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MOLECULAR MECHANISM OF THE CAROTENOID BIOSYNTHESIS ACTIVATION IN THE PRODUCER *Streptomyces globisporus* 1912

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The aim of research was a comparative analysis of the sequences of the carotenoid biosynthetic gene clusters of the initial inactive strain *S. globisporus* 1912-2 and spontaneously arising, carotenoid producing mutant 1912-4Crt, and comparison of these sequences with the known sequences of the *crt* genes of the other representatives of streptomycetes. *Streptomyces globisporus* 1912 is a producer of the antitumor antibiotic landomycin E and new regulator of diketopiperazine nature. Comparative analysis of the obtained DNA sequences using the GenBank data allowed localization of 7 carotenoid biosynthetic *crt* genes of *S. globisporus* 1912 in one cluster. This cluster, similar to other *crt* clusters of different *Streptomyces* species, consists of two convergent operons from 4 and 3 *crt* genes. The high homology (93%) of the *crt* gene clusters of *S. globisporus* 1912 and *S. griseus* IFO 13350 was shown. Two non-punctual repeats of 21 b. p. were found in the sequence of *crtY* gene coding lycopene cyclase. It was shown that the deletion of 117 b.p. including the sequence between non-punctual repeats of 96 b. p. and one NPR from 5′-side activated the *crt* gene cluster and production of beta-carotene (6.91 mg/l) and lycopene (3.24 mg/l) by the mutant 1912-4Crt. A hypothesis about the site-specific recombination between two non-punctual repeats as the cause of the deletion in *crtY* gene of strain 1912-4Crt was proposed. The obtained strain 1912-4Crt is essential for the next genetic selection of the more effective carotenoid producers. Deletion of 86 b. p. was revealed in the regulatory gene *lndRR* resulting in the deficiency of landomycin E production by the strain 1912-4Crt. The DNA sequences of *crt* and *lnd* genes of *S. globisporus* 1912 were submitted to the NCBI database with accession numbers KM349312 and KJ645792, respectively.

Key words: *Streptomyces globisporus*, spontaneous deletion, lycopene cyclase, *crt* gene cluster.

Natural carotenoid pigments synthesized by plants, some bacteria, streptomycetes and fungi play an important role in the life of animal and humans as a biostimulant, vitamin A substitute, antioxydant, coloring and tumor inhibiting compounds [1]. Soil bacteria of the genus *Streptomyces* are known to be the main industrial producers of the different antibiotics widely used in medicine, veterinary sciences and agriculture [2]. Genetic study of the representatives of the different species of *Streptomyces* showed the presence of the carotenoid biosynthetic gene clusters in their genomes in the functionally inactive state. Activation of the transcription of these cryptic *crt* genes in *S. coelicolor* A3(2) and *S. griseus* IFO 13350 requires induction of a stress-responsible sigma factor by illumination the culture with blue light [3] or increasing copy number of a *crtS* gene [4]. In some rare cases the carotenoid producing mutants can appear in a spontaneous manner in *S. globisporus* 1912 [5] and *S. albus* J1074 [6]. We explained the spontaneously activated carotenogenesis by the chromosome rearrangement in the region of TIR sequences localized in the ends of a linear chromosome in the neighborhood of the *crt* gene clusters [7]. Recently this hypothesis was confirmed by experimental data [6]. So, the production of carotenoids in the genus *Streptomyces* is inducible by sigma factor, constitutive, or completely absent. The genomes of two mutants of this strain: 1912-2, the more efficient antibiotic producer, and 1912-4Crt, the spontaneous producer of beta-carotene and lycopene, and defective in landomycin E biosynthesis, were sequenced by Illumina.

The aim of research was the comparative analysis of the sequences of the carotenoid biosynthetic gene clusters of the initial inactive strain *S. globisporus* 1912-2 and spontaneously arising, carotenoid producing mutant 1912-4Crt, and the comparison of these sequences with known sequences of the *crt* genes of the other representatives of streptomycetes.

Materials and Methods

Strains and culture conditions. The initial wild-type strain *S. globisporus* 1912 was isolated from a soil sample from Armenia and stored in the Ukrainian Collection of Microorganisms at the Institute of Microbiology and Virology of NASU as the strain *S. globisporus* Ac-2098 [http://www.imv.kiev.ua/images/doc/catalog/UCM_catalog.pdf]. Two derivative mutant strains, obtained from 1912, were use in this study. The strain 1912-2 producing about 200 mg/l of the antitumor antibiotic landomycin E and the new regulator of antibiotic biosynthesis and morphogenesis in streptomycetes of diketopiperazine nature (Fig. 1, *a*) [8, 9] was isolated by action of nitrosoguanidine on the spores and mycelium fragments of the strain 1912 [10]. The strain 1912-4Crt, the spontaneous mutant of 1912, produced beta-carotene (7.0 mg/l) and lycopene (3.24 mg/l) in shaking flasks after 72 h growth in the corn-soy liquid medium at 28 °C (Fig. 1, *b*) [11]. It lost the ability to synthesize landomycin E. The cultures of both strains were grown at 28 °C in 20 ml medium S [7] in 250 ml shake flasks during 48 h. The sensibility of the cell wall of the mycelium to the action of the lysozyme was increased by the addition of glycine to the medium at concentration of 1.0%.

Isolation of chromosomal DNA. The young mycelium of the strains 1912-2 and 1912-4Crt after sedimentation by centrifugation was washed by TE buffer, pH 8.0 and resuspended in the same buffer containing 300 mg lysozyme in 1.0 ml. The procedure of mycelium lysis, DNA deproteinisation and purification followed the standard protocol of the Kirby method [7]. The precipitated DNA was dissolved in TE buffer, pH 8.0 and stored at 4 °C. The A_{260}/A_{280} index of the DNA preparations was equal to 2.1–2.3 and electrophoresis showed one compact and high-situated strip of DNA in agarose gel. These data confirm the high molecular weight of the DNA preparations.

DNA sequencing. The sequencing of the genome DNA of the strains 1912-2 and 1912-4Crt was carried out in BaseClear B.V., Leiden, Netherlands using the following procedures. The FASTQ sequence reads were generated according to Illumina Casava pipeline version 1.8.3. Initial quality assessment was based on

a *b* *c*

Fig. 1. **Pigmentation of the colonies of *S. globisporus*:** 1912-2 (*a*), 1912-4Crt (*b*) and 1912-Hp7 (*c*) producing landomycin E (dark-blue), beta-carotene (orange) and lycopene (pink), correspondingly

data passing the Illumina Chastity filtering. Subsequently, reads containing adapters and/or PhiX control signal were removed using an in-house filtering protocol. The second quality assessment was based on the remaining reads using the FASTQ quality control tool version 0.10.0. The quality of the FASTQ sequences was enhanced by trimming off low-quality bases using the «Trim sequences» option of the CLC Genomics Workbench version 6.0.4. The quality-filtered sequence reads were puzzled into a number of contig-sequences. The analysis has been performed using the «De novo assembly» option of the CL C Genomics Workbench version 6.0.4. The optimal k-mer size was automatically determined using KmerGenie [12]. The contigs were linked and placed into scaffolds or supercontigs. The orientation, order and distance between the contigs were estimated using the insert size between the paired-end and/or matepair reads. The analysis has been performed using the SSPACE Premium scaffolder version 2.3 [13]. The gapped regions within the scaffolds are (partially) closed in an automated manner using GapFiller version 1.10 [14].

Results and Discussion

The Illumina paired end sequencing data showed 1438 and 917 contigs of different size composing 7,125 kb and 7,368 kb of the genomes of the strains 1912-2 and 1912-4Crt, respectively. These sizes represent about 90% of the middle streptomycete chromosome.

Identification of the *crt* genes in the contigs of the strains 1912-2 and 1912-4Crt was carried out by means of the BLAST program [www.ncbi.nlm.nih.gov/blast] using sequences of *crt* genes of different *Streptomyces* species in GenBank data (Table 1). First of all it is necessary to note the 100% identity of all *crt* genes 1912-2 and 1912-4Crt strains, except for the sequences of *crtY* and *lndRR* genes. The sizes of *crt* genes of streptomycetes are approximately equal with the exception of the smaller length *crtI* of *S. coelicolor* A3(2) and

Table 1. **Characteristic of the carotenoid biosynthetic genes of *Streptomyces***

NN	Strain (GenBank access N)	*crt* gene, size and identity with 1912 *crt* gene (%)						
		crtE	*crtI*	*crtB*	*crtV*	*crtU*	*crtT*	*crtY*
1	*S. coelicolor* A3(2) (AL939104.1)	1572 (68)	996 (76)	1179 (75)	1011 (75)	1569 (74)	741 (69)	1218 (69)
2	*S. griseus* NRBC 13350 (AF272737.1)	1278 (84)	1524 (92)	1029 (92)	1017 (95)	1554 (93)	729 (94)	1242 (93)
3	*S. avermitilis* MA-4680 (AB070934.1)	1140 (66)	1542 (78)	1029 (75)	1080 (75)	1503 (76)	732 (70)	1347 (69)
4	*S. albus* J1074 (NC_020990)	1239 (68)	1542 (78)	963 (75)	999 (75)	1560 (74)	- (72)	1209 (72)
5	*S. fulvissimus* DSM 40593 (NC_021177.1)	1251 (74)	1518 (86)	1034 (83)	1023 (82)	1563 (85)	714 (80)	1230 (77)
6	*S. globisporus* 1912 (KM349312)	1206	1518	1029	1005	1554	717	1239

Notes: products of the *crt* genes: *crtE* — geranylgeranyl pyrophosphate synthase, *crtI* — phytoene synthase, *crtB* — phytoene dehydrogenase, *crtV* — methylesterase, *crtU* — dehydrogenase, *crtT* — methyltransferase, *crtY* — lycopene cyclase.

crtB of *S. albus* J1074. There is a considerably greater difference between the homology of *crt* genes. All *crt* genes of *S. globisporus* 1912 have very high identity with the corresponding *crt* genes of *S. griseus* NRBC 13350 (92–95%) and a smaller identity with the *crt* genes of *S. fulvissimus* DSM 40593 (80–86%). The sizes of the *crtB* and *crtU* of 1912 and NRBC 13350 strains are the same, 1029 b. p. and 1554 b. p., respectively. The difference between the length of *crtI* and *crtY* of both strains is very low (3–6 b. p.). There is also similarity between all *crt* gene clusters in organization and direction of transcription. These clusters are presented by two convergent operons (Fig. 2). It is necessary to note the frequent presence of the overlapping start-stop GTGA (*crtE-crtF*, *crtB-crtV*) and ATGA (*crtI-crtB*) or stop-start TGATG (*crtT-crtU*) codons. The main result of the present work was the discovery of the deletion of 117 b. p. in the *crtY* gene coding lycopene cyclase. The length of *crtY* in 1912-2 strain with the silent *crt* gene cluster was 1239 b. p., whereas in 1912-4Crt strain, producing beta-carotene and lycopene, it was reduced to 1122 b. p. The *crtY* gene in the strain 1912-2 has two non-punctual repeats (NPRs) of 21 b. p., flanking the sequence of 96 b. p. The deletion begins from 669 b. p. and last to 785 b. p. Both repeats are not identical. NPR from 3′-side of the (+) DNA strand contained four base substitutions (underlined letter) (Table 2). The first 6 b. p. from the 5′-side are the same in both NPRs (GGGGCG) and may be the site for site-specific recombination resulting in the deletion of the NPR from 3′-side and the flanking sequence of 96 b. p. The rearrangement *crtY* gene in the strain 1912-4Crt has 1122 b. p. and only one NPR with the overlapping stop-start codon TGATG [CATCA in the (+) DNA strand].

The comparison of the *crtY* gene sequences of the different bacteria and streptomycetes showed the beginning of their homology from this TGATG codon and finishing at 150 and 50 b. p. from the end of gene, correspondingly. We suppose that the deletion in *crtY* gene of 1912-4Crt strain permits the correct transcription of the silent *crtY* gene. The comparison of the *crtY* genes of the different species of *Streptomyces* genus and bacteria showed the absence of the site 5′-GGGGCG-3′ in the both NPRs. This underlined site is present only in one NPR of NRBC 13350 and DSM 40593 strains. The sequence CGGAAGTCCATCA is more stable and present in all NPRs from 5′-side of the compared strains (Table 2).

One can suppose that this difference in both NPRs prevented the site-specific recombination in the *crtY* gene with next activation of the silent *crt* cluster.

Many representatives of bacteria (genus *Nocardiopsis*, *Micromonospora, Salinospora, Actinoplanes*) also have similar NPRs in the *crtY* genes. One NPR is the same as in 1912-4Crt or slightly changed but the second one has more significant changes in their sequences

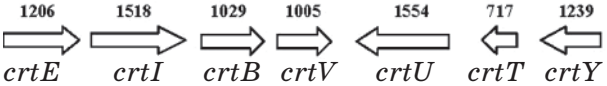

Fig. 2. **Organization of the carotenoid biosynthetic gene cluster of *S. globisporus* 1912:**
the arrows and numbers showed the direction of gene transcription and their length in b. p.

Table 2. **Non-punctual repeats in *crtY* gene of *Streptomyces***

Strain	Non-punctual repeat sequences
S. globisporus 1912-2	5′– GGGGCGTGCGGAAGTCCATCA…. ….GGGGCGGGCGGGAGTCGAACA – 3′
S. coelicolor A3(2)	5′ – GGGGCACGCGGAAGTCCATCA…. ….GGGAAGCGGCCGTGAGTCGAA –3′
S. griseus NRBC 13350	5′ – GGGACGTGCGGAAGTCCATCA…. ….GGGGCGGACGGGAGTCCAACA – 3′
S. avermitilis MA-4680	5′ – GGGGTGTGCGGAAGTCCATGA…. ….CGAGCGGGCGCGAGTCGAAGA – 3′
S. albus J1074	5′ – GTGCGGTGCGGAAGTCCATCA…. ….GGGTAGCGGGCGCGAGTCGAA – 3′
S. fulvissimus DSM 40593	5′ – GCGGTGTGCGGAAGTCCATCA…. ….GGGGCGGGCGGGAGTCGAAGA – 3′
S. cattleya NRRL pSAT	5′ – GGCGGGTACGGAAGTCCATCA…. ….TCGGCGGCCGGGAGTCGAACG – 3′

(deletion and base substitutions). The strain 1912-4Crt lost the ability to synthesize the antibiotic landomycin E. It contains the deletion of 86 underlined b. p. in the gene *lndRR* coding the putative response regulator of the two-component system involved in biosynthesis of landomycin E [GenBank access number KJ645792]:

31681 TTCTGGCGGGTGGTTCCGCAA CGGCGCGGGGGCAGCTGCCGTGATCTCAC GGCAGCTGCC

31741 CCCGCGCCGCGGTTCCCACGATC TGACGCGGGGATTCAGCCAGGCCGTCG CTACGGGGGC.

In this study we have confirmed the earlier published hypothesis about the chromosome rearrangement as the cause of the carotenoid biosynthetic gene cluster activation in *S. globisporus* 1912 [5]. It was shown that the spontaneous deletion of 117 b. p. in the *crtY* gene leads to activation of the carotenoid production in the mutant strain 1912-4Crt. The carotenoids in this mutant were identified as the beta-carotene and lycopene by means of thin layer chromatography, specific maxima of absorption, HPLC and mass-spectrometry [11]. The output of the carotenoids after culture growing in the shake flasks in corn-meal medium was high, 6.91 mg/l of beta-carotene and 3.24 mg/l of lycopene. The level of the produced lycopene was increased to 50.9 mg/l in the selected mutant 1912-7Hp, the spontaneous derivative of 1912-4Crt (Fig. 1, *c*). These strains present technological interest as possible candidates for further improvement of the carotenoid production. It is the first case of the spontaneous activation of the *crt* genes of *Streptomyces*. The carotenogenesis in *S. coelicolor* A3(2) and *S. griseus* IFO 13350 may be activated by the different sigma factors induced by the blue light illumination of culture or by introduction of the *crtS* gene on the a high-copy-number plasmid into the cells [3, 4]. In both cases the production of beta-carotene and isorenieratene was very low. A very similar analog of *crtS* gene of *S. setonii* and *S. griseus* IFO 13350, consisting of 780 b.p. and 94 % homology and coding sigma factor of RNA polymerase, was identified in the 1912-4Crt strain. Participation of this sigma factor in the transcription of the *crt* gene cluster of 1912-4Crt in the stress conditions is not clear. The illumination of the culture of the 1912-4Crt strain by blue light did not resulted in activation of the carotenoid biosynthetic gene cluster. The second example of the genome rearrangement was found in *S. albus* J1074, defective in *SalG1* restriction-modification system [6]. The spontaneous mutants with activated carotenoid gene cluster contain the deletion leading to carotenoid gene cluster amplification from the right terminal 0.42 Mb chromosomal region. The level of carotenoid production in this mutant was also low in contrast to 1912-4Crt strain. The silent *crtY* gene of the 1912-2 strain has 1239 b.p. and 93 % homology with the *crtY* (1242 b.p.) of *S. griseus* IFO 13350. The sequence of *crtY* 1912-4Crt contains two direct repeats of 21 b.p. each, flanking a region of 96 b.p.. Deletion of this region together with one NPR from 5′-side leads to the activation of the carotenoid gene cluster. We suppose that site-specific recombination between two NPRs into GGGGCG site may cause the appearance of this deletion. The deleted sequence of 117 b. p. has homology with the genes of two-component his kinase system (676–706 b. p.) and transposase (722-747 b. p.) of *S. albus* J1074, and transmembrane protein (698–750 b.p.) and oxidoreductase (764–789 b. p.) of *S. coelicolor* A3(2). The homology of the different bacterial *crtY* begins from overlapped stop-start codon GTAGT of NPR from 3′-side. One can suppose that NPRs were introduced into *crtY* genes of different bacteria in the distant past and their function is not clear today. One of the hypotheses is the increase of

complexity of the carotenoid gene clusters of streptomycetes throughout evolution. Directly before the start codon of *crtY* 1912-4Crt the gene of putative transcriptional regulator (300 b. p.) is localized. The product of this gene may participate in the regulation of carotenoid biosynthesis in 1912-4Crt strain. One can think that the region of 117 b. p. between two NPRs may present the barrier for this regulation or contain unnecessary genetic information not suitable for the active display of the lycopene cyclase. Bacteria and streptomycetes have classical monomeric lycopene β-cyclase as opposed to the order *Actinomycetales* possesing heterodimeric enzyme and two *crtYc and crtYd genes* [15]. The sequence of *crtY* 1912-4Crt does not have homology with carotenoid genes of *Mycobacterium* representatives. Unfortunately, the information about purified lycopene cyclase of streptomycetes was not found in literature. The gene *crtY* of 1912-4Crt has 1122 b.p. corresponding to 344 amino acids of lycopene cyclase. In bacteria one can find similar data. Scientists use metabolic engineering in order to increase the biotechnological production of carotenoids in non-carotenogenic microorganisms [16]. The results of these investigations are until poor. We suppose that the rearrangement of the genomes of the carotenoid producing streptomycetes appeared in a spontaneous manner or in the laboratory experiments may also lead to more effective results in comparison with the metabolic engineering approach. The sequence of the *crt* gene cluster of *S. globisporus* 1912 has been submitted to the GenBank with the access number KM349312.

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МОЛЕКУЛЯРНИЙ МЕХАНІЗМ АКТИВАЦІЇ БІОСИНТЕЗУ КАРОТИНОЇДІВ У ПРОДУЦЕНТА *Streptomyces globisporus* 1912

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Метою роботи був порівняльний аналіз послідовностей кластерів генів біосинтезу каротиноїдів вихідного неактивного штаму *S. globisporus* 1912-2 і спонтанного мутанта, продуцента каротиноїдів 1912-4Crt, а також порівняння цих послідовностей з відомими для генів *crt* інших представників стрептоміцетів. Порівняльний аналіз одержаних послідовностей ДНК з даними GenBank дав змогу локалізувати 7 генів *crt* біосинтезу каротиноїдів *S. globisporus* 1912 в одному кластері. Цей кластер, подібно до інших генів *crt* різних видів *Streptomyces*, складається з двох конвергентних оперонів із 4 і 3 генами *crt*. Встановлено високу гомологію (93 %) кластерів генів *crt S. globisporus* 1912 і *S. griseus* IFO 13350. Знайдено два непунктуальних повтори із 21 п. о. у послідовності гена *crtY*, який кодує лікопінциклазу. Показано, що делеція зі 117 п. о., яка включає послідовність між двома непунктуальними повторами із 96 п.о. і один непунктуальний повтор із 5′- кінця, активує кластер генів *crt*, синтез бета-каротину (6,91 мг/л) і лікопіну (3,24 мг/л) штамом 1912-4Crt. Запропоновано гіпотезу про сайт-специфічну рекомбінацію між двома непунктуальними повторами як причину появи делеції в гені *crtY* штаму 1912-4Crt. Одержаний штам 1912-4Crt є важливим для наступної генетичної селекції більш ефективних продуцентів каротиноїдів. Виявлено наявність делеції з 86 п. о. у регуляторному гені *lndRR*, яка призводить до втрати біосинтезу ландоміцину Е штамом 1912-4Crt. Послідовності ДНК генів *crt* і *lnd S. globisporus* 1912 представлено у базі даних NCBI номерами доступу KM349312 і KJ645792, відповідно.

Ключові слова: *Streptomyces globisporus*, спонтанна делеція, лікопінциклаза, кластер генів *crt*.

МОЛЕКУЛЯРНЫЙ МЕХАНИЗМ АКТИВАЦИИ БИОСИНТЕЗА КАРОТИНОИДОВ У ПРОДУЦЕНТА *Streptomyces globisporus* 1912

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Целью работы был сравнительный анализ последовательностей кластеров генов биосинтеза каротиноидов исходного неактивного штамма *S. globisporus* 1912-2 и спонтанного мутанта, продуцента каротиноидов 1912-4Crt, а также сравнение этих последовательностей с известными для генов *crt* других представителей стрептомицетов. Сравнительный анализ полученных последовательностей ДНК с данными GenBank дал возможность локализовать 7 генов *crt* биосинтеза каротиноидов *S. globisporus* 1912 в одном кластере. Этот кластер, подобно другим генам *crt* разных видов *Streptomyces*, состоит из 2 конвергентных оперонов с 4 и 3 генами *crt*. Установлена высокая гомология (93%) кластеров генов *crt S. globisporus* 1912 и *S. griseus* IFO 13350. Найдены 2 непунктуальных повтора из 21 п. н. в последовательности гена *crtY*, который кодирует ликопинциклазу. Показано, что делеция из 117 п. о., включающая последовательность между двумя непунктуальными повторами с 96 п. о. и один непунктуальный повтор с 5′-конца, активирует кластер генов *crt* и синтез бета-каротина (6,91 мг/л) и ликопина (3,24 мг/л) штаммом 1912-4Crt. Предложена гипотеза о сайт-специфической рекомбинации между двумя непунктуальными повторами в качестве причины появления делеции в гене *crtY* штамма 1912-4Crt. Полученный штамм 1912-4Crt важен для последующей генетической селекции более эффективных продуцентов каротиноидов. Показано наличие делеции с 86 п. о. в регуляторном гене *lndRR*, которая приводит к потере биосинтеза ландомицина Е штаммом 1912-4Crt. Последовательности ДНК генов *crt* и lnd S. globisporus 1912 представлены в базе данных NCBI номерами доступа KM349312 и KJ645792, соответственно.

Ключевые слова: *Streptomyces globisporus*, спонтанная делеция, ликопинциклаза, кластер генов *crt*.