Heterologous Expression of Novobiocin and Clorobiocin Biosynthetic Gene Clusters

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A method was developed for the heterologous expression of biosynthetic gene clusters in different *Streptomyces* strains and for the modification of these clusters by single or multiple gene replacements or gene deletions with unprecedented speed and versatility. λ -Red-mediated homologous recombination was used for genetic modification of the gene clusters, and the attachment site and integrase of phage ϕ C31 were employed for the integration of these clusters into the heterologous hosts. This method was used to express the gene clusters of the aminocoumarin antibiotics novobiocin and clorobiocin in the well-studied strains *Streptomyces coelicolor* and *Streptomyces lividans*, which, in contrast to the natural producers, can be easily genetically manipulated. *S. coelicolor* M512 derivatives produced the respective antibiotic in yields comparable to those of natural producer strains, whereas *S. lividans* TK24 derivatives were at least five times less productive. This method could also be used to carry out functional investigations. Shortening of the cosmid's inserts showed which genes are essential for antibiotic production.

The development of new antimicrobial agents is crucial to reduce the future clinical impact of resistant pathogens (35). Natural products play a dominant role in antimicrobial drug discovery (19), and streptomycetes produce two-thirds of the clinically useful antibiotics of natural origin (12). Structural modification of these natural products is often necessary in drug development, e.g., for improvements in efficacy and pharmacokinetics (32). The manipulation of genes which encode enzymes of the biosynthetic pathways represents a promising approach for introducing structural changes (33). The functional analysis of biosynthetic genes is, however, a prerequisite for such approaches. Moreover, a principal limitation is often presented by the lack of suitable protocols for the genetic manipulation of natural producers (11).

The aminocoumarin antibiotics novobiocin (Albamycin; Pharmacia & Upjohn) and clorobiocin (chlorobiocin) (Fig. 1) are very potent inhibitors of DNA gyrase and are produced by different *Streptomyces* strains (17). Novobiocin was licensed in the United States for the treatment of human infections with multiresistant gram-positive bacteria such as *Staphylococcus aureus* and *S. epidermidis* (21, 22, 34). Novobiocin and its derivatives have also been investigated as potential anticancer drugs (16, 23, 30). The aminocoumarin antibiotics are closely related in structure and their biosynthetic gene clusters have been cloned and sequenced, and therefore, they offer excellent possibilities for the generation of new antibiotics via genetic engineering (20, 28).

However, the development of efficient protocols for the genetic manipulation of aminocoumarin antibiotic-producing

Streptomyces strains is time consuming and, e.g., for the novobiocin producer *S. spheroides*, gives unsatisfactory results. This problem presents a severe limitation for genetic engineering and strain improvement.

In order to overcome this problem, we have developed a method which quickly allowed the heterologous expression of the entire novobiocin and clorobiocin biosynthetic gene clusters in the well-studied *Streptomyces coelicolor* and *S. lividans* strains (1, 12). By means of λ -Red-mediated recombination (4, 9), we introduced the integrase gene (*int*) and the attachment site (*attP*) of phage ϕ C31 into cosmids containing the biosynthetic gene clusters of novobiocin or clorobiocin. The modified cosmids were site-specifically integrated into the chromosome of the heterologous hosts (31).

Prior to heterologous expression, the DNA sequence of the biosynthetic gene clusters could be easily modified in *Escherichia coli* by gene replacements or gene deletions, allowing functional investigations. As examples, we have removed several nonessential sequences from the cosmid inserts.

MATERIALS AND METHODS

Bacterial strains, cosmids, and culture conditions. *Streptomyces coelicolor* M512 ($\Delta redD \Delta actII-ORF4$ SCP1[−] SCP2[−]) (7) was kindly provided by E. Takano (Tübingen, Germany) and Janet White (Norwich, United Kingdom), *S. lividans* TK24 (*str-6* SLP2[−] SLP3[−]) was provided by D. A. Hopwood (Norwich, United Kingdom), *S. roseochromogenes* var. *oscitans* DS 12.976 was provided by Aventis, and *S. spheroides* NCIMB 11891 was provided by E. Cundliffe (Leicester, United Kingdom). The strains were cultured as described previously (5, 12, 28).

Escherichia coli XL1-Blue MRF' (Stratagene, Heidelberg, Germany) was used for cloning experiments and grown as described previously (24).

The REDIRECT technology kit for PCR targeting (9) was obtained from Plant Bioscience Limited (Norwich, United Kingdom).

Kanamycin (15 μ g/ml in liquid medium and 50 μ g/ml in solid medium for *Streptomyces* and 50 μ g/ml for *E. coli*), chloramphenicol (25 to 50 μ g/ml), apramycin (50 μ g/ml), carbenicillin (50 to 100 μ g/ml), and thiostrepton (15 μ g/ml in liquid medium and 50 μ g/ml in solid medium) were used for selection of recombinant strains.

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FIG. 1. Structures of novobiocin and clorobiocin.

Cosmids 10-9C and D1A8 contained the novobiocin and the clorobiocin biosynthetic gene cluster in the SuperCos1 vector, respectively (20, 28).

Construction of pIJ787. The tetracycline resistance gene (*tet*) from pBR328 was amplified using primers pIJ782forw (5'-CTA TGA TCG ACT GAT GTC ATC AGC GGT GGA GTG CAA TGT CAT GAA ATC TAA CAA TGC GC-3') and pIJ782rev (5'-GAA CTT CAT GAG CTC AGC CAA TCG ACT GGC GAG CGG CAT CTC AGG TCG AGG TGG CGG GG-3') (the beginning and the end of the coding region of the *tet* sequence are underlined). This fragment was used to replace the apramycin resistance gene in pIJ773 using λ -Red-mediated recombination (4, 9), generating pIJ782. The structural gene *tet* was thereby placed under control of the apramycin resistance promoter, resulting in slightly lower resistance levels (5 μ g/ml instead of 10 μ g/ml tetracycline). *tet* together with the apramycin resistance promoter was then amplified from pIJ782 using primers TetAatIIforw (5'-AAA AAA AGA CGT CTT GAA TGG GTT CAT GTG-3') and TetAatII rev (5'-AAA AAA AGA CCG CTC AGG TCG AGG TGG CCC-3') (the AatII restriction site is underlined). After digestion of the PCR product with AatII, it was cloned into the same site of pSET152. A 4,590-bp MscI-PvuI fragment obtained from these clones was ligated into the ScaI-PvuI sites (i.e., into the ampicillin resistance gene *bla*) of SuperCos1 to give pIJ787.

Construction of pUG019. pUG019, containing an apramycin resistance cassette and flanked by XbaI and SpeI recognition sites, was generated by PCR amplification of two fragments from pIJ773 (REDIRECT technology kit). The first fragment of 97 bp was amplified using primers FRT_P01f (5'-CTG CAG GAA TTC GAT ATT CCG GGG ATC TCT AGA TCT-3') (the EcoRI, XbaI, and BglII restriction sites are underlined) and FRT_P01r (5'-TGG CGG GGA TAT CGA AGT TCC-3') (the EcoRV restriction site is underlined). After digestion with EcoRI and EcoRV, this fragment was ligated into the same sites of pBluescript SK(-) (Stratagene, Heidelberg, Germany) to give pUG017. The second fragment of about 1 kb containing the apramycin resistance gene *aac(3)IV* was amplified using primers apra_P03f (5'-GGG GAT GAT ATC TTT ATC ACC ACC GAC TAT TTG-3') (the EcoRV restriction site is underlined) and apra_P02r (5'-TCG ATA AGC TTG ATG ACT AGT CTG GAG CTG GAG CTG CTT CGA-3') (the HindIII and SpeI restriction sites are underlined). After digestion with EcoRV and HindIII, this fragment was ligated into the same sites of pUG017 to give pUG019.

DNA isolation, manipulation, and cloning. Standard procedures for DNA isolation and manipulation were performed as described previously by Sambrook and Russell (24) and Kieser et al. (12). Isolation of DNA fragments from agarose gel and purification of PCR products were carried out with the NucleoSpin 2 in 1 Extract kit (Macherey-Nagel, Düren, Germany). Isolation of cosmids and plasmids was carried out with ion-exchange columns (Nucleobond AX kits; Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. Genomic DNA was isolated from *Streptomyces* strains using the Kirby mix procedure (12).

Southern blot analysis was performed on Hybond-N nylon membranes (Amersham, Braunschweig, Germany) with a digoxigenin (DIG)-labeled probe by using the DIG High Prime DNA labeling and detection starter kit II (Roche Molecular Biochemicals).

PCRs were carried out using the Expand High Fidelity PCR system (Roche Molecular Biochemicals) according to the manufacturer's instructions.

Heterologous expression. The DraI-BsaI fragment of pIJ787, containing the integrase cassette and flanked by about 100 bp of *bla* sequence on one site and about 300 bp of *bla* sequence on the other side, was used to replace the respective *bla* gene in the SuperCos1 backbone of cosmids 10-9C and D1A8, via λ -Red-mediated recombination (4, 9), generating nov-BG1 and clo-BG1, respectively.

Because of the potent methylation restriction system of *S. coelicolor*, cosmid DNA had to be passed through a nonmethylating host. We used *E. coli* ET12567 for this purpose (14). The modified cosmids nov-BG1 and clo-BG1, still carrying the kanamycin resistance gene *neo*, were then introduced into *S. coelicolor* M512 and *S. lividans* TK24 via polyethylene glycol-mediated protoplast transformation (12). Kanamycin-resistant clones were checked for site-specific integration into the genome by Southern blot analysis.

Protocol for single or multiple deletions within the cosmids. The apramycin resistance cassette (approximately 1 kb) was excised from pUG019 by digestion with EcoRI and HindIII and amplified by PCR using the forward primer 5'-(N)₃₉ ATT CCG GGG ATC TCT AGA TCT-3' and the reverse primer 5'-(N)₃₉ ACT AGT CTG GAG CTG CTT C-3' [(N)₃₉ represents 39 nucleotide extensions for λ -Red-mediated recombination, homologous to the regions upstream and downstream of the DNA fragment to be deleted; underlined are the XbaI and SpeI restriction sites]. Amplification was performed using the Expand High Fidelity PCR system (Roche Molecular Biochemicals) as described previously (9), with the following modification: annealing temperatures were 45°C and 48°C, respectively. The PCR product was used for gene replacement in cosmids via λ -Red-mediated recombination as described previously (9). For excision of the resistance cassette, cosmid DNA was isolated from *E. coli* ET12567 and digested with XbaI and SpeI, and 100 ng of DNA was religated overnight at 4°C. *E. coli* XL1-Blue MRF' cells were transformed with the ligation reaction. Apramycin-sensitive kanamycin-resistant clones were analyzed by restriction enzyme digestion and gel electrophoresis. For subsequent gene deletions, the identical procedure was used.

Using this procedure, e.g., for the generation of cosmids nov-AE6 and nov-AE4, first, the region downstream of the gyrase B resistance gene (Fig. 2) was removed from nov-BG1 using primers PhovgyrB_f (5'-GGT TCC TCC AGC GTG GCC ACG ACC ATG ACC GGG AGG TCG ATT CCG GGG ATC TCT AGA TC-3') and PT3SC1_r (5'-GTC TTC AAG AAT TCG CGG CCG CAA TTA ACC CTC ACT AAA ACT AGT CTG GAG CTG CTT C-3') (underlined letters represent the 39 nucleotide extensions homologous to the region downstream of the gyrase B resistance gene and to the T3 promoter of SuperCos1, respectively), generating cosmid nov-AE2. Subsequently, *ORF21* was deleted from nov-AE2 using primers PT7SC1_f (5'-ACA TGA GAA TTC GCG GCC GCA TAA TAC GAC TCA CTA TAG ATT CCG GGG ATC TCT AGA TC-3') and Phov20_r (5'-CTT CCC GAG GTT CAA TTC CGC CGC GCA CGT CAG CTC CTC ACT AGT CTG GAG CTG CTT C-3') (sequences homologous to the T7 promoter region of SuperCos1 and to the region downstream of *ORF20*, respectively, are underlined), generating cosmid nov-AE6. Alternatively, the entire region upstream of *novE* was deleted from nov-AE2 using primers PT7SC1_f (see above) and PhovE_r (5'-GCT GGA ATG CGC GGC TGC CGT CGC CGG GAC GGT CCC GGC ACT AGT CTG GAG CTG CTT C-3') (the sequence homologous to the region directly downstream of *novD* is underlined), generating cosmid nov-AE4. The insert of nov-AE6 comprises the DNA sequence from positions 5619 to 6339 of GenBank accession number AY227005, all of the sequence from GenBank accession number AF170880 (positions 1 to 25617), and from positions 1 to 2490 of GenBank accession number AF205854. The insert of nov-AE4 comprises the sequence from positions 4628 to 25617 of Gene Bank accession number AF170880 and from positions 1 to 2490 of GenBank accession number AF205854.

clo-AE2, containing genes from *cloE* to the gyrase B resistance gene, was generated from clo-BG1 in the same way using primers Pcloorf9_f (5'-TAG TAT GGC GAA ATT GGG TGA TCT GCT TGC CGC CGT CGA ATT CCG GGG ATC TCT AGA TC-3') (the sequence homologous to the region directly downstream of *ORF9* is underlined) and PT3SC1_r (see above). The insert of clo-AE2

FIG. 2. Cosmid constructs containing the novobiocin biosynthetic gene cluster and their integration into the *S. coelicolor* chromosome. (a) Cosmid constructs nov-BG1, nov-AE6, and nov-AE4. P, PstI restriction site; T3 and T7, T3 and T7 promoter of the SuperCos1 vector; *tet*, tetracycline resistance gene; *neo*, neomycin/kanamycin resistance gene; *int* and *attP*, integrase gene and attachment site of phage ϕ C31; *gyrB^R*, gyrase B resistance gene. Fragment sizes resulting from digestion with PstI are indicated. The cosmid backbone is out of scale. (b) Schematic representation of site-specific integration of constructs nov-BG1, nov-AE6, and nov-AE4 (see reference 31 for details of the integration mechanism). (c) Southern blot analysis of the *S. coelicolor* (S. c.) M512 parental strain; *S. coelicolor* M512 integration mutants harboring nov-BG1, nov-AE6, or nov-AE4; and the respective cosmid constructs. M, DIG-labeled DNA Molecular Weight Marker VII (Roche). Genomic and cosmid DNA were digested with PstI. The DIG-labeled cosmid nov-BG1 was used as a probe. The 14.0-kb band resulting from site-specific integration overlaps with the 13.8-kb band from the cosmid inserts.

comprises the DNA sequence from positions 9200 to 40573 of GenBank accession number AF329398 and from positions 1 to 2238 of GenBank accession number AY136281.

Production and analysis of secondary metabolites. Transformants and parental strains of *S. coelicolor* and *S. lividans*, as well as *S. spheroides* and *S. roseochromogenes*, were cultured and assayed for novobiocin or clorobiocin production by high-performance liquid chromatography (HPLC) as described previously (5, 6).

Negative-ion fast atom bombardment mass spectra were recorded with a TSQ70 spectrometer (Finnigan, Bremen, Germany) using diethanolamine as matrix. ^1H nuclear magnetic resonance (NMR) spectra were measured with either an AC 250 or an AMX 400 spectrometer (Bruker, Karlsruhe, Germany) using CD_3OD as a solvent.

In the case of strains harboring nov-BG1, nov-AE6, and nov-AE4 (comprising the novobiocin cluster), the isolated compound showed a molecular ion $[\text{M-H}]^-$ at m/z 611 (novobiocin, $\text{C}_{31}\text{H}_{36}\text{N}_2\text{O}_{11}$; molecular weight, 612). The isolated substance gave ^1H NMR signals identical to those obtained from authentic novobiocin (6).

Strains harboring clo-BG1 and clo-AE2 (comprising the clorobiocin cluster) showed four peaks in the HPLC analysis (see Fig. 4d). The first peak (peak 1), with the shortest retention time, showed a molecular ion $[\text{M-H}]^-$ at m/z 661 and the following isotopic pattern [mass (percent intensity)]: 661 (100.0%), 662 (39.9%), 663 (11.9%). Furthermore, this compound gave ^1H NMR signals identical to those obtained for C-8'-deschloro-clorobiocin (novclorobiocin 101; described in reference 5). The second peak (peak 2) showed a molecular ion $[\text{M-H}]^-$ at m/z 695 and the following typical isotopic pattern caused by the chlorine isotopes ^{35}Cl and ^{37}Cl [mass (percent intensity)]: 695 (100.0%), 696 (36.1%), 697 (39.5%), 698 (14.6%). This compound gave ^1H NMR signals identical to those obtained for clorobiocin (5). The third peak (peak 3) had the same mass as the first one, molecular ion $[\text{M-H}]^-$ at m/z 661, and showed the same isotopic pattern (mass [percent intensity], 661 [100.0%], 662 [30.2%], 663 [17.5%]), indicating the absence of a chlorine atom. The fourth peak (peak 4) had the same mass as clorobiocin, molecular ion $[\text{M-H}]^-$ at m/z 695, and the isotopic pattern caused by the chlorine isotopes ^{35}Cl and ^{37}Cl (mass [percent intensity]: 695 [100.0%], 696 [37.6%], 697 [36.0%], and 698 [14.6%]). Substances 3 and 4 are therefore likely to represent structural isomers of substances 1 and 2, which carry the acyl group at 2-OH rather than 3-OH of the deoxysugar, as identified previously (8).

RESULTS

Heterologous expression. Cosmids containing the entire biosynthetic gene clusters of novobiocin and clorobiocin had been obtained previously (20, 28) by employing the widely used cosmid vector SuperCos1 (Stratagene), which contains an ampicillin (*bla*) and a neomycin/kanamycin (*neo*) resistance gene.

We now replaced the *bla* gene within the SuperCos1 backbone of cosmids 10-9C and D1A8 (containing the novobiocin and clorobiocin cluster, respectively) with a cassette containing the integrase gene (*int*) and the attachment site (*attP*) of phage ϕC31 , as well as a selectable marker (tetracycline resistance), using λ -Red-mediated recombination in *E. coli* (see Materials and Methods). This one-step procedure readily yielded the desired modified cosmids which were termed nov-BG1 and clo-BG1, respectively. They were introduced into *S. coelicolor* M512 (ΔredD $\Delta\text{actII-ORF4}$ SCP1^- SCP2^-) and *S. lividans* TK24 (*str-6* SLP2^- SLP3^-) by protoplast transformation. Selection for kanamycin resistance resulted in the desired integration mutants (approximately 10^3 mutants per microgram cosmid DNA for *S. coelicolor* and 10^5 mutants for *S. lividans*). Southern blot analysis showed that the entire cosmids had integrated site specifically into the *attB* site of the chromosome in both *Streptomyces* strains (Fig. 2c and 3c; only results for *S. coelicolor* strains are shown).

Integration mutants and parental host strains were cultured in production media. The analysis of secondary metabolites by HPLC (Fig. 4) showed that, in contrast to the untransformed

host strains, the integration mutants accumulated novobiocin and clorobiocin, respectively. The identity of these substances was confirmed, after preparative isolation, by negative-ion fast atom bombardment mass spectrometry and ^1H NMR analysis (see Materials and Methods). *S. coelicolor* strains expressing the clorobiocin cluster additionally accumulated three clorobiocin analogs (Fig. 4d) identical to those observed in the natural clorobiocin producer *S. roseochromogenes* (5) (see Materials and Methods).

The productivity of the integration mutants of *S. coelicolor* was comparable to that of the original producers *S. spheroides* and *S. roseochromogenes* (Table 1). *S. lividans*, as a host, was much less productive. Therefore, *S. coelicolor* was used in the subsequent experiments.

Removal of nonessential DNA regions from the cosmid inserts. Based on the λ -Red recombination system, we developed a two-step procedure which allowed us to readily produce single or multiple deletions at any desired site within the cosmid insert and which can be utilized for functional investigations.

The apramycin resistance cassette from the previously described plasmid pIJ773 (9) is flanked by FLP recombinase recognition targets (FRT). After gene replacement, this cassette can be conveniently removed by action of FLP recombinase, leading to the excision of the DNA region in between the FRT sites and leaving an 81-bp "scar" sequence. However, the presence of this scar sequence makes further knockouts in the same cosmid difficult, because it represents a functional FRT site as well as a target for λ -Red-mediated recombination.

To overcome this problem, an apramycin resistance cassette (Fig. 5) was generated by PCR using primers with either an XbaI or an SpeI recognition site between the apramycin resistance marker and the 39-bp flanking sequence for λ -Red-mediated recombination (9). XbaI and SpeI sites are rare in the GC-rich *Streptomyces* genome. After gene replacement, this cassette can be removed by digestion with XbaI and SpeI and religation of the resulting compatible ends (Fig. 5). This procedure leaves a minimal in-frame "scar" of 18 nucleotides which is not recognized by XbaI or SpeI and which does not interfere with further gene deletions or replacements.

The biosynthetic gene cluster of clorobiocin is flanked on one side by the gyrase B resistance gene and the topoisomerase IV resistance gene (26). Of the latter 2.1-kb gene, only 123 bp are contained in cosmid clo-BG1 (Fig. 3a, right-hand side of the insert). On the opposite end, the gene *cloE* may represent the border of the cluster (6, 26). We now deleted all genes upstream of *cloE* from cosmid clo-BG1 using the two-step procedure explained above (see Materials and Methods). The insert of the resulting cosmid, clo-AE2, starts 148 bp upstream of the start codon of *cloE* (Fig. 3).

This cosmid was transformed into *S. coelicolor* M512, and integration mutants were selected. Site-specific integration of the entire cosmid was confirmed by Southern blot analysis (Fig. 3c, lanes 5 and 6). When these mutants were cultured in production medium, HPLC analysis clearly showed that clorobiocin was still produced in significant amounts (Table 1), proving that the 34-kb sequence from *cloE* to the gyrase B resistance gene contains all genes required for clorobiocin biosynthesis.

A similar experiment was performed with the novobiocin cluster contained in cosmid nov-BG1. It has been suggested previously that *novE* and the gyrase B resistance gene (Fig. 2)

FIG. 3. Cosmid constructs containing the clorobiocin biosynthetic gene cluster and their integration into the *S. coelicolor* chromosome. (a) Cosmid constructs clo-BG1 and clo-AE2. B, BglIII restriction site; T3 and T7, T3 and T7 promoter of the SuperCos1 vector; gyrB^R, gyrase B resistance gene; parY^R, topoisomerase IV resistance gene. Fragment sizes resulting from digestion with BglIII are indicated. The cosmid backbone is out of scale. (b) Schematic representation of site-specific integration of constructs clo-BG1 and clo-AE2 (see reference 31 for details of the integration mechanism). (c) Southern blot analysis of *S. coelicolor* (S. c.) M512 parental strain, of *S. coelicolor* M512 integration mutants harboring clo-BG1 or clo-AE2, and of the respective cosmid constructs. M, DIG-labeled DNA Molecular Weight Marker VII (Roche). Genomic and cosmid DNA were digested with BglIII. The DIG-labeled cosmid clo-BG1 was used as a probe.

FIG. 4. HPLC analyses of secondary metabolites. (a) *S. coelicolor* M512 parental strain (detection at 305 nm). (b) *S. coelicolor* harboring nov-BG1 (c) *S. coelicolor* M512 parental strain (detection at 340 nm). (d) *S. coelicolor* harboring clo-BG1.

may delineate the left and right border of this cluster, respectively (6, 26). However, *novA*, which is located outside of this region, shows sequence similarity to ABC transporters and has been suggested to be involved in novobiocin transport (18, 26). Therefore, we decided to produce two different shortened cosmids (see Materials and Methods): nov-AE4, containing only the genes from *novE* to the gyrase B resistance gene, and nov-AE6, additionally containing the genes *novABCD* and

TABLE 1. Novobiocin and clorobiocin production by *S. coelicolor* and *S. lividans*

Strain	Production (mg/liter)	
	Novobiocin ^a	Clorobiocin ^{a,b}
Parental strains		
<i>S. spheroides</i>	35 (30–40)	
<i>S. roseochromogenes</i>		25 (20–30)
<i>S. coelicolor</i> M512		
<i>S. lividans</i> TK24		
Strains carrying full-length cosmids		
<i>S. coelicolor</i> (nov-BG1)	31 (20–42)	
<i>S. lividans</i> (nov-BG1)	<1	
<i>S. coelicolor</i> (clo-BG1)		26 (18–34)
<i>S. lividans</i> (clo-BG1)		5 (4–6)
Strains carrying shortened cosmids		
<i>S. coelicolor</i> (nov-AE6)	24 (23–25)	
<i>S. coelicolor</i> (nov-AE4)	1.5 (0.8–2.2)	
<i>S. coelicolor</i> (clo-AE2)		14 (8–19)

^a Mean values (range) from at least two independent experiments.
^b Total amount of clorobiocin and the three major analogs (Fig. 4d).

ORF20 (Fig. 2). *ORF20* may encode a regulator of *novA*, as suggested by sequence comparison with database entries. Cosmids nov-AE4 and nov-AE6 were transformed into *S. coelicolor* M512, and site-specific integration of the entire cosmids was confirmed by Southern blot analysis (Fig. 2). Cultivation in production medium and HPLC analysis revealed that integration mutants containing the larger cosmid, i.e., nov-AE6, still produced two-thirds of the novobiocin amount found in the natural producer, *S. spheroides*. In contrast, mutants containing the smaller cosmid nov-AE4 produced only 6% of the amount accumulated by *S. coelicolor* (nov-AE6). This proves that the DNA region from *ORF20* to *novD* contains elements which are required for a high productivity of novobiocin.

FIG. 5. Gene deletion using an apramycin resistance cassette containing flanking XbaI and SpeI recognition sites. *aac(3)IV*, apramycin resistance gene; P, promoter of the apramycin resistance gene.

DISCUSSION

The present work describes an efficient method for the heterologous expression of biosynthetic gene clusters in different *Streptomyces* strains. λ -Red-mediated recombination in *E. coli* was used for the introduction of integration functions into the cosmid backbones, which allowed subsequent site-specific integration into the genome of the heterologous host. Single or multiple gene replacements and gene deletions could be created at any chosen site within the gene clusters.

During our procedure, the stability of the cosmids containing the gene clusters was quite satisfactory. Southern blot analysis of the integration mutants, using the entire cosmids as probes, proved that in more than 80% of the mutants, no rearrangements or deletions had taken place within the integrated sequence, and this was confirmed by the functional studies, i.e., by the formation of the respective antibiotics. We cannot exclude, however, that in certain other clusters, instability may present a more significant problem, e.g., in clusters containing type I polyketide synthase genes with repeated DNA sequences.

The complete genome sequence of strain M145 of *S. coelicolor* has been published previously (1). *S. coelicolor* M145 is able to produce three antibiotics, i.e., prodiginines (Red), actinorhodin (Act), and calcium-dependent antibiotic. To avoid interference in the chemical and biological analysis of antibiotic formation, we expressed the novobiocin and clorobiocin biosynthetic gene clusters in *S. coelicolor* M512, which is derived from M145 but which is unable to produce Red and Act (7).

Historically, *S. lividans* has been distinguished phenotypically from its close relative *S. coelicolor* by the inability of the former to produce significant amounts of antibiotics. Yet functional biosynthetic Red and Act gene clusters are present in *S. lividans*, suggesting different regulation of the onset of secondary metabolism in the two species (13, 15, 27). A strain of *S. lividans*, TK24 (*str-6*), was found to overproduce antibiotics in comparison to the parent strain TK21. Shima et al. (27) proposed that the *str-6* mutation (point mutation in the *rpsL* gene encoding ribosomal protein S12) leads to a change in the ribosomal structure which gives rise to initiation of the onset of secondary metabolism. Therefore, to test *S. lividans* as a host, strain TK24 was chosen. However, *S. coelicolor* M512 showed much higher productivity than *S. lividans* TK24 and was therefore used for subsequent experiments.

It remains unclear whether the higher productivity observed for *S. coelicolor* M512-derived strains is due to the Act and Red mutations or whether *S. coelicolor* in general is a better producer strain than *S. lividans*.

After testing different strains for heterologous expression, optimization of the fermentation conditions will next be an important task in order to achieve improvement in productivity.

Using the method developed in this study, we proved, for the first time, that the DNA region from *cloE* to the gyrase B resistance gene (Fig. 3) contains all genes necessary for clorobiocin production. This finding is of special interest since no candidate gene has been identified which may code for the enzyme responsible for the introduction of the ring oxygen of the coumarin moiety of the aminocoumarin antibiotics (2, 6,

10). Unless this oxygenation is carried out by primary metabolic enzymes of *S. coelicolor* (a rather unlikely scenario), this catalytic function will have to be identified in one of the proteins encoded in cosmid clo-AE2.

The principal resistance gene of the novobiocin producer *S. spheroides* is the gyrase B resistance gene, encoding for an aminocoumarin-resistant gyrase B subunit (25, 26, 29). Since this gene is contained within the novobiocin cluster (Fig. 2), the *S. coelicolor* mutants generated in this study were able to tolerate the accumulation of the antibiotic.

The clorobiocin producer *S. roseochromogenes* contains, besides the gyrase B resistance gene, an additional resistance gene, that for topoisomerase IV, which encodes an aminocoumarin-resistant topoisomerase IV subunit (25, 26). Only 123 bp of the 2.1-kb topoisomerase IV resistance gene was contained in the cosmids clo-BG1 and clo-AE2 (Fig. 3). Nevertheless, clorobiocin was produced in good yield by the mutants expressing these cosmids, consistent with our previous finding that the gyrase B resistance gene alone is sufficient to provide resistance against aminocoumarin antibiotics (26).

Vectors containing the integrase gene and the *attP* site of ϕ C31 can integrate not only as a single copy but also in tandem (3). Tandem integration of cosmids was observed in approximately 90% of all integration mutants obtained in this study, as identified in Southern blot analysis by a hybridizing fragment containing the unchanged *attP* site (e.g., 3.9 kb in Fig. 2c). Mutants with tandem integration showed somewhat higher antibiotic production than mutants with single-copy integration, but the difference, and the number of available strains, was not large enough to draw unequivocal conclusions. In contrast, the 16-fold-higher productivity of strains bearing cosmid nov-AE6, compared to strains harboring nov-AE4 (Table 1 and Fig. 2), was observed in more than 10 independent mutants each and clearly proves that the DNA sequence from *ORF20* to *novD* contains elements required for a high novobiocin production. The gene *novA*, which codes for an ABC transporter, may be responsible for this effect.

The heterologous expression experiments described here have given rise to strains which, in contrast to the natural producers, are highly amenable for genetic manipulation and strain improvement. These methods may be useful both for basic research on microbial secondary metabolism and for drug discovery programs from streptomycetes, allowing, e.g., the exploitation of biosynthetic gene clusters from organisms which are difficult to cultivate.

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