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GENOMICS, TRANSCRIPTOMICS, PROTEOMICS

Genome rearrangements of *Streptomyces albus* J1074 lead to the carotenoid gene cluster activation

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Abstract *Streptomyces albus* J1074 is a derivative of the *S. albus* G1 strain defective in *Sal*G1 restriction–modification system. Genome sequencing of *S. albus* J1074 revealed that the size of its chromosome is 6.8 Mb with unusually short terminal arms of only 0.3 and 0.4 Mb. Here we present our attempts to evaluate the dispensability of subtelomeric regions of the *S. albus* J1074 chromosome. A number of large site-directed genomic deletions led to circularization of the *S. albus* J1074 chromosome and to the overall genome reduction by 307 kb. Two spontaneous mutants with an activated carotenoid cluster were obtained. Genome sequencing and transcriptome analysis indicated that phenotypes of these mutants resulted from the right terminal 0.42 Mb chromosomal region deletion, followed by the carotenoid cluster amplification. Our results indicate that the right terminal 0.42 Mb fragment is dispensable under laboratory conditions. In contrast, the left terminal arm of the *S. albus* J1074 chromosome contains essential genes and only 42 kb terminal region is proved to be dispensable. We identified overexpressed carotenoid compounds and determined fitness costs of the large genomic rearrangements.

Keywords Genome reduction · Genomic rearrangements · Cluster amplification · Carotenoids

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Introduction

Streptomyces albus J1074 can be referred to as one of the *Streptomyces* model strains, which is known mostly for its outstanding heterologous host properties (Baltz 2010). Successful expression of numerous gene clusters responsible for production of natural compounds ranging from marine actinomycetes secondary metabolites to potent anticancer agents was reported in this strain (Baltz 2010; Lombó et al. 2006). Heterologously produced antibiotic compounds in *S. albus* J1074 include steffimycin, fredericamycin, isomigrastatin, napyradiomycin, cyclooctatin, thiocoraline, moenomycin (Feng et al. 2009; Gullón et al. 2006; Kim et al. 2009; Lombó et al. 2006; Makitrynskyy et al. 2010; Wendt-Pienkowski et al. 2005; Winter et al. 2007). *S. albus* J1074 is defective in the *Sal*I (*Sal*GI) restriction–modification system which enables effective introduction of heterologous DNA (Chater and Wilde 1980). Various genetic modification approaches were developed making this strain open to all kinds of manipulations (Makitrynskyy et al. 2010; Rodriguez et al. 2000). Genetic accessibility and perfect heterologous host properties of *S. albus* J1074 make this strain a good candidate for the development of an "all-purpose" heterologous host, so-called chassis strain.

Minimization of chromosomes by deleting nonessential DNA regions is a promising approach for generating improved strains with better growth rates and reduced levels of genetic instability (Pósfai et al. 2006). In this respect identification of dispensable stretches of DNA in the genome of interest is important. Members of the *Streptomyces* genus usually contain numerous secondary metabolite clusters in their chromosome, most of which are cryptic under standard laboratory conditions (Bentley et al. 2002; Ohnishi et al. 2008; Omura et al. 2001). These clusters are primary candidates for deletion on the way to an optimized heterologous host. Bioinformatics tools for identification of genes involved in biosynthesis of secondary metabolites are publicly available, simplifying

identification of these genes (Medema et al. 2011). Terminal arms are regarded to contain long stretches of unessential DNA, thereby having a potential for genome minimization. Lengths of terminal arms vary greatly among different species. Based on comparison to the genomes of other streptomycetes, *S. albus* J1074 has some of the shortest arms — only 0.4 and 0.3 Mb long (Zaburannyi, unpublished data). Although terminal arms often contain secondary metabolite clusters and examples of their successful deletion are known, dispensability of these regions cannot be easily predicted and requires experimental verification (Komatsu et al. 2010; Zhou et al. 2012).

In a previous study we sequenced a complete genome of *S. albus* J1074 (Zaburannyi, unpublished data). We are currently attempting to construct the genome-reduced chromosome of *S. albus* J1074 in order to improve its heterologous host properties. The aim of this study was to obtain additional information about the dispensability of subtelomeric chromosomal regions of this strain. In this paper we describe construction of *S. albus* J1074 mutants with deleted terminal chromosomal regions. In two steps we succeeded in deleting 307 kb and circularizing the chromosome. During the study we obtained two spontaneous mutants of *S. albus* J1074 with large chromosomal deletions accompanied by the silent carotenoid gene cluster activation and amplification. As a result of this study we identified terminal chromosomal regions of overall length of 464 kb which are dispensable for *S. albus* J1074 when cultivated under laboratory conditions. Results of transcriptome and fitness analyses of the different mutants are discussed in this article as well.

Materials and methods

Strains, plasmids, and culture conditions

All of the strains and plasmids used in this study are listed in Table 1. Solid medium MS and liquid media TSB, NL410 (glucose 10 g/l, glycerol 10 g/l, oat flour 5 g/l, soya flour 10 g/l, yeast extract 5 g/l, bacto casaminoacids 5 g/l, $CaCO_3$ 1 g/l) and NL333 (glucose 5 g/l, soluble starch 10 g/l, malt extract 10 g/l, yeast extract 3 g/l, bacto pepton 3 g/l, ammonium nitrate 3 g/l, $CaCO_3$ 1 g/l) were used for the cultivation of *S. albus* strains (Kieser et al. 2000). L broth and L agar were used for the cultivation of *Escherichia coli* strains (Kieser et al. 2000). The following antibiotics were added to the medium in standard concentrations when required: apramycin, kanamycin, hygromycin, thiostrepton, and phosphomycin (Kieser et al. 2000; Green and Sambrook 2012).

DNA manipulation

Isolation of DNA and all subsequent manipulations were performed according to the standard protocols (Green and Sambrook 2012). All primers used in this study are listed in Table S1.

Plasmid construction

To construct pR6KThyglox, the *R6Kγ* replicon was amplified from pALHim with the R6Kfor/R6Krev primers, and hygromycin resistance gene with *oriT* was amplified from pIJ10700 with primers 10700rev/10700for (Bilyk et al. 2013; Gust et al. 2004). The PCR products were PCR-fused using primers R6Kfor/10700rev, and the resulting fragment was cloned into the *Eco*RV site of the pKC1139 plasmid (Kieser et al. 2000).

To prove the deletions in *S. albus* Del1 and Del2 strains rescue plasmids pDel1 and pDel2 were constructed. In both strains cassette containing hygromycin resistance gene, *oriT*, *R6Kγ* and single *loxP* resided on the place of the deleted fragment. To clone and sequence regions flanking deletion, rescue plasmids were constructed. For this purpose, chromosomal DNA of *S. albus* mutants was digested with restriction enzymes which did not cut cassette but had their recognition sites in its flanking regions. In case of pDel1, *Sca*I was used and in case of pDel2 – *Nco*I. Digested DNA was precipitated, dissolved and self-ligated. Ligation mixtures were transformed into *E. coli* Transformax, and transformants were selected on L agar supplemented with 100 μg/ml hygromycin. *E. coli* Transformax contains replication initiator gene *pir*, able to initiate replication of *R6Kγ*-containing plasmids. Only those cells which were transformed with self-ligated fragment containing cassette gave rise to colonies on the selective medium.

Recombinant BAC construction

To construct 3D5Am, the cassette, containing apramycin resistance gene *aac(3)IV*, origin of conjugal transfer *oriT* and single *loxP*, was amplified from pIJ774 using primers AmloxF and AmloxR (Gust et al. 2004). The amplified fragment was used for the RedET recombineering of the 3D5 BAC as described previously (Gust et al. 2004).

To construct 1C15Hyg, the cassette, containing hygromycin resistance gene *hph*, *oriT*, origin of replication *R6Kγ* and single *loxP*, was amplified from pR6KThyglox using primers 11Hyglox13uni and 11Hyglox13rev. The amplified fragment was used for the RedET recombineering of the 1C15 BAC.

To construct 2E8Am, 1J6Am and 2O21Am, the cassette containing *aac(3)IV*, *oriT* and single *loxP*, was amplified from pIJ774 using three pairs of primers 3AmloxF/3AmloxR, Circ1for/Circ1rev, and Circ2for/Circ2rev (Gust et al. 2004). The PCR products were used for the recombineering of the BACs 2E8, 1 J6 and 2O21, respectively.

Table 1 Strains and plasmids

Strain or plasmid	Characteristics	Reference or source
Streptomyces strains		
S. albus J1074	*S. albus* G1 (DSM 41398) derivative with the defective *Sal*GI restriction modification system heterologous host	Chater and Wilde (1980)
S. albus 3D5Am	WT with the first *loxP* introduced	This work
S. albus 3D5Am 1C15Hyg	*S. albus* 3D5Am with the second *loxP* introduced	This work
S. albus Del1	WT with the deletion of 204 kb	This work
S. albus Del2	WT with the circularised chromosome	This work
S. albus Pink	WT with the 422 kb deletion and amplification of the carotenoid gene cluster	This work
S. albus Orange	WT with the 420 kb deletion and amplification of the carotenoid gene cluster	This work
E. coli strains		
XL1-Blue	General cloning host	Stratagene, Santa Clara, CA, USA
ET12567 pUZ8002	Strain for intergeneric conjugation	Kieser et al. (2000)
Transformax EC100D	Cloning host	Epicentre, Madison, WI, USA
Plasmids		
pIJ10700	Ap^r, Hyg^r; contains *hph* and *oriT* cassette flanked with *frt* sites	Gust et al. (2004)
pIJ774	Ap^r, Am^r; contains *aac(3)IV* and *oriT* cassette flanked with *loxP* sites	Gust et al. (2004)
pUWLcre	Ap^r, Tsr^r; Cre recombinase expression vector	Fedoryshyn et al. (2008)
pKC1139	Am^r; *E. coli-Streptomyces* shuttle vector	Kieser et al. (2000)
pALHim	Am^r; transposon mutagenesis vector	Bilyk et al. (2013)
pR6KThyglox	pKC1139 with *hph*, *oriT*, *R6Kγ*, *loxP*	This work
pDel1	Hyg^r; rescue plasmid from *S. albus* Del1	This work
pDel2	Hyg^r; rescue plasmid from *S. albus* Del2	This work
Bacs		
3D5Am	Derivative of 3D5 with a cassette from pIJ774	This work
1C15Hyg	Derivative of 1C15 with a cassette from pR6KThyglox	This work
2E8Am	Derivative of 2E8 with a cassette from pIJ774	This work
1J6Am	Derivative of 1 J6 with a cassette from pIJ774	This work
2O21Am	Derivative of 2O21 with a cassette from pIJ774	This work

Construction of *S. albus* strains with deleted clusters

The BACs 3D5Am and 1C15Hyg were subsequently used to achieve the deletion of 204 kb in the *S. albus* Del1 mutant. These BACs were introduced in the *S. albus* J1074 strain via conjugation (Kieser et al. 2000). Screening for double-crossover mutants was performed using blue-white screen on the MS agar supplemented with 50 μg/ml of apramycin or hygromycin and 70 μg/ml of X-gluc (Myronovskyi et al. 2011). The backbone of all BACs used in this study contained *gusA* gene, which predetermined characteristic blue pigmentation of single crossover colonies when grown on media supplemented with X-gluc. Hygromycin resistance cassette from 1C15Hyg was introduced in the chromosome of *S. albus* J1074 (Genbank accession number NC_020990) between nucleotides 6576395 and 6576406. Apramycin resistance cassette from 3D5Am was introduced in the chromosome of *S. albus* J1074 between nucleotides 6781200 and 6781205. Cre recombinase was expressed in the double knockout strain *S. albus* 3D5Am 1C15Hyg from the pUWLcre plasmid (Fedoryshyn et al. 2008). Deletion of the 204-kb fragment in *S. albus* Del1 was confirmed via construction and sequencing of the rescue plasmid pDel1.

To construct *S. albus* Del2, 1J6Am was transferred into *S. albus* Del1. The double knockout mutant was selected using blue-white screen. Apramycin resistance cassette from 1J6Am was introduced in the chromosome of *S. albus* J1074 (Genbank accession number NC_020990) between nucleotides 42061 and 42072. Circularisation of the chromosome was performed by expression of the Cre recombinase from the pUWLCre plasmid. Circularisation of the chromosome in *S. albus* Del2 was confirmed via construction and sequencing of the rescue plasmid pDel2.

Competition experiments

Fitness costs of the mutants relative to the wild type were estimated by competition experiments. Spore suspensions of

the mutant and wild type strains were mixed 1:1 and grown in a NL410 medium at 28 °C 180 rpm. After 48 h, the culture was inoculated in the new medium with 100-fold dilution. This procedure was repeated three times. Ratios of mutants to the wild type were measured after 48 h of incubation, before inoculation in the new medium. All experiments were performed in three parallels. Selection coefficients were estimated using the regression model $s=[\ln(R(t)/R(0))]/[t]$ (Dykhuizen 1990).

Analysis of natural compounds production

For the production of secondary metabolites, the strains of *S. albus* were cultivated in NL333 medium for 6 days at 28 °C and 180 rpm. Mycelium was separated from supernatant by centrifugation. Supernatant was extracted with ethyl acetate and mycelium – with methanol. Extracts were combined and vacuum dried. The dry extract was dissolved in methanol. Separation of compounds was performed on 100 mm BEH C18 column (Waters, Milford, USA) using the flow rate of 0.55 ml/min with the following gradient: 0 min 5 % B, 18 min 95 % B (solvent A: water + 0.1 % formic acid, solvent B: acetonitrile + 0.1 % formic acid).

For the production of carotenoids, the strains of *S. albus* were cultivated in NL333 medium for 6 days at 28 °C and 180 rpm. Mycelium was separated from supernatant by centrifugation. Carotenoids were extracted from the supernatant with chloroform and from the mycelium with chloroform/methanol (1:1 v/v). Extracts were then mixed and evaporated. The dry extract was washed three times with methanol. The remaining material was dissolved in chloroform.

Separation of carotenoids was performed on Phenomenex Luna C18 HST column (100×2 mm, 2.5 μm) (Aschaffenburg, Germany) with acetonitrile/methanol/2-propanol (85:10:5) at a flow rate of 0.5 ml/min and a detection wavelength of $\lambda=$ 440 nm (Schumann et al. 1996).

Ultra performance convergence chromatography (UPCC) of carotenoids was performed on a Waters Acquity UPC2 using column BEH, HSS SB (100×3.0 mm) (Milford, USA) with CO_2 and MeOH as co-solvent in a gradient from 10 % MeOH to 40 % MeOH in 3.7 min, further increasing to 50 % MeOH in 1 min. The flow was 1.5 ml/min and column temperature was 45 °C. Carotenoids were detected at a wavelength of λ=440 nm.

Genome and transcriptome sequencing, genome assembly, and analysis

Both strains were sequenced using two Illumina MiSEQ libraries (San Diego, CA, USA) — short-insert (paired-end, PE) and long-insert (mate pair, MP). For the Orange strain, PE insert size was 582 with a standard deviation of 96.4 (726,698 read pairs), and MP insert size was 8,437 with a deviation of 1,482.2 (606,953 read pairs). For the Pink strain, PE insert size was 592 with a deviation of 105.4 (850,929 read pairs), and MP insert size was 8,938 with a deviation of 1,511.4 (588,604 read pairs). Insert sizes and standard deviations were calculated by mapping the reads to the full *S. albus* J1074 genome with Novoalign V3.00.04 (Novocraft, Selangor, Malaysia).

Pink and Orange strain genomes were assembled with Newbler v2.8. Orange assembly has a total of 341 contigs (277 are large), and two final scaffolds — 6,436,668 and 3,192 bp. Pink assembly has 338 contigs (261 are large) and also two final scaffolds — 6,421,314 and 3,194 bp.

Structural variations analysis was performed with Breakdancer version 1.2.6 (Chen et al. 2009). Sequencing reads coverage against the *S. albus* J1074 genome (Genbank accession number NC_020990) was examined with Geneious 6.1.6 (Biomatters Ltd, Auckland, New Zealand).

RNA sequencing was performed by Vertis Biotechnologie AG (Freising, Germany). Briefly, ribosomal RNA depletion of isolated total RNA was performed with Ribo-Zero Bacteria kit from Epicentre (Madison, USA). The rRNA depleted RNA was fragmented with Rnase III and 5′PPP structures were removed using RNA 5′ polyphosphatase (Epicentre). Afterwards, RNA fragments were poly(A)-tailed using poly(A) polymerase. First-strand cDNA synthesis was performed using an oligo(dT)-adapter primer and M-MLV reverse transcriptase. The resulting cDNA was amplified and purified, and fragments in the length range 250–350 were sequenced on an Illumina HiSeq 2000 machine (San Diego, CA, USA) with 100 bp read length. A total of 10,030,273 (Pink), 8,601,646 (Del1), and 7,141,882 (Orange) non-ribosomal reads were successfully mapped using Novoalign V3.00.04 to the *S. albus* J1074 genome. Differential expression analysis was performed with DeSeq package for the R statistical environment (Anders and Huber 2010).

Results

Generation of the *S. albus* mutants

The *S. albus* J1074 chromosome is 6.841 Mb long with 0.3- and 0.4-Mb terminal arms (Zaburannyi, unpublished data). Antismash analysis (Medema et al. 2011) of the *S. albus* J1074 genome revealed the presence of three large hybrid PKS-NRPS gene clusters of 141, 50, and 35 kb in its right chromosomal arm (Fig. 1a). The third cluster overlaps with the 30 kb terminal inverted repeat. To delete the DNA fragment containing the first two clusters we introduced two *loxP* sites in the chromosome of *S. albus* J1074 on their outer borders, flanking the 204-kb region (Fig. 1b). We used two *loxP*-containing antibiotic cassettes. The first cassette was amplified from pIJ774 and contained apramycin resistance gene *aac(3)IV*, origin of conjugal transfer *oriT* and single

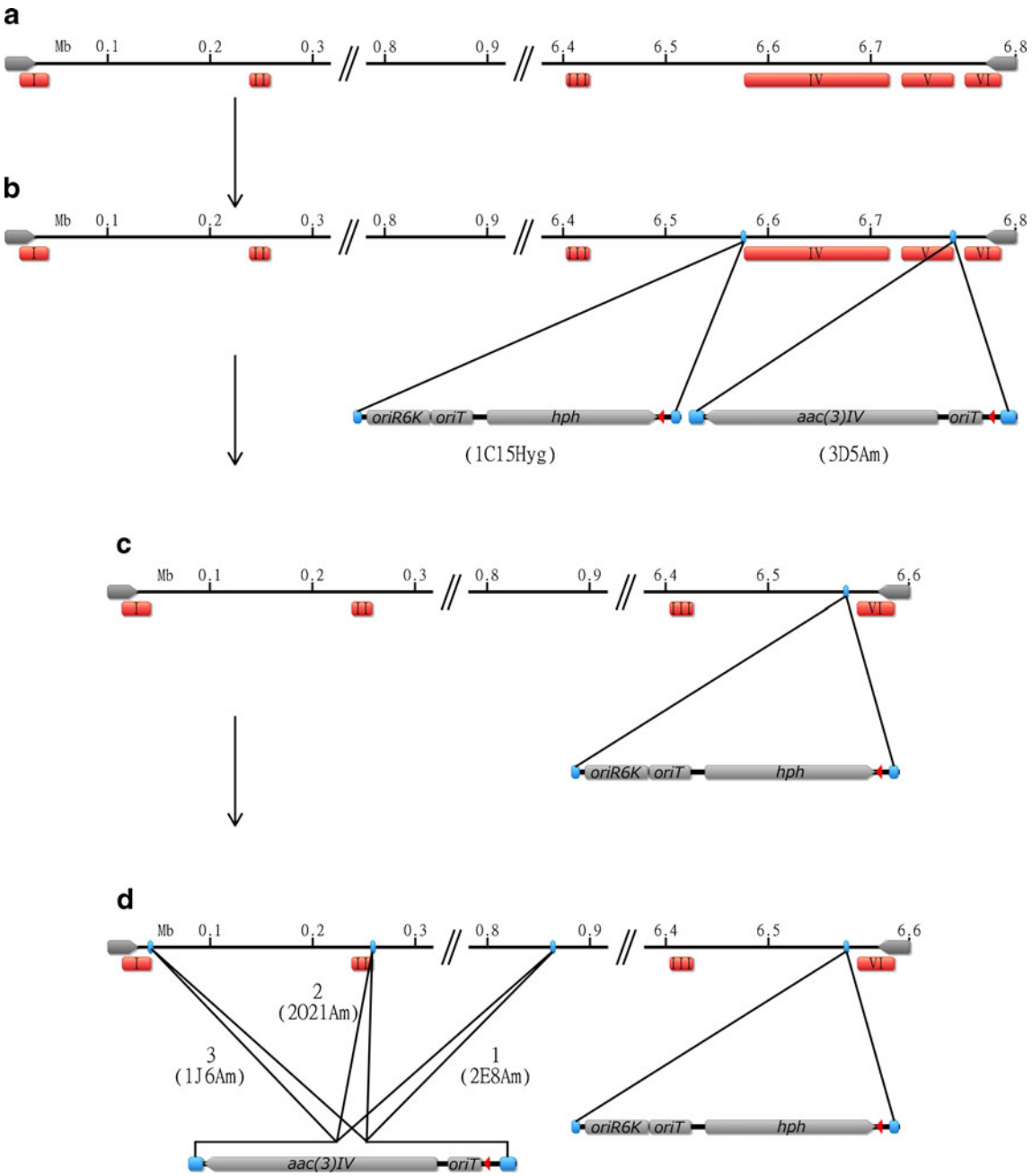

Fig. 1 Schematics of the genome engineering steps towards generation of genome minimized *S. albus* mutants. Secondary metabolite clusters are marked with *red colour*, terminal inverted repeats—with *grey*, sites of cassette integration — with *blue*, *loxP* sites — with *red*. Clusters I, II, IV, V, VI were annotated as hybrid PKS-NRPS according to the results of Antismash, cluster III — carotenoid. **a** The chromosome of the wild type *S. albus* J1074. **b** The chromosome of *S. albus* 3D5Am 1C15Hyg with two antibiotic resistance cassettes. Each of the cassettes contains *loxP* site. **c** The chromosome of *S. albus* Del1 with the deletion of the 204-kb DNA fragment. Deletion was achieved by expressing Cre recombinase and recombination between two *loxP* sites. Cassette, containing *hph*, *oriT*, *R6Kγ* and *loxP*, resides on the place of the deletion. **d** Three attempts to circularize the chromosome of *S. albus* Del1. Antibiotic cassette, containing *loxP*, was introduced in three different sites in the chromosome (1, 2 and 3) and Cre recombinase was expressed. Insertion of the cassette in site no. 3 caused circularization of the chromosome after the expression of Cre

loxP. The second cassette was constructed de novo and contained hygromycin resistance gene *hph*, *oriT*, *R6Kγ* origin of replication and single *loxP*. These cassettes were utilized for the RedET recombineering of two BACs, 3D5 and 1C15, which contained DNA fragments flanking the targeted 204-kb chromosomal region from both sides. Resulting BACs, 3D5Am and 1C15Hyg, were applied one after another for introduction of the *loxP*-containing antibiotic cassettes into the *S. albus* chromosome. The BACs were transferred into *S. albus* by conjugation, and double-crossover mutants were

selected using blue-white screen. Resulting strain with both cassettes flanking the 204-kb region was called *S. albus* 3D5Am 1C15Hyg (Fig. 1b). As expected, expression of Cre recombinase caused excision of the 204 kb fragment containing two PKS-NRPS clusters together with the apramycin cassette (Fig. 1c). The hygromycin resistance gene, *oriT*, *R6Kγ* and single *loxP* site resided in the chromosome of the *S. albus* Del1 mutant instead of the deleted fragment. Deletion was confirmed by cloning and sequencing of the rescue plasmid derived from *S. albus* Del1. *S. albus* Del1 showed no differences in growth or morphology compared to the wild type.

After the successful deletion of the 204-kb fragment, we evaluated the essentiality of the remaining 60 kb located downstream from the deleted fragment, as well as the left chromosomal arm of *S. albus*. Since the chromosome of *S. albus* Del1 already contained *loxP* site within hygromycin cassette, insertion of only one additional *loxP* was required to perform the next deletion. We checked the feasibility of deleting the terminal 864 kb region of the left chromosomal arm together with the terminal 60 kb fragment from the right arm. Apramycin cassette with a single *loxP* was used for the RedET-mediated recombination of Bac 2E8. Mutated BAC 2E8Am was used for inserting the *loxP*-containing cassette into the left chromosomal arm of *S. albus* Del1 via double crossover, yielding *S. albus* Del1 2E8Am (Fig. 1d). Recombination of the two *loxP* sites should cause circularization of the chromosome accompanied by the loss of the terminal 864 kb from the left arm and 60 kb from the right arm of *S. albus* Del1. Such mutant should be resistant to hygromycin and sensitive to apramycin due to the deletion of apramycin cassette. However, after Cre recombinase expression we failed to detect clones with the expected resistance profile, which indicated that genes in either the left terminal 864 kb or in the right terminal 60 kb were essential.

During the next attempt to circularize the chromosome of *S. albus* Del1 we decided to decrease the length of the left terminal region which should be deleted in order to exclude essential genes from the targeted fragment. The above described strategy was used to circularize the chromosome. Recombined Bac 2O21Am was utilized for inserting the apramycin cassette with the single *loxP* into the chromosome of *S. albus* Del1, yielding *S. albus* Del1 2O21Am (Fig. 1d). As mentioned above, recombination between two *loxP* sites should result in circularization of the chromosome and deletion of the terminal regions. In the second chromosome cyclization approach, the length of the left terminal region to be deleted was decreased to 259 kb, while the length of the right one remained 60 kb. The 259-kb fragment contained two hybrid PKS-NRPS clusters of 27 and 20 kb. Expression of Cre recombinase in *S. albus* Del1 2O21Am also did not generate expected DNA recombination, indicating that essential genes were present within the 259-kb fragment of the left terminal region. However, during this circularization attempt two colonies of different colour (pink and orange) were detected on the white lawn of *S. albus* Del1 2O21Am pUWLCre. These coloured colonies were picked as a monoclonal culture. The corresponding pure cultures were called *S. albus* Pink and *S. albus* Orange according to their colours (Fig. 2). When the resistance phenotypes of both strains were checked, it turned out that the mutants were apramycin-resistant and hygromycin-sensitive. This phenotype is opposite to the expected one in the case of successful recombination between two *loxP* sites in *S. albus* Del1 2O21Am—resistance to hygromycin and sensitivity to apramycin. Interpretation of the events which underlie emergence of *S. albus* Pink and Orange are further discussed in this article.

The third attempt to circularize chromosome of *S. albus* Del1 was performed by further decreasing the left terminal region which should be deleted. Using the recombined Bac 1J6Am, *loxP* site within an apramycin resistance cassette was introduced into the chromosome of *S. albus* Del1 at the end of the first PKS-NRPS cluster (Fig. 1d). After expression of Cre recombinase apramycin-sensitive and hygromycin-resistant colonies were detected. Such phenotype indicates that the circularization of the chromosome was successful. Obtained strain *S. albus* Del2 contains deletion of the left-hand terminal 42 kb and right-hand terminal 60 kb compared to *S. albus* Del1. *S. albus* Del2 strain is reduced by 307 kb in comparison to parental *S. albus* J1074. Differences in morphological differentiation and growth characteristics of *S. albus* Del2 were not detected when grown in a form of confluent lawn. When plated in dilutions, *S. albus* Del2 gave rise to bold colonies with approximate frequency of 0.1 %, what was not observed in case of the *S. albus* J1074.

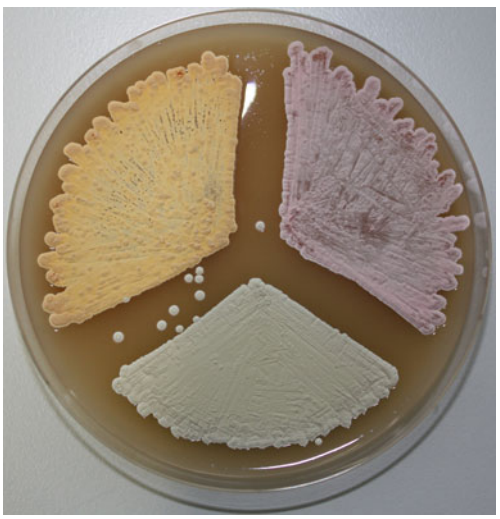

Fig. 2 Different pigmentation of *S. albus* Del1, *S. albus* Orange, and *S. albus* Pink

To find the reason for our failure to delete left terminal DNA fragments longer than 42 kb, we tried to identify possible essential genes in the left subtelomeric region of the *S. albus* J1074 chromosome. For this purpose, translated products of all coding sequences located in the left terminal 260-kb-long chromosomal fragment were uploaded to the KAAS, automatic genome annotation and pathway reconstruction server (Moriya et al. 2007). Our results indicate that genes which can be potentially essential were indeed located within the analyzed fragment. We identified nine genes whose products are participating in carbon metabolism, fatty acid metabolism, and biosynthesis of amino acids. However, all genes (except for XNR_0091, encoding L-threonine aldolase) have corresponding paralogs in the genome of *S. albus* J1074. Besides genes involved in primary metabolism, genes encoding 50S ribosomal protein L32 (XNR_0047), alkylated DNA repair protein (XNR_0074), and uracil-DNA glycosylase (XNR_0112) are also located in the analyzed DNA fragment. The latter genes are also presented in two and more copies in the chromosome of *S. albus* J1074. Analysis of transcriptome data showed that from all abovementioned genes only one encoding L-threonine aldolase (XNR_0091) was highly expressed.

Genome analysis of *S. albus* Pink and *S. albus* Orange

To find out the reasons which underlie phenotypic changes in *S. albus* Pink and *S. albus* Orange, their paired-end genomic libraries were created and sequenced. By mapping the sequencing reads to the genome of *S. albus* J1074, we discovered that both *S. albus* Pink and *S. albus* Orange lack the right chromosomal terminal region including the inverted terminal repeat, while the rest of the chromosome was unaffected. Interestingly, the deletions in both strains were of almost identical size: 420,240 bp in the case of *S. albus* Orange and 422,388 bp in the case of *S. albus* Pink. Sequencing data analysis gives evidence that the linear topology of the chromosome was not affected in *S. albus* Pink and *S. albus* Orange stains.

Further analysis of sequence coverage revealed that in the case of *S. albus* Pink and *S. albus* Orange it was not uniform along their chromosomes. In both cases the coverage of DNA fragment which became terminal in consequence of deletion was much higher than of other chromosomal regions (Table 2). Such increase in the coverage can be explained only by the amplification of the respective DNA fragment, since we did not observe such differences in coverage when *S. albus* J1074 was sequenced. Analysis of insert sizes of paired reads using Breakdancer confirmed this assumption, indicating that both *S. albus* Pink and *S. albus* Orange contain amplified DNA sequences (ADSs) composed of directly repeated copies of amplifiable unit of DNA (AUD) (Table S2).

Table 2 Coverage statistics for the amplified fragment in the Pink and Orange strains

Strain	Library	Mean coverage, amplified fragment	Mean coverage, rest of the genome	Fold difference
Pink	MP	3647.8	24.4	149.5
Pink	PE	6333.5	36.7	172.6
Orange	MP	3570.7	26	137.3
Orange	PE	5687.1	33.8	168.2

For the Pink strain, amplified fragment is at 6404757–6419260; for the Orange strain, amplified fragment is at 6409922–6421408. Both coordinates are 1-based from the *S. albus* J1074 genome. The rest of the genome is defined as the genome sequence between coordinates 1 and the beginning of the amplified fragment of the respective strain

The AUDs from the mutant strains are overlapping but they differ in size. Both AUDs include carotenoid biosynthetic cluster, and this gives a hint about the nature of the coloured compounds produced by the obtained mutants. The AUD from *S. albus* Orange has a length of 11.487 kb and includes the whole carotenoid cluster, while the AUD from *S. albus* Pink a the length of 14.504 kb and encompasses the mentioned cluster only partially (Fig. 3a). We failed to reveal long direct or inverted repeats inside AUDs from *S. albus* Orange and *S. albus* Pink.

Database search indicated that the amplified carotenoid biosynthetic gene cluster from *S. albus* J1074 is homologous to the isorenieratene clusters of *Streptomyces griseus* and *Streptomyces coelicolor* which share the same organization (Krügel et al. 1999; Takano et al. 2005). Similar to the isorenieratene clusters, carotenoid cluster of *S. albus* J1074 consists of two convergent operons *crtEIBV* and *crtYU* (Fig. 3b). Besides the similarities in the organization of all three clusters, translational products of their genes also share considerable level of homology (Table S3). However, the cluster of *S. albus* J1074 differs from the one from *S. griseus* by the absence of *crtT* gene which is not involved in biosynthesis of isorenieratene together with *crtV* gene (Fig. 3) (Krügel et al. 1999). Another difference of the cluster from *S. albus* J1074 is that the additional genes encoding lysine exporter, IS1647-like transposase, hypothetical protein and putative transcriptional regulator are present within its central part (Fig. 3).

In order to find possible reasons for the difference in colour of *S. albus* Pink and *S. albus* Orange strains, the differences in their ADSs were surveyed. From the homology of translational products and the similarity in their organization, we supposed that the genes of the native carotenoid cluster of *S. albus* J1074 are likely to be involved in biosynthesis of isorenieratene from farnesyl pyrophosphate and isopentenyl pyrophosphate (Fig. S1). The amplified chromosome region of *S. albus* Pink does not contain the entire isorenieratene gene cluster. Sequencing data show that the *crtY* gene in

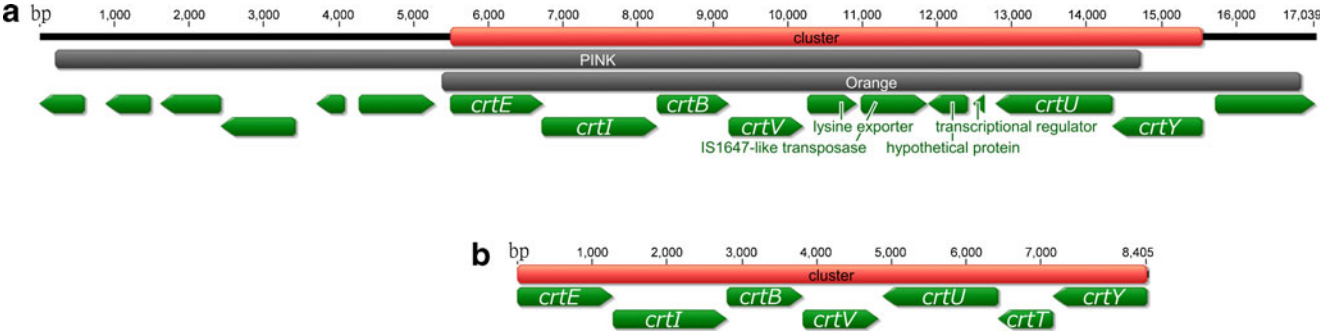

Fig. 3 Fragments of *S. albus* and *S. griseus* chromosomes with the homologous carotenoid cluster. **a** Region of the *S. albus* chromosome with the carotenoid cluster marked with *red*. Chromosomal regions which were amplified in *S. albus* Pink and *S. albus* Orange are shown in *grey*. **b** The carotenoid cluster involved in biosynthesis of isorenieratene in *S. griseus*. Genes *crtE–crtY* of *S. albus* correspond to loci XNR_5683–XNR_5692

S. albus Pink is truncated within the amplified region (Fig. 3). This gene encodes lycopene cyclase which converts lycopene to β-carotene (Fig. S1). This reaction precedes conversion of β-carotene to isorenieratene. Deficiency in lycopene cyclase activity blocks the isorenieratene biosynthetic pathway and should lead to the accumulation of lycopene (Krügel et al. 1999).

Transcriptome analysis of the *S. albus* Pink and *S. albus* Orange

To decide if the observed phenotypic changes of the Pink and Orange strains are the result of the respective ADSs and/or the changes in the expression regulation, we performed RNA-sequencing of the Pink, Orange, and Del1 strains. First, similarly to the genome sequencing coverage analysis, we compared mean coverage of the ADSs to the coverage of the rest of the two genomes, and found that ADSs in both Pink and Orange indeed have 50- to 70-fold higher coverage than the rest of the genome (Table S4). However, such a comparison is not sufficiently precise, as it does not take into account mean gene density, which is higher within gene clusters. Also, without the reference to compare to, we cannot be totally sure that observed coverage deviations are not simply an intrinsic feature of the studied genome (though we can discard the GC-bias argumentation, as GC content of ADSs in both genomes is higher than genome average).

Taking the aforementioned considerations into account, we performed a differential expression analysis of the Pink and Orange strains versus their parent strain Del1. Quite expectedly, fold-changes of genes within the amplified regions were much higher than those of the genes immediately nearby, reaching fold-change values as high as 110-fold (Table 3). It is quite clear from the table that genome boundaries of increased gene expression perfectly match the identified start/end coordinates of the ADSs. Taking into account fold-change expression values of *S. albus* Pink and *S. albus* Orange versus *S. albus* Del1, we conclude that the overexpression of the carotenoid gene cluster is solely due to its amplification within ADS.

Phenotypic analysis and fitness studies

Based on sequencing results, we supposed that the pigmentation of *S. albus* Pink and *S. albus* Orange strains was caused by the production of lycopene and isorenieratene, respectively. To verify this hypothesis, both mutant strains were cultivated in NL333 medium with subsequent extraction of compounds and their analysis. We expected the production of carotenoids in our mutants; therefore, lycopene and β-carotene were purchased and used as the reference materials. UPCC analysis of the extracts from *S. albus* Pink and *S. albus* Orange together with the reference compounds revealed the presence of β-carotene in both extracts (Fig. S2). However, β-carotene did not constitute a major fraction in any of the extracts. Comparison of the retention times and UV spectra unambiguously indicates that the major peak of the *S. albus* Pink extract corresponds to lycopene (Fig. S3). As expected, the major peak from *S. albus* Orange extract did not correspond to any of our reference compounds. However, UV–Vis spectrum of this major peak corresponds to the spectrum of isorenieratene giving us strong evidence of the identity of both compounds (Fig. S4) (Fuciman et al. 2010; Takaichi 2000). The results of quantitative measurements indicated that the *S. albus* Pink and *S. albus* Orange strains produce approximately 90 and 210 mg/l of carotenoids, respectively.

To detect the effect of large genomic deletions in *S. albus* Del1 and Del2 strains on the production of secondary metabolites, compounds were extracted and analyzed. Comparison of mutant HPLC traces with the one of *S. albus* J1074 did not reveal any differences, indicating that deletions had no effect on the production of secondary metabolites.

We did not detect any obvious changes in the growth of Pink, Orange, and Del2 strains on agar medium, as compared with *S. albus* J1074. To address this question in more details,

Table 3 Log2 fold-changes of gene expression of Pink and Orange versus Del1, inside the amplified regions

Deduced protein function	Thioredoxin	Oxidoreductase	Integral membrane pr.	Hypothetical protein	29 kDa antigen cfp29	Secreted Dyp-type peroxidase	Hypothetical protein	DNA polymerase subunit beta	Geranylgeranyl-PP synthase	Phytoene dehydrogenase	Phytoene synthase
Gene ID	XNR_5675	XNR_5676	**XNR_5677**	XNR_5678	XNR_5679	XNR_5680	XNR_5681	XNR_5682	**XNR_5683**	XNR_5684	XNR_5685
Pink	−0.9	−0.6	5.4	5.7	5.5	5.3	5.1	5.0	2.9	3.0	3.4
Orange	0.5	0.1	0.6	0.6	0.5	0.5	0.6	0.5	6.6	6.6	6.4

Deduced protein function	CrtV methyltransferase	Lysine exporter protein	IS1647-like transposase	Hypothetical protein	Transcriptional regulator	Dehydrogenase CrtU	Lycopene cyclase	Stress protein	Hypothetical protein	Divalent cation-transporter
Gene ID	XNR_5686	XNR_5687	XNR_5688	XNR_5689	XNR_5690	XNR_5691	**XNR_5692**	**XNR_5693**	XNR_5694	XNR_5695
Pink	3.7	5.1	5.8	4.8	5.0	4.8	4.3	−0.3	−0*	−0*
Orange	6.4	5.5	5.1	5.0	4.0	4.4	5.6	6.8	−0*	−4.2

Data in *bold*: genes 5677 and 5683, the first genes of the amplified region in the Pink and Orange strains, respectively; genes 5692 and 5693, the last amplified genes in the Pink and Orange strains, respectively. The *−0** fold-change denotes low-read-count genes from Del1 which had zero reads in Orange/Pink strains

we determined the fitness costs of the genome rearrangements in our mutant strains in competition assays. For this purpose, *S. albus* J1074 was inoculated with each of the Pink, Orange and Del2 strains in NL410 medium. Obtained co-cultures were cultivated for three passages and the ratio of individual strains was calculated after each passage. Both *S. albus* Pink and *S. albus* Orange strains showed reduced fitness compared to the parental strain with the average costs of $s_P = -1.46 \pm 0.16$ and $s_O = -1.08 \pm 0.04$. Interestingly, we could not calculate confidently selection coefficient for Del2 despite repeating the experiment several times. It was obvious that the fitness of Del2 strain was reduced, but the values of selection coefficient varied strongly between different repeats and experiments. We also observed that, despite no pronounced differences in the appearance, the spore titers of Del2 strain were approximately 100 times lower compared to those of *S. albus* J1074.

Discussion

We reported the 6.841 Mb complete genome sequence of *S. albus* J1074. Comparison of this genome with the genomes of other streptomycetes revealed the presence of 6.1 Mb core region flanked with the arms of 0.3 and 0.4 Mb, respectively (Zaburannyi, unpublished data). Our initial aim was to evaluate the dispensability of the arm regions of the *S. albus* J1074 chromosome. Antismash analysis revealed the presence of twenty secondary metabolite clusters in the chromosome of *S. albus* J1074. Three large clusters were located in the right terminal arm with the third one overlapping with terminal inverted repeat (Fig. 1a). Terminal repeats are thought to play an important role in the initiation of terminal replication, therefore it was decided to delete the 204-kb region containing only the two first clusters (Huang et al. 1998; Qin and Cohen 1998). For this purpose two *loxP* sites were introduced in the regions flanking the targeted fragment and Cre-recombinase was subsequently expressed (Fig. 1b). Successful deletion of the 204-kb fragment of the right arm in the Del1 mutant without any obvious phenotypic changes indicates its dispensability for *S. albus* J1074.

To further assess the essentiality of the remaining terminal chromosomal regions several attempts to circularize the chromosome of *S. albus* Del1 were undertaken. After deleting the 204 kb fragment a single *loxP* site resided in the right chromosomal arm. Therefore, for circularization to occur, an insertion of the second *loxP* site in the left chromosomal arm was required. The second *loxP* site was introduced in three different positions of the left chromosomal shoulder of Del1 strain: 864, 259, and 42 kb downstream from the terminus (Fig. 1d). Expression of Cre-recombinase failed to circularize the chromosome when the second *loxP* site was introduced in the first two positions, indicating that essential genes were

present within the 864- and 259-kb fragments. Only in the third case, when the second *loxP* site was introduced 42-kb downstream from the left terminus, did the expression of Cre-recombinase lead to chromosomal circularization. The generated Del2 strain has a circular chromosome and contains deletions of the 42-kb left-terminal and 60-kb right-terminal chromosomal fragments. Our data indicate that the right terminal 264 kb are dispensable for *S. albus* J1074, supporting the bioinformatically predicted presence of the 0.4-Mb right-terminal chromosomal arm in the genome of *S. albus* J1074. In contrast, our failure to delete large chromosomal fragments in the left chromosomal region demonstrates that the left arm is shorter than predicted due to the presence of essential genes (Zaburannyi, unpublished data). In order to find these essential genes, translated products of all coding sequences from the 260 kb left terminal region of the *S. albus* J1074 chromosome were analyzed using KAAS automatic genome annotation and pathway reconstruction server (Moriya et al. 2007). This analysis revealed the presence of nine genes involved in primary metabolism, eight of which were present in two and more copies in the genome of *S. albus* J1074. The gene present in a single copy encodes L-threonine aldolase. This enzyme participates in glycine, serine, and threonine metabolism. We also identified three genes whose products participate in protein synthesis and DNA repair, but each of these genes was also present in multiple copies in the chromosome. Analysis of transcriptome data revealed that the gene encoding L-threonine aldolase was highly expressed, providing additional evidence about its essentiality.

The pigmented mutants named *S. albus* Pink and *S. albus* Orange were obtained during the attempt to circularize the chromosome of *S. albus* Del1 by deleting 259-kb left-terminal and 60-kb right-terminal DNA fragments (Fig. 2). Resistance phenotypes of *S. albus* Pink and Orange could not be explained by recombination between the *loxP* sites introduced in *S. albus* Del1 2O21Am and resulted from the unexpected DNA rearrangements. Analysis of genome sequencing data revealed that both mutants have undergone very similar chromosomal rearrangements. Compared to the genome sequence of *S. albus* J1074, the chromosomes of Pink and Orange strains have lost the right terminal chromosomal region of 422,388 and 420,240 bp, respectively. We suppose that DNA rearrangements in the *S. albus* Pink and Orange strains are the result of salvation reaction in response to our attempt to delete essential genes. *S. albus* Del1 2O21Am, which is parental to both Orange and Pink strains, has two *loxP* sites within the left and the right-terminal DNA fragments of the chromosome. Recombination between these two *loxP* sites would lead to the deletion of essential genes, and, therefore, the loss of one *loxP* stabilizes the chromosome of *S. albus*.

However, the loss of the right terminal chromosomal region in *S. albus* Pink and *S. albus* Orange does not explain the onset of the production of coloured compounds. A more in-depth analysis of sequencing coverage revealed the amplification of relatively short DNA sequence directly at the right chromosomal termini of Pink and Orange strains. Our results indicate that the ADSs in both Pink and Orange strains are composed of directly repeated copies of the AUD. DNA amplifications, very similar to those observed in Pink and Orange strains, were previously reported. Most often, these amplifications occur when large chromosomal deletion events lead to the loss of one of terminal inverted repeats (Fischer et al. 1997; Rauland et al. 1995). In general, AUDs of two different types are described – type I and type II. The characteristic feature of type II AUDs is the presence of long direct repeats of at least 0.8 kb in their terminal regions (Volff and Altenbuchner 1998). Our attempts to detect long direct or inverted repeats inside AUDs or even their flanking regions failed. Therefore we consider that the AUDs from *S. albus* Orange and *S. albus* Pink do not belong to type II AUDs. AUDs of type I are less conservative in their structure and can overlap with each other. Moreover, these AUDs amplify themselves directly in the regions where they were originally located, and ADSs observed in individual derivatives of the same strain often differ (Volff and Altenbuchner 1998). Taking into account the features of type I AUDs, we considered that AUDs discovered in *S. albus* Orange and *S. albus* Pink belong to the type I.

The AUDs from Pink and Orange strains overlap and differ in size. In both cases they include the carotenoid biosynthetic gene cluster, which is similar to isorenieratene biosynthetic cluster of *S. coelicolor* and *S. griseus* (Krügel et al. 1999; Takano et al. 2005). Similarities in the architecture (Fig. 3) and homology of the genes suggest the origin of pigmentation of mutant strains. The AUD of *S. albus* Orange contains the entire carotenoid gene cluster, while the one from *S. albus* Pink – truncated cluster. This can explain the difference in colours of both mutant strains. We suspected *S. albus* Orange strain to produce isorenieratene. *S. albus* Pink strain contains truncated *crtY* gene encoding lycopene-cyclase. This enzyme is responsible for converting lycopene into β-carotene—precursor of isorenieratene. Hence we assumed that production of lycopene caused characteristic colour of the Pink strain. UPCC analysis of extracts of the *S. albus* Pink and Orange strains confirmed production of lycopene by the first one. Homology of the amplified genes to the genes encoding isorenieratene pathway, as well as similarity of UV–Vis spectra gives strong evidence that the *S. albus* Orange strain does produce isorenieratene.

It is worth mentioning that spontaneous genome rearrangement events which led to the amplification of secondary metabolite clusters were previously reported for kanamycin, oxytetracyclin, and actinorhodin clusters as the best studied examples (Gravius et al. 1993; Widenbrant et al. 2008; Yanai et al. 2006). Amplification of carotenoid cluster described in

this article indicates once more that production of natural compounds belongs to unstable phenotypes.

Production of carotenoids is often associated with environmental stress (Lee et al. 2001; Moradas-Ferreira et al. 1996; Olson and Krinsky 1995; Sandmann et al. 1998). To find out whether activation of the carotenoid gene cluster in the Pink and Orange strains was caused by its amplification or by the disorders in the regulation of genes expression, differential expression analysis of these two strains versus their parent strain Del1 was performed. We found that transcription levels of the amplified genes were much higher than those of the genes immediately flanking amplification, reaching fold-change values as high as 110-fold (Table 3). All the genes in the amplified AUD showed the same levels of overexpression regardless of biosynthetic pathway they belong to. However, levels of this uniform overexpression were not higher than levels of AUD amplification (Table 2), giving us evidence that no changes occurred in the carotenoid gene cluster expression regulation. Therefore, we conclude that amplification of the DNA fragment containing carotenoid gene clusters is the only reason for the overproduction of lycopene and isorenieratene.

No changes in the growth of Pink, Orange, and Del2 strains compared with *S. albus* J1074 were detected when cultivated on the agar medium in a form of confluent lawn. However, large deletions and genome rearrangements, which these mutants have undergone, should affect their physiology and, consequently, change their fitness. Competition assays performed with *S. albus* J1074 indeed indicate that the fitness of Pink, Orange, and Del2 strains was decreased. The average costs of $s_P = -1.46 \pm 0.16$ and $s_O = -1.08 \pm 0.04$ were determined for Pink and Orange strains, respectively. We were unable to calculate the fitness cost for Del2 strain, due to strong variations between individual experiments and parallel repeats. Such behavior of Del2 strain in competition assays can be explained by its high genetic variability, which is further supported by the fact that Del2 strain gives rise to bold colonies when plated in dilutions. For some strains, evidence exists that chromosome circularization triggers genome rearrangements; therefore, such circularized strains are often very unstable. It is proposed that DNA regions where two replication forks meet are subjected to rearrangements (Volff and Altenbuchner 1998).

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References

Anders S, Huber W (2010) Differential expression analysis for sequence count data. Genome Biol 11:R106

Baltz RH (2010) *Streptomyces* and *Saccharopolyspora* hosts for heterologous expression of secondary metabolite gene clusters. J Ind Microbiol Biotechnol 37:759–772

Bentley SD, Chater KF, Cerdeño-Tárraga A-M, Challis GL, Thomson NR, James KD, Harris DE, Quail MA, Kieser H, Harper D, Bateman A, Brown S, Chandra G, Chen CW, Collins M, Cronin A, Fraser A, Goble A, Hidalgo J, Hornsby T, Howarth S, Huang C-H, Kieser T, Larke L, Murphy L, Oliver K, O'Neil S, Rabbinowitsch E, Rajandream M-A, Rutherford K, Rutter S, Seeger K, Saunders D, Sharp S, Squares R, Squares S, Taylor K, Warren T, Wietzorrek A, Woodward J, Barrell BG, Parkhill J, Hopwood DA (2002) Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). Nature 417:141–147

Bilyk B, Weber S, Myronovskyi M, Bilyk O, Petzke L, Luzhetskyy A (2013) In vivo random mutagenesis of streptomycetes using mariner-based transposon Himar1. Appl Microbiol Biotechnol 97: 351–359

Chater KF, Wilde LC (1980) *Streptomyces albus* G mutants defective in the *Sal*GI restriction–modification system. J Gen Microbiol 116: 323–334

Chen K, Wallis JW, McLellan MD, Larson DE, Kalicki JM, Pohl CS, McGrath SD, Wendl MC, Zhang Q, Locke DP, Shi X, Fulton RS, Ley TJ, Wilson RK, Ding L, Mardis ER (2009) BreakDancer: an algorithm for high-resolution mapping of genomic structural variation. Nat Methods 6:677–681

Dykhuizen DE (1990) Experimental studies of natural selection in bacteria. Annu Rev Ecol Syst 21:373–398

Fedoryshyn M, Welle E, Bechthold A, Luzhetskyy A (2008) Functional expression of the Cre recombinase in actinomycetes. Appl Microbiol Biotechnol 78:1065–1070

Feng Z, Wang L, Rajski SR, Xu Z, Coeffet-LeGal MF, Shen B (2009) Engineered production of iso-migrastatin in heterologous *Streptomyces* hosts. Bioorg Med Chem 17:2147–2153

Fischer G, Decaris B, Leblond P (1997) Occurrence of deletions, associated with genetic instability in *Streptomyces ambofaciens*, is independent of the linearity of the chromosomal DNA. J Bacteriol 179: 4553–4558

Fuciman M, Chábera P, Zupcanová A, Hríbek P, Arellano JB, Vácha F, Psencík J, Polívka T (2010) Excited state properties of aryl carotenoids. Phys Chem Chem Phys 12:3112–3120

Gravius B, Bezmalinović T, Hranueli D, Cullum J (1993) Genetic instability and strain degeneration in *Streptomyces rimosus*. Appl Environ Microbiol 59:2220–2228

Green MR, Sambrook J (2012) Molecular cloning: a laboratory manual, 4th edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Gullón S, Olano C, Abdelfattah MS, Braña AF, Rohr J, Méndez C, Salas JA (2006) Isolation, characterization, and heterologous expression of the biosynthesis gene cluster for the antitumor anthracycline steffimycin. Appl Environ Microbiol 72:4172–4183

Gust B, Chandra G, Jakimowicz D, Yuqing T, Bruton CJ, Chater KF (2004) λ Red-mediated genetic manipulation of antibiotic-producing *Streptomyces*. Adv Appl Microbiol 54:107–128

Huang CH, Lin YS, Yang YL, Huang SW, Chen CW (1998) The telomeres of *Streptomyces* chromosomes contain conserved palindromic sequences with potential to form complex secondary structures. Mol Microbiol 28:905–916

Kieser T, Bibb M, Buttner M, Chater K, Hopwood D (2000) Practical *Streptomyces* genetics. John Innes Foundation, Norwich

Kim S-Y, Zhao P, Igarashi M, Sawa R, Tomita T, Nishiyama M, Kuzuyama T (2009) Cloning and heterologous expression of the cyclooctatin biosynthetic gene cluster afford a diterpene cyclase and two p450 hydroxylases. Chem Biol 16:736–743

Komatsu M, Uchiyama T, Omura S, Cane DE, Ikeda H (2010) Genome-minimized *Streptomyces* host for the heterologous expression of secondary metabolism. Proc Natl Acad Sci U S A 107:2646–2651

Krügel H, Krubasik P, Weber K, Saluz HP, Sandmann G (1999) Functional analysis of genes from *Streptomyces griseus* involved in the synthesis of isorenieratene, a carotenoid with aromatic end groups, revealed a novel type of carotenoid desaturase. Biochim Biophys Acta 1439:57–64

Lee HS, Ohnishi Y, Horinouchi S (2001) A σ^B-like factor responsible for carotenoid biosynthesis in *Streptomyces griseus*. J Mol Microbiol Biotechnol 3:95–101

Lombó F, Velasco A, Castro A, de la Calle F, Braña AF, Sánchez-Puelles JM, Méndez C, Salas JA (2006) Deciphering the biosynthesis pathway of the antitumor thiocoraline from a marine actinomycete and its expression in two *streptomyces* species. Chembiochem Eur J Chem Biol 7:366–376

Makitrynskyy R, Rebets Y, Ostash B, Zaburannyi N, Rabyk M, Walker S, Fedorenko V (2010) Genetic factors that influence moenomycin production in streptomycetes. J Ind Microbiol Biotechnol 37:559–566

Medema MH, Blin K, Cimermancic P, de Jager V, Zakrzewski P, Fischbach MA, Weber T, Takano E, Breitling R (2011) AntiSMASH: rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences. Nucleic Acids Res 39:W339–346

Moradas-Ferreira P, Costa V, Piper P, Mager W (1996) The molecular defences against reactive oxygen species in yeast. Mol Microbiol 19:651–658

Moriya Y, Itoh M, Okuda S, Yoshizawa AC, Kanehisa M (2007) KAAS: an automatic genome annotation and pathway reconstruction server. Nucleic Acids Res 35:W182–185

Myronovskyi M, Welle E, Fedorenko V, Luzhetskyy A (2011) β-Glucuronidase as a sensitive and versatile reporter in actinomycetes. Appl Environ Microbiol 77:5370–5383

Ohnishi Y, Ishikawa J, Hara H, Suzuki H, Ikenoya M, Ikeda H, Yamashita A, Hattori M, Horinouchi S (2008) Genome sequence of the streptomycin-producing microorganism *Streptomyces griseus* IFO 13350. J Bacteriol 190:4050–4060

Olson JA, Krinsky NI (1995) Introduction: the colorful, fascinating world of the carotenoids: important physiologic modulators. FASEB J 9: 1547–1550

Omura S, Ikeda H, Ishikawa J, Hanamoto A, Takahashi C, Shinose M, Takahashi Y, Horikawa H, Nakazawa H, Osonoe T, Kikuchi H, Shiba T, Sakaki Y, Hattori M (2001) Genome sequence of an industrial microorganism *Streptomyces avermitilis*: deducing the ability of producing secondary metabolites. Proc Natl Acad Sci U S A 98:12215–12220

Pósfai G, Plunkett G 3rd, Fehér T, Frisch D, Keil GM, Umenhoffer K, Kolisnychenko V, Stahl B, Sharma SS, de Arruda M, Burland V, Harcum SW, Blattner FR (2006) Emergent properties of reduced-genome *Escherichia coli*. Science 312:1044–1046

Qin Z, Cohen SN (1998) Replication at the telomeres of the *Streptomyces* linear plasmid pSLA2. Mol Microbiol 28:893–903

Rauland U, Glocker I, Redenbach M, Cullum J (1995) DNA amplifications and deletions in *Streptomyces lividans* 66 and the loss of one end of the linear chromosome. Mol Gen Genet 246:37–44

Rodriguez L, Oelkers C, Aguirrezabalaga I, Braña AF, Rohr J, Méndez C, Salas JA (2000) Generation of hybrid elloramycin analogs by combinatorial biosynthesis using genes from anthracycline-type and macrolide biosynthetic pathways. J Mol Microbiol Biotechnol 2: 271–276

Sandmann G, Kuhn S, Böger P (1998) Evaluation of structurally different carotenoids in *Escherichia coli* transformants as protectants against UV-B radiation. Appl Environ Microbiol 64:1972–1974

Schumann G, Nürnberger H, Sandmann G, Krügel H (1996) Activation and analysis of cryptic *crt* genes for carotenoid biosynthesis from *Streptomyces griseus*. Mol Gen Genet 252:658–666

Takaichi S (2000) Characterization of carotenes in a combination of a C_{18} HPLC column with isocratic elution and absorption spectra with a photodiode-array detector. Photosynth Res 65:93–99

Takano H, Obitsu S, Beppu T, Ueda K (2005) Light-induced carotenogenesis in *Streptomyces coelicolor* A3(2): identification of an extracytoplasmic function sigma factor that directs photodependent transcription of the carotenoid biosynthesis gene cluster. J Bacteriol 187:1825–1832

Volff JN, Altenbuchner J (1998) Genetic instability of the *Streptomyces* chromosome. Mol Microbiol 27:239–246

Wendt-Pienkowski E, Huang Y, Zhang J, Li B, Jiang H, Kwon H, Hutchinson CR, Shen B (2005) Cloning, sequencing, analysis, and heterologous expression of the fredericamycin biosynthetic gene cluster from *Streptomyces griseus*. J Am Chem Soc 127:16442–16452

Widenbrant EM, Tsai H-H, Chen CW, Kao CM (2008) Spontaneous amplification of the actinorhodin gene cluster in *Streptomyces coelicolor* involving native insertion sequence IS466. J Bacteriol 190:4754–4758

Winter JM, Moffitt MC, Zazopoulos E, McAlpine JB, Dorrestein PC, Moore BS (2007) Molecular basis for chloronium-mediated meroterpene cyclization: cloning, sequencing, and heterologous expression of the napyradiomycin biosynthetic gene cluster. J Biol Chem 282:16362–16368

Yanai K, Murakami T, Bibb M (2006) Amplification of the entire kanamycin biosynthetic gene cluster during empirical strain improvement of *Streptomyces kanamyceticus*. Proc Natl Acad Sci U S A 103:9661–9666

Zhou M, Jing X, Xie P, Chen W, Wang T, Xia H, Qin Z (2012) Sequential deletion of all the polyketide synthase and nonribosomal peptide synthetase biosynthetic gene clusters and a 900-kb subtelomeric sequence of the linear chromosome of *Streptomyces coelicolor*. FEMS Microbiol Lett 333:169–179