

A σ^B -Like Factor Responsible for Carotenoid Biosynthesis in *Streptomyces griseus*

Han-Seung Lee,¹ Yasuo Ohnishi
and Sueharu Horinouchi*

Department of Biotechnology, Graduate School of Agriculture and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan

¹Present address: Bioproducts Research Center, Yonsei University, Seodaemon-ku, Seoul 120-749, Korea

Abstract

Self-cloning experiments with a high-copy-number plasmid and *Streptomyces griseus* IFO13350 led to the cloning of a 11-kb DNA fragment that conferred yellow pigment production on the host. The cloned fragment contained a gene cluster for carotenoid biosynthesis, in which two polycistrons, *crtE* (encoding geranylgeranyl pyrophosphate synthase)-*crtI* (phytoene dehydrogenase)-*crtB* (phytoene synthase)-*crtV* (functionally unknown methyltransferase-like protein) and *crtY* (lycopene cyclase)-*crtT* (functionally unknown methyltransferase-like protein)-*crtU* (β -carotene dehydrogenase), were present in a convergent way. Since strain IFO13350 produced no detectable amount of carotenoids, an increase in the copy number of the *crt* gene cluster led to production of carotenoids at a detectable level. Overexpression of the stress-responsive σ^B -like protein CrtS from *Streptomyces setonii* also activated the cryptic *crt* genes in *S. griseus* and conferred pigmentation. A CrtS homologue (σ^{CrtS}) in *S. griseus*, which was predicted by a computer-aided homology search, caused carotenogenesis to the same extent as CrtS of *S. setonii*, indicating that the two σ^B -like proteins were functionally the same. Yellow pigment production by *S. griseus* containing *crtS* under the control of a strong promoter on a high-copy-number plasmid resulted from activation of transcription of the *crt* genes, because overexpression of σ^{CrtS} in *S. griseus* led to transcriptional activation of the promoters in front of *crtE* and *crtY*. S1 nuclease mapping showed that *crtS* itself was transcribed at a low level under the laboratory conditions, which may account for undetectable production of carotenoids. The *crt* genes were suggested to locate very near one end of the linear chromosome, since they were completely deleted in mutant HH1 having large deletions at both ends. The gene organization of *crt* in *S. griseus* is similar to that in *S. coelicolor* A3(2) where the whole *crt* gene set is near one end of the chromosome.

Introduction

During our study on A-factor (2-isocapryloyl-3R-hydroxymethyl- γ -butyrolactone) in *Streptomyces griseus* IFO13350 (Horinouchi and Beppu, 1992; 1994), we happened to clone a DNA fragment that conferred yellow pigment production on the *S. griseus* host. The pigment turned out to be a carotenoid compound, because the cloned DNA fragment contained the whole gene set for carotenoid biosynthesis. This meant that we activated the "cryptic" carotenoid biosynthetic genes by increasing their copy number by placing them on a high-copy-number plasmid, since *S. griseus* IFO13350 does not produce a detectable amount of carotenoids under the laboratory conditions. In the bacterial genus *Streptomyces*, carotenoid production is light-inducible, constitutive, or completely absent (Kato *et al.*, 1995). Our interest in A-factor that switches on secondary metabolite formation and aerial mycelium formation at an extremely low concentration prompted us to determine possible dependence of carotenoid production on A-factor, because carotenoids are regarded as secondary metabolites.

Carotenoids comprise a major class of pigment molecules in all photosynthetic organisms from bacteria to plants, as well as non-photosynthetic bacteria, fungi, and yeasts (Goodwin, 1980; Armstrong, 1997). The carotenoid compounds protect cells against photooxidative damage and harmful radicals formed when cells are illuminated. The biosynthetic genes for carotenoids have been cloned from various organisms and the biosynthetic enzymes have been characterized (for review, see Armstrong, 1994; 1997). Alternative sigma factors of RNA polymerase responsible for transcription of carotenoid genes in some bacteria have been studied; an extracytoplasmic function (ECF) sigma factor, CarQ, that is essential for light-induced carotenoid synthesis in *Myxococcus xanthus* (Gorham *et al.*, 1996; Martinez-Argudo *et al.*, 1998) and stress-responsive σ^B -like CrtS that probably activates transcription of the carotenoid biosynthetic genes in *Streptomyces setonii* (Kato *et al.*, 1995) are examples. We then started to identify a sigma factor responsible for transcription of the carotenoid biosynthetic genes in *S. griseus*. This paper deals with the characterization of the carotenoid production genes, the map location of the genes on the chromosome, and the identification and characterization of a σ^B -like sigma factor responsible for transcription of the genes in *S. griseus*.

Results

Cloning and Nucleotide Sequencing of Carotenoid Biosynthesis Gene Cluster

During our shot-gun cloning of the chromosomal DNA, partially digested with *Sau3A*I, from *S. griseus* IFO13350 by using a high-copy-number plasmid pIJ702 (40 to 100

*For correspondence. Email asuhori@mail.ecc.u-tokyo.ac.jp; Tel. +81 3 5841 5123; Fax. +81 3 5841 8021.

copies per genome) as a vector and an *adpA*-disrupted strain derived from strain IFO13350 as the host, we found a transformant that produced a yellow pigment. AdpA is a transcriptional activator in the A-factor regulatory cascade and a mutation in *adpA* results in the loss of secondary metabolite production and aerial mycelium formation (Horinouchi and Beppu, 1992; 1994; Ohnishi *et al.*, 1999; Yamazaki *et al.*, 2000). The $\Delta adpA$ strain therefore produced neither streptomycin nor an A-factor-dependent diffusible yellow pigment. However, preliminary characterization of the yellow pigment produced by the transformant was apparently different from the A-factor-dependent yellow pigment (data not shown). In addition, the pigment was not detected in the wild-type strain IFO13350 when the culture broth was examined by HPLC. This meant that the cloned fragment at a high copy-number activated some gene(s), either a biosynthetic or regulatory gene, responsible for yellow pigment production.

Recombinant plasmid pYA1 containing the cloned 11-kb *Sau3AI* fragment in the *BglII* site of pIJ702 also conferred yellow pigment production at almost the same yield on the wild-type strain IFO13350 (Figure 1). The "Frame Plot" analysis of the nucleotide sequence revealed the presence of eight open reading frames (Figure 1A). All these open reading frames showed great homology with known proteins; FadD, long chain fatty acid CoA ligase; CrtE, geranylgeranyl pyrophosphate synthase; CrtI, phytoene dehydrogenase; CrtB, phytoene synthase; CrtV, functionally unknown methyltransferase-like protein; CrtU, β -carotene dehydrogenase; CrtT, functionally unknown methyltransferase protein; and CrtY, lycopene cyclase. The seven Crt proteins comprise a set of carotenoid biosynthetic enzymes found in many bacteria and a biosynthetic pathway is shown in Figure 2 (Armstrong, 1994; 1997). The degree of the homology of each of the gene products with those from *S. griseus* IMET JA3933 (Schumann *et al.*, 1996; Krügel *et al.*, 1999) is shown in percentage in Figure 1A. Plasmid pYA4F1 containing *crtE-crtI-crtB-crtV* on pIJ702 conferred production of a pink pigment on the hosts (Figure 1). The pink pigment was assumed to be lycopene, because, as seen from the biosynthetic pathway, lycopene is formed by the actions of CrtE, CrtB and CrtI. In addition to the high similarity in amino acid sequence and in size to the known carotenoid biosynthetic enzymes, these observations showed that the cloned fragment contained functional carotenoid production genes.

We introduced pYA1 into an A-factor-deficient mutant strain HH1 to see the effect of A-factor on the yellow pigment production. Mutant HH1 does not produce A-factor because of complete deletion of *afsA* probably encoding an A-factor biosynthetic enzyme (Lezhava *et al.*, 1997). Mutant HH1 harbouring pYA1 produced the yellow pigment at almost the same yield as the wild-type strain IFO13350 and the $\Delta adpA$ strain both containing pYA1 (data not shown), showing that A-factor or the transcriptional activator AdpA did not influence the yellow pigment production.

Deletion of Carotenoid Biosynthetic Gene Cluster in Mutant HH1

In *S. coelicolor* A3(2), carotenoid biosynthetic genes are located near the ends of the linear chromosome (Redenbach *et al.*, 1996; <http://www.sanger.ac.uk/Projects/>

S. coelicolor). We carried out Southern hybridization with the *crt* gene sequence against the chromosomal DNA from mutant HH1, on the assumption that the *crt* genes might be deleted in mutant HH1 if located near the end of the linear chromosome of *S. griseus*. The chromosomal ends of mutant HH1, including *afsA* that locates 150-kb from one end, were shown to be deleted (Lezhava *et al.*, 1997). A 2.6-kb *BamHI-SphI* fragment containing *crtE* and *crtI* and a 2.1-kb *BamHI* fragment containing *crtT* and *crtY* were used as probes for Southern hybridization against the *BamHI*-digested chromosomal DNA. No signals for the chromosomal DNA from mutant HH1 were detected, whereas a distinct signal was seen in the chromosomes from the wild-type and $\Delta adpA$ strains (Figure 3). This finding suggests that the *crt* genes are near one of the chromosomal ends, which are known to be readily deleted.

A Sigma-Like Factor Responsible for Transcription of *crt* Genes

A stress-responsive sigma factor σ^B -like protein in *S. setonii* (Kato *et al.*, 1995) and an ECF σ factor in *M. xanthus* (Gorham *et al.*, 1996) are responsible for transcription of *crt* genes. Kato *et al.* (1995) observed that *crtS* encoding a σ^B -like protein activated carotenoid production in *S. setonii* when placed on a plasmid. Schumann *et al.* (1996) also observed that the *S. setonii crtS* gene caused carotenoid production in *S. griseus* JA3933/956/2 having cryptic *crt* genes. To examine whether the *S. setonii crtS* gene on a plasmid causes carotenoid production in *S. griseus* IFO13350, we introduced p6P7S containing *crtS* under the control of the *tipA* promoter in pIJ702 into this strain (Figure 4A) and observed pigmentation. The *S. setonii crtS* gene was kindly provided by F. Kato (Kato *et al.*, 1995). Strain IFO13350 harbouring p6P7S produced a yellow pigment, whereas that harbouring the vector p6F7 did not (Figure 4B). This implied that the *S. setonii crtS* gene activated the cryptic *crt* genes in *S. griseus* IFO13350. The transformant harbouring p6P7S formed less abundant spores, the reason of which is unclear. We then searched the DNA databases for σ^B homologues from *S. griseus* and found a CrtS homologue. The *crtS* homologue in *S. griseus* has been registered as functionally unknown ORF1 (the GenBank accession number AB023642) that locates near *craA* responsible for carbon-source-dependent differentiation (Ueda *et al.*, 1999). ORF1 shows 94% identity in amino acid sequence to CrtS. ORF1 is 259 amino acids long and shows end-to-end similarity to σ^B in *Bacillus subtilis* responsible for stress responses (Hecker *et al.*, 1996), σ^F responsible for stationary-phase stress-response in *Mycobacterium tuberculosis* (DeMaio *et al.*, 1996), σ^F responsible for spore pigment formation in *Streptomyces aureofaciens* (Rezuchová *et al.*, 1997), and σ^F in *S. coelicolor* A3(2) responsible for sporulation (Potuckova *et al.*, 1995). We then placed the ORF1 sequence, kindly provided by K. Ueda (Ueda *et al.*, 1999), under the control of the thiostrepton-inducible *tipA* promoter in pIJ702 (plasmid p6P7B) and introduced it into the wild-type *S. griseus* IFO13350 (Figure 4A and B). *S. griseus* IFO13350 containing ORF1 on the plasmids produced a yellow pigment at the same amount as that containing the *S. setonii crtS* gene (plasmid p6P7S). Introduction of the plasmids into mutant HH1 having complete deletion of the

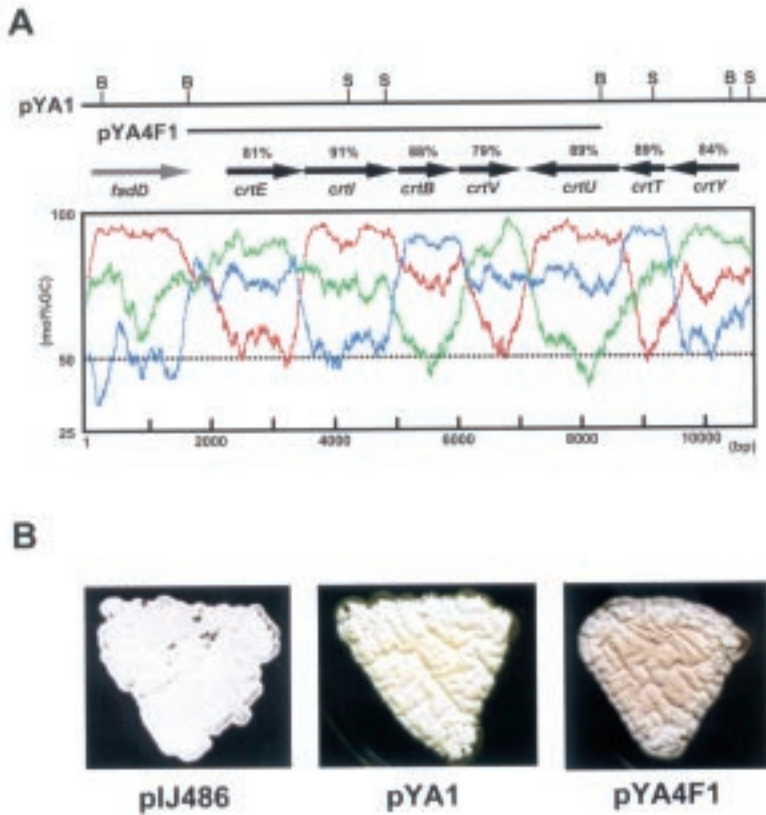


Figure 1. The Positions and Directions of Carotenoid Biosynthetic Genes in the Cloned 11-kb *Sau3A*I Fragment on pYA1 (A) and Carotenogenesis of *S. griseus* IFO13350 Transformants (B)

(A) The 6.8-kb *Bam*HI fragment on pYA4F1 conferring production of a pink compound on the host is also shown. The positions of open reading frames were deduced on the basis of the FRAME analysis of the nucleotide sequence (Bibb *et al.*, 1984; Ishikawa and Hotta, 1999) and a computer-aided homology search (BLAST, <http://www.ncbi.nlm.nih.gov/blast/blast.cgi>). The amino acid identity of each of the gene products to the corresponding gene product in *S. griseus* IMET JA3933 (Schumann *et al.*, 1996) is indicated on each of the open reading frames. Restriction enzymes abbreviated are: B, *Bam*HI; and S, *Sph*I. (B) *S. griseus* IFO13350 harbouring the vector pJ486 forms white spores (left), whereas that harbouring pYA1 or pYA4F1 produces a pink or yellow pigments, respectively. The pigments diffused from mycelium dye spores.

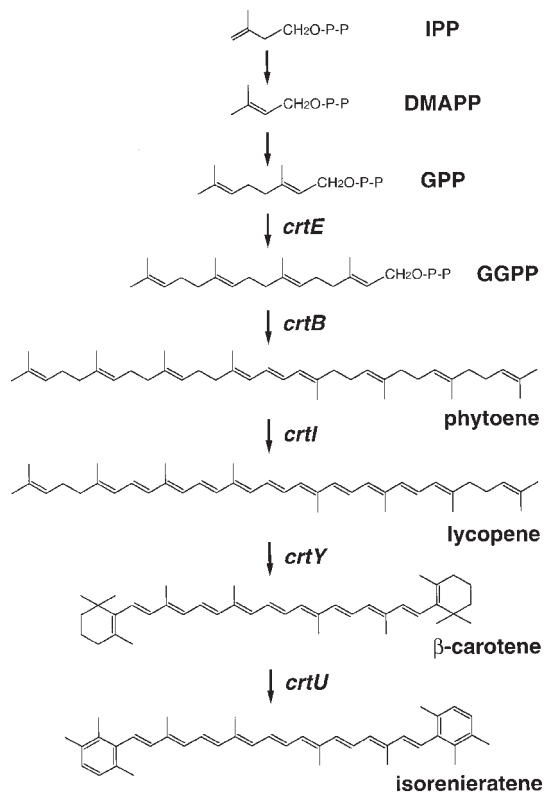


Figure 2. Scheme of the Biosynthetic Pathway of Carotenoids in *S. griseus* IFO13350 Abbreviations used are: IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; GPP, geranyl pyrophosphate; and GGPP, geranylgeranyl pyrophosphate.

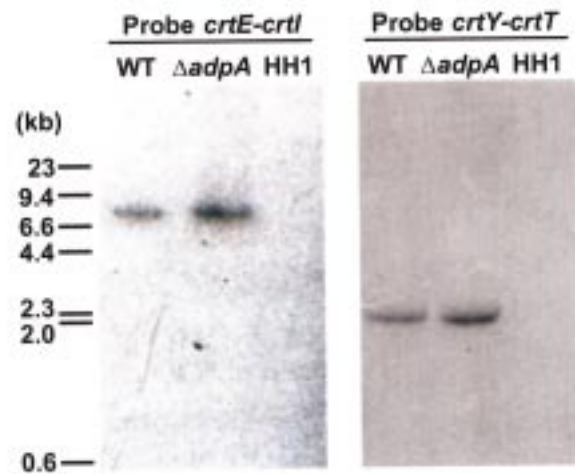


Figure 3. Southern Hybridization Showing Deletion of the Whole *crt* Genes The *crtE-crtI* and *crtY-crtT* regions are both lost in the chromosome of mutant HH1, whereas both regions are present in the wild-type IFO13350 (WT) and $\Delta adpA$ strains.

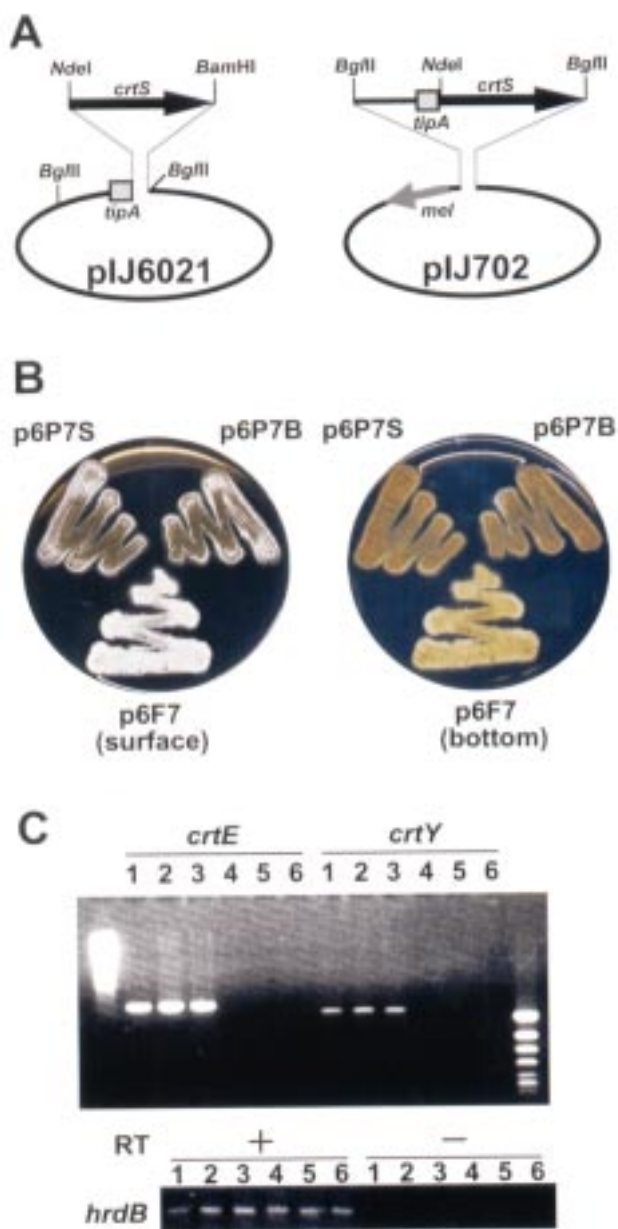


Figure 4. Structures of Plasmids for Overexpression of the *crtS* Genes from *S. setonii* and *S. griseus* (A), Carotenogenesis of the Transformants Containing the *crtS* Genes (B), and Detection of Transcripts for *crt* by RT-PCR (C)

(A) The ATG codon was placed downstream of the thiostrepton-inducible *tipA* promoter in pIJ6021. The *Bgl*II fragment containing the *tipA* and *crtS* sequences was then inserted in the *Bgl*II site of pIJ702. p6P7S constructed in this way contained *crtS* of *S. setonii*, and p6P7B contained *crtS* of *S. griseus*. (B) *S. griseus* IFO13350 harbouring p6P7S and p6P7B produces a yellow pigment, whereas that harbouring a control plasmid p6F7 does not. p6F7 contained the original *Bgl*II fragment on pIJ6021 in the *Bgl*II site of pIJ702. These plasmids do not confer melanin production because the *Bgl*II site is in the essential region of *mel*. (C) The primers were designed to detect transcripts starting at the points in front of *crtE* and *crtY*. RNA was isolated from *S. griseus* IFO13350 harbouring p6P7B grown in liquid medium for 12 h (lane 1), 24 h (lane 2), and 36 h (lane 3). RNA was similarly prepared from *S. griseus* IFO13350 harbouring pIJ702 grown for 12 h (lane 4), 24 h (lane 5), and 36 h (lane 6). Size markers are *Hind*III-digested λ and *Hinc*II-digested ϕ X174 DNAs. Transcription of *hrdB* was monitored to judge quality and quantity of the RNA used. When reverse transcriptase (RT) was omitted, no signal was detected, showing that the RNA samples contained no DNA.

crt genes caused no such pigmentation. Thus, we assumed that the *S. griseus orf1* and the *S. setonii crtS*, both of which were under the control of the strong promoter on a high-copy-number plasmid, activated transcription of the silent *crt* genes in *S. griseus*, resulting in production of carotenoids at a detectable level. The pigment, probably isorenieratene, was yellow, which suggested that the whole *crt* genes were activated. The *S. griseus orf1* was therefore functionally the same as *crtS* in *S. setonii* and named *crtS* (sigma factor responsible for carotenoid biosynthesis).

We next examined whether the putative sigma factor σ^{crtS} actually activates transcription of *crt* genes. Each of the convergently oriented gene units, *crtE-crtI-crtB-crtV* and *crtY-crtT-crtU*, was expected to form a polycistron because all the initiation codons of *crtIBV* and *crtTU* are overlapped with the termination codons of the preceding genes. We first attempted to detect transcripts starting from the regions upstream of *crtE* and *crtY* by S1 nuclease mapping with RNA prepared from *S. griseus* IFO13350 harbouring p6P7B. However, no signals were detected, suggesting that the amounts of transcripts were still very small. We then tried to detect transcripts by RT (reverse transcription)-PCR. As shown in Figure 4C, a signal was detected for both *crtE* and *crtY* in the RNA sample prepared from *S. griseus* IFO13350 harbouring p6P7B, but not from strain IFO13350 with the vector pIJ702. The transcripts for *crtE* and *crtY* were 1.27-kb and 1.24-kb in length, respectively, as were expected. Transcription of *hrdB* that encodes σ^{HrdB} and is transcribed throughout growth (Shinkawa *et al.*, 1995) was used to monitor the quantity and quality of the RNA samples used. It was thus concluded that production of carotenoids in *S. griseus* containing *crtS* on a plasmid resulted from transcriptional activation of the *crt* genes by σ^{crtS} as a sigma factor.

Transcriptional Start Point of *crtS*

We prepared RNA from *S. griseus* IFO13350 and determined the transcriptional start point of *crtS* by S1 nuclease mapping (Figure 5). A single start point was assigned to be the G residue six nucleotides upstream of the translational start codon ATG. The amount of the *crtS* transcript was expected to be small since *S. griseus* containing the intact *crt* genes produced no detectable amount of carotenoids. Consistent with this idea, the signal was faint. Upstream of the transcriptional start point, no sequences similar to the promoter consensus sequences are present. In addition, the *crtS* transcript contains no ribosome-binding sequence. Translation of leaderless transcripts is, however, not uncommon in *Streptomyces*. For example, the 23S rRNA methylase gene mediating erythromycin resistance in *Streptomyces erythraeus* (Bibb *et al.*, 1986), the streptothricin acetyltransferase gene in *Streptomyces lavendulae* (Horinouchi *et al.*, 1987), and *afsA* probably encoding an A-factor biosynthetic enzyme in *S. griseus* (Horinouchi *et al.*, 1989) are transcribed from leaderless transcripts.

Discussion

We awakened the cryptic or silent carotenoid biosynthetic gene cluster in *S. griseus* by increasing its copy number by means of cloning it on a high-copy-number plasmid.

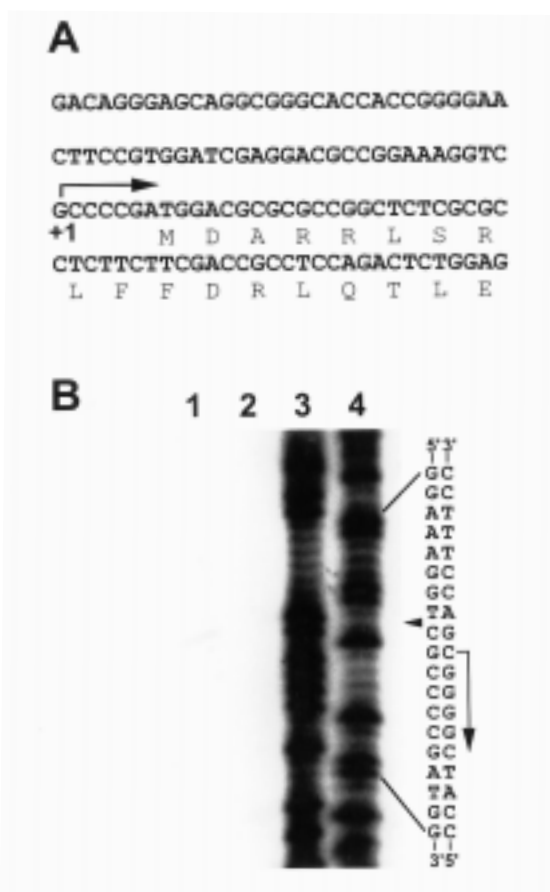


Figure 5. S1 Nuclease Mapping of *crtS* in *S. griseus*. RNA was prepared from *S. griseus* IFO13350 grown for 24 h in liquid medium. S1-protected fragments (lane 1) were run in parallel with the Maxam-Gilbert sequencing ladders, G+A (lane 3) and C+T (lane 4). Lane 2 is a control lane in which only the ^{32}P -probe was run. The position of the S1-protected fragment is shown by a triangle and the transcriptional start point is assigned to the G residue indicated because the fragments generated by the chemical sequencing reactions migrate 1.5 nucleotides further than the corresponding fragments generated by S1 nuclease digestion of the DNA-RNA hybrids (half a residue from the presence of the 3'-terminal phosphate group and one residue from the elimination of the 3'-terminal nucleotide) (Sollner-Webb and Reeder, 1979).

Introduction of the σ^{B} -like genes, *crtS* from *S. setonii* and *S. griseus*, on a high-copy-number plasmid into *S. griseus* also caused carotenogenesis. The *crt* transcripts were detected by RT-PCR in the *S. griseus* strain containing either the *crt* genes on a high-copy-number plasmid or *crtS* under the control of a strong promoter, but not in the wild-type strain. It is therefore apparent that an increase in transcription of the carotenoid biosynthetic genes causes production of carotenoids in a detectable yield. Schumann *et al.* (1996) also observed similar activation of cryptic carotenoid genes of *S. griseus* by cloning part of the genes on a plasmid and Kato *et al.* (1995) identified a sigma factor by cloning its gene that conferred carotenoid production on a carotenoid-nonproducing mutant. These observations suggest that the functional, whole set of carotenoid biosynthetic genes in some *Streptomyces* species are sleeping because of very weak transcription of the genes.

Carotenoids provide crucial protection against photooxidative damage resulting from the combination of visible or near-UV light, singlet oxygen, and endogenous lipophilic photosensitizers in photosynthetic and nonphotosynthetic bacteria (Armstrong, 1994; 1997). Because of these roles of carotenoids, *M. xanthus* produces carotenoids in response to light illumination via an ECF sigma factor CarQ (Martinez-Argudo *et al.*, 1998). This is reasonable for the cells to survive in the ecosystem. Also reasonable is that carotenoids are produced in response to a σ^{B} -like sigma factor σ^{CrtS} in *S. griseus* and *S. setonii*, since the sigmas belonging to this family are stress-responsive (Wösten, 1998; Kovács *et al.*, 1998). Thus, σ^{B} -like σ^{CrtS} and an ECF σ factor CarQ fulfill a role in the onset of carotenogenesis. Although a *crtS* transcript was detected in *S. griseus* IFO13350 grown under usual conditions, a larger amount of transcript appears to be needed for transcription of *crt* sufficient for production of carotenoids in a detectable amount. We have not observed carotenogenesis in *S. griseus* under various conditions, but this does not exclude the possibility that σ^{CrtS} is induced by some environmental stimuli and carotenoids are produced as a result of transcription of *crt* genes by the induced σ^{CrtS} , but in an undetectable amount. Speculatively, *crtS* may be induced by some unknown stress in the soil, which cannot be reproduced on agar medium under laboratory conditions.

In the A-factor-deficient mutant *S. griseus* HH1, the whole *crt* genes are deleted. Mutant HH1 has large-scale deletions at both ends (Lezhava *et al.*, 1997). Because the *crt* genes in *S. coelicolor* A3(2) are located near the ends of the linear chromosome, we assume that the *crt* genes in *S. griseus* are also near one end of the linear chromosome. The ends of the linear chromosomes in *Streptomyces* have terminal inverted repeats ranging from 24-kb (*S. griseus*; Lezhava *et al.*, 1995) to 550-kb (*S. rimosus*; Pandza *et al.*, 1997), which are subject to a high degree of genetic instability, such as deletion, amplification, and chromosomal arm replacement (Chen *et al.*, 1993; Pandza *et al.*, 1997; Fischer *et al.*, 1998). Genetic instability of carotenogenesis in some *Streptomyces* spp. can be explained in terms of the location of *crt* genes close to the ends of the linear chromosome.

Experimental Procedures

Bacterial Strains, Plasmids, and Media

S. griseus strains IFO13350 and an A-factor-deficient mutant HH1 were previously described (Horinouchi *et al.*, 1984). *S. griseus* ΔadpA was also described (Ohnishi *et al.*, 1999). *S. griseus* strains were grown on YMPD medium and R2YE medium (Hopwood *et al.*, 1985). High-copy-number plasmids pIJ702 containing the thiostrepton resistance gene and the melanin gene (Katz *et al.*, 1983) and pIJ486 containing the thiostrepton and neomycin resistance genes (Ward *et al.*, 1986) were used as *Streptomyces* cloning vectors. A high-copy-number plasmid pIJ6021 containing a thiostrepton-inducible *tipA* promoter, obtained from M.J. Bibb, John Innes Centre (Takano *et al.*, 1995), was used for expression of the *crtS* genes. These three plasmids have their copy number of 40 to 100 per genome. *E. coli* JM109 and pUC19 were used for DNA manipulation (Yanisch-Perron *et al.*, 1985). *E. coli* strains were grown on 2 x YT medium (Maniatis *et al.*, 1982).

General Recombinant DNA Techniques

Restriction endonucleases, bacterial alkaline phosphatase, T4 DNA ligase, and Taq DNA polymerase were purchased from Takara Shuzo. Thiostrepton was a gift from Asahi Chemical Company. DNA fragments were purified from agarose gels with a GeneClean kit (Bio 101). Nucleotide sequences were determined by the dideoxynucleotide chain termination method on an

automated fluorescence DNA sequencer. DNA manipulations in *E. coli* were as described by Maniatis *et al.* (1982) and those in *Streptomyces* were as described by Hopwood *et al.* (1985).

Cloning of a Carotenoid Biosynthesis Gene Cluster

Chromosomal DNA from *S. griseus* IFO13350 was digested partially with *Sau3A*I and 5 to 12-kb fragments were purified by agarose gel electrophoresis. The fragments were ligated with *Bgl*II-digested pJ702 and the reaction mixture was used to transform protoplasts of an *adpA*-disrupted mutant derived from *S. griseus* IFO13350. After protoplast regeneration on R2YE medium, transformants were transferred to YMPD medium containing 50 µg/ml of thiostrepton. From more than 15,000 thiostrepton resistant transformants, one colony producing a yellow pigment was picked for further study. The plasmid contained in this transformant was named pYA1, which carried a 11-kb *Sau3A*I fragment in the *Bgl*II site of pJ702 (Figure 1A). Plasmid pYA4F1 was constructed by insertion of a 6.8-kb *Bam*HI fragment containing *crtE*IBV in the *Bam*HI site of pJ486.

Construction of p6P7S, p6P7B, and p6F7

Plasmid pJ6021 contained the thiostrepton-inducible *tipA* promoter in front of an ATG translational start codon. The ATG codon was included in an *Nde*I cleavage sequence, CATATG. The *S. griseus crtS* sequence was prepared by PCR with the High-Fidelity PCR kit (Boehringer Mannheim) and the following two primers: 5'-CGCggtaccATATGGACGC GCGCCGGCTCTCGCGCC-3' (the underline and the lower-case letters indicate an *Nde*I and *Bam*HI sites, respectively; the italic letters indicate the translational start codon of *crtS*) and 5'-GCGggtaccTCACTCCTGGA CGAGCATCCCCG-3' (the lower-case letters indicate a *Bam*HI site and the italic letters indicate the termination codon of *crtS*). For the *S. setonii crtS* sequence, 5'-CGCggtaccATATGGACGC GCGCCGGCTCTC-3' (the underline and the lower-case letters indicate an *Nde*I and *Bam*HI sites, respectively; the italic letters indicate the translational start codon of *crtS*) and 5'-GCGggtaccTCACTCCTGGGTGAGCATCCC-3' (the lower-case letters indicate a *Bam*HI site and the italic letters indicate the termination codon of *crtS*) were used. Each of the *crtS* sequences from *S. griseus* and *S. setonii* was isolated as an *Nde*I-*Bam*HI fragment and placed between the *Nde*I and *Bam*HI sites of pJ6021, resulting in p6B and p6S, respectively (see Figure 4A). p6B was digested with *Bgl*II and a 1.6-kb DNA fragment containing *tipA* and the *crtS* sequence of *S. griseus* was inserted in the *Bgl*II site of pJ702, resulting in p6P7B. Similarly, p6P7S containing *tipA* and the *crtS* sequence of *S. setonii* was constructed. As a control plasmid, a 1.0-kb *Bgl*II fragment from pJ6021 was ligated with *Bgl*II-digested pJ702, resulting in p6F7. This plasmid conferred no melanin production because of insertional inactivation of *mel* (Katz *et al.*, 1983).

RT-PCR

S. griseus IFO13350 containing p6P7B or pJ702 was cultured for various periods at 30°C in YMPD liquid medium, and total RNA was prepared with the RNAqueous phenol-free total RNA isolation kit (Ambion). For detection of transcripts for *crtE*IBV by RT-PCR, 5'-ATGGCCGCTCGCCAGCCT GAGGAACC-3' (the italic ATG indicates the translational start codon of *crtE*) and 5'-TCACCTGGTGGTACCTCCTTCCGCCG-3' (the italic TCA indicates the termination codon of *crtE*) were used as primers. For *crtY*TL, 5'-GTGCCCGCCGACTTCGACGTCGTGATCGTG-3' (the italic GTG indicates the translational start codon of *crtY*) and 5'-TCATGGGGTGCTCT CCTCGGTGTCGGTTCG-3' (the italic TCA indicates the termination codon of *crtY*) were used. For *hrdB* as a control, 5'-GTGTCCGCCAGCACATCCCGTACGC-3' (the italic GTG indicates the initiation codon of *hrdB*; Shinkawa *et al.*, 1995) and 5'-CTAGTCCGAGGTAGTCCGCGCAGCACC-3' (the italic CTA indicates the termination codon of *hrdB*) were used. cDNA was synthesized with Super Script II reverse transcriptase (Gibco BRL) with the purified RNAs and the reverse primers, according to the manual of the supplier. RNA was removed by digestion with RNase H. cDNAs were then used to synthesize double-stranded DNAs by PCR with LA-*Taq* polymerase according to supplier's manual. PCR conditions were: initiation at 94°C for 5 min, denaturation at 98°C for 30 sec, annealing at 62°C for 30 sec, and polymerization at 72°C for 90 sec in a total of 30 cycles.

Southern Hybridization

*Bam*HI-digested chromosomal DNAs from *S. griseus* strains IFO13350, Δ *adpA*, and HH1 were separated by agarose gel electrophoresis, alkali denatured and blotted to an Amersham Hybond N⁺ membrane. The 2.6-kb *Bam*HI-*Sph*I fragment containing *crtE* and a 5'-portion of *crtI* and the 2.1-kb *Bam*HI fragment containing *crtY* and *crtT* were used as probes (Figure 1). The ECL direct nucleic acid labelling and detection systems (Amersham) were used for probe labelling, prehybridization, hybridization, and signal detection.

S1 Nuclease Mapping of *crtS* in *S. griseus*

The 5'-end of a 27-mer oligonucleotide, 5'-AGAGTCTGGAGGCGGT CGAAGAAGAGG-3' (nucleotide positions from +56 to +30 with respect to the transcriptional start point of *crtS*, which was later determined by S1 mapping), was labelled with [γ -³²P]ATP by T4 polynucleotide kinase. For preparation of ³²P-labelled 208-bp probe, PCR was carried out with this oligonucleotide and a nonlabelled 27-mer oligonucleotide, 5'-CTCTGGGAAGGCGAACAGACCTCAGGG-3' (nucleotide positions from -152 to -126), under standard conditions. RNA was prepared from *S. griseus* IFO13350 grown for 24 h in YMPD liquid culture. RNA (50 µg) was hybridized with 100,000 Cerenkov cpm of the probe. Maxam-Gilbert sequencing ladders on a 6% polyacrylamide sequencing gel were generated with the same ³²P-labelled fragment (Maxam and Gilbert, 1980).

DNA Sequence Accession Number

The nucleotide sequence reported in this paper has been registered in the DDBJ, EMBL, and GenBank databases under accession number AF272737.

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