that the transcriptional order of the subcluster *crtQ-crtP* negatively affects the expression of CrtP. Therefore, we constructed and coexpressed two synthetic expression modules, *crtN2-crtM* and *crtP*, in *E. coli* (**Fig. 3F**). As with the expression of the two subclusters, *crtN1-crtN2-crtM* and *crtQ-crtP*, 4,4'-diaponeurosporene-4-al was not detected. Further research is

underway to determine why there was no CrtP activity, including an investigation of the possible misannotation of the *aldH*-like gene as *crtP*.

The complementation of the heterologous pathway enzymes is a useful criterion for characterizing the target pathway enzyme(s), including their substrate specificity, and for optimizing engineered pathways in heterologous hosts (Kim et al., 2016). Therefore, the complementation capabilities of CrtN1, CrtN2, and CrtM of *P. faecalis* AJ003^T were investigated by coexpression with heterologous CrtN_{SA} and CrtM_{SA} of *S. aureus* (**Table 1**). We detected very little 4,4 $\square$ -diapolycopene or 4,4 $\square$ -diaponeurosporene when CrtN1 and CrtM_{SA} were coexpressed in *E. coli* (**Fig. 3G**), which suggests that CrtN1 is an auxiliary enzyme. Unlike CrtN1, when CrtN2 was coexpressed with CrtM_{SA} in *E. coli*, both 4,4 $\square$ -diapolycopene and 4,4 $\square$ -diaponeurosporene were detected in considerable quantities (**Fig. 3H**), proving that CrtN2 is the main 4,4 $\square$ -diapophytone desaturase. As with the observed complementation of CrtN1 and CrtN2, CrtM exhibited complementation with heterologous CrtN_{SA} in *E. coli* (**Fig. 3F**). Therefore, the observed complementation of CrtN2 and CrtM can be used as an expression module for identifying the missing carotenogenic genes from putative candidates, and for extending C30 carotenoid pathways (Kim et al., 2016). In conclusion, we computationally annotated the genes encoding the enzymes of the C30 carotenoid pathway and its precursor MEP pathway, based on the completely sequenced genome of C30 carotenoid-producing *P. faecalis* AJ003^T. Although at least six carotenogenic genes are required to produce C30 carotenoid glycosyl-4,4 $\square$ -diaponeurosporen-4 $\square$ -ol-4-oic acid in *P. faecalis* AJ003^T (**Fig. 2B**), the identified carotenoid pathway gene cluster comprises five genes, of which *crtN1* and *crtN2* encode 4,4 $\square$ -diapophytone desaturase. Therefore, two missing genes encoding AldH-like aldehyde dehydrogenase and CrtA-like monooxygenase, which are remote from the cluster, need to be mined.

The products of three genes, *crtN1*, *crtN2*, and *crtM*, exhibited complementation with the heterologous CrtM_{SA} and CrtN_{SA} of *S. aureus*. This complementation could assist the identification of the two missing carotenogenic genes from putative candidates in reconstructed carotenoid pathways, and could be used to confirm the functions of the computationally annotated pathway enzymes CrtP and CrtQ.

The two genes encoding AldH-like aldehyde dehydrogenase and CrtA-like monooxygenase are thought to be located away from the core gene cluster. Locating and identifying these missing genes could provide insight into the genome mobility of *P. faecalis* AJ003^T, and could elucidate the mechanism by which glycosyl-4,4'-diaponeurosporen-4'-ol-4-oic acid is produced. Furthermore, engineered *E. coli* cells expressing complete pathway genes could be used to produce glycosyl-4,4'-diaponeurosporen-4'-ol-4-oic acid and/or pathway intermediates for biological studies.

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NUCLEOTIDE SEQUENCE AND STRAIN ACCESSION NUMBERS

The complete genome sequence of *P. faecalis* AJ003^T has been deposited at GenBank under accession number **CP019401**, and the strain has been deposited at the Korean Collection Type Culture (KCTC) under accession number **KCTC 33580^T**.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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Table 1. Bacterial strains and plasmids used in this study

Strains or plasmids	Relevant properties	Source or reference
Strains		
<i>E. coli</i> XL1-blue	endA1 gyrA96(nal ^R) thi-1 recA1 relA1 <i>lac</i> glnV44 F $\square$ [:Tn10 proAB ⁺ lacI ^q (Δ lacZ)M15] hsdR17(r _K m _K ⁺)	Stratagene
<i>Planococcus faecalis</i> AJ003 ^T	A type strain of genus <i>Planococcus</i>	Kim et al., 2015
Plasmids		
pUC19	Cloning vector. pMB1 origin. Inducible <i>lac</i> promoter, Amp ^R	NEB
pUCM	Cloning vector modified from pUC19. Constitutive <i>lac</i> promoter, Amp ^R	This study
pSTVM	Expression vector modified form pSTV28; deleted <i>lacZ</i> fragment and <i>lac</i> promoter, Cm	Kim et al., 2016
pACM	Expression vector modified form pACYC184; deleted <i>lacZ</i> fragment and <i>lac</i> promoter, Cm	Kim and Lee, 2012
pACM_crtN _{SA}	Constitutively expressed <i>crtN</i> gene from <i>Staphylococcus aureus</i>	Kim and Lee, 2012

pACM_crtM _{SA}	Constitutively expressed <i>crtM</i> gene from <i>S. aureus</i>	Kim and Lee, 2012
pUCM_crtN1	Constitutively expressed <i>crtN1</i> gene from <i>P. faecalis</i> AJ003 ^T	This study
pUCM_crtN2	Constitutively expressed <i>crtN2</i> gene from <i>P. faecalis</i> AJ003 ^T	This study
pUCM_crtM	Constitutively expressed <i>crtM</i> gene from <i>P. faecalis</i> AJ003 ^T	This study
pUCM_crtP	Constitutively expressed <i>crtP</i> gene from <i>P. faecalis</i> AJ003 ^T	This study
pUCM_Cluster	Constitutively expressed a whole gene cluster consisting of <i>crtM</i> , <i>crtN1</i> , <i>crtN2</i> , <i>crtP</i> , and <i>crtQ</i> of <i>P. faecalis</i> AJ003 ^T	This study
pUCM_crtN1_crtN2_crtM	Constitutively expressed <i>crtN1</i> , <i>crtN2</i> , and <i>crtM</i> of <i>P. faecalis</i> AJ003 ^T	This study
pACM_crtN2_crtM	Constitutively expressed <i>crtN2</i> and <i>crtM</i> of <i>P. faecalis</i> AJ003 ^T	This study
pSTVM_crtP	Constitutively expressed <i>crtP</i> of <i>P. faecalis</i> AJ003 ^T	This study
pSTVM_crtP_crtQ	Constitutively expressed <i>crtP</i> and <i>crtQ</i> of <i>P. faecalis</i> AJ003 ^T	This study

FIGURES AND LEGENDS

Fig. 1 Circular representation (A) and features of the *Planococcus faecalis* AJ003^T genome (B).

From the outer to inner circle: predicted protein-coding sequences (colored according to clusters of orthologous groups (COG) categories) on the plus strand; predicted protein-coding sequences (colored according to COG categories) on the minus strand; RNA genes (tRNAs, blue; rRNAs, red); GC content (blue/black); and GC skew (red/black).

Fig. 2. Proposed MEP and C30 carotenoid pathways of *Planococcus faecalis* AJ003^T (A) and the C30 carotenoid biosynthesis gene cluster (B).

(A) The proposed MEP and carotenoid pathways are based on genome annotation and information on the chemical structure of glycosyl-4,4'-diaponeurosporen-4'-ol-4-oic acid, as reported in a previous study (Kim et al., 2015). The two question marks (?) in the carotenoid pathway indicate that two enzymes involved in the reactions were not identified in the genome based on the annotation analysis.

Fig. 3. High-performance liquid chromatography (HPLC) analysis of extracts of

recombinant *Escherichia coli* strains expressing carotenoid pathway genes. Expression of the whole gene cluster comprising *crtN1*, *crtN2*, *crtM*, *crtP*, and *crtQ* under the control of a constitutive modified *lac* promoter (A); expression of one expression module of *crtN1*-*crtN2*-*crtM* (B); coexpression of two expression modules, *crtN1* and *crtM* (C); coexpression of two expression modules, *crtN2* and *crtM* (D); coexpression of two expression modules, *crtN1*-*crtN2*-*crtM* and *crtP*-*crtQ* (E); coexpression of two expression modules, *crtN2*-*crtM* and *crtP* (F). Heterologous complementation of *crtN1* of *P. faecalis* AJ003^T (CrtN1_{PF}) and *crtM* of *Staphylococcus aureus* (CrtM_{SA}) (G); *crtN2* of *P. faecalis* AJ003^T (CrtN2_{PF}) and *crtM* of *S. aureus* (CrtM_{SA}) (H); and *crtM* of *P. faecalis* AJ003^T (CrtM_{PF}) and *crtN* of *S.*

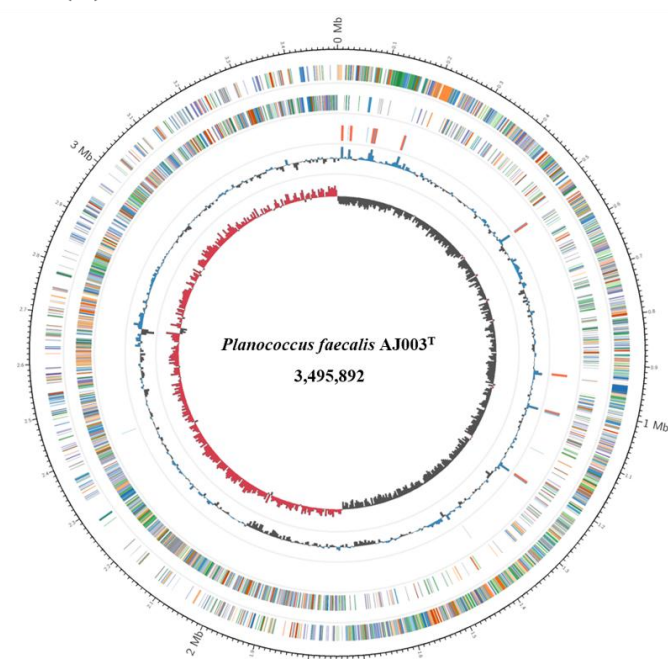
aureus (CrtN_{SA}) (I). The insert UV/VIS spectra in the panels correspond to peaks in the HPLC chromatogram. Peak 1, 4,4'-diapolycopene; peak 2, 4,4'-diaponeurosporene.

ACCEPTED MANUSCRIPT

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Figr-1

(A)

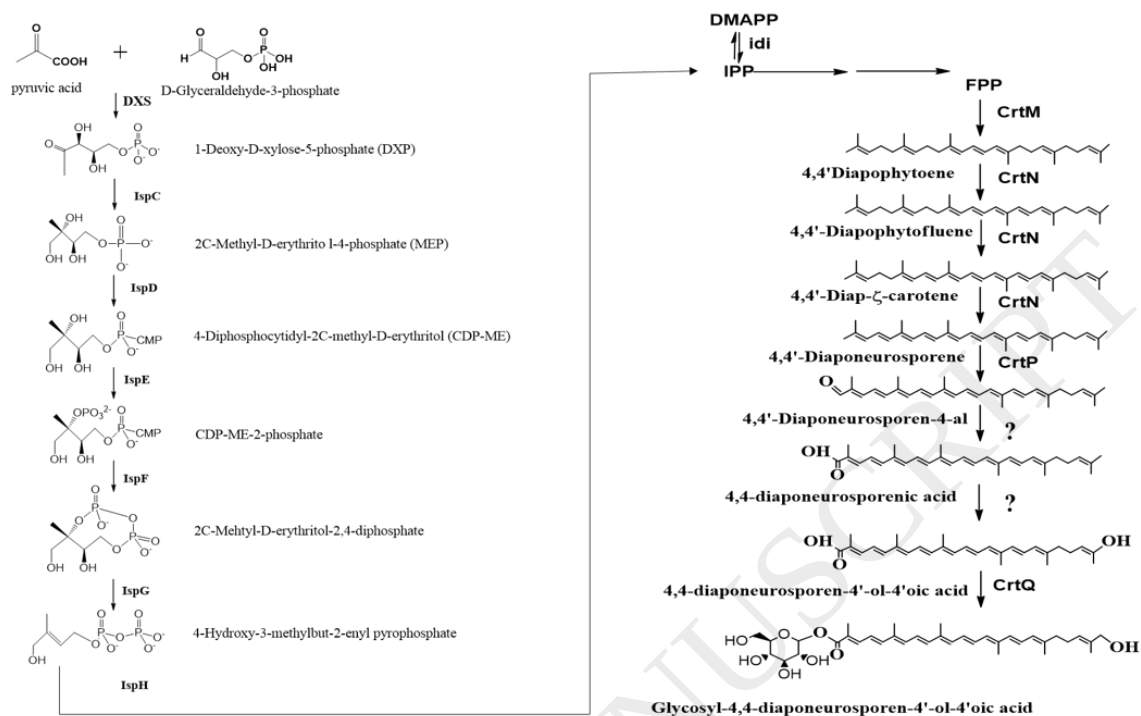


(B)

Attribute	Chromosome
Genome size (bp)	3,495,892
Number of Contigs	1
G + C content (%)	40.9
CDS	3511
rRNA(5S, 16S, 23S)	27
tRNA	72

Figr-2

(A)



(B)

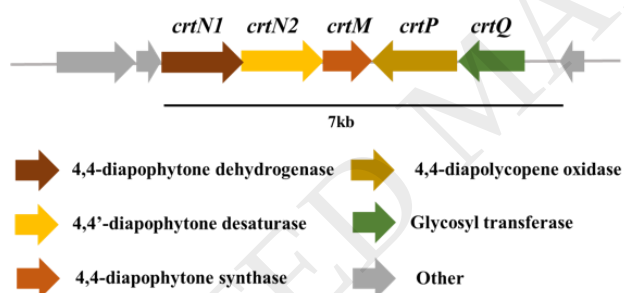
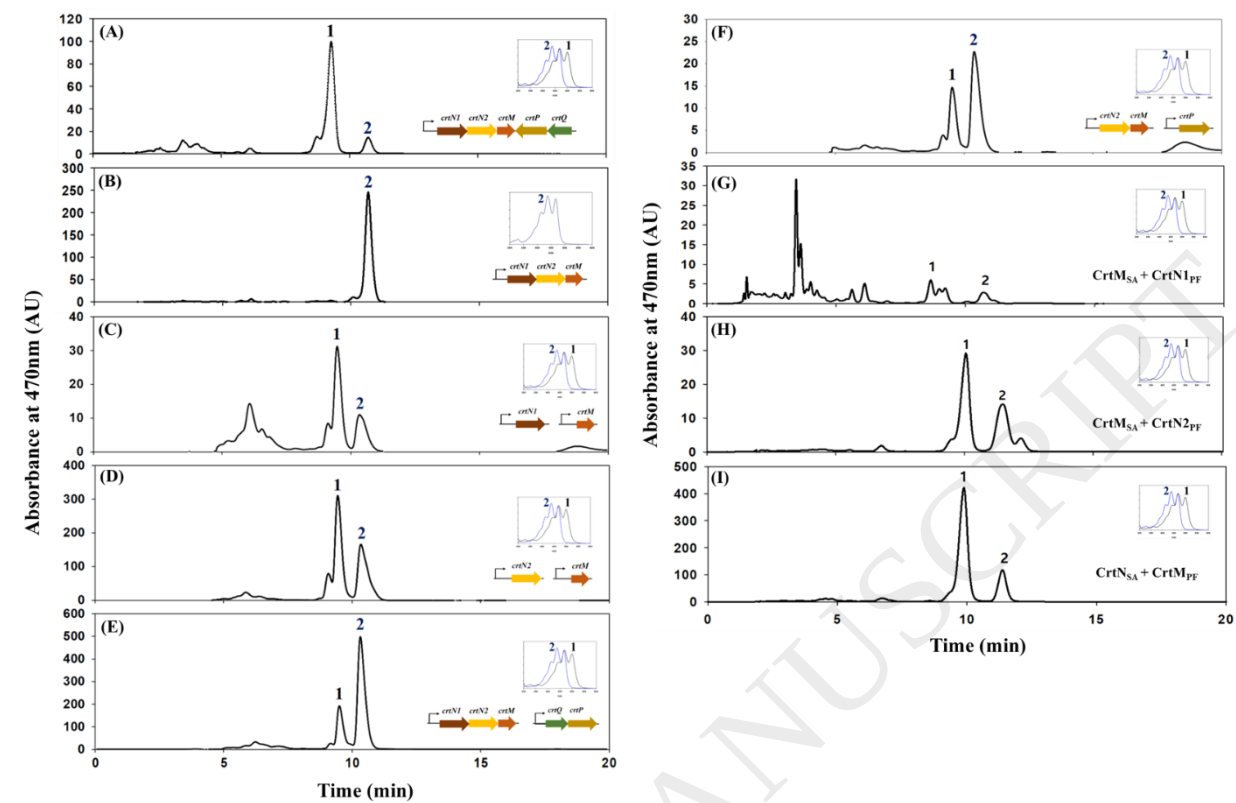


Fig-3



Supplementary table S1

Primer	Sequence (5' → 3')	Enzyme site
<i>crtN1_F</i>	GCTCTAGAAGGAGGATTACAAAATGCCGGAAAAAATGATTGT	<i>XbaI</i>
<i>crtN1_R</i>	GCGAATTCTCATGCAAATCCCTCCATT	<i>EcoRI</i>
<i>crtN2_F</i>	GCTCTAGAAGGAGGATTACAAAATGAAAATAGCTATGG TCGGTG	<i>XbaI</i>
<i>crtN2_R</i>	GCGAATTCTCATGCGTTGCCC	<i>EcoRI</i>
<i>crtM_F</i>	TCCCCCGGGAGGAGGATTACAAAATGGACTTAAAAGATGCTTAT	<i>XmaI</i>
<i>crtM_R</i>	GGAATTCCATATGTTACAAGCAAGCT GGATT	<i>NdeI</i>
<i>crtP_F</i>	GCTCTAGAAGGAGGATTACAAAATGAAAAAATCGTTATAGTCG	<i>XbaI</i>
<i>crtP_R</i>	GCGAATTCTTAAGGAAAACCTTTTTTAGAA	<i>EcoRI</i>
<i>crtQ_F</i>	GCTCTAGAAGGAGGATTACAAAATGATGGCTAGCTATATCG	<i>XbaI</i>
<i>crtQ_R</i>	GGAATTCATATGTCATGAATAATGCCTTCCTT CTGAGAACGACGAGC	<i>NdeI</i>