J Ind Microbiol Biotechnol (2003) 30: 516–522
DOI 10.1007/s10295-003-0064-y

ORIGINAL PAPER

Zhihao Hu · David A. Hopwood · C. Richard Hutchinson

Enhanced heterologous polyketide production in *Streptomyces* by exploiting plasmid co-integration

Received: 9 January 2003 / Accepted: 21 April 2003 / Published online: 21 June 2003

Abstract A plasmid named pSMALL was discovered in a *Streptomyces coelicolor* strain that significantly enhanced the levels of production of 15-methyl-6-deoxyerythronolide B, a polyketide lactone normally produced in low amounts by engineered polyketide synthase (PKS) genes. It is a co-integrate between a conventional SCP2*-derived *Streptomyces* expression plasmid, pJRJ2, and SCP2@, a variant of the parental SCP2* plasmid. SCP2@ has a 45-bp deletion 35 bp upstream of the start codon of the *repI* gene in the replication region; and this correlated with an enhanced plasmid copy number and polyketide overproduction by its derivatives. This discovery was exploited to construct pBOOST, a high-copy-number cloning vector that can be used to achieve greatly elevated (at least 25-fold), stable metabolite production by PKS genes cloned in SCP2*-derived vectors by forming co-integrates with them.

Keywords Co-integration · SCP2 · Heterologous expression

Introduction

Production of novel polyketide metabolites by the expression of recombinant polyketide synthase (PKS) gene sets in a suitably engineered host strain is becoming a well established approach to the manufacture of useful therapeutic agents. A widely used host–vector system for this purpose consists of *Streptomyces coelicolor* CH999 as host with the SCP2*-derived pRM5 plasmid vector [16] and later derivatives of it. In this system, the cloned genes are expressed under the control of the *act*I and/or *act*III promoters at a low copy number, activated by the native *act*II-ORF4 pathway-specific regulator, in a host deleted for the actinorhodin (*act*) gene cluster. Following the first cloning and expression of the entire *Saccharopolyspora erythraea* 6-deoxyerythronolide B synthase (DEBS) gene set [8] and the 6-methylsalicylic acid synthase gene of *Penicillium patulum* [1], this host–vector system has provided an efficient, successful and expedient platform for combinatorial biosynthesis. It has been used to create a large number of ″unnatural natural products″ [17, 21, 25] and to express sets of tailoring genes, such as those for deoxysugar and 3-amino-5-hydroxybenzoic acid biosynthesis [23, 26]. Some of the same constructs have also been expressed in the closely related *Streptomyces lividans* K4-114, an analogue of CH999 and a strain with the advantage of lacking the methylation-dependent restriction system of *S. coelicolor* [15]. K4-114 produces about the same titer of 6-deoxyerythronolide B (6-dEB) as CH999 when it contains the DEBS gene set [27]. SCP2*-derived plasmid vectors like pRM5 have a broad host range [2, 14] and have been widely used for heterologous expression, not only in *S. coelicolor* and *S. lividans*, but also in *S. parvulus* [12], *S. venezuelae* (R. McDaniel, personal communication) and *Saccharopolyspora erythraea* [18].

The parental SCP2* plasmid consists of three functional regions, responsible for replication, stability and self-transfer (the latter is also called the fertility region because it confers on the plasmid its ability to promote the exchange of chromosomal markers between the parents in a mating). The plasmid also contains a functionally uncharacterized region, between *Pst*I sites 18 and 37 (see Fig. 1 and the map of SCP2* in reference [11]), which is absent from all SCP2-based vectors. Both the stability region [3] and the fertility region [14] are important for stable inheritance of SCP2*, the former presumably aiding the distribution of plasmid copies among daughter cells and the latter probably ensuring re-infection of any hyphal compartments that fail to inherit plasmid copies.

Z. Hu (✉) · D. A. Hopwood · C. R. Hutchinson
Kosan Biosciences, 3832 Bay Center Place, Hayward, CA 94545, USA
E-mail: hu@kosan.com
Tel.: +1-510-7328400
Fax: +1-510-7328401

Present address: D. A. Hopwood
John Innes Centre, Department of Molecular Microbiology, University of East Anglia, Norwich, NR4 7UH, UK

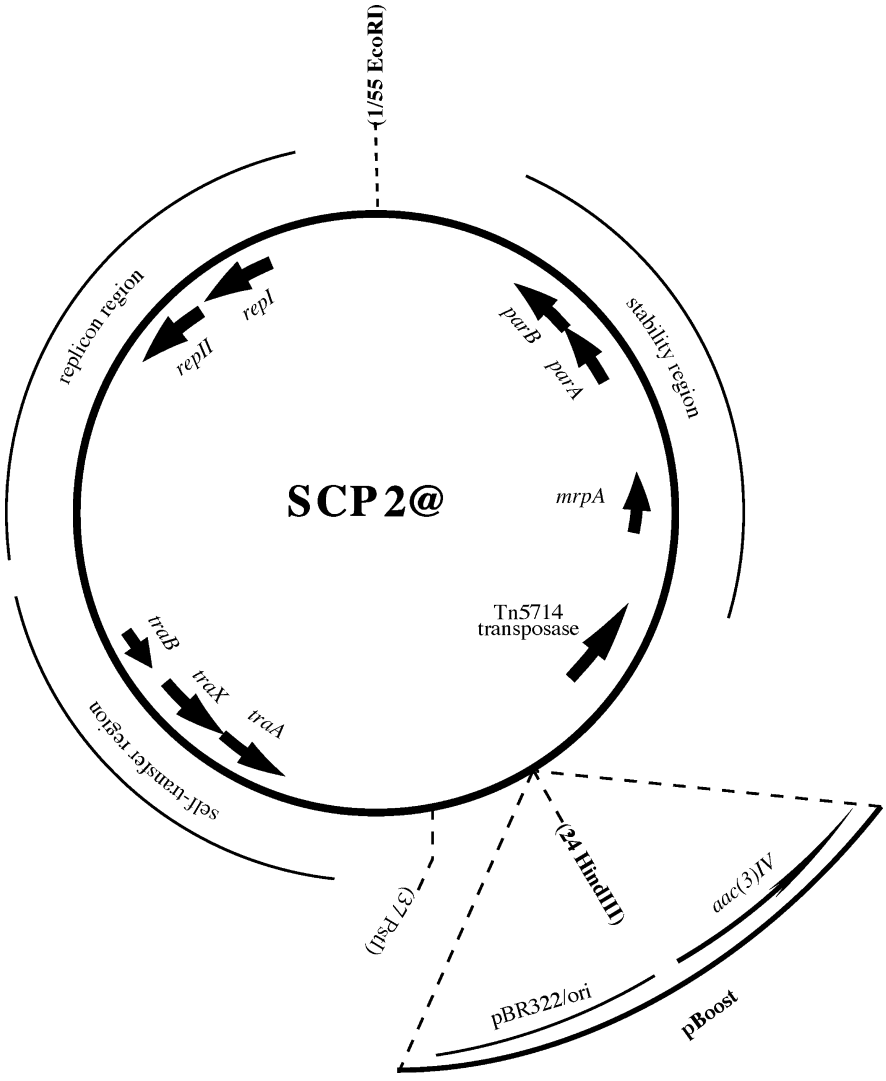

Fig. 1 Map of SCP2@ and pBOOST. Selected open reading frames of SCP2@ are *indicated by arrows* and named according to the annotated sequence of SCP2* (see [4] and Materials and methods). The unique *Eco*RI, *Hin*dIII and one of the relevant *Pst*I sites are shown, too. *aac(3)IV* apramycin-resistance gene, *pBR322/ori* pBR322 replicon. *The numbers in front of restriction sites* correspond to the SCP2* map [11]

Most vectors derived from SCP2*, including pRM5, are present in *Streptomyces* spp at a low and rather constant copy number of ca. 1–5 per chromosome, which makes them very suitable for the cloning of the large gene clusters (>30 kb) typical of multi-module PKS gene sets. Although many plasmids, such as pIJ6021, have been derived from the naturally high-copy-number pIJ101 [10] for the heterologous expression of individual genes in *Streptomyces* [20], vectors derived from pIJ101, or other high-to-medium copy-number replicons such as pJV1 or pSG5 [11], have been found unsuitable for cloning DNA inserts greater than 30 kb [25]. Certain SCP2* derivatives, which contain only a part of the replication region [11], have a copy number of around ten, or even many hundreds for pHJL203 [13]. Unfortunately, pHJL203 appears to be very unstable. The only stable high-copy-number SCP2* derivatives so far reported (40–100 copies per chromosome) are co-integrates containing not only the SCP2* replicon but also that of pIJ101. Such co-integrates arose by homologous recombination between SCP2* and pIJ101 derivatives during conjugal transfer, but not after co-transformation [24]. Here, we report the discovery of a variant of SCP2*, named SCP2@, with a 45-bp deletion, and of co-integrates between the SCP2-derived pJRJ2 and SCP2@. The co-integrates have a high copy number and, when used as vectors for the expression of PKS genes, cause a large increase in the production of polyketide metabolites. This discovery led to the development of pBOOST, which can be used to form co-integrates intentionally with other SCP2* derivatives by co- or step-wise transformation. The resulting increase in the copy number of SCP2* derivatives thus enhances the expression of the PKS genes that they carry. This furnishes a valuable tool for the production of novel compounds otherwise available only at low levels.

Materials and methods

Bacterial strains and plasmids

The *Streptomyces* and *Escherichia coli* strains and plasmids used are listed in Table 1.

Table 1 Bacterial strains and plasmids used in this study. *dEB*Deoxyerythronolide B, *DEBS* 6-deoxyerythronolide B synthase, *PKS* polyketide synthase

Strain	Genotype/relevant characteristics	Reference/source
Streptomyces coelicolor CH999	*proA1*, *argA1 redE60* Δ*act*::*ermE*SCP$^-$, SCP2$^-$	[16]
S. coelicolor 1147	Wild type	[11]
S. lividans K4-114	*str-6*, SLP2$^-$, SLP3$^-$, Δ*act*::*ermE*	[27]
CH999/pSMALL	15-Methyl-6-dEB high-producing isolate of CH999/pJRJ2	Kosan Biosciences
pJRJ2	pRM5 derivative carrying the KS1^o mutant DEBS genes	[7]
SCP2*	High-fertility variant of SCP2,*tra*$^+$, *par*$^+$	[2]
pIJ2581	Plasmid containing the *glkA* gene from *S. coelicolor* and the *tsr* gene from *S. azureus*	[22]
pKOS97–152a	pRM5 derivative carrying modified *meg* PKS genes with the KS1^o mutation	[6]
pBOOST	*Hin*dIII-linearized pHU152 inserted into the *Hin*dIII site of SCP2@	This study
pSMALL	Co-integrate between SCP2@ and pJRJ2	This study
pHU152	pBR322 derivative with the *bla* gene replaced by the *aac(3)IV* apramycin-resistance gene; *Nde*I site filled-in by treatment with T4 DNA polymerase	This study
SCP2@	Derived from SCP2* with 45 bp missing at positions 29,549–29,505	This study

Media, chemicals and growth conditions

R5 medium was used for propagation and protoplast transformation of *Streptomyces* strains. TSB medium was used to culture *Streptomyces* strains for DNA preparation and as seed medium; R6 medium was used for fermentations. LB medium was used for growing *E. coli*. The recipes for R5, TSB, LB and trace element solution in R6 medium are from [11]. R6 medium consists of (per liter): 103 g sucrose, 0.25 g K_2SO_4, 10.12 g $MgCl_2{\cdot}6H_2O$, 0.96 g sodium propionate, 0.1 g Difco casamino acids, 5.0 g Difco yeast extract, 28.2 g bis-Tris propane and 2.0 ml trace element solution. After autoclaving, the following were added (per liter): 10 ml KH_2PO_4 (0.5% w/v), 8 ml $CaCl_2{\cdot}2H_2O$ (2.5 M) and 15 ml L-proline (20% w/v). The final concentrations of antibiotics used in all media were (per liter): 60 mg apramycin, 50 mg carbenicillin, 25 mg chloramphenicol, 50 mg kanamycin and 50 mg thiostrepton. The diketide was (2*S*,3*R*)-2-methyl-3-hydroxyhexanoate *N*-propionyl cysteamine thioester.

Fermentation and quantification of 15-methyl-6-dEB

Streptomyces transformants were picked into 6 ml of TSB liquid medium with the appropriate antibiotic and grown at 30 °C. After sufficient growth (normally 3–4 days), they were transferred to 250-ml flasks containing 50 ml of R6 medium [supplemented with the appropriate antibiotics and the (2*S*,3*R*)-2-methyl-3-hydroxyhexanoate *N*-propionyl cysteamine thioester at a final concentration of 1 g l^{-1}]. The flasks were shaken at 30 °C for about 7 days, after which 1 ml of culture was withdrawn and spun down. A 200-µl sample of the supernatant, or a dilution of it, was subjected to HPLC-MS analysis. Analysis and quantitative determination were according to [25] with unpublished modifications, using 15-methyl-6-dEB as standard.

Results

A novel plasmid in a high-producing strain

The titers of 6-dEB and its various analogues produced by recombinant *S. coelicolor* CH999 strains carrying SCP2*-derived plasmids are reproducibly about 30–40 mg l^{-1} in R5 or R6 medium. Similar titers of 6-dEB analogues, such as 15-methyl-6-dEB, were produced by CH999-carrying plasmids, such as pJRJ2 with a DEBS1 KS1 null (KS1^o) mutation [7] and fed with the "diketide" (2*S*,3*R*)-2-methyl-3-hydroxyhexanoate-*N*-propionyl cysteamine thioester. Unexpectedly, one particular isolate carrying pJRJ2 used to produce 15-methyl-6-dEB for further modifications was found to yield the product at 100–160 mg l^{-1} in R5 or R6 medium. In a high-producing medium under optimized conditions, it could make up to 25-fold more 15-methyl-6-dEB than the parent (500 mg l^{-1}, compared with 30–40 mg l^{-1}).

By introducing the plasmid (called pSMALL) present in the novel high-producing CH999 strain into alternative hosts, it was shown that the plasmid was responsible for the high productivity and that it was not due to a genetic change in the CH999 host. For example, when *S. lividans* K4-114 (a suitable alternative host to CH999 for producing recombinant 6-dEB and its analogues) was transformed with pSMALL, the resulting strain produced up to 160 mg 15-methyl-6-dEB l^{-1} when fed with the diketide, compared with the normal level of 30–40 mg l^{-1} (Table 2). When pSMALL was introduced into the wild-type strain, *S. coelicolor* 1147, by conjugation from CH999/pSMALL, the resulting strain had a productivity as high as CH999/pSMALL (Table 2).

Plasmids like pJRJ2 normally have a copy number of ca. 1–5 per chromosome in *S. coelicolor* and *S. lividans*. In contrast, DNA bands of pSMALL were visible in agarose gels generated from total DNA of CH999/pSMALL digested with *Pst*I plus *Bam*HI against the background "smear" of digested chromosomal DNA

Table 2 Titers of polyketides produced by representative *Streptomyces* strains

Host strain	Plasmid vector	Polyketide produced	Titer (mg l^{-1})
S.coelicolor			
CH999	pJRJ2	15-Methyl-6-dEB	30–40
CH999	pSMALL	15-Methyl-6-dEB	100–160
1147	pSMALL	15-Methyl-6-dEB	100–160
S. lividans			
K4–114	pSMALL	15-Methyl-6-dEB	100-160
K4–114	pJRJ2	15-Methyl-6-dEB	30–40

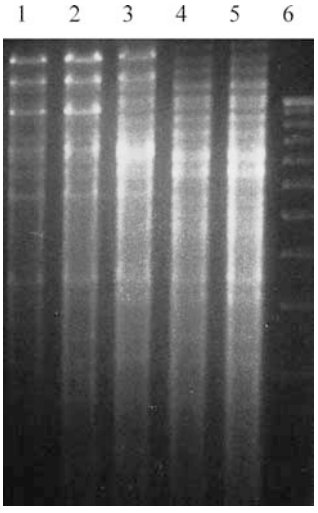

Fig. 2 Agarose gel of total DNA samples digested with *Pst*I and *Bam*H1. *Lanes 1, 2* Two isolates of CH999/pSMALL, *lanes 4, 5* CH999 control, *lane 6* 1-kb ladder (GibcoBRL). Note the intense bands in *lanes 1 and 2*, attributed to the presence of pSMALL at a high copy number

(Fig. 2), suggesting that pSMALL was present in CH999 at an elevated copy number. To confirm this conclusion, a *Xba*I fragment containing the *tsr* and *glkA* genes—which are approximately the same length—from pIJ2581 [22] was used to measure the relative (but not absolute) abundance of *tsr* on pSMALL and the *glkA* gene in the host chromosome by Southern blot hybridization. The results show, by comparing the relative intensity of the signal from the single copy *glkA* and the multicopy *tsr* genes, that pSMALL was present in CH999 at ca. 100–125 copies per chromosome, whereas pJRJ2 was present at ca. 5–10 copies per chromosome (Fig. 3). This result negates the generalization that a high copy number is unsuitable for the expression of large PKS gene clusters (see Introduction). Significantly, whereas pJRJ2 was lost very rapidly without selective pressure, pSMALL was more stably maintained in its host (0.05% of the colonies were thiostrepton-resistant after three serial transfers in the absence of selection for pJRJ2, compared with 8.3% for pSMALL).

The plasmid, pSMALL, in the CH999 high-producing strain was found to be larger than the expected size (ca. 80 kb instead of 49 kb). The structure of pSMALL was analyzed by restriction mapping and partial sequencing. These results (data not shown) established that the ″extra″ DNA in pSMALL consisted of SCP2* sequences and indicated that pSMALL was a co-integrate between the original pJRJ2 plasmid and a plasmid similar or identical to SCP2*.

The presence of a plasmid related to SCP2* in strain CH999 was unexpected, because SCP2* was believed to have been cured from the parent of CH999 [9, 16]. To re-examine this belief, three cultures of CH999 that had been sub-cultured separately for many transfers and isolates of two of its progenitors, strains B385 [19] and CH1 [9], were tested phenotypically for the presence of SCP2-related plasmids. B385 (as expected) gave characteristic SCP2 pocks on a lawn of a SCP2- strain and was resistant to pocking by a control SCP2* strain, while two of the three CH999 isolates gave SCP2* pocks and one isolate lacked the plasmid (H.M. Kieser, personal communication). The presence of a plasmid

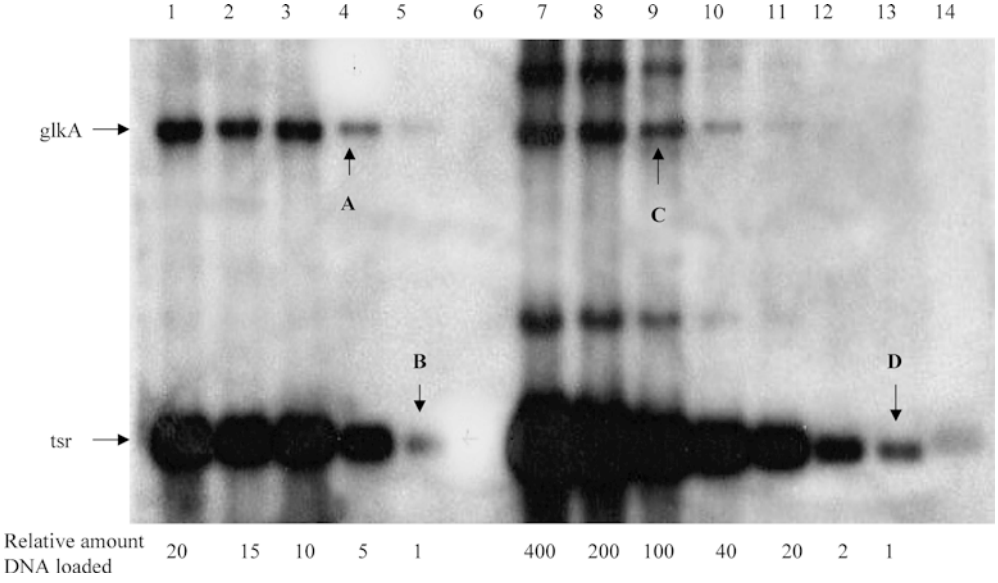

Fig. 3 Measurement of plasmid copy number. The total DNA of CH999/pJRJ2 and CH999/pSMALL was digested with *Pst*I and *Bam*HI and then loaded onto an agarose gel in serial dilutions. The figure shows a Southern blot hybridization of the gel with a [^{32}P]dCTP-labeled *Xba*I fragment containing the *tsr* and *glkA* genes (from pIJ2581). *Lanes 1–5* were loaded with CH999/pJRJ2 DNA at 20, 15, 10, 5 and 1 µl, respectively. *Lane 6* is blank. *Lanes 7–14* were loaded with CH999/pSMALL DNA at 400, 200. 100, 40, 20, 2 and1 µl, respectively. *Arrows* show the positions of bands hybridizing to the *tsr* and *glkA* genes. The intensity of the bands hybridizing to *glkA*, *indicated by arrows A and C*, is almost equal to the intensity of the bands *indicated by arrows B and D* (which hybridized to *tsr*). The copy numbers of pJRJ2 and pSMALL were therefore estimated as ca. 5 and 100 copies per chromosome, respectively

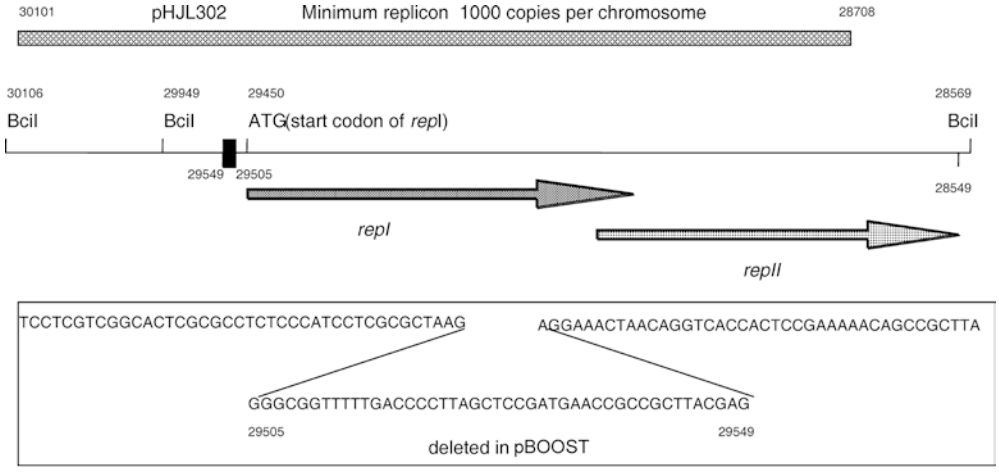

Fig. 4 DNA sequences and deduced open reading frames of a portion of the replication region of SCP2 and pBOOST. Only restriction sites of interest are shown. The sequence of the region upstream of *repI* containing the 45-bp deletion, at positions 29,549–29,505 (compared with its parent, SCP2@) is *shown beneath the physical map* of the region of SCP2* at positions 28,569–30,106. The corresponding region of the ultra-high-copy-number plasmid pHJL302 [13] is also *shown above the restriction map* of SCP2*

with a size characteristic of SCP2* was confirmed by the isolation of plasmid DNA from one of the CH999 cultures.

Sequencing of a derivative of pSMALL and comparison with the SCP2* sequence (GenBank Accession No. AL645771 [4]) revealed a 45-bp deletion in the SCP2 replication region (Figs. 1, 4). The deletion was 35 bp upstream of the start site of the *repI* gene that encodes a putative DNA-binding protein [4]. The *repI* gene, along with *repII* downstream of it and the 650-bp non-coding region upstream of *repI* (postulated to be the replication origin of SCP2*), are essential for plasmid replication [4, 13]. We speculate that the 45-bp deletion disrupts the promoter for *repI* transcription.

We reasoned that the 45-bp deletion must have been present in the SCP2-derived mutant plasmid (named SCP2@ here) which was unexpectedly found in CH999 and thought to have given rise to the original co-integrate derivatives. This was confirmed by PCR analysis (data not shown). Further PCR analysis with DNA from several different plasmids confirmed that all the plasmids with a high copy number carried the same 45-bp deletion.

Creation of a plasmid to enhance PKS gene expression from pRM5-related vectors

Since pJRJ2 and SCP2* share 7.2 kb of identical sequence, pSMALL was expected to carry a 7.2-kb duplication and was found to be an impractical vector because of its large size and instability (due to rearrangement between duplicated parts) in *E. coli* strains, in which homologous recombination led to resolution of the two parts of the co-integrate. Attempts were therefore made to construct a convenient plasmid that could be used to increase the copy number and hence the polyketide productivity of any SCP2*-derived clone. One of these, pBOOST, was made by directly inserting the linearized (by *Hin*dIII) plasmid pHU152, which contains an apramycin-resistance gene cassette (Table 1), into the unique *Hin*dIII site of SCP2@ to give pBOOST. Co-integrate plasmids were formed with pBOOST both in the K4-114 and CH999 hosts and in the CH999/pKOS97-152a strain (Table 1) into which pBOOST had been introduced by transformation. These strains exhibited an 8- to 12-fold enhancement of 15-methyl-6-dEB production (data not shown) when compared with their parental strains.

To test whether the metabolite production was stable, two representative CH999/pKOS97-152a + pBOOST transformants were grown in liquid TSB medium with only thiostrepton at a concentration that maintains CH999/pSMALL. After 3 days, a 100-µl portion of the culture was transferred into fresh growth medium and the culture was grown for a further 3 days. The rest of the original culture was mixed with an equal volume of 20% glycerol in water and stored at −80 °C. This process was repeated twice. The titers of 15-methyl-6dEB among these serially transferred cultures differed by only a small amount (data not shown).

Discussion

The results reported here have important implications for the production of novel polyketides—and perhaps new molecules of other chemical families of pharmaceuticals. Although it is now becoming routine to generate large numbers of polyketide ″unnatural natural products″ by genetic engineering, very often the levels of production are much lower than those of the original metabolites. Thus, technologies for increasing productivity would be valuable. We describe the creation of pBOOST, a plasmid able to conveniently be used to enhance significantly the production of recombinant erythromycin aglycones when allowed to form co-integrates with conventional SCP2*-derived expression plasmids.

In this study, we detected the presence of a new SCP2* derivative in an isolate of strain CH999. This strain was derived from CH1, which was presumed to have been cured of SCP2 (or SCP2*) in its origin from strain B385 [16]. The curing was performed in a routine

fashion [5] by introducing a highly unstable SCP2* derivative, pIJ80, with selection on neomycin-containing agar, followed by spontaneous loss of pIJ80 on a non-selective medium. Evidently, the normal incompatibility between two SCP2-derived replicons must have failed to be expressed, so that the expected curing did not occur. The 45-bp deletion now known to be present on the ″surviving″ SCP2 derivative, SCP2@, which is associated with the special properties described in this paper, may have allowed it to co-exist with pIJ80, rather than being displaced by that plasmid.

When CH999/SCP2@ was transformed with pJRJ2, we presume that co-integrate plasmids did not arise in the transformed hyphal compartments, because transformation using these plasmids was subsequently found not to give rise to co-integrates at detectable frequency. Instead, SCP2@ is presumed to be able to transfer itself by conjugation into one or more plasmid-free cells in the culture, thereby creating the opportunity for the formation of co-integrates. These were eventually detected because of the elevated metabolite titer that resulted from the expression of the DEBS genes carried on the plasmids. Presumably, the original co-integrates had one wild-type origin of replication, from pJRJ2, and one mutant origin, with the 45-bp deletion, from SCP2@. Since the co-integrate plasmids examined were found to carry the 45-bp deletion in both their origin regions, we suggest that homogenotization occurred either by gene conversion or by reciprocal double crossing-over followed by segregation, as depicted in Fig. 5.

In conclusion, we discovered SCP2@ (a mutant SCP2* derivative) and a co-integrate between pJRJ2 and SCP2@, named pSMALL, both of which are high-copy-number plasmids in *S. coelicolor* and *S. lividans* and probably also in other *Streptomyces* spp. The elevated metabolite production conferred when genes are cloned in this high-copy-number co-integrate is the result of an increased gene dosage and greater plasmid stability. We speculate that the 45-bp DNA deletion upstream of *repI* in SCP2@ is responsible for the increased copy-number of the co-integrant plasmids and that a gene(s) flanking the *Hin*dIII site somehow governs co-integration between SCP2@ (and its derivatives) and SCP2*-derived plasmids. It also is likely that genes in the plasmid transfer and fertility regions (Fig. 1) are important to maintain high metabolite productivity upon expression of large PKS gene clusters in heterologous hosts. During our investigation of pSMALL, we developed a plasmid, pBOOST, that can mimic SCP2@ and form co-integrates with SCP2* derivatives. pBOOST can be used reliably to achieve a large increase in metabolite production from cloned PKS genes (several different examples have been used at Kosan Biosciences) and presumably other types, as a consequence of the co-integrate plasmids formed during protoplast-mediated transformation.

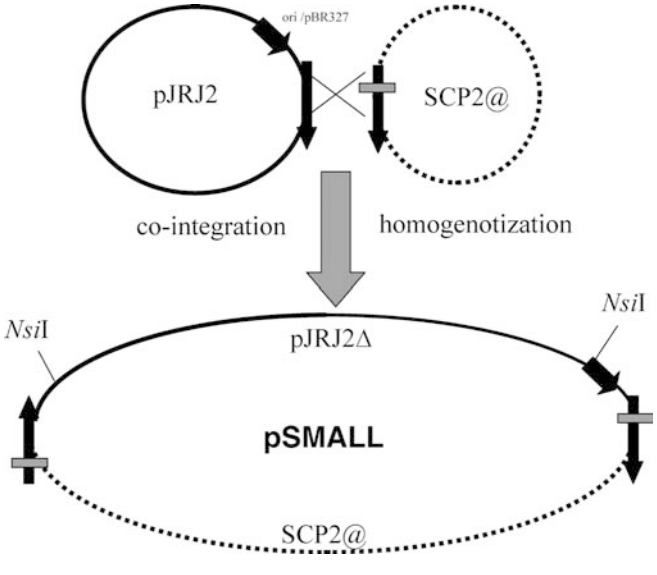

Fig. 5 Model for the origin of pSMALL by co-integration between pJRJ2 and a SCP2* variant called SCP2@ found to be present in CH999. *The bar* shows the 45-bp deletion in SCP2@. It is believed that the original co-integrate contained one copy of the replication region from SCP2@ and one from SCP2*. The final pSMALL was found to contain only the mutant replication region of SCP2@, presumably through homogenotization (see Discussion)

Acknowledgements We thank Dr. Helen Kieser (John Innes Centre) for the phenotypic tests of SCP2 status in strain CH999 and its progenotors. We also thank Dr. Stephen Bentley (Sanger Institute, Cambridge, UK) for the sequence of SCP2, now deposited as AL645771, and John Carney and Nina Viswanathan (Kosan Biosciences) for assistance with HPLC-MS analysis.

References

1. Bedford DJ, Schweizer E, Hopwood DA, Khosla C (1995) Expression of a functional fungal polyketide synthase in the bacterium *Streptomyces coelicolor* A3(2). J Bacteriol 177:4544–4548
2. Bibb MJ, Hopwood DA (1981) Genetic studies of the fertility plasmid SCP2 and its SCP2* variants in *Streptomyces coelicolor* A3(2). J Gen Microbiol 126:427–442
3. Bibb M, Schottel JL, Cohen SN (1980) A DNA cloning system for interspecies gene transfer in antibiotic-producing *Streptomyces*. Nature 284:526–531
4. Haug I, et al (2003) *Streptomyces coelicolor* A3(2) plasmid SCP2*: deductions from the complete sequence. Microbiology 149:505–513
5. Hopwood DA, Lydiate DJ, Malpartida F, Wright HM (1985) Conjugative sex plasmids of *Streptomyces*. Basic Life Sci 30:615–634
6. Hu Z, et al (2003) Approaches to stabilization of inter-domain recombination in polyketide synthase gene expression plasmids. J Ind Microbiol Biotechnol 30:161–167
7. Jacobsen JR, Hutchinson CR, Cane DE, Khosla C (1997) Precursor-directed biosynthesis of erythromycin analogs by an engineered polyketide synthase. Science 277:367–369
8. Kao CM, Katz L, Khosla C (1994) Engineered biosynthesis of a complete macrolactone in a heterologous host. Science 265:509–512
9. Khosla C, Ebert-Khosla S, Hopwood DA (1992) Targeted gene replacements in a *Streptomyces* polyketide synthase gene cluster: role for the acyl carrier protein. Mol Microbiol 6:3237–3249
10. Kieser T, Hopwood DA, Wright HM, Thompson CJ (1982) pIJ101, a multi-copy broad host-range *Streptomyces* plasmid:

functional analysis and development of DNA cloning vectors. Mol Gen Genet 185:223–228
11. Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA (2000) Practical *Streptomyces* genetics. John Innes Centre, Norwich
12. Kim ES, Cramer KD, Shreve AL, Sherman DH (1995) Heterologous expression of an engineered biosynthetic pathway: functional dissection of type II polyketide synthase components in *Streptomyces* species. J Bacteriol 177:1202–1207
13. Larson JL, Hershberger CL (1986) The minimal replicon of a streptomycete plasmid produces an ultrahigh level of plasmid DNA. Plasmid 15:199–209
14. Lydiate DJ, Malpartida F, Hopwood DA (1985) The *Streptomyces* plasmid SCP2*: its functional analysis and development into useful cloning vectors. Gene 35:223–235
15. MacNeil DJ, et al (1992) Analysis of *Streptomyces avermitilis* genes required for avermectin biosynthesis utilizing a novel integration vector. Gene 111:61–68
16. McDaniel R, Ebert-Khosla S, Hopwood DA, Khosla C (1993) Engineered biosynthesis of novel polyketides. Science 262:1546–1550
17. McDaniel R, et al (1999) Multiple genetic modifications of the erythromycin polyketide synthase to produce a library of novel ″unnatural″ natural products. Proc Natl Acad Sci USA 96:1846–1851
18. Rowe CJ, Cortes J, Gaisser S, Staunton J, Leadlay PF (1998) Construction of new vectors for high-level expression in actinomycetes. Gene 216:215–223
19. Rudd BAM (1978). Genetics of pigmented secondary metabolites in *Streptomyces coelicolor*. PhD thesis, University of East Anglia, Norwich
20. Takano E, White J, Thompson CJ, Bibb MJ (1995) Construction of thiostrepton-inducible, high-copy-number expression vectors for use in *Streptomyces* spp. Gene 166:133–137
21. Tang L, McDaniel R (2001) Construction of desosamine containing polyketide libraries using a glycosyltransferase with broad substrate specificity. Chem Biol 8:547–555
22. Wezel GP van, Bibb MJ (1996) A novel plasmid vector that uses the glucose kinase gene (*glkA*) for the positive selection of stable gene disruptants in *Streptomyces*. Gene 182:229–230
23. Wohlert S, et al (2001) Insights about the biosynthesis of the avermectin deoxysugar L-oleandrose through heterologous expression of *Streptomyces avermitilis* deoxysugar genes in *Streptomyces lividans*. Chem Biol 8:681–700
24. Xiao J, Melton RE, Kieser T (1994) High-frequency homologous plasmid–plasmid recombination coupled with conjugation of plasmid SCP2* in *Streptomyces*. Mol Microbiol 14:547–555
25. Xue Q, Ashley G, Hutchinson CR, Santi DV (1999) A multiplasmid approach to preparing large libraries of polyketides. Proc Natl Acad Sci USA 96:11740–11745
26. Yu TW, et al (2001) Mutational analysis and reconstituted expression of the biosynthetic genes involved in the formation of 3-amino-5-hydroxybenzoic acid, the starter unit of rifamycin biosynthesis in *Amycolatopsis mediterranei* S699. J Biol Chem 276:12546–12555
27. Ziermann R, Betlach MC (1999) Recombinant polyketide synthesis in *Streptomyces*: engineering of improved host strains. Biotechniques 26:106–110