Appl Microbiol Biotechnol (2016) 100:2797–2808
DOI 10.1007/s00253-015-7271-0

METHODS AND PROTOCOLS

New ΦBT1 site-specific integrative vectors with neutral phenotype in *Streptomyces*

Nathaly Gonzalez-Quiñonez[1] · María Teresa López-García[1] · Paula Yagüe[1] · Beatriz Rioseras[1] · Annalisa Pisciotta[2] · Rosa Alduina[2] · Ángel Manteca[1]

Received: 29 September 2015 / Revised: 15 December 2015 / Accepted: 19 December 2015 / Published online: 13 January 2016

Abstract Integrative plasmids are one of the best options to introduce genes in low copy and in a stable form into bacteria. The ΦC31-derived plasmids constitute the most common integrative vectors used in *Streptomyces*. They integrate at different positions (*attB* and pseudo-*attB* sites) generating different mutations. The less common ΦBT1-derived vectors integrate at the unique *attB* site localized in the *SCO4848* gene (*S. coelicolor* genome) or their orthologues in other streptomycetes. This work demonstrates that disruption of *SCO4848* generates a delay in spore germination. *SCO4848* is co-transcribed with *SCO4849*, and the spore germination phenotype is complemented by *SCO4849*. Plasmids pNG1–4 were created by modifying the ΦBT1 integrative vector pMS82 by introducing a copy of *SCO4849* under the control of the promoter region of *SCO4848*. pNG2 and pNG4 also included a copy of the P_{ermE}* in order to facilitate gene overexpression. pNG3 and pNG4 harboured a copy of the *bla* gene (ampicillin resistance) to facilitate selection in *E. coli*. pNG1–4 are the only integrative vectors designed to produce a neutral phenotype when they are integrated into the *Streptomyces* genome. The experimental approach developed in this work can be applied to create phenotypically neutral integrative plasmids in other bacteria.

Keywords *Streptomyces* · ΦBT1 integrative vector · Heterologous expression · Neutral phenotype

Introduction

Streptomycetes are important industrial bacteria, which produce two thirds of all clinically relevant secondary metabolites (Hopwood 2007; Davies 2014). They have complex life cycles that include sporulation and developmentally associated programmed cell death, and they are considered multicellular prokaryotic models (reviewed in Yagüe et al. 2013).

Streptomyces genetic methodologies have been extensively optimized during the last decades (Kieser et al. 2000). Vectors including the ΦC31 *attP* site were the first and the most commonly used integrative plasmids designed to work in *Streptomyces* (Bierman et al. 1992). The major limitation of these types of vectors is that they integrate at different positions in the *Streptomyces* genome, resulting in different mutations and phenotypes (Combes et al. 2002). This issue is especially relevant when ΦC31 plasmids are used to introduce genes in *Streptomyces* mutants in order to complement their mutations: phenotypes can be a consequence of the integration of the plasmid instead to the function of the gene that is being harboured by the plasmid.

The alternatives to the ΦC31 integrative site in *Streptomyces* are the ΦBT1 (Gregory et al. 2003), TG1 (Morita et al. 2009) and SV1 (Fayed et al. 2014) integrative sites. The ΦBT1 derived vectors integrate into the *attP* site localized into the *SCO4848* gene (*S. coelicolor* genome) (Gregory et al. 2003), while the TG1 plasmids integrate at *SCO3658* (Fayed et al. 2014) and SV1 plasmids at *SCO4383* (Fayed et al. 2014). To

Electronic supplementary material The online version of this article (doi:10.1007/s00253-015-7271-0) contains supplementary material, which is available to authorized users.

✉ Ángel Manteca
mantecaangel@uniovi.es

[1] Área de Microbiología, Departamento de Biología Funcional e IUOPA, Facultad de Medicina, Universidad de Oviedo, 33006 Oviedo, Spain

[2] Department of Biological, Chemical and Pharmaceutical Sciences and Technologies, University of Palermo, Palermo, Italy

our knowledge, there are no reports describing the phenotype generated by the integration of TG1- and SV1-derived plasmids. However, in the case of ΦBT1 plasmids, it was reported that integration into *SCO4848* leads to the expression of a modified but functional version of *SCO4848*, generating a neutral phenotype once they are integrated (Gregory et al. 2003).

In this work, a detailed phenotypic analysis of *S. coelicolor* harbouring the ΦBT1 integrative vector pMS82 (Gregory et al. 2003) resulted in the identification of a novel phenotype consisting of a delay in spore germination. It was demonstrated that the disruption of *SCO4848* affected the expression of *SCO4849*, and the detected phenotype is complemented by *SCO4849*. *SCO4848* and *SCO4849* encode for putative transmembrane proteins with unknown functions which are highly conserved in streptomycetes.

Four plasmids were created modifying the ΦBT1 integrative vector pMS82 (Gregory et al. 2003) in order to restore *SCO4849* expression: pNG1, in which the *SCO4849* gene was introduced under the control of the promoter region of *SCO4848* into pMS82 to complement the phenotype generated by the integration of the plasmid; pNG2, in which the P_{ermE}* promoter was introduced into pNG1 to facilitate heterologous gene expression; pNG3, in which the *bla* gene for ampicillin resistance was included in pNG1 to facilitate selection in *E. coli*; and pNG4, in which the *bla* gene was introduced into pNG2. As discussed below, these plasmids are the only integrative vectors designed to produce a neutral phenotype when they are integrated in *Streptomyces*.

Materials and methods

Bacterial strains and media

Streptomyces coelicolor M145, *S. griseus* IFO 13350, *S. clavuligerus* ATCC 27064 and *S. lividans* 1326 were the reference strains used in this work (Table 1). Petri dishes (8.5 cm) with 25 ml of solid medium were covered with cellophane discs (cellulose acetate films were from Sadipal), inoculated with 100 μl of a spore suspension (1×10^8 viable spores/ml), and incubated at 30 °C. Glucose, yeast/malt (GYM) extract (Novella et al. 1992) was the medium used for *S. coelicolor*, *S. griseus* and *S. lividans* cultures. *Streptomyces clavuligerus* germinates very slowly in GYM, and for this reason, Trypticase soy agar (TSA) (Kieser et al. 2000) was used for spore germination of this strain. Unless otherwise stated, soya flour mannitol (SFM) (Kieser et al. 2000) was the medium used to obtain spores of *S. coelicolor*, *S. lividans* and *S. griseus*. *Streptomyces clavuligerus* do not sporulate well in SFM, and therefore, MA (Sánchez and Braña 1996) was used for sporulation of this strain.

Liquid cultures were performed in R5A sucrose-free liquid medium (Fernandez et al. 1998). Laboratory flasks of 500 ml were filled with 100 ml of culture medium, inoculated with 1×10^7 spores/ml and incubated at 200 rpm and 30 °C.

The influence of the medium used to raise the spores was tested in *S. coelicolor*, comparing germination of spores obtained in SFM or GYM. The effect of the medium used for germination was analysed testing germination of all the strains in two additional media: MM with glucose and R2YE (Kieser et al. 2000).

E. coli strains were grown at 37 °C in solid (2 % agar) or liquid 2xYT medium (Sambrook and Russell 2001) supplemented with the appropriate antibiotics (Table 1): ampicillin was used at 100 μg/ml, kanamycin at 50 μg/ml and hygromycin at 100 μg/ml. The antibiotic used for selection in *Streptomyces* was hygromycin at the final concentration of 200 μg/ml.

Viability staining

Culture samples were obtained and processed for microscopy at different incubation time points, as previously described (Manteca et al. 2006). Cells were stained with propidium iodide and SYTO 9 (LIVE/DEAD BacLight Bacterial Viability Kit, Invitrogen, L-13152). The samples were observed under a Leica TCS-SP2-AOBS confocal laser scanning microscope at wavelengths of 488 and 568 nm excitation and 530 nm (green) or 640 nm (red) emissions (Manteca et al. 2006).

Spore germination

Germination was quantified as previously reported (de Jong et al. 2009). Briefly, germination was quantified in solid media with cellophane discs. At different developmental time points, pieces of cellophane discs were cut and processed for confocal microscopy as described in the previous paragraph. Three biological replicates of the cultures were analysed at different developmental time points. The percentage of germination was assessed from at least 100 spores at each time point. Spores were considered to be germinating when the germ tubes were visible under the confocal microscope.

Antibiotic quantification

Undecylprodigiosin and actinorhodin were quantified spectrophotometrically according to Tsao et al. (1985) and Bystrykh et al. (1996). In order to measure the total amount of actinorhodin (intracellular and extracellular), cells were ruptured in their culture medium by adding KOH 0.1 N. Cellular debris was discarded by centrifugation, and actinorhodin was quantified spectrophotometrically with a UV/visible spectrophotometer (Shimadzu, Model UV-1240), applying the linear Beer–Lambert relationship to estimate concentration

Table 1 Bacterial strains, plasmids and primers used in this study

Strain, plasmid, cosmid	Description	Origin or reference
Bacterial strains		
S.coelicolor M145	SCP1$^-$ SCP2$^-$	Kieser et al. (2000)
S. griseus	IFO 13350	IFO
S. clavuligerus	ATCC 27064	ATCC
S. lividans	1326	Kieser et al. (2000)
ΔSCO0995	*S. coelicolor* SCO0995 mutant: SCO0995::Tn5062, Am^R	This study
E. coli TOP10	F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL (Str^R) endA1 λ$^-$	Invitrogen
E. coli ET12567	dam-13::Tn9, dcm-6	MacNeil et al. (1992)
E. coli ET12567/pUZ8002	*E. coli* ET12567 containing plasmid pUZ8002, a not self-transmissible plasmid which can mobilize other plasmids	Flett et al. (1997)
Plasmids and cosmids		
pCR™-Blunt II-TOPO®	Zero Blunt® TOPO® PCR Cloning Kit, Km^R	Invitrogen
pQM5062	Plasmid containing *eGFP* Tn5062	Bishop et al. (2004)
pTOPO-$P_{SCO4848}$	$P_{SCO4848}$ cloned in Zero Blunt® TOPO®, Km^R	This study
pTOPO-SCO4849	SCO4849 cloned in Zero Blunt® TOPO®, Km^R	This study
pTOPO-$P_{SCO4848}$ + SCO4849	$P_{SCO4848}$SCO4849 cloned in Zero Blunt® TOPO®, Km^R	This study
pMS82	Cloning vector, Hyg^R	Gregory et al. (2003)
pNG1	$P_{SCO4848}$ + SCO4849 cloned into pMS82/*Nsi*I/*Hin*dIII, Hyg^R	This study; accession KR131848
pNG1'	pNG1 without the *Nde*I cutting site, Hyg^R	This study
pNG2	P_{ermE}* + RBS + MCS + *fd-ter* cloned into pNG1'/*Eco*RV/*Spe*I Hyg^R	This study; accession KR131849
pNG3	*bla* cloned into pNG1/*Hin*dIII/*Avr*II Hyg^R, Amp^R	This study; accession KR131850
pNG4	*bla* cloned into pNG2/*Hin*dIII/*Avr*II Hyg^R, Amp^R	This study; accession KR131851
2StG2.1.G12	StG2 cosmid carrying Tn5062 transposon interrupting *SCO0995* ORF	Fernández-Martínez et al. (2011)
pNG2-SCO0995	pNG2 plasmid containing the ORF of SCO0995 under the control of P_{ermE}*	
Primers		
PSCO4848F	5′ GGAAGCTTGCGAACAGCATCTTCAGGG 3′	This study
PSCO4848R	5′ AACATATGGGTTTCAGTATCGCCGCAC 3′	This study
SCO4849F	5′ GGCATATGGCGACGAGGACGTCACGGA 3′	This study
SCO4849R	5′ AAATGCATGGGGATGACGAAGGTGATG 3′	This study
Bla-F	5′ AAACCTAGGAAATATGTATCCGCTCATG 3′	This study
Bla-R	5′ GGGAAGCTTAGGGATTTTGGTCATGAG 3′	This study
SCO4848F	5′ CGTCGTATCCCCTCGGTTG 3′	This study
pMS82R	5′ GAGCCGGGAAAGCTCATTCA 3′	This study
SCO0995F	5′ CATATGGCACGAACGGGCGGGTT 3′	This study
SCO0995R	5′ ACTAGTATGACCCGCTGATACTACGG 3′	This study
RT4848F	5′ GGATCCGGACAGCGTGCG 3′	This study
RT4849F	5'ATGGTGGTCGTCTTCGTGCT 3′	This study
RT4849R	5′ CAGCGGCCGGACGACCT 3′	This study
RT0995F	5′ GAACTGCTCGACACCTCTCC 3′	This study
RT0995R	5′ GTCTGCACCATCAGGTCCTC 3′	This study

(e640 = 25,320). Undecylprodigiosin was measured after vacuum drying the culture (including the mycelium and culture medium) followed by extraction with methanol, acidification with HCl (to 0.5 M) and spectrophotometric assay at 530 nm, again using the Beer–Lambert relationship to estimate concentration (e530 = 100,500). In all cases, for the high concentration solutions, dilutions were performed to conduct the analysis in the linear Beer–Lambert region.

Protein quantification Samples (half millilitre) were collected at different developmental time points, and stored at −20 °C until they were analysed. Half millilitre of NaOH 1 M was

added to the half millilitre samples (0.5 M final concentration of NaOH) and boiled for 10 min. Cellular debris was removed by centrifugation (at 7740×*g* for 15 min at 4 °C), in order to obtain the intracellular samples. Protein was quantified using the Bradford method (Bradford 1976) with bovine serum albumin (Sigma) as the standard.

Confirmation of the ΦBT1 integration site

Two primers were designed that hybridize to the *SCO4848* 5′ end (SCO4848F, hybridizing 33 bp upstream of the SCO4848 ORF) and the DNA region flanking *attP* in pMS82 (pMS82R, hybridizing at 368 bp downstream of the pMS82 *attP*). These primers amplify a fragment of 617 bp only if pMS82 or their derived plasmids (pNG1–4) are integrated at the *attB* site of SCO4848.

RT-PCR

Total RNA samples were extracted from 24- and 72-h cultures of *S. coelicolor* growing in GYM solid media with cellophane. RNA samples were obtained using phenol extraction and the RNeasy Midi Kit (Qiagen) and chromosomal DNA was removed after DNase I (Qiagen) and Turbo DNase (Ambion) treatments. RNA integrity was verified by means of the 2100 Bioanalyzer (Agilent). Reverse transcription (RT)-PCR analysis was performed using the SuperScript one-step RT-PCR system with Platinum *Taq* DNA polymerase (Invitrogen), using 200 ng of total RNA as template. Positive controls were performed using chromosomal DNA as template.

RNA obtained at 72 h and primers RT4848F/RT4849R (hybridizing at position −15 of *SCO4848* ORF and 285 of *SCO4849* ORF, respectively) were used for the analysis of *SCO4848* and *SCO4849* co-transcription (623-bp amplicon). Reverse transcription was performed at 55 °C for 30 min followed by an initial denaturation at 94 °C for 2 min. Then, a touchdown was performed in 10 cycles during which the annealing temperature was reduced by 1 °C in each cycle: 94 °C for 15 s, 65 °C (Δ−1 °C) for 30s and 68 °C for 35 s. For the next 35 cycles (94 °C for 15 s, 55 °C for 30s and 68 °C for 35 s), the annealing temperature was set at 55 °C with a final extension step at 68 °C for 5 min.

RNA obtained at 24 h and primers RT0995F/RT0995R were used for the analysis of *SCO0995* gene expression (420-bp amplicon). First-strand complementary DNA (cDNA) synthesis was performed at 50 °C for 30 min followed by an initial denaturation at 94 °C for 2 min. Then, a touchdown was performed in 6 cycles with a 1 °C reduction in the annealing temperature in each cycle: 94 °C for 15 s, 66 °C (Δ−1 °C) for 30s and 68 °C for 45 s. An annealing temperature of 60 °C was used in the next 35 cycles (94 °C for 15 s, 55 °C for 30s and 68 °C for 45 s) with a final extension step at 68 °C for 5 min.

Fig. 1 Effect of pMS82 in spore germination and integration in the *S. coelicolor* chromosome. **a** Percentage of germination in *S. coelicolor* M145 wild-type strain and *S. coelicolor* harbouring pMS82 growing in GYM medium. Germination values correspond to the average ± SD of three biological replicates. **b** Confocal microscope images of *S. coelicolor* and *S. coelicolor* pMS82 cultures at 4 and 7 h. *Arrows* point to ungerminated spores. **c** Scheme of the integration of pMS82 into the *S. coelicolor* chromosome. Primers SCO4848F and pMS82R are indicated. *Int1* and *int2* indicate the mutated versions of SCO4848 generated by the integration of the plasmid. **d** PCR confirming the integration of pMS82 into the SCO4848 *attB* site. **e** Comparison of the amino acid sequences of SCO4848 and their mutated versions (*int1* and *int2*). **f** Scheme of the chromosomal region around SCO4848 illustrating the position of the primers RT4848F and RT4849R. **g** RT-PCR with primers RT4848F and RT4849R demonstrating the co-transcription of *SCO4848* and *SCO4849* (*RNA lane*); the *DNA lane* corresponds to PCR using chromosomal DNA as template (positive control). *M* molecular weight marker. **h** Scheme of the chromosomal region around SCO4848 illustrating the position of the primers RT4849F and RT4849R. **i** RT-PCR illustrating the alteration of the transcription of SCO4849 in the *S. coelicolor* strain harbouring pMS82

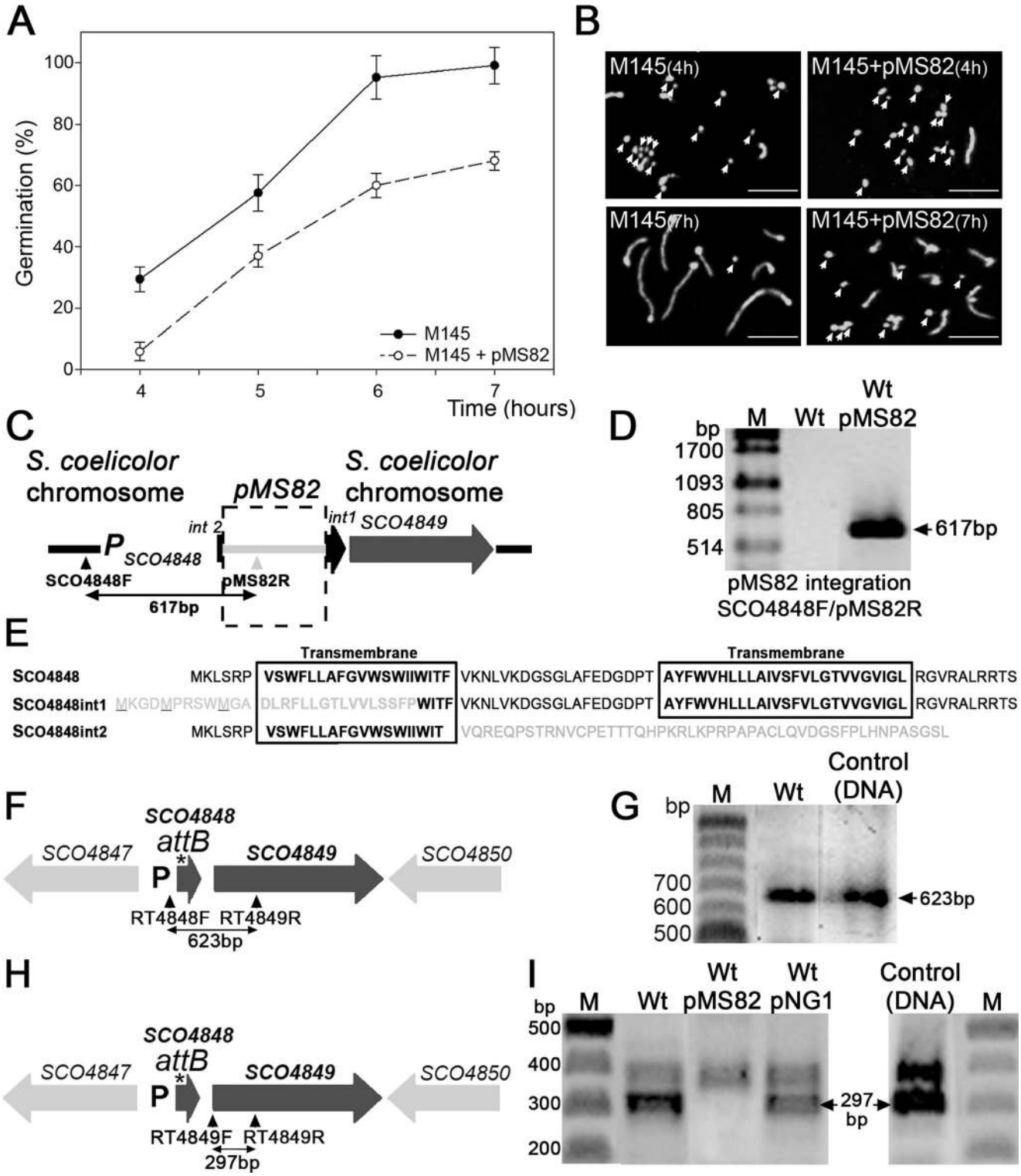
RNA obtained at 24 h and primers RT4849F/RT4849R (hybridizing at position 1 and 285 of *SCO4849*) were used for the analysis of *SCO4849* transcription (297-bp amplicon). RT-PCR conditions were as follows: first-strand cDNA synthesis was performed at 60 °C for 30 min and denaturation at 94 °C for 2 min. PCR amplification in 40 cycles of 94 °C for 15 s, 65 °C for 30s and 68 °C for 15 s and final extension at 68 °C for 5 min. This RT-PCR generated unspecific amplicons, but the identity of the 297-bp amplicon was confirmed by Sanger DNA sequencing.

Gene synthesis

The synthesis of *SCO4848* including silent mutations in the *attB* site (Fig. 1d) (accession number KR150757) and P_{ermE}*+RBS+MCS+*fd-ter* (accession number KR131846) were ordered from GeneCust Europe.

pNG1 construction

The fragment $P_{SCO4848}$+*SCO4849* was obtained in three steps: first, the promoter region of *SCO4848* ($P_{SCO4848}$) was amplified from the *S. coelicolor* genome using primers $P_{SCO4848}$F/R (incorporating *Hin*dIII and *Nde*I restriction sites at the 5′ and 3′ ends, respectively) and cloned into pCR™-Blunt II-TOPO® (pTOPO-$P_{SCO4848}$); secondly, the ORF of *SCO4849* was amplified with primers SCO4849 F/R (incorporating *Nde*I and *Nsi*I restriction sites at the 5′ and 3′ ends, respectively) and cloned into pCR™-Blunt II-TOPO® selecting for the plasmid in which the 5′ end of the *SCO4849* gene incorporating the *Nde*I restriction site was orientated to the *Hin*dIII side of the pCR™-Blunt II-TOPO® (pTOPO-SCO4849); thirdly, $P_{SCO4848}$ was released from pTOPO-$P_{SCO4848}$ using *Hin*dIII and *Nde*I and cloned



into pTOPO-SCO4849 digested with the same enzymes, generating pTOPO-$P_{SCO4848}$+SCO4849. Fragment *$P_{SCO4848}$+SCO4849* was released from pTOPO-$P_{SCO4848}$+SCO4849 using *Hin*dIII and *Nsi*I, and it was religated into pMS82 (Gregory et al. 2003) digested with the same enzymes, generating pNG1 (accession number KR131848). pMS82 containing *$P_{SCO4848}$+SCO4848+SCO4849* was constructed by cloning the synthetized *SCO4848* gene

(accession number KR150757) harbouring synonymous mutations (Fig. 1d) into pNG1 (both digested with *Nde*I). As detailed below, this construction was unstable.

pNG2 construction

pNG1 was digested with *Nde*I. *Nde*I single-stranded overhangs were digested with nuclease S1 (Thermo Scientific), and the plasmid was religated using T4 DNA ligase (Invitrogen) to generate plasmid pNG1' without the *Nde*I restriction site. The synthetized fragment P_{ermE}*+RBS+MCS+*fd-ter*, digested with *Eco*RV-*Xba*I, was cloned into pNG1' digested with *Eco*RV-*Spe*I (*Xba*I and *Spe*I are compatible enzymes) generating pNG2 (accession number KR131849).

pNG3 construction

The *bla* gene conferring resistance to ampicillin was amplified from plasmid pBR322 (Fermentas) using primers Bla-F and Bla-R (Table 1) and Phusion High-Fidelity DNA Polymerase (Thermo). The amplified gene was cloned into pCR™-Blunt II-TOPO®. The *bla* gene was released from pCR™-Blunt II-TOPO with *Hin*dIII/*Avr*II and cloned into pNG1 digested with the same enzymes, generating pNG3 (accession number KR131850).

pNG4 construction

The *bla* gene was released from pCR™-Blunt II-TOPO with *Hin*dIII/*Avr*II and cloned into pNG2 digested with the same enzymes, generating pNG4 (accession KR131851).

Disruption and overexpression of SCO0995

The transposon insertion single-gene knockout library created by Prof. P. Dyson's research group (Fernández-Martínez et al. 2011) was used for mutagenesis of *SCO0995*. Cosmid 2StG2.1.G12 was used for constructing the SCO0995 mutant strain (ΔSCO0995) with the SCO0995 gene interrupted by the Tn5062 transposon (Table 1). Gene disruption was carried out by obtaining double crossovers via conjugation using *E. coli* ET12567/pUZ8002 as a donor strain and following the protocol described in Kieser et al. (2000). Mutant strains were confirmed by Southern blotting, using chromosomal DNA digested with *Sal*I. Southern hybridization was carried out by established procedures using the digoxigenin-labelled 3442-bp Tn5062 *Pvu*II fragment from plasmid pQM5062 (Bishop et al. 2004) as a probe.

The ORF of *SCO0995* gene was amplified from *S. coelicolor* genome using primers SCO0995F and SCO0995R primers and cloned in pNG2 (into the *Nde*I-*Spe*I restriction sites) under the control of *PermE**.

Bioinformatic analyses

Transmembrane topology of the SCO4848 and SCO4849 genes was analysed by Phobius software (http://phobius.sbc.su.se/). The rest of the bioinformatics and phylogenetic analyses were performed using the Phylemon 2.0 bioinformatic tools (Sánchez et al. 2011).

Orthologous sequences to SCO4848 and SCO4849 from other streptomycetes were obtained from the databases at the National Center for Biological Information (http://www.ncbi.nlm.nih.gov). SCO4848 was not annotated in the genomes of *S. lividans* or *S. griseus*; however, the ORF was present in their respective genomes (StrepDB database). The sequences selected were SLI_5122 (*S. lividans*), SAV_3412 and SAV_3411 (*S. avermitilis*); SVEN_4521 and SVEN_4522 (*S. venezuelae*); SCAB35521, SCAB35511 (*S. scabies*), SGR_2696 (*S. griseus*) and SCLAV_3766, SCLAV_3767 (*S. clavuligerus*). Amino acid sequences were aligned using MUSCLE software, and amino acid similarities were estimated by Lalign software (http://www.ch.embnet.org/software/LALIGN_form.html).

Results

Phenotype caused by the integration of pMS82 into the *S. coelicolor* genome

Key phenotypes of the *S. coelicolor* strain harbouring pMS82 were analysed and compared with *S. coelicolor* wild-type strain. Spore germination, first compartmentalized mycelium, second multinucleated mycelium without (substrate) or with (aerial) hydrophobic covers, and sporulation were analysed in solid cultures (Supplementary Figures S1, S2). Antibiotic production (undecylprodigiosin and actinorhodin) was analysed in liquid cultures (Supplementary Figure S3). As discussed below, the only phenotypic difference observed in the *S. coelicolor* harbouring pMS82, with respect to the wild-type strain, was a delay in spore germination (Fig. 1a, b).

Spore germination timing depends on the culture medium used for germination (GYM, R2YE or MM) (Fig. 1a and Supplementary Figure S4). However, the delay in germination generated by the integration of ΦBT1 plasmids was observed in all the strains and all the media tested.

The effect of the medium used to raise the spores was tested in *S. coelicolor*, comparing the differences in germination of spores obtained from SFM or GYM (Fig. 1a and Supplementary Figure S4). In both cases, pMS82 reduced spore germination. Differences in the percentage of germination between the two batches of spores were not significant. It is notable that germination in *S. coelicolor* is very fast (starting at 4 h and finishing at around 7 h) (Fig. 1a), and small variations in the cultures can generate differences in the exact

percentage of germination observed at a specific developmental time point.

ΦBT1 integration in *S. coelicolor* chromosome

The integration site of ΦBT1 plasmids into the *S. coelicolor* genome was reported to be in the *SCO4848* gene (Gregory et al. 2003). To our knowledge, there are no studies describing the existence of alternative ΦBT1-*attB* sites in *Streptomyces*, which is in contrast to the sites for ΦC31 integration (Combes et al. 2002). Two primers were designed in this work, hybridizing at the *SCO4848* 5′ end and the DNA region flanking *attP* in pMS82 (Fig. 1c). These primers amplify a fragment of 617 bp only if pMS82 or their derived plasmids are integrated at the *attB* site of *SCO4848* (Figs. 1d). The pMS82- and the pMS82-based plasmids created in this work (pNG1–pNG4, see below) were used in our lab in 24 independent experiments to complement wild phenotypes in different *S. coelicolor* mutants, and in all of them, the integration was found at the *SCO4848 attB* site (Supplementary Figure S5). This suggests that *SCO4848* harbours the only ΦBT1-*attB* site in *S. coelicolor*, which, as discussed below, is one of the key requirements to construct a phenotypically neutral integrative plasmid in *Streptomyces*.

Integration of the ΦBT1 plasmid interrupts *SCO4848* by modifying its 5′ end. Integration generates a modified SCO4848 ORF (SCO4848int1 in Fig. 1e), which retains a putative signal peptide, and the key features of SCO4848 and was predicted to be functional (Gregory et al. 2003). However, as discussed below, the *SCO4848int1* gene and the promoter region of *SCO4848* are separated by the integrated plasmid, and the transcription of *SCO4848int1* is very unlikely (Fig. 1c). *SCO4849* is located downstream of *SCO4848* (Fig. 1f), and RT-PCR experiments performed in this work demonstrated that *SCO4848* is co-transcribed with *SCO4849* in the *S. coelicolor* wild-type strain (Fig. 1g). Thus, the integration of ΦBT1 plasmids into *SCO4848* might also affect the expression of *SCO4849*. The alteration of *SCO4849* transcription was confirmed by RT-PCR using primers RT4849F and RT4849R (Fig. 1h, i). This RT-PCR amplified more than one amplicon; however, the expected amplicon (297 bp) was present in *S. coelicolor* wild type, and it was absent in the strain harbouring pMS82 (Fig. 1i). As stated in "Materials and methods" section, the identity of this 297-bp amplicon was confirmed by DNA sequencing (data not shown).

Construction of ΦBT1 integrative plasmids restoring the expression of *SCO4849*

In order to create ΦBT1 integrative plasmids restoring the expression of *SCO4848* and *SCO4849*, after the plasmid had integrated into the *Streptomyces* genome, two genetic constructs were synthetized (Fig. 2a): the first one, containing a 423-bp fragment upstream of the ATG of the SCO4848 gene, large enough to encompass the promoter region of the *SCO4848* gene ($P_{SCO4848}$), together with the ORFs of *SCO4848* and *SCO4849* ($P_{SCO4848}$+*SCO4848*+*SCO4849*); and the second one, consisting of the promoter of *SCO4848* and the ORF of *SCO4849* ($P_{SCO4848}$+*SCO4849*). In order to try to inactivate the *attB* site of *SCO4848* and to prevent the recombination with the *attP* site of the plasmid, the SCO4848 amino acid sequence was retained, but the nucleotides of the *attB* site were changed by synonymous mutations (Fig. 2a).

$P_{SCO4848}$+*SCO4848*+*SCO4849* and $P_{SCO4848}$+*SCO4849* were introduced into the ΦBT1plasmid pMS82 (Gregory et al. 2003); however, only the second one ($P_{SCO4848}$+*SCO4849*) was stable, generating plasmid pNG1 (Fig. 3a). Introduction of $P_{SCO4848}$+*SCO4848*+*SCO4849* into pMS82 generated plasmid reorganizations and deletions in pMS82 (Supplementary Figure S6), indicating that the mutated *attB* site synthetized (Fig. 2b) was still functional after recombining with the *attP* site of the plasmid.

The phenotype in spore germination generated by the integration of ΦBT1 integrative plasmids into SCO4848 (see above and Fig. 1a, b) was complemented by the $P_{SCO4848}$+*SCO4849* fragment included in pNG1 (Fig. 2c), and the restauration of the *SCO4849* expression in the *S. coelicolor* strain harbouring pNG1 was demonstrated by RT-PCR (Fig. 1i). These results demonstrated that the phenotype detected was a consequence of the alteration of *SCO4849* expression due to the plasmid integration.

To facilitate work in *Streptomyces*, three more plasmids were constructed based on pNG1: pNG2, in which the strong constitutive promoter P_{ermE}* (Bibb et al. 1994), the RBS of the gene *tuf-1* (Vijgenboom et al. 1994), a cloning site including single-cutter restriction enzymes, and the terminator of the fd phage (Gentz et al. 1981) were introduced into pNG1 to facilitate heterologous gene expression and protein translation (Fig. 3b); pNG3, in which the *bla* gene for ampicillin resistance was included in pNG1 in order to facilitate selection in *E. coli* (Fig. 3c); and pNG4, in which the *bla* gene was introduced into pNG2 (Fig. 3d).

The functionality of the P_{ermE}* and RBS site included in pNG2 and pNG4 was tested using pNG2 to overexpress the ORF of SCO0995 (plasmid pNG2-SCO0995) (Table 1), encoding a putative DNA-methyltransferase, into a ΔSCO0995 strain (mutated in *SCO0995*) (Fig. 4a). The integration of the pNG2-SCO0995 plasmid and the presence/absence of the SCO0995 in the *S. coeliocolor* wild-type strain, the ΔSCO0995 mutant and the mutant strain harbouring pNG2-SCO0995 were confirmed by PCR (Figs. 4b, c). As expected, the *SCO0995* mRNA was detected in the wild-type and the mutant strain complemented with plasmid pNG2-SCO0995, but not in the ΔSCO0995 mutant strain (Fig. 4e).

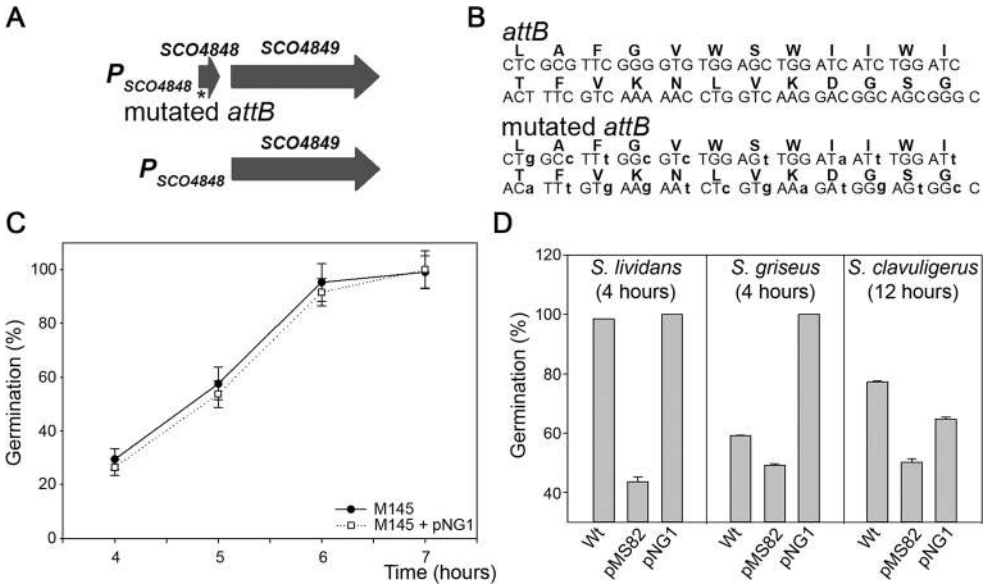


Fig. 2 Construction of ΦBT1 integrative plasmids restoring the expression of *SCO4849*. **a** Genetic constructs to restore the expression of *SCO4848* and *SCO4849*. The *attB* integrative site is indicated by an asterisk. **b** Detail of the sequence of the *attB* and the mutated *attB* harbouring synonymous mutations. **c** Percentage of germination (average ± SD) in *S. coelicolor* M145 wild-type strain and *S. coelicolor* harbouring pNG1 growing in GYM medium. **d** Percentage of spore germination (average ± SD) in *S. griseus*, *S. lividans* and *S. clavuligerus* with and without pMS82 or pNG1. GYM was used in the case of *S. griseus* and *S. lividans*; TSA was used for *S. clavuligerus*

ΦBT1 integration in other streptomycetes

The SCO4848 and SCO4849 orthologues are highly conserved in streptomycetes, 96 % of similarity in the case of SCO4848, 95 % in the case of the metallophosphatase domain of SCO4849 and 86 % in the case of the transmembrane domain of SCO4849 (Fig. 5).

The effect of the integration of ΦBT1 vectors was analysed in other key streptomycetes including *S. lividans*, *S. griseus* and *S. clavuligerus* (Fig. 2d). *Streptomyces lividans* and *S. griseus* wild-type strains germinated much faster than *S. coelicolor*, reaching 100 and 59.2 % of spore germination at 4 h, in comparison to the 30 % observed in *S. coelicolor* (Fig. 2c). *Streptomyces clavuligerus* wild-type strain germination was slower than in *S. coelicolor*, showing 77 % of germination at 12 h, in comparison to the 100 % observed in *S. coelicolor* at this developmental time point (Fig. 2c, d). Integration of pMS82 delayed spore germination in all the strains, and the SCO4849 gene included in pNG1 compensated for this delay in *S. lividans* and *S. griseus*, but not in *S. griseus* (100 % of spore germination of the strain harbouring pNG1 vs. the 59.2 % observed in the wild-type strain) (Fig. 2d). As discussed below, this result indicates that the effect of SCO4849 in accelerating spore germination is stronger than the delay generated by the mutation of its *S. griseus* orthologue caused by the integration of the vector. Similar results were obtained using different culture media for germination (Supplementary Figure S4).

Discussion

The ΦC31 integrative plasmids are widely used in streptomycetes; however, they have severe limitations due to the existence of several integration sites in the *Streptomyces* genome, which cause different mutations resulting in different phenotypes (Bierman et al. 1992). The most common alternative in *Streptomyces* to ΦC31 integrative plasmids are the ΦBT1-derived vectors which integrate at the unique *attB* site localized in the ORF of *SCO4848* (*S. coelicolor* genome). Mutation of *SCO4848* was reported to produce an ORF that remained functional (*SCO4848int1* in Fig. 1e) and to be neutral in terms of phenotype (Gregory et al. 2003). However, the *SCO4848int1* gene and the promoter region of *SCO4848* are separated by the integrated plasmid (Fig. 1c), and the transcription of *SCO4848int1* is very unlikely. The other ORF generated by the integration of the ΦBT1plasmids (*SCO4848int2*) has 30 % of the amino acids of SCO4848 protein (24 amino acids of a total of 74) (Fig. 1e) under the control of the SCO4848 promoter, and it might conserve part of the function of the SCO4848 protein. In this work, we further characterized the phenotype generated by the integration of ΦBT1 plasmids into the *Streptomyces* genome, discovering the existence of a previously uncharacterized phenotype during the germination stages consisting of a delay in spore germination. This phenotype was demonstrated to be a consequence of an effect on *SCO4849* expression, which encodes a putative integral membrane protein that is co-transcribed together with *SCO4848*. Further work is required to identify

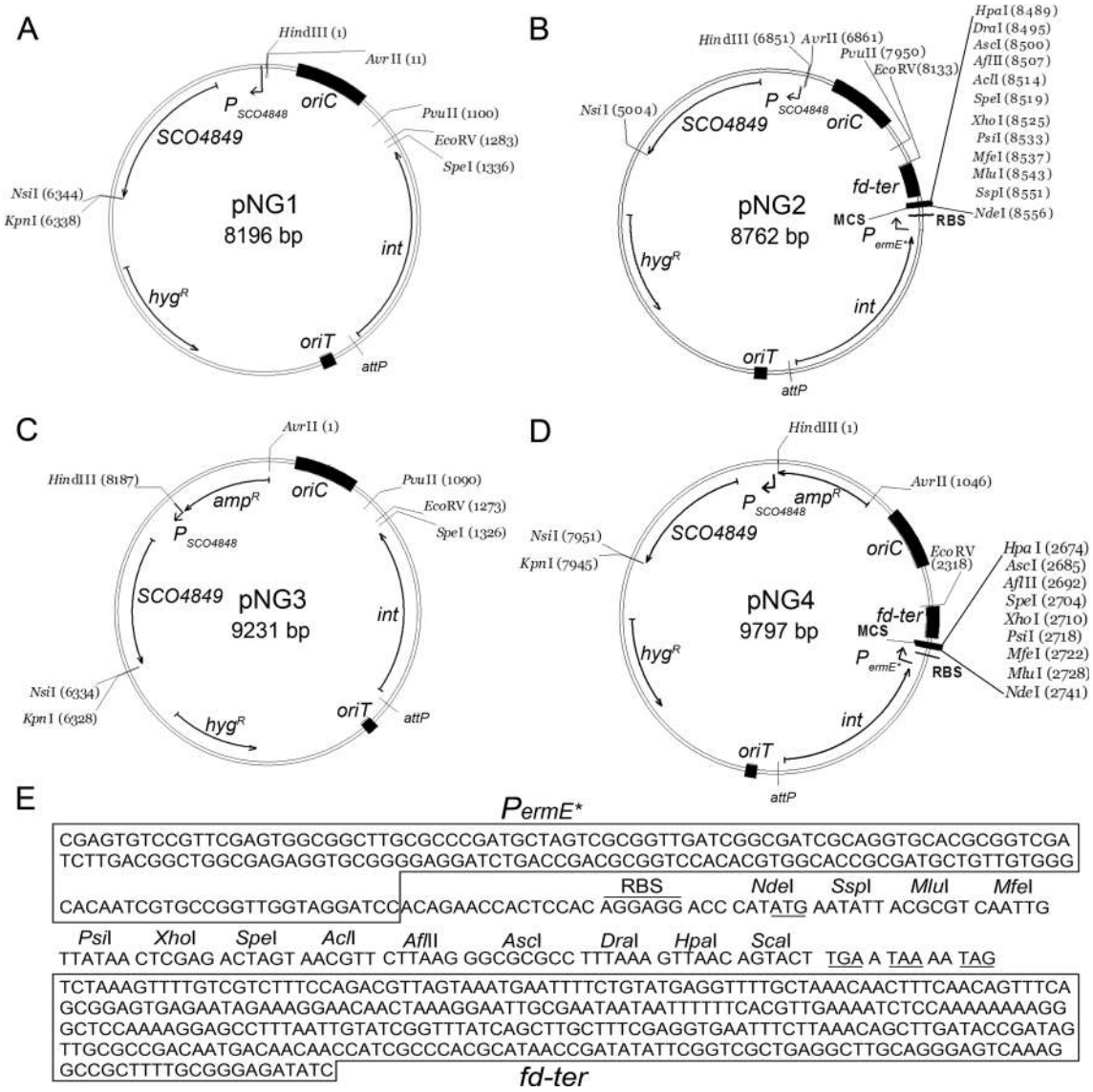


Fig. 3 ΦBT1 integrative plasmids pNG1, pNG2, pNG3 and pNG4. The basis of all these plasmids is pMS82 (Gregory et al. 2003). **a** pNG1 harbours $P_{SCO4848}$+*SCO4849*. **b** pNG2 harbours $P_{SCO4848}$+*SCO4849* and P_{ermE}*+RBS+MCS+*fd-ter*. **c** pNG3 harbours $P_{SCO4848}$+*SCO4849* and *bla* (resistance to ampicillin). **d** pNG4 harbours $P_{SCO4848}$+*SCO4849*, P_{ermE}*+RBS+MCS+*fd-ter* and *bla*. **e** Sequence of the P_{ermE}*+RBS+MCS+*fd-ter* region. *oriC* origin of replication, *Int* ΦBT1 integrase, *oriT* origin of transfer, *hyg* hygromycin resistance gene. Single-cutter enzymes are shown

the biological function, if any, of *SCO4848*, and to characterize the biochemical mechanism by which SCO4849 controls spore germination. Other phenotypes, such as antibiotic production, growth rate or sporulation, were not significantly affected by the integration of ΦBT1 vectors, consistent with the results previously reported by Gregory et al. (2003).

The aim of this work was the construction of phenotypically neutral integrative plasmids for use in *Streptomyces*. These plasmids needed to meet two main characteristics: first, they should have a unique and conserved integrative site in streptomycetes; and second, the mutation generated by the integration of the plasmid should not generate any phenotype.

Concerning the existence of a unique integrative site, ΦBT1 integrative plasmids represent a good alternative (Gregory et al. 2003). To our knowledge, no studies are available of the existence of alternative ΦBT1-*attB* sites different from the *attB* site located at SCO4848. In this work, it was demonstrated in 24 independent integrations (Supplementary Figure S5) that pMS82 and their derived plasmids were always integrated into SCO4848, suggesting that SCO4848 harbours a unique ΦBT1-*attB* site in *S. coelicolor*. In fact, a BLAST of the SCO4848 *attB* sequence against the *S. coelicolor* genome did not retrieve alternative putative *attB* sites (data not shown).

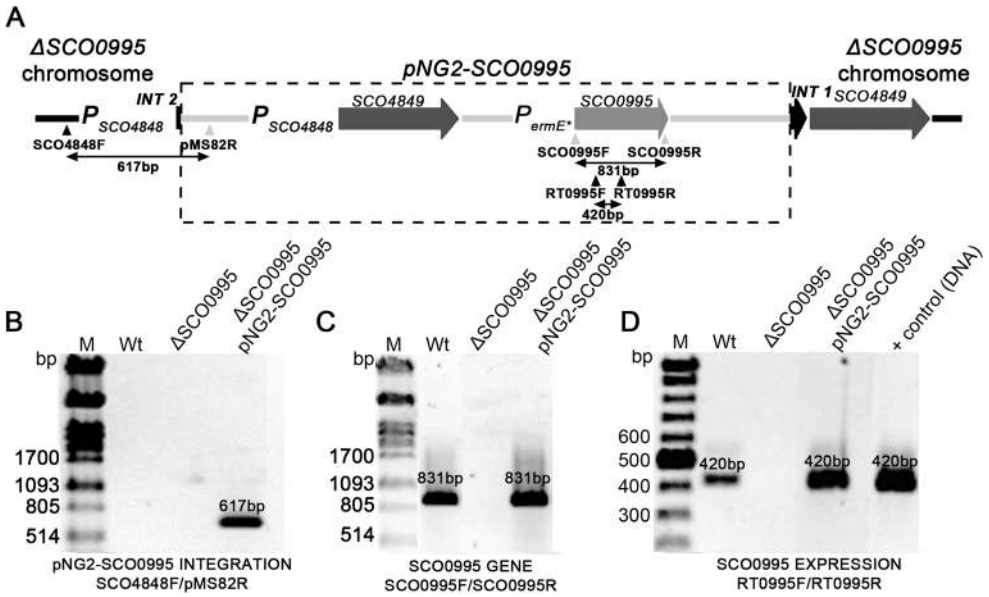


Fig. 4 Functionality of the expression vector pNG2 to restore SCO0995 expression in the ΔSCO0995 mutant. **a** Scheme illustrating the integration of pNG2-SCO0995 into the ΔSCO0995 chromosome. **b** PCR using primers SCO4848F and pMS82R confirming plasmid integration into SCO4848. **c** PCR using primers SCO0995F and SCO0995R confirming the presence of the SCO0995 gene into the ΔSCO0995 strain harbouring pNG2-SCO0995. **d** RT-PCR demonstrating the functionality of the expression vector pNG2. *SCO0995* is expressed in the SCO0995 mutant strain (Δ*SCO0995*) harbouring the pNG2-SCO0995 plasmid

Fulfilling the second requirement, the absence of a phenotype after plasmid integration is more difficult to achieve because general integrative sites need to be conserved, and conserved genes are usually important, and their mutations lead to phenotypes. As discussed above, the integration of ΦBT1 plasmids into the *S. coelicolor* genome generates a spore germination phenotype. The most obvious approach to construct a phenotypically neutral integrative plasmid is to introduce the chromosomal gene/s interrupted by the integration, into the integrative plasmid. However, this was not possible in the case of pMS82 due to the interaction between the *attP* site of the plasmid and the *attB* site of the gene to be complemented, which made these plasmids unstable (Supplementary Figure S6). Fortunately, the phenotype detected during germination after the integration of ΦBT1 plasmids was a consequence of the absence of the gene located downstream of the *attB* integration site (*SCO4849* in the case of *S. coelicolor*). This allowed us to construct a ΦBT1 integrative plasmid including the promoter of the *SCO4848* gene fused with the ORF of *SCO4849* (pNG1), which complements the phenotype in germination generated by the integration of the plasmid into *SCO4848*. pNG1 is in fact the first integrative plasmid in *S. coelicolor* demonstrated to be phenotypically neutral. The P_{ermE}* promoter, a RBS, a multiple cloning site and the terminator of the fd phage (*fd-ter*) were introduced in pNG1 to facilitate heterologous gene expression, creating pNG2. Both plasmids showed a resistance to hygromycin which is effective in *Streptomyces*. In *E. coli*, however, false resistant colonies can grow, albeit at a slower rate than the *E. coli* harbouring the plasmids with the resistance, making hygromycin selection challenging. For this reason, the *bla* gene coding for ampicillin resistance was included into pNG1 and pNG2 in order to facilitate selection in *E. coli*, thus generating plasmids pNG3 and pNG4.

SCO4848 and *SCO4849* orthologues are highly conserved in streptomycetes (Fig. 5), and the delay in spore germination as a consequence of the integration of ΦBT1 plasmids into *SCO4848* and their orthologues, may be common in *Streptomyces* (Fig. 2c, d). pNG1–4 are useful in other streptomycetes beyond *S. cocelicolor*; however, the usefulness of these plasmids requires verification for each specific *Streptomyces* species because they do not always complement the wild phenotypes. For instance, pNG1 accelerates the germination in *S. griseus* with respect to the wild-type strain, indicating that the effect of the *S. coelicolor SCO4849* is stronger than that of the *S. griseus* orthologue mutated by the integration of the plasmid. In these types of strains, alternative ΦBT1 plasmids harbouring the *SCO4849* orthologue of the specific host strain might be created and tested.

Overall, this work describes the construction of plasmids pNG1–4, the first integrative vectors designed to restore the expression of the genes interrupted by their integrations, producing neutral phenotypes in *S. coelicolor*, *S. lividans* and *S. clavuligerus.* These vectors facilitate the interpretation of the phenotypes generated by the integration of heterologous DNA, limiting the interference of the phenotypes caused by the integration vectors. The experimental approach developed in this work to create phenotypically neutral ΦBT1integrative plasmids in *S. coelicolor* might be applied to other bacterial integrative vectors.

Fig. 5 Sequence alignment of *SCO4848* and *SCO4849* (*S. coelicolor* numeration) and their orthologues in other model streptomycetes. **a** *SCO4848* orthologues. **b** *SCO4849* orthologues. Putative transmembrane regions, average similarities and conserved database domain references (*CDD*) are indicated

Acknowledgments Our thanks to Maggie Smith (University of York) for providing the pMS82 plasmid, Beatriz Gutiérrez Magán (Universidad de Oviedo, Dpto. Biología Funcional, Área de Microbiología) for the laboratory assistance, and Proof-Reading-Service.com for proofreading the text.

Compliance with ethical standards

Ethical statement

Funding This research was funded by an ERC Starting Grant (Strp-differentiation 280304). NGQ was funded by a Severo Ochoa fellowship (FICYT, Consejería de Educación y Ciencia, Spain).

Conflict of interest The authors declare that they have no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Bibb MJ, White J, Ward JM, Janssen GR (1994) The mRNA for the 23S rRNA methylase encoded by the *ermE* gene of *Saccharopolyspora erythraea* is translated in the absence of a conventional ribosome-binding site. Mol Microbiol 14:533–545

Bierman M, Logan R, O'Brien K, Seno ET, Rao RN, Schoner BE (1992) Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. Gene 116:43–49

Bishop A, Fielding S, Dyson P, Herron P (2004) Systematic insertional mutagenesis of a streptomycete genome: a link between osmoadaptation and antibiotic production. Genome Res 14:893–900

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Bystrykh LV, Fernandez-Moreno MA, Herrema JK, Malpartida F, Hopwood DA, Dijkhuizen L (1996) Production of actinorhodin-related "blue pigments" by *Streptomyces coelicolor* A3(2). J Bacteriol 178:2238–2244

Combes P, Till R, Bee S, Smith MC (2002) The *Streptomyces* genome contains multiple pseudo-attB sites for the (phi)C31-encoded site-specific recombination system. J Bacteriol 184:5746–5752

Davies J (2014) Specialized microbial metabolites: functions and origins. J Antibiot (Tokyo) 66:361–364

Fayed B, Younger E, Taylor G, Smith MC (2014) A novel *Streptomyces* spp. Integration vector derived from the *S. venezuelae* phage, SV1. BMC Biotechnol 14:51

Fernandez E, Weissbach U, Sanchez-Reillo C, Braña AF, Mendez C, Rohr J, Salas JA (1998) Identification of two genes from *Streptomyces argillaceus* encoding glycosyltransferases involved in transfer of a disaccharide during biosynthesis of the antitumor drug mithramycin. J Bacteriol 180:4929–4937

Fernández-Martínez LT, Del Sol R, Evans MC, Fielding S, Herron PR, Chandra G, Dyson PJ (2011) A transposon insertion single-gene knockout library and new ordered cosmid library for the model organism *Streptomyces coelicolor* A3(2). Antonie Van Leeuwenhoek 99:515–522

Flett F, Mersinias V, Smith CP (1997) High efficiency intergeneric conjugal transfer of plasmid DNA from *Escherichia coli* to methyl DNA-restricting streptomycetes. FEMS Microbiol Lett 155:223–229

Gentz R, Langner A, Chang AC, Cohen SN, Bujard H (1981) Cloning and analysis of strong promoters is made possible by the downstream placement of a RNA termination signal. Proc Natl Acad Sci U S A 78:4936–4940

Gregory MA, Till R, Smith MC (2003) Integration site for *Streptomyces* phage phiBT1 and development of site-specific integrating vectors. J Bacteriol 185:5320–5323

Hopwood DA (2007) *Streptomyces* in nature and medicine: the antibiotic makers. Oxford University Press, New York

de Jong W, Manteca A, Sanchez J, Bucca G, Smith CP, Dijkhuizen L, Claessen D, Wösten HA (2009) NepA is a structural cell wall protein involved in maintenance of spore dormancy in *Streptomyces coelicolor*. Mol Microbiol 71:1591–1603

Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA (2000) Growth and preservation of *Streptomyces*. In Practical *Streptomyces* genetics, Chapter 2, pp. 43–61. Norwich, UK: The John Innes Foundation

MacNeil DJ, Gewain KM, Ruby CL, Dezeny G, Gibbons PH, MacNeil T (1992) Analysis of *Streptomyces avermitilis* genes required for avermectin biosynthesis utilizing a novel integration vector. Gene 111:61–68

Manteca A, Fernandez M, Sanchez J (2006) Cytological and biochemical evidence for an early cell dismantling event in surface cultures of *Streptomyces antibioticus*. Res Microbiol 157:143–152

Morita K, Yamamoto T, Fusada N, Komatsu M, Ikeda H, Hirano N, Takahashi H (2009) The site-specific recombination system of actinophage TG1. FEMS Microbiol Lett 297:234–240

Novella IS, Barbes C, Sánchez J (1992) Sporulation of *Streptomyces antibioticus* ETHZ 7451 in liquid culture. Can J Microbiol 38: 769–773

Sambrook J, Russell D (2001) Molecular cloning: a laboratory manual, Ed 3. Cold spring harbour laboratory press. Cold Spring Harbour, NY

Sánchez L, Braña AF (1996) Cell density influences antibiotic biosynthesis in *Streptomyces clavuligerus*. Microbiology 142:1209–1220

Sánchez R, Serra F, Tárraga J, Medina I, Carbonell J, Pulido L, de María A, Capella-Gutíerrez S, Huerta-Cepas J, Gabaldón T, Dopazo J, Dopazo H (2011) Phylemon 2.0: a suite of web-tools for molecular evolution, phylogenetics, phylogenomics and hypotheses testing. Nucleic Acids Res 39:470–474

Tsao SW, Rudd BA, He XG, Chang CJ, Floss HG (1985) Identification of a red pigment from *Streptomyces coelicolor* A3(2) as a mixture of prodigiosin derivatives. J Antibiot (Tokyo) 38:128–131

Vijgenboom E, Woudt LP, Heinstra PW, Rietveld K, van Haarlem J, van Wezel GP, Shochat S, Bosch L (1994) Three tuf-like genes in the kirromycin producer *Streptomyces ramocissimus*. Microbiology 140:983–998

Yagüe P, López-García MT, Rioseras B, Sánchez J, Manteca A (2013) Pre-sporulation stages of *Streptomyces* differentiation, state-of-the-art and future perspectives. FEMS Microbiol Lett 342:79–88