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Isolation and Characterization of Carotenoid Biosynthesis Genes from *Pantoea agglomerans* pv. *millettiae* Wist 801

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Summary : *Pantoea agglomerans* pv. *millettiae* produces a yellow pigment and is the causal agent of wisteria stem galls. We cloned and sequenced a 6.8-kb DNA fragment containing the yellow pigment gene. Analysis of the nucleotide sequence revealed that this clone contains five open reading frames (ORFs) transcribed in the same direction, and one terminal ORF transcribed in the opposite orientation. Comparison of the nucleotide and predicted amino acid sequences of these ORFs showed that they correspond to the carotenoid biosynthesis (*crt*) genes *crtE*, *-X*, *-Y*, *-I*, *-B* and *-Z*, respectively, in *P. ananatis* pv. *uredovora* 20D3, and *P. agglomerans* strains Eho 10 and Eho13. It was suggested that the carotenoid biosynthesis pathway of *P. agglomerans* pv. *millettiae* is basically identical to that of the three other *Pantoea* strains. When the *crt* genes from *P. agglomerans* pv. *millettiae* were expressed in *E. coli* WP2, this bacterium also displayed a yellow phenotype. The carotenoid pigments are known for their ability to protect various organisms against UV-induced damage, however, there were no significant differences in mutagenesis and survival after UV irradiation between yellow-pigmented and non-pigmented *E. coli* WP2 strains.

Key words : *Pantoea agglomerans* pv. *millettiae*, Carotenoid biosynthesis, UV irradiation.

Introduction

Carotenoids are important natural pigments distributed widely in plants, algae, and photosynthetic bacteria, where they play a critical role in photosynthesis. They also occur in some non-photosynthetic bacteria such as the epiphytic *Pantoea*, yeasts, and molds, where they may play a protective function against damage by light and oxygen (Misawa *et al.* 1996 ; Armstrong *et al.* 1997). The genes encoding carotenoid biosynthesis in *Pantoea ananatis* pv. *uredovora* 20D3 (Misawa *et al.* 1990), *Pantoea agglomerans* Eho10 (Hundle *et al.* 1994) and *P. agglomerans* Eho13 (To *et al.* 1994) have been cloned and sequenced. The func-tion of these crt genes has also been determined. The *crt* genes of the above strains were chromosome encoded. However, the yellow-pigment genes of *P. agglomerans* Eh112Y were on a plasmid (Gantotti *et al.* 1982). The biological role of the carotenoid pigment of the genus *Pantoea*, which was expressed in *E. coli*, has been shown to be protection against near-UV light (Becker-Hapak *et al.* 1997 ; Tuveson *et al.* 1988 ; Sandmann *et al.* 1998).

Pantoea agglomerans pv. *millettiae* is a yellow-pigmented bacterium that causes stem galls on Japanese (*Wisteria floribunda*) and Chinese wisteria (*W. sinensis*) in Japan and the United States (Goto *et al.* 1980 ; Opgenorth *et al.* 1994). Strains of *P. agglomerans* pv. *millettiae* lost yellow pigmentation when grown at elevated temperature (37 °C) (Kamiunten 1987). However, no plasmid loss was observed in any of the pigmentless variants. This result suggested that the pigment genes are located on the chromosome of *P. agglomerans* pv. *millettiae*. However, genetic and biological analyses of *crt* genes of this bacterium have not been carried out.

In this paper, we report on the cloning of *crt* genes from a genomic library of *P. agglomerans* pv. *millettiae* Wist 801 and the effect of ultraviolet irradiation on the mutation and survival of *E. coli* WP2

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transformants carrying *crt* genes.

Materials and Methods

Bacterial strains, plasmids, culture conditions and chemicals

The bacterial strains and plasmids used in this study are listed in Table 1. *P. agglomerans* pv. *millettiae* and *E. coli* strains were routinely cultured in YP medium (Kamiunten, 1990) at 30°C and 37°C, respectively. Mutation and survival were assayed on SEM agar (minimal agar supplemented with 0.5 % glucose and 0.4 % nutrient broth) and nutrient broth (Difco), respectively. The final concentrations of antibiotics were as follows: tetracycline (Tc), 30 μg/ml ; ampicillin (Ap), 50 μg/ml ; nalidixic acid (Nal), 50 μg/ml.

DNA manipulations and DNA sequencing

The genomic DNA of *P. agglomerans* pv. *millettiae* Wist 801 was partially digested with *Sau* 3A. DNA fragments within a size range of 25 to 40 kb were isolated by gradient ultracentrifugation (10–40 % sucrose, 75,000 × *g*, 22h) and ligated to the cosmid vector pLAFR3, which had been digest-ed with *Bam*HI and then treated with alkaline phosphatase. The ligated DNA mixture was packaged *in vitro* using a LAMBDA INN packaging kit (Nippon gene) and then introduced into *Escherichia coli* DH5. The cosmid clones were plated and selected on YP agar containing Tc.

The nucleotide sequence of the insert DNA was determined by primer extension sequencing at Sawaday Technology (Tokyo, Japan). The nucleotide and deduced amino acid sequences were compared with sequences in the database using Genetyx programs (Tokyo, Japan).

UV mutagenesis and survival assay

E. coli WP2 was used in mutagenicity and survival tests because of its high sensitivity to many kinds of mutagens. For UV mutagenesis assays, overnight cultures of transformant strains of WP2 were washed twice and resuspended in 1/15 M phosphate buffer (pH 7.0). Two-milliliter lots of the bacterial suspension were irradiated in petri dishes with stirring in a dark room. Irradiation was carried out using a 253 nm UV lamp (HITACHI-GL15), 300 nm UV lamp (FUNAKOSHI-F15T8UV-B) and 360 nm UV lamp (TOSHIBA-FL15BLB) under various conditions of both irradiation time and distance. The UV-irradiated bacterial suspension (0.2 ml) was mixed with the overlay agar (2.0 ml), and immediately poured onto an SEM agar plate. To avoid photoactivation, plates were immedi-ately wrapped in foil and then incubated at 37°C. After 3 days, number of Trp^+ revertant colonies was counted. For survival assays, bacteria grown in nutrient broth medium at 37°C to *ca.* $\times 5 \times 10^8$ cells/

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference or source
Pantoea agglomerans pv. *millettiae*		
Wist 801	Pathogen of wisteria, wild type	Goto and Takikawa[a]
Wist 801W	Pigmentless mutant of Wist 801, Nal^r	This study
E. coli		
DH5	Competent cell	Nippongene Co., Ltd
WP2	Tryptophan-requiring mutant	IFO[b]
Plasmids		
pLAFR3	Broad-host-range cosmid vector, Tc	Staskawicz *et al.* (1987)
pRK2013	Kn^r Tra^+ Mob^+ ColE1 replicon	Ditta *et al.* (1980)
pBR322	Cloning vector, Ap^r, Tc^r	Takara Bio Inc.
pUC19	Cloning vector, Ap^r	Takara Bio Inc.
pW92	40-kb genomic DNA containing *crt* genes in pLAFR3	This study
pPST2	11-kb genomic DNA containing *crt* genes in pUC19	This study
pECO1	8-kb genomic DNA containing *crt* genes in pBR322	This study
pSAU1	6.8-kb genomic DNA containing *crt* genes in pBR322	This study

[a] Shizuoka University
[b] Institute for fermentation, Osaka

ml were centrifuged and resuspended in 0.1 M sodium phosphate buffer (pH 7.4). The bacterial suspension was diluted to *ca.* $5 \times 10^3 - 1 \times 10^4$ cells/ml. Aliquots (0.1 ml) of diluted culture were plated on nutrient agar plates containing Tc and then exposed to UV irradiation. Plates were wrapped in foil and incubated at 37°C. Clonies were counted on second day after incubating.

Results and Discussion

Cloning of yellow pigment genes

A cosmid library was constructed from the genomic DNA of yellow-pigmented *P. aglomerans* pv. *millettiae* Wist 801 (Fig. 1A). Genomic library constructs were introduced into *E. coli* DH5 (Fig. 1B). Of 6,000 cosmid clones, only one was yellow (Fig. 1C). This yellow clone contained a recombinant plasmid designated as pW92 and carrying 40-kb of insert DNA. Transfer of pW92 from *E. coli* DH5 to a pigmentless mutant strain of *P. agglomerans* pv. *millettiae* Wist 801 (Wist 801W ; Fig. 1D) was conducted by triparental mating with the helper plasmid pRK2013 ; the resulting trans-conjugant showed yellow pigmentation (Fig. 1E). As the yellow pigment genes were expressed in both *E. coli* and *P. agglomerans* pv. *millettiae*, subsequent subcloning experiments were conducted using *E. coli*. The pW92 plasmid was subjected to *Pst*I partial digestion, ligated into the *Pst*I site of pUC19, and transformed into *E. coli* DH5. The resulting yellow clone had a plasmid, designated pPST2, containing approximately 11 kb of insert DNA. The pPST2 plasmid was further subjected to *Eco*RI digestion and ligated into the *Eco*RI site of pBR322. The resulting plasmid, pECO1, contained the yellow pigment genes in approximately 8 kb of insert DNA. Further deletion of pECO1 by *Sau*3AI partial digestion yielded a plasmid, designated pSAU1, containing approximately 6.8 kb of insert DNA. This plasmid was still able to produce yellow pigment (Fig. 1F) ; however, further deletion of pSAU1 by *Sau*3AI partial digestion resulted in a loss of yellow pigmentation.

Nucleotide sequence analysis

The nucleotide sequence of the insert in pSAU1 was determined. The results showed that the entire sequence was 6815 bp long. The sequences of the *crt* genes in *P. agglomerans* pv. *millettiae* Wist 801 have been deposited in DDBJ under accession No. AB07662.

Nucleotide sequence analysis revealed six open

Fig. 1. Pigmentation of *P. agglomerans* pv. *millettiae* Wist801 and *E. coli* DH5 containing crt genes. **A** : *P. agglomerans* pv. *milletitae* Wist 801 (wild-type), **B** : *E. coli* DH5, **C** : *E. coli* DH5 (pW92), **D** : *P. agglomerans* pv. *millettiae* Wist 801W (pigmentless mutant), **E** : *P. agglomerans* pv. *millettiae* 801W (pW92), **F** : *E. coli* DH5 (pSAU1).

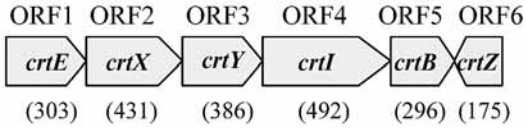

Fig. 2. Genetic organization of the *crt* genes of *P. agglomerans* pv. *millettiae* Wist 801. The arrowhead indicates the deduced direction of transcription for the operon. The length of the amino acid sequence is shown in parentheses.

Table 2. Sequence similarities of *crt* genes of *P. agglomerans* pv. *millettiae* Wist801

ORF (bp)	Gene	Homology (%)					
		P. a. pv. 20D3[a]		*P. a.* Eho10[b]		*P. a.* Eho13[c]	
		DNA	Peptide	DNA	Peptide	DNA	Peptide
ORF-1(912)	*crt E*	75.0	80.1	60.5	55.1	73.8	78.1
ORF-2(1296)	*crt X*	67.1	70.7	59.8	47.8	69.1	70.7
ORF-3(1161)	*crt Y*	70.8	73.0	62.0	55.8	70.1	73.3
ORF-4(1479)	*crt I*	77.6	88.0	74.5	77.5	78.0	88.2
ORF-5(891)	*crt B*	75.6	83.1	68.0	64.9	74.7	82.1
ORF-6(528)	*crt Z*	79.2	86.3	69.7	66.7	—[d]	—

a) *Pantoea ananatis* pv. *uredovora* 20D3
b) *Pantoea agglomerans* Eho10
c) *Pantoea agglomerans* Eho13
d) *crt Z* has not been reported.

reading frames (ORFs) arranged contiguously. The first five ORFs were transcribed in the same direction while the sixth was transcribed in the opposite direction (Fig. 2). Organization of the *crt* gene in *P. agglomerans* pv. *millettiae* Wist 801 was similar to that in *P. ananatis* pv. *uredovora* 20D3 (Misawa *et al.* 1990). However, the *crt* gene cluster of *P. agglomerans* Eh10 contained an additional ORF6 with unknown function between *crt* E and *crt* Z. All six ORFs contained in the insert of pSAU1 were preceded by a putative ribosome binding site and began with an ATG codon. The ORFs had significant similarity to known *crt* genes (Table 2). The percentages of sequence similarity among each *crt* gene at the protein level ranged from 47.8 to 88.2 %. Thus, the six ORFs were designated *crtE*, *-X*, *-Y*, *-I*, *-B* and *-Z*, respectively.

The function of the *crt* genes was clarified by analyzing carotenoids accumulated in *E. coli* containing partially deleted or mutated *crt* gene clusters from the genus *Pantoea* (Misawa *et al.* 1990, 1996 ; Funter *et al.* 1994 ; Armstrong *et al.* 1997). From sequence analysis, *P. agglomerans* pv. *millettiae* Wist 801 might be expected to follow a carotenoid biosynthesis pathway similar to that of *P. ananatis.* pv. *uredovora* 20D3 (Fig. 3).

UV mutability and survival of *E. coli* WP2 carrying *crt* genes

When the *crt* genes from *P. agglomerans* pv. *millettiae* Wist 801 were introduced into *E. coli* WP2, the cells became yellow (Fig. 4). To analyze the role of the carotenoids in protection against UV light, both *E. coli* WP2 (pSAU1) producing yellow pigment and pigmentless *E. coli* WP2 (pBR322) were exposed to UV irradiation. In preliminary experiments, the highest mutation frequencies were observed at UV-irradiation under the following conditions ; 253 nm UV lamp for 15 sec at a distance of 50 cm and 300 nm UV lamp for 10 sec at a distance of 20 cm. Therefore, the mutagenicity experiments were carried out under these irradiation conditions. However, no mutation was observed after 360 nm UV light exposure in preliminary tests. Mutation rates of *E. coli* WP2 (pSAU1) and WP2 (pBR322) following UV irradiation (253 and 300 nm) were 24×10^{-3}, 1×10^{-3}, 37×10^{-3} and 4×10^{-3} %, respectively (Table 3). There was no significant difference in mutation frequency between the two strains.

To investigate the UV sensitivity of *E. coli* WP2 (pSAU1) and WP2 (pBR322), cells were exposed to 253, 300 and 360 nm UV lamps for various times. The survival rates of the two strains were 0－98.5 %

Farnesyl pyrophosphate
↓ ***crtE*** (geranylgeranyl pyrophosphate synthase)
Geranylgeranyl pyrophosphate
↓ ***crt B*** (phytoene synthase)
Phytoene
↓ ***crt I*** (phytoene desaturase)
Lycopen
↓ ***crt Y*** (lycopene cyclase)
ß-carotene
↓ ***crt Z*** (ß-carotene hydroxylase)
Zeaxanthin
↓ ***crt X*** (zeaxanthin glucosylase)
Zeaxanthin diglucoside

Fig. 3. Schematic representation of the carotenoid biosynthesis pathway of *P. agglomerans* pv. *millettiae* Wist 801. The enzymes encoded by each gene are indicated in parentheses.

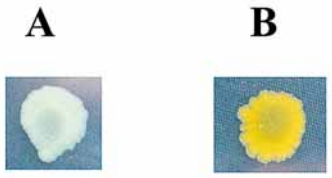

Fig. 4. Pigmentation of *E. coli* WP2 containing *crt* genes.
A : *E. coli* WP2 (pBR322)
B : *E. coli* WP2 (pSAU1)

Table 3. Effect of UV irradiation on the reverse mutation of *E. coli* WP2 containing *crt* genes

Strains	The rate of reverse mutation (%)[a] UV wavelength		
	253 nm[b]	300 nm[c]	360 nm[d]
	($\times10^{-3}$)	($\times10^{-3}$)	($\times10^{-3}$)
E. coli WP2(pBR322)	37±5.1	4±1.4	0±0
E. coli WP2(pSAU1)	24±6.0 NS	1±0.7 NS	0±0 NS

a) Data are mean ± SD of 3－5 replicates. NS, not significant ($p>0.05$).
b) HITACHI-GL15 lamp(50 cm, 15 sec)
c) FUNAKOSHI - F15T8 UV- B lamp(20 cm, 10 sec)
d) TOSHIBA - FL15BLB lamp(20 cm, 90 se)

Table 4. Effect of UV irradiation on the survival of *E. coli* WP2 containing *crt* genes

Strains	Survival rate (%)[a]										
	UV 253 nm[b] (sec)			UV 300 nm[c] (sec)				UV 360 nm[d] (min)			
	5	10	15	5	10	15	30	5	10	15	20
E. coli WP2 (pBR322)	11.7±2.7	0.8±0.3	0±0	81.7±3.3	73.5±8.4	44.9±10.2	9.2±2.4	98.1±2.7	97.2±4.0	98.5±1.6	96.9±4.4
E. coli WP2 (pSAU1)	11.1±4.5	0.8±0.3	0±0	80.6±9.1	70.0±6.9	43.1±9.2	9.1±1.6	96.7±4.6	93.6±9.1	97.4±2.0	96.7±4.6

a) Data are mean ± SD of 3 replicates. There was no significant difference between the two strains (all $p>0.05$).
b) HITACHI - GL15 lamp (70 cm)
c) FUNAKOSHI - F15T8 UV-B lamp (30 cm)
d) TOSHIBA - FL15BLB lamp (10 cm)

(Table 4). However, the survival rates were almost the same in two strains.

In the present studies, the carotenoid pigments produced in *E. coli* WP2 did not play an important role in cellular protection from UV irradiation. In the future, it would be necessary to conduct UV irradiation experiments under various conditions using *P. agglomerans* pv. *millettiae* strains originally carrying *crt* genes in order to elucidate the biological roles of carotenoids

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フジこぶ病細菌から分離したカロテノイド生合成遺伝子

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要 約

黄色色素を産生するフジこぶ病細菌（*Pantoea agglomerans* pv. *millettiae*）Wist 801からカロテノイド生合成遺伝子をクローニングし，全塩基配列を決定した．カロテノイド生合成遺伝子は６つのORFからなり，それぞれの塩基配列および想定されるアミノ酸配列の相同性から*crtE, crtX, crtY, crtI, crtB, crtZ*と同定され，カロテノイドの合成経路は他の*Pantoea*属細菌と基本的に同じであることが示唆された．カロテノイド色素はUVから細胞を保護することが知られており，分離した*crt*遺伝子群を復帰変異が検出できる大腸菌WP2に導入・発現させ，UV照射後の変異率および生存率を調べた．しかし，ベクターのみを導入したWP2（白色）との有意差は認められなかった．

キーワード：フジこぶ病細菌，カロテノイド生合成遺伝子，UV照射