# Kim, C.- G. et al. Biosynthesis of rubradirin as an ansamycin antibiotic from Streptomyces achromogenes var. rubradiris NRRL3061. Arch. Microbiol. 189, 463-473

**ARTICLE** *in* ARCHIVES OF MICROBIOLOGY · JUNE 2008

Impact Factor: 1.67 · DOI: 10.1007/s00203-007-0337-3 · Source: PubMed

Arch Microbiol (2008) 189:463–473
DOI 10.1007/s00203-007-0337-3

ORIGINAL PAPER

# Biosynthesis of rubradirin as an ansamycin antibiotic from *Streptomyces achromogenes* var. *rubradiris* NRRL3061

**Chun-Gyu Kim · Janardan Lamichhane · Kwang-Il Song · Van D. Nguyen · Dong-Hee Kim · Tae-Sung Jeong · Sung-Ho Kang · Kyoung-Wook Kim · Jyoti Maharjan · Young-Soo Hong · Jae Seon Kang · Jin-Cheol Yoo · Jung-Joon Lee · Tae-Jin Oh · Kwangkyoung Liou · Jae Kyung Sohng**

Received: 27 July 2006 / Revised: 1 August 2007 / Accepted: 26 November 2007 / Published online: 14 December 2007


**Abstract** The four overlapping cosmids from the rubradirin producer, *Streptomyces achromogenes* var *rubradiris* NRRL 3061, have 58 ORFs within a 105.6 kb fragment. These ORFs harbored essential genes responsible for the formation and attachment of four distinct moieties, along with the genes associated with regulatory, resistance, and transport functions. The PKS (*rubA*) and glycosyltransferase (*rubG2*) genes were disrupted in order to demonstrate a complete elimination of rubradirin production. The rubradirin biosynthetic pathway was proposed based on the putative functions of the gene products, the functional identification of sugar genes, and the mutant strains.

**Keywords** Ansamycin · Biosynthesis · Polyketide synthase · Rubradirin · Rubranitrose · *Streptomyces*

**Abbreviations**

| | |
|---|---|
| AHBA | 3-Amino-5-hydroxybenzoic acid |
| AMC | 3-Amino-4-hyrdoxy-7-methoxycoumarin |
| DHDP | 3,4-Dihydroxydipicolinate |
| NRPS | Nonribosomal polypeptide synthetase |
| PKS | Polyketide synthase |

## Introduction

Rubradirin is an ansamycin antibiotic that can be isolated from *Streptomyces achromogenes* var. *rubradiris* NRRL3061 and exhibits profound levels of activity against a wide variety of Gram-positive bacteria, especially against multiple-antibiotic-resistant strains of *Staphylococcus aureus* (Bhuyan et al. 1964). Rubradirin is comprised of four distinct moieties; rubransarol, 3-amino-4-hydroxy-7-methoxycoumarin (AMC), 3,4-dihydroxydipicolinate (DHDP), and D-rubranitrose (2,3,6-trideoxy-3-*C*-4-*O*-dimethyl-3-*C*-nitro-D-*xylo*-hexose) (Fig. 1). The structural moieties of rubradirin actively participate in its various modes of action. Rubradirin inhibits the function of microbial ribosomes via the selective prevention of translation chain initiation during protein synthesis (Reusser 1973). However, the rubradirin aglycon has been shown to act as a potent inhibitor of bacterial RNA polymerase distinct from the termination of the initiation of RNA synthesis exploited by the

Communicated by Jean-Luc Pernodet.

The GeneBank accession number for the sequence reported in this paper is AJ871581.

C.-G. Kim · K.-I. Song · V. D. Nguyen · D.-H. Kim · T.-S. Jeong · S.-H. Kang · K.-W. Kim · J. S. Kang
Department of Pharmaceutical Engineering, Inje University, Kimhae, Kyung-nam 621-749, Republic of Korea

J. Lamichhane · J. Maharjan · T.-J. Oh · K. Liou · J. K. Sohng
Institute of Biomolecule Reconstruction, Department of Pharmaceutical Engineering, Sun Moon University, #100, Kalsan-ri, Tangjeong-myeon, Asansi, Chungnam 336-708, Republic of Korea

Y.-S. Hong · J.-J. Lee
Korea Research Institute of Bioscience and Biotechnology, KIST, Daejeon 305-600, Republic of Korea

J.-C. Yoo
Department of Pharmacy, Chosun University, Kwangju 501-759, Republic of Korea

J. K. Sohng (✉)
Department of Pharmaceutical Engineering, Sun Moon University, #100, Kalsan-ri, Tangjeong-myeon, Asansi, Chungnam 336-708, Republic of Korea
e-mail: sohng@sunmoon.ac.kr

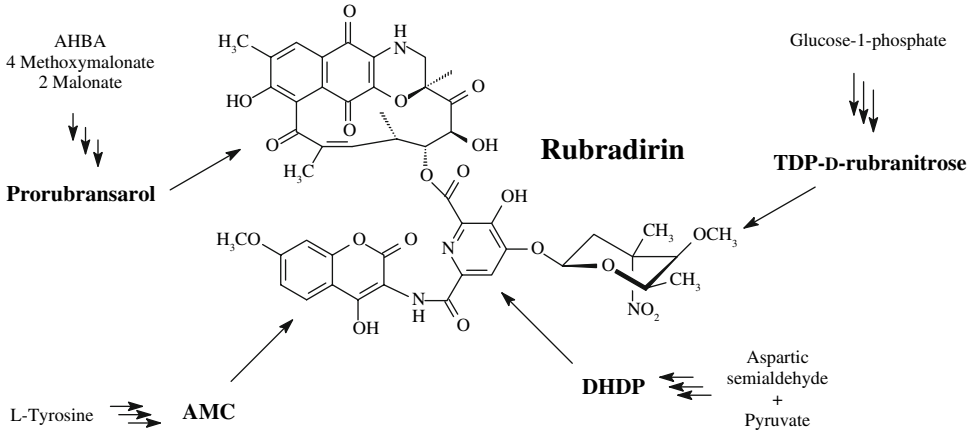


**Fig. 1** Structure of rubradirin. *AHBA* 3-amino-5-hydroxybenzoic acid; *AMC* 3-amino-4-hydroxy-7-methoxycoumarin; *DHDP* 3,4-dihydroxydipicolinate

other ansamycins including rifamycins, streptovaricins and tolypomycins (Wherli 1977). The rubradirin aglycon has also been found to exert some inhibitory effects against protein synthesis (Reusser 1979). Rubransarol does not inhibit bacterial RNA polymerase or ribosomal functions. It is also worth noting that the AMC–DHDP moiety also exhibits a moderate degree of inhibitory activity against RNA synthesis (Reusser 1979). The multifunctional nature of rubradirin, which is dependent on the presence of its various structural moieties, is a hallmark characteristic of this compound. The rubradirin aglycon, which acts as a potent inhibitor of human immunodeficiency virus (HIV) reverse transcriptase, provides further evidence for the multifunctional nature of rubradirin (Russer et al. 1988).

Minimal attention has been paid to the biosynthetic origins of rubradirin. However, relevant biosynthetic pathways can be deduced from previously published information related to the biosynthetic pathways of ansamycins, dideoxysugars, aminocoumarin, and dipicolinate, since the biosynthetic origins of all of these structures have been extensively studied (Fig. 1). Rubransarol can be derived from the well-known polyketide biosynthetic pathway, using 3-amino-5-hydroxybenzoic acid (AHBA) as a starter unit, and methylmalonate and malonate as the extender units (Wherli 1977). D-rubranitrose can be derived from the 6-deoxysugar that acts as a structural moiety in a number of antibiotics (He and Liu 2002).

In a previous report, we cloned the components of the AHBA synthase and TDP-D-glucose 4,6-dehydratase genes from the genomic DNA of a rubradirin producer via PCR (Sohng et al. 1997). The two PCR products were used as gene probes in the isolation of cosmid clones following the construction of a genomic DNA library from the total DNA of *S. achromogenes*. In the present study, we describe the functional identification, sequencing, and analysis of this rubradirin biosynthetic gene cluster.

## Materials and methods

### Bacterial strains, plasmids, and vectors

*Streptomyces achromogenes* var. *rubradiris* NRRL3061 is a wild-type obtained from the ARS collection (Peoria, IL). *Escherichia coli* XL1-blue MRF (Stratagene, La Jolla, CA) and *E. coli* ET12567/pUZ8002 were routinely used as the hosts for plasmid preparation and for the preparation of methylase-negative plasmid DNA, respectively (Kieser et al. 2000). The pGEM-3Zf (Promega) was utilized as a cloning vector. The total DNA for the library of *S. achromogenes* var. *rubradiris* was constructed using the pOJ446 cosmid vector (Bierman et al. 1992). The synthetic oligonucleotides were synthesized by GenoTec (Daejeon, Korea).

### Chemicals and enzymes

All chemicals used were of reagent grade; [α-$^{32}$P]dCTP was purchased from Amersham Pharmacia Biotech. All other essential chemicals used in this study were purchased from Sigma, United States Biochemical, and Amersham. All enzymes used in this study were obtained from Promega (Madison, WI) and Takara (Japan).

### Genetic manipulation

The *S. achromogenes* genomic cosmid library was screened by chromosome walking with overlapping DNA fragments. Colony and Southern hybridizations were conducted as previously described (Sambrook and Russell 2001). The hybridizations were conducted in the presence of 30% (vol/vol) formamide in 5× SSPE at 50°C. The DNA was labeled with [α-$^{32}$P]dCTP using a random-labeling kit (Amersham). The nylon membranes were washed with 5× SSPE/0.1% SDS for 30 min at 55°C. Autoradiography was conducted at −70°C using Fuji film with intensifying screens. Any

radioactive matter was stripped from the membranes via washing with 0.4 N NaOH at 42°C for 30 min.

### DNA sequencing and sequence analysis

Sequencing was conducted via dideoxy-chain termination using the dye terminator kit from Amersham. Sequencing was conducted on an ABI 377 automated DNA sequencer (Perkin Elmer). The BLAST program (NCBI) was utilized for sequence analysis and homology searches in the GenBank database, respectively.

### Insertional inactivation of *rubA* and *rubG2*

A 1 kb *Bcl*I DNA fragment of pIJ702, which harbored the thiostrepton resistance gene, was routinely used as the selection marker to construct the gene inactivation plasmids. A 2.5 kb *Kpn*I/*Bam*HI fragment of the pRub4 cosmid, which contained a portion of the *rubA*, was cloned into pGEM3(+), resulting in the formation of pSB525. The 1 kb *Bcl*I thiostrepton resistance fragment was blunt-ligated into *Not*I-digested pSB525, generating pSB525T. The 3.5 kb *Hin*dIII/*Eco*RI fragment of the *rubA* from pSB525T was cloned into pKC1139, yielding pJC525T. The DNA of pJC525T was then isolated from ET12567/pUZ8002 (Kieser et al. 2000), and was used in the transformation of *S. achromogenes* protoplasts. Recombinants found harboring the disrupted *rubA* were probed via Southern hybridization. The 2.8 kb *Apa*I fragment containing the glycosyltransferase (*rubG2*) gene obtained from pRub6 was subcloned into pGEM7, yielding pJLPG2. A thiostrepton resistance gene (*tsr*) with a *Bcl*I overhang was inserted into a unique site in this subcloned recombinant, thus generating pJLPTG2. The *Apa*I fragment of pJLPTG2 was then ligated to pKC1139, which yielded pJL001. The PCR product, coupled with *Nco*I and *Hin*dIII, was used as a probe for Southern hybridization of the mutated DNA.

### Fermentation and isolation of rubradirin and its related compounds

*Streptomyces achromogenes* was cultivated as described in the literature (Marshall et al. 1990). Both the wild-type and mutant were grown on an ISP4 medium. The fermentation broth was filtered at a harvest pH of 7.6. After the filtrate was adjusted to a pH of 3.2 using 6 N $H_2SO_4$, the mixtures that included the antibiotics were extracted with $CH_2Cl_2$ and were then concentrated under reduced pressure. The mixtures of rubradirin and its related compounds were then analyzed via HPLC at 335 nm, using an ODS column (150 × 4.6 mm, Japan, ODS-H80) with a gradient system of 30 to 85% methanol in 50 mM ammonium acetate (pH 4.0) at a flow rate of 1 ml/min for 55 min. The HPLC profiles were then further analyzed using ESI-MASS (Spectra-Physics Co.).

## Results

### Isolation and sequencing of the rubradirin biosynthetic gene cluster

The pRub4 cosmid clone which was described in a previous report was fully sequenced (Sohng et al. 1997). Since the genes in pRub4 are presumably insufficient for rubradirin production, we cloned the cosmids from the DNA library using the 4.6 kb *Eco*RI/*Spe*I DNA fragment, which is located at one of the ends of pRub4, as a gene probe by hybridization. The pRub44 clone includes 17 ORFs and a component of the PKS gene. Since the PKS gene from pRub44 did not contain all of the necessary modules for rubransarol production, two additional cosmid clones were isolated pRub32 (which has some genes in common with pRub44) and pRub6 (which shares some genes with pRub32). Nine ORFs were assigned in pRub32, and 18 ORFs were assigned in pRub6. The four cosmid clones extended jointly over 105.6 kb of DNA (Fig. 2). Fifty-eight ORFs were assigned in the gene cluster and the sequence data was compared with the data contained in the NCBI gene database. The putative functions of the genes within the cluster are shown in Table 1. The putative functions of a total of 23 genes were determined via sequence comparison. Eight genes, *rubK*, *L*, *M*, *N*, *G*, *H*, *J*, and *L*, were implicated in the biosynthesis of the common ansamycin precursor; two PKS genes, *rubA* and *B*, which consisted of six modules, were involved in rubransarol biosynthesis; eight genes, *rubN1* to *N8*, were putatively related to TDP-D-rubranitrose biosynthesis; and five genes, *rubC1* to *C5*, were involved in AMC and DHDP biosynthesis.

### Genes related to AHBA biosynthesis

A set of the AHBA biosynthetic genes were determined to be similar to those involved in the rifamycin, ansamitocin, and geldanamycin biosynthetic gene clusters (August et al. 1998; Yu et al. 2002; Rascher et al. 2003). Four genes, *rubK*, *rubL*, *rubM*, and *rubN*, appear to be linked to a single operon. The *rubG* and *rubH* are also transcriptionally linked, as determined using the sequence data exclusively, however *rubJ* is actually interrupted in comparison with the other clusters. Six of the genes, with the exception of *rubN*, exhibit a high degree of homology with the known products of the AHBA biosynthetic genes, *rifK*, *L*, *M*, *N*, *G*, *H*, and *J*, all of which were found in a rifamycin-generating strain. The functions of the six gene products, except for RifL, have been reported (Kim et al. 1998), and the AHBA

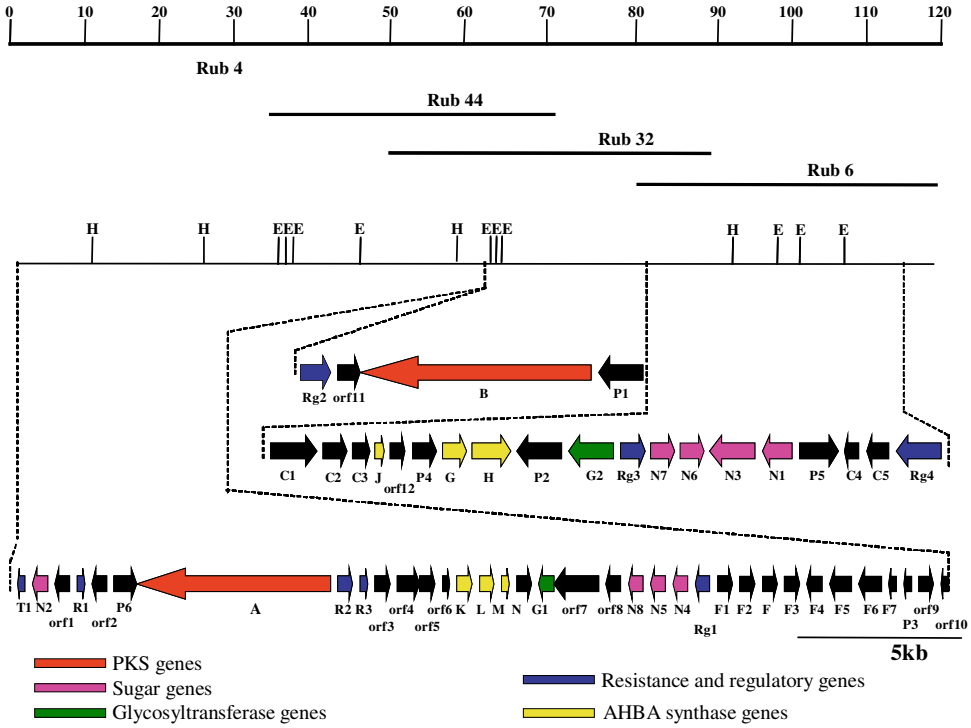


**Fig. 2** Organization of the rubradirin biosynthetic gene cluster from *S. achromogenes* var. *rubradiris* NRRL3061. Isolated cosmid clones are shown above the map. ORFs are shown as *arrows* indicating the size and direction of transcription

biosynthetic pathway from kanosamine to AHBA was clearly elucidated (Guo and Frost 2002) (Fig. 3).

PKS genes related to rubransarol biosynthesis

Two large ORFs, *rubA* and *rubB*, were separated by approximately 37 kb. Each of the ORFs harbored three modules, each of which encoded multifunctional PKSs as shown in Fig. 4. The two proteins harbor distinct domains typical of a type I PKS, the active sites of which closely resemble those identified in animal fatty acid synthases. RubA contains a loading domain presumed to accept the AHBA precursor. Based on the amino acid sequence criteria reported in a previous study (Tang et al. 1998), the AT domain in modules 2 and 5 presumably accepts malonyl-CoA, and the AT domains in modules 1, 3, 4, and 6 appear to accept methylmalonyl-CoA. Modules 1, 4, 5, and 6 in the rubradirin PKSs appear to harbor a DH domain. However, the consensus sequence of the DH domain, $H(X)_3G(X)_4P$, was not found to be conserved in any of the domains, with the exception of the DH domain in module 4. KR domains were detected in modules 1, 3, 4, 5, and 6. The consensus KR domain sequences, $GXG(X)_2G(X)_3A$ and RXG, were identified in modules 1, 4, and 5, and were found to have been modified in modules 3 and 6. It was quite surprising that the chain-terminating gene of ansamacrolide, which assists in the formation of the macrolactam amide bond, was not detected immediately downstream of *rubB*. The C-terminus of the fully-extended ansamycin chain is known to be transferred to the aromatic amino acid, resulting in the formation of macrolactam by the *rifF* gene product during rifamycin biosynthesis. All of the genes homologous to *rifF* have been detected immediately downstream of all of the ansamycin PKS genes (August et al. 1998; Yu et al. 2002; Rascher et al. 2003). The *rubF*, which is quite similar to *rifF*, was assigned from 13 kb upstream of *rubB*.

Genes related to AMC and DHDP biosynthesis

The gene cluster exhibited four genes that were similar to those involved in aminocoumarin biosynthesis. RubC1 exhibits amino acid sequence similarity to NovL and NovH, which function in the formation of the amide bond and in the adenylation of tyrosine, respectively. NovL and NovH were identified as separate proteins and the *rubC1* was detected as a single gene harboring both the NovL and NovH domains. RubC2 resembles NovI, which functions in the conversion of tyrosyl-NovH to hydroxytyrosyl-NovH (Chen and Walsh 2001). RubC3 is similar to NovJ, which, together with NovK, has been loosely implicated in the conversion of hydroxytyrosine to keto-tyrosine, although no NovK homologue was detected in the *rub* gene cluster (Chen et al. 2000). NovC, a putative flavine-dependent monooxgenase, is believed to hydroxylate the ortho position of the phenyl ring of keto-tyrosine, thereby facilitating cyclization. However, there were no gene products homologous to NovC detected in the *rub* gene cluster. RubC4 is similar to NovE, which is not essential to novobiocin biosynthesis (Eustaquio et al. 2003). A methyltransferase gene that methylates 4-hydroxy-aminocoumarin and the

**Table 1** Deduced functions of ORFs in the rubradirin biosynthetic gene cluster

| ORFs | Amino acid | Proposed function | % Identity acid | Accession number |
|---|---|---|---|---|
| RubT1 | 320 | ABC transporter (ATP binding) | 43 | AE000972 |
| RubN2 | 338 | TDP-glucose 4,6-dehydratase | 70 | AB103463 |
| ORF1 | 272 | Reductase | 23 | AP005049 |
| RubR1 | 74 | Translation initiation factor | 82 | AP005023 |
| ORF2 | 334 | Glucose-6-phosphate dehydrogenase | 69 | AF152397 |
| RubP3 | 427 | Cytochrome P450 | 42 | AB072568 |
| RubA | 5,349 | Polyketide synthase | 56 | AF040570 |
| | Loading domain | CoA ligase ACP | | |
| | Module1 | KS ATmm DH KR ACP | | |
| | Module2 | KS Atm ACP | | |
| | Module3 | KS Atmm KR* ACP | | |
| RubR2 | 254 | 30S Ribosomal protein modification | 41 | AE008914 |
| RubR3 | 74 | Translation initiation factor | 67 | AP005041 |
| ORF3 | 333 | Acetamidase | 35 | AE005929 |
| ORF4 | 521 | Aminocoumarin ligase | 44 | AF321122 |
| ORF5 | 209 | Methyltransferase | 30 | AE013220 |
| ORF6 | 207 | Methyltransferase | 34 | AE010900 |
| RubK | 386 | AHBA synthase | 75 | AF040570 |
| RubL | 364 | Oxidoreductase | 48 | AAS07754 |
| RubM | 221 | Phosphatase | 60 | AF040570 |
| RubN | 307 | Kinase | 38 | AF040570 |
| RubG1 | 310 | Glycosyltransferase | 41 | CAE17547 |
| ORF7 | 1,096 | Rhs core protein | 41 | AP005021 |
| ORF8 | 280 | SnoK | 24 | AF187532 |
| RubN8 | 424 | Unknown | 61 | AX195934 |
| RubN5 | 411 | Hypothetical, protein | 67 | AX195930 |
| RubN4 | 373 | Aminotransferase (sugar) | 72 | AB103463 |
| RubRg1 | 266 | Regulator | 42 | AB070945 |
| RubF1 | 265 | SnoK | 26 | AF187532 |
| RubF2 | 280 | Quinone oxidoreductase | 39 | AY223810 |
| RubF | 264 | Amide synthase | 38 | AF040570 |
| RubF3 | 428 | Rif20 | 22 | AF040570 |
| RubF4 | 406 | Cytochrome P450 | 39 | AF040570 |
| RubF5 | 557 | Acid AMP ligase | 37 | AF196567 |
| RubF6 | 529 | Aminocoumarin ligase | 41 | AF321122 |
| RubP7 | 218 | F420-dependent NADP reductase | 30 | AL939112 |
| RubP3 | 270 | Hydroxylase | 38 | AF187532 |
| ORF9 | 382 | Unknown | 43 | AL939104 |
| ORF10 | 211 | Unknown (putative reductase) | 46 | AX005171 |
| RubRg2 | 429 | Transcription regulator | 33 | AF040570 |
| ORF11 | 251 | Unknown | 49 | AL939132 |
| RubB | 5,066 | Polyketide synthase | 47 | AF040570 |
| | Module4 | KS ATmm DH KR ACP | | |
| | Module5 | KS ATm DH KR ACP | | |
| | Module6 | KS ATmm DH KR ACP | | |
| RubP1 | 512 | Hydroxyphenylpropionate hydroxylase (Rif19) | 55 | AF040570 |
| RubC1 | 1,317 | CoA ligase | 50 | AF321122 |

**Table 1** continued

| ORFs | Amino acid | Proposed function | % Identity acid | Accession number |
|---|---|---|---|---|
| RubC2 | 409 | Cytochrome P450 | 61 | AF324838 |
| RubC3 | 249 | Oxoacyl-(acyl carrier protein) reductase | 45 | AF321122 |
| RubJ | 151 | aDHQ dehydratase | 73 | AF040570 |
| ORF12 | 260 | Thioesterase | 65 | AF040570 |
| RubP4 | 270 | Hydroxylase | 37 | AF187532 |
| RubG | 350 | aDHQ synthase | 69 | AF040570 |
| RubH | 407 | aDAHP synthase | 63 | AF040570 |
| RubP2 | 400 | Cytochrome P450 | 43 | AB072568 |
| RubG2 | 398 | Glycosyltransferase | 37 | AF497482 |
| RubRg3 | 290 | DnrI | 34 | A43306 |
| RubN7 | 308 | Unknown | 60 | AX196129 |
| RubN6 | 329 | 4-Ketoreductase | 49 | AB088119 |
| RubN3 | 458 | TDP-glucose-2,3-dehydratase | 62 | AF080235 |
| RubN1 | 345 | TDP-glucose synthase | 54 | AJ011500 |
| RubP5 | 530 | Mitomycin resistance (MitR) | 47 | AF127374 |
| RubC4 | 226 | LmbU, | 55 | X79146 |
| RubC5 | 431 | Putative secreted protein, monooxygenase | 33 | AL939127 |
| RubRg4 | 755 | afsR-g | 31 | AB025225 |

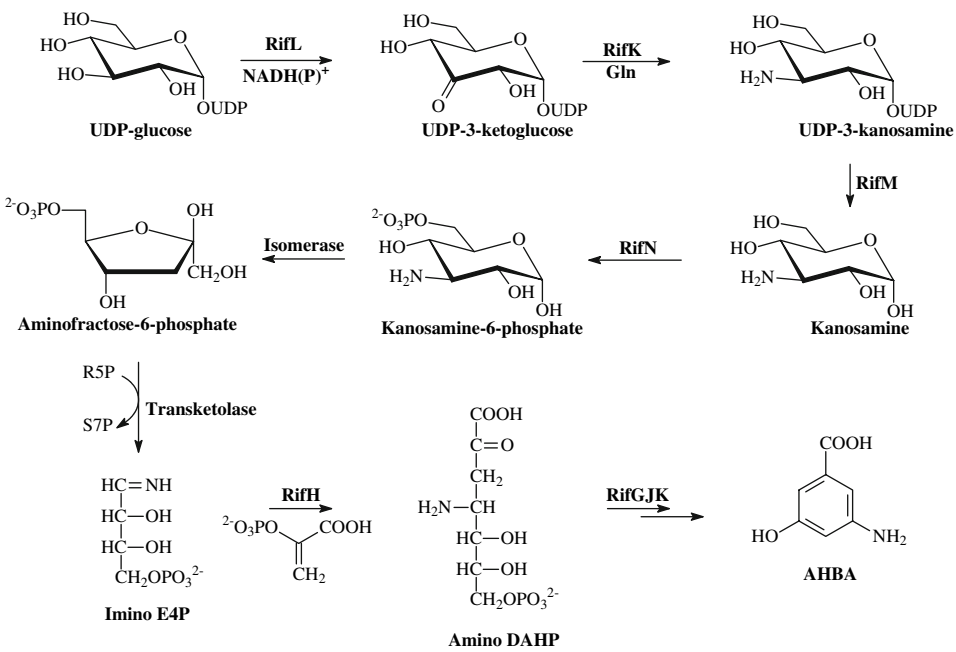


**Fig. 3** Biosynthetic pathway of 3-amino-5-hydroxybenzoic acid (*AHBA*). *DAHP* deoxy-arabinoheptulosonate 7-phosphate; *E4P* erythrose 4-phosphate; *PEP* phosphoenol pyruvate; *UDP* uridine-5′-diphosphate

dipicolinate synthase gene (Daniel and Errington 1993) have not been discovered within the sequenced gene cluster thus far.

## Regulatory and resistance genes

Two translation initiation factors, RubR1 and RubR3, were found within the gene cluster. Considering the fact that rubradirin acts as an inhibitor protein chain initiation, and these genes are expected to exert a resistance function (Reusser 1973). A putative transporter gene was found in the cluster. RubT1 shows homology to ABC transporter ATP-binding proteins. Three putative transcription regulator genes were detected in the cluster. RubRg1 and RubRg2 are similar to the bacterial transcription activators, which include DnrI, RedD, and AfsR, and RubRg3 encodes for a response regulator containing a helix-turn-helix DNA-binding domain.

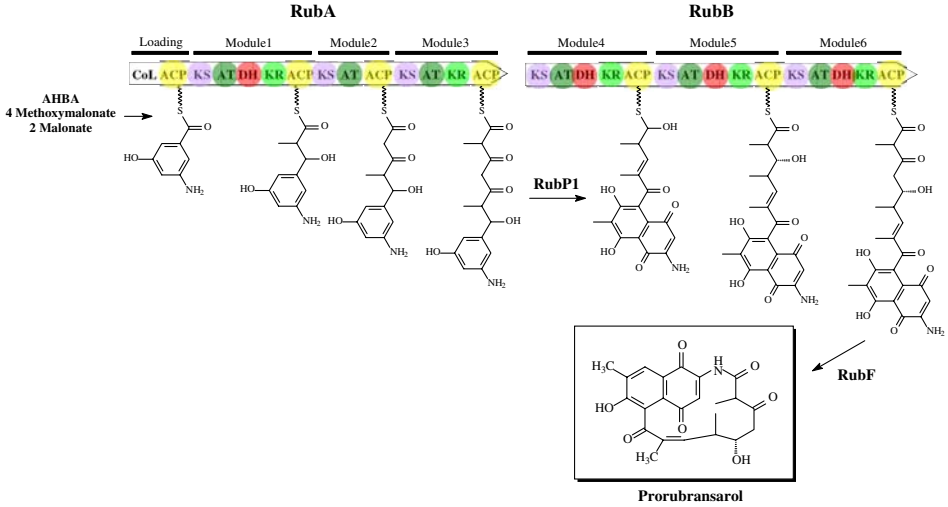


**Fig. 4** Proposed pathway of prorubransarol biosynthesis. Domain organization and intermediates for RubA and RubB are shown at the *top*. Domain designation: *KS* ketosynthase; *AT* acyltransferase; *ACP* acyl carrier protein; *DH* dehydratase; *KR* ketoreductase; *ER* enoyl reductase. DH and KR domains in *bold* are assumed to be inactive. Single enzymatic steps are indicated by the presence of an enzyme name on each *arrow*. *AHBA* 3-amino-5-hydroxybenzoic acid

## Genes for D-rubranitrose biosynthesis

Sequence analysis revealed that the region spanning from RubN1 to N8 is putatively involved in the biosynthesis of TDP-D-rubranitrose. The biosynthetic pathway to D-rubranitrose is thought to require seven steps (Fig. 5, Discussion). These steps may include TDP-D-glucose synthase (RubN1), 4,6-dehydratase (RubN2) (Yoo et al. 2000), 2,3-dehydratase (RubN3) (Maharjan et al. 2003), 3-aminotransferase (RubN4), 4-ketoreductase (RubN6) (Lamichhane, et al. 2006), 3-*C*-methyltransferase (RubN5) and *O*-methyltransferase (RubN7). RubN5 is similar to the 3-*C*-methyltransferases involved in the biosynthesis of L-evernitrose in *Micromonospora carbonacea* (an everninomicin producer) (Staffa et al. 2001) and L-epivancosamine in *Amycolatopsis orientalis* (a chloroeremomycin producer), and exhibits 67 and 62% amino acid sequence identity with the respective 3-*C*-methyltransferases. RubN7 shows a high degree of

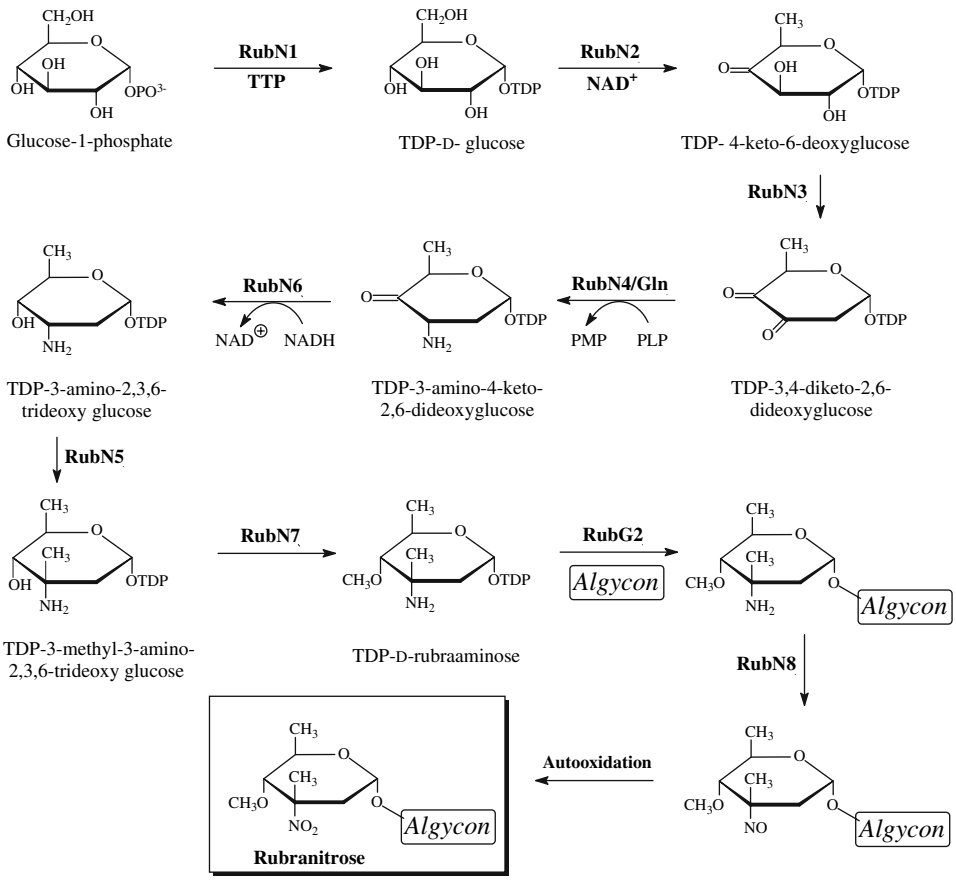


**Fig. 5** Proposed biosynthetic pathway of D-rubranitrose

similarity to the 4-*O*-methyltransferase involved in the biosynthesis of L-evernitrose and *O*-methyl-L-rhodinose in the nanchangmycin producer, *S. nanchangensis* (Sun et al. 2003) however, it does not exhibit any meaningful similarity with any other *O*-methyltransferases associated with deoxysugar pathways. Two glycosyltransferase analogues, RubG1 and G2, were detected in the *rub* gene cluster.

### Inactivation of PKS and *rubG2* Gene

An inactivation experiment was conducted in the rubradirin polyketide gene (*rubA*) by pJC525T to determine whether the gene cluster was responsible for rubradirin biosynthesis. The pJC525T was introduced into the protoplasts of *S. achromogenes*. The selected clones (525T1/11 and 525T1/33), which were the products of homologous recombination, were isolated and analyzed via Southern hybridization with the *Kpn*I/*Bam*HI 2.5 kb probe and the *Bcl*I 1 kb thiostrepton resistance probe from pIJ702. The expected hybridization bands appeared at 3.5 kb following digestion with *Kpn*I/*Bam*HI and at 5.5 kb after digestion with *Bam*HI, which confirmed the integration of the *Bcl*I 1 kb fragment into the chromosomal DNA via double-crossing-over recombination (Fig. 6a, b). The result verified that the expected disruption occurred in the chromosome of *S. achromogenes*.

The pJL0012 was constructed by inserting the thiostrepton marker within the *rubG2* gene in order to inactivate the glycosyltransferase gene (data not shown). The pJL0012 was transferred into *S. achromogenes*. The phenotypic changes in the strains after 5–6 generations of culture at 39°C with continuous resistance against $Ap^r/Ts^r$ ensured that the transformants had undergone a single crossover event, thereby transferring the disrupted allele, coupled with the resistance marker, into the native chromosomal DNA. The allele transformation in the chromosomal DNA was confirmed by using a *rubG2* probe to determine the sizes (7.8 and 7.8/10.3 kb) of the hybridization fragments detected in the *Bam*HI-digested wild-type and mutant-type genomic DNA, respectively (data not shown).

### Analysis of mutants

The *rubA* gene-inactivated mutant (525T1/33) was grown on a plate containing thiostrepton, and was found to have completely lost its ability to produce rubradirin (Fig. 6d) which confirmed that the PKS gene was deactivated. Similarly, the inactivation of *rubG2* and the product analysis of the mutant provided a plausible explanation for the function of the *rubG2* in rubradirin biosynthesis. The mutant (SJK2) clearly showed the absence of rubradirin in comparison to the wild-type (data not shown).

## Discussion

We sequenced and analyzed 105.6 kb of DNA related to rubradirin biosynthesis and knocked out two genes of this gene cluster. Two important factors are essential for the processing of the rubradirin PKS one of which is the origin of the C-4 hydroxyl group of rubransarol and the other is the DH domain functionality of the first module. The hydroxyl group at C-4 may be introduced via prorubransarol hydroxylation, which is a malonyl-CoA-accepting AT domain incorporating methoxymalonate rather than a hydroxymalonate. However, the amino acid sequence analysis of the AT domain in module 5 revealed that it is a typical malonyl-CoA-accepting domain, in which HAFH is one of the consensus amino acid sequences (Kato et al. 2002), which precludes the acceptance of glycolate at this domain (data not shown). FAGH is the consensus amino acid sequence typically found in the glycolate-accepting AT domains in the PKS of ansamycin, geldanamycin, or ansamitocin producers. The consensus sequence of the DH domain, $H(X)_3G(X)_4P$, is not conserved and would fail to function in module 1. The C-13 hydroxyl group in prorubransarol (Fig. 1) is, therefore, spontaneously dehydrated during folding in the PKS, or via some post-PKS reaction, but not by the DH domain in module 1. The dehydrogenase gene, which reduces 1,4-dihydroproansamycin, has not been detected in the rubradirin cluster. Therefore, on the basis of this domain analysis, we suggest that Fig. 4 represents the structure of prorubransarol.

All of the amide synthase genes related to macrolactam formation of ansamycins were found immediately downstream of last module in the ansamycin PKS genes (August et al. 1998; Yu et al. 2002; Rascher et al. 2003) except for *rubF*, which was found 13 kb upstream of *rubB*. The macrolactam of rubransarol is structurally different from those found in ansamycins in that the carbonyl group of the amide is fully reduced. The *rubF* is presumed to be linked to a gene or to genes that affect the reduction of the macrolactam amide bond, and is not believed to be linked to *rubB*. Consistent with this assumption, *rubF2* and *rubF3* exhibit homologies to an alcohol dehydrogenase and the adenylation domain of nonribosomal polypeptide synthetase (NRPS), which is located in the gramidicin biosynthetic gene cluster (Kessler et al. 2004). RubF2 and RubF3 are presumably found within one operon upstream and downstream of *rubF*. Therefore, the C-terminus of the fully extended polyketide chain, which is attached to the conserved Cys-73 residue on RubF (Payton et al. 2001), is thought to be transferred and adenylated by RubF3 and reduced to cyclic amine by RubF2. The manner for the formation of the morpholine ring of rubransarol remains unclear, however, the *rubF4* to *rubF7* and *rubP3* genes might play a role in this process.

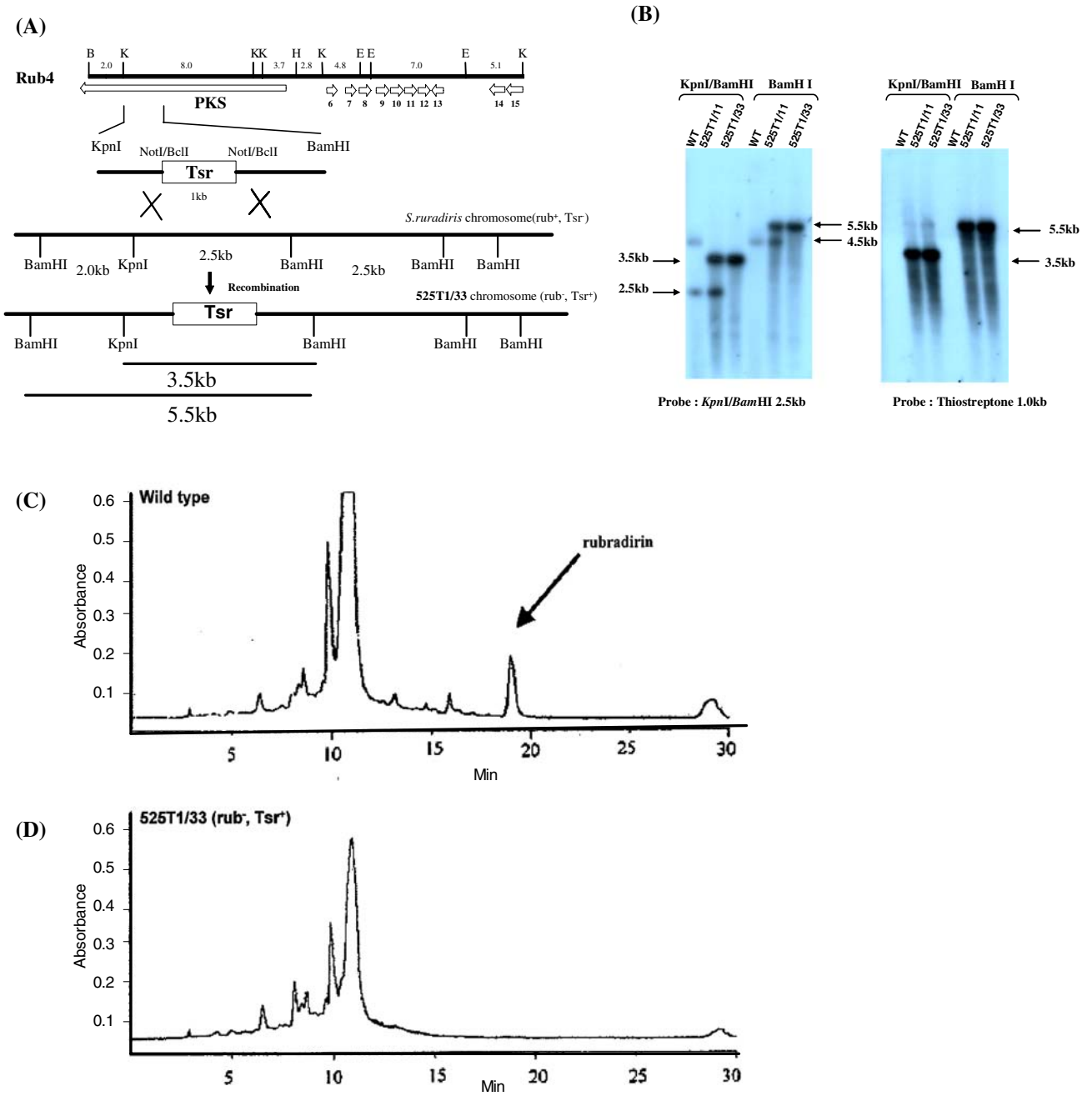


**Fig. 6** Generation of a 525T1/33 mutant via double-crossover integration of pJC525/T **a**, analysis of the chromosomal DNA by Southern blot analysis of wild-type and mutant **b**, HPLC analysis of the rubradirin produced by the wild-type **c** and pJC525/T1/33 mutant **d**

Rubradirin is known to harbor an unusual nitrosugar moiety, which has also been identified in several other natural products, including the everninomicins from *Micromonospora carbonacea*, kijanimicin from *Actinomadura kijaniata*, and decilorubicin from *S. virginiae*. The putative pathway of D-rubranitrose biosynthesis, which was determined based on sequence analysis and experimental evidences is shown in Fig. 5. The conversion of TDP-4-keto-6-deoxyglucose to TDP-3-amino-2,3,6-trideoxyglucose via the consecutive catalysis of 2,3-dehydratase (RubN3), 4-ketoreductase (RubN6), and 3-aminotransferase (RubN4), has been reported in conjunction with the biosynthesis of TDP-L-daunosamine, which is a component of the antiparsitic agent megalomicin (Volchegursky et al. 2000; Maharjan et al. 2003; Lamichhane et al. 2006). RubN5 exhibits a very low degree of similarity to 3-*C*-methyltransferase TylC3 (26% amino acid sequence identity), which is associated

with L-mycarose biosynthesis in tylosin. The minimal homology of RubN5 to TylC3 may be attributable to the opposite stereochemistry exhibited by the 3-*C*-methyl groups in D-rubranitrose and L-mycarose. We putatively allocated RubN8 to amine oxidase. Since the nitro group of D-rubranitrose was found to be an oxidation product of the nitroso group, which is easily oxidized by air (Bannister and Zapotocky 1992), RubN8 is thought to work on the final step of the biosynthesis. It is reasonable to believe that RubN8 should operate after glycosylation of the D-rubraminose on the rubradirin aglycon. The absolute stereochemistry of D-rubranitrose remains somewhat controversial, but the synthesis of D- and L-rubranitrose confirmed that this sugar has been appropriately assigned to the D-series (Brimacombe and Rahman 1983). In accordance with this finding, no 3-epimerase was detected in the rubradirin biosynthetic gene cluster.

The PKS gene-inactivated double crossover mutant was tested via HPLC analysis in order to characterize the production of secondary metabolites. The *rubA* gene-inactivated mutant (525T1/33) was found to have completely lost its ability to produce rubradirin (Fig. 6d), thereby confirming the deactivation of the PKS gene. However, in another case of *rubG2* inactivation, we made little progress in our attempt to construct the double crossover mutant, despite the exhaustive effort by our own and other laboratories. Due to the difficulty in the construction of a double crossover mutant, we have shifted our efforts to the demonstration of the absence of rubradirin from single crossover mutant (SJK2). The inactivation of the *rubG2* was demonstrated by the absence of rubradirin in comparison to the SJK2 and wild-type by HPLC. Moreover, the products of SJK2 exhibited a distinct mass of the rubradirin aglycon ($m/z^+ = 812$, positive mode) and a rubransarol peak ($m/z^+ = 442$, positive mode) based on the results of LC–MS analysis (data not shown). This result clearly indicates that the function of *rubG2* is as a glycosyltransferase in rubradirin biosynthesis. We suggest that this abolition of rubradirin can only be attributed to the polar effect of the integrated plasmid on the genes located upstream of the integration site in the single crossover-type mutants.

Although some functional genes involved in 4-hydroxyaminocoumarin and the dipicolinate synthase gene have yet to be discovered, the availability of the *rub* gene cluster has now set the stage for the investigation of the molecular basis between the other ansamycins, including rifamycins, streptovaricins, tolypomycins, ansamitocin and geldanamycin in hybrid natural product biosynthesis and for the engineering of novel rubradirin analogues by the genetic manipulation of genes governing rubradirin biosynthesis.

**Acknowledgments** This study was supported by the 21C Frontier Microbial Genomics and Application Center Program, Ministry of Science & Technology (Grant MG02-0301-004-2-3-1), Republic of Korea. The standard rubradirin sample was supplemented by Pfizer Global Research and Development Strategic Alliances, Eastern Point Road, Gorton, Connecticut.

## References

August PR, Tang L, Yoon YJ, Ning S, Muller R, Yu TW, Taylor M, Hoffmann D, Kim CG, Zha-ng X, Hutchinson CR, Floss HC (1998) Biosynthesis of the ansamycin antibiotic rifamycin: deductions from the molecular analysis of the *rif* biosynthetic gene cluster of *Amycolatopsis mediterranei* S699. Chem Biol 5:69–79

Bannister B, Zapotocky BA (1992) Protorubradirin, an antibiotic containing a C-nitroso-sugar fr-agment, is the true secondary metabolite produced by *Streptomyces achromogenes* var. *rubradiris*. Rubradirin, described earlier, is its photo-oxidation product. J Antibiot (Tokyo) 45:1313–1324

Bhuyan BK, Owen SP, Dietz A (1964) Rubradirin, a new antibiotic. I. Fermentation and biological properties. Antimicrobi Agents Chemother 10:91–96

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

Brimacombe JS, Rahman KMM (1983) Synthesis of L-rubranitrose (2,3,6-trideoxy-3-*C*-methyl-4 -*O*-methyl-3-nitro-L-*xylo*-hexopyranose) and naturally occuring D-enantiomer. Carbohydr Res 114:C1–C2

Chen H, Thomas MG, Hubbard BK, Losey HC, Walsh CT, Burkart MD (2000) Deoxysugars in glycopeptide antibiotics: enzymatic synthesis of TDP-L-epivancosamine in chloroeremomycin biosynthesis. Proc Natl Acad Sci USA 97:11942–11947

Chen H, Walsh CT (2001) Coumarin formation in novobiocin biosynthesis: beta-hydroxylation of the aminoacyl enzyme tyrosyl-*S*-NovH by a cytochrome P450 NovI. Chem Biol 8:301–312

Daniel RA, Errington J (1993) Cloning, DNA sequence, functional analysis and transcriptional regulation of the genes encoding dipicolinic acid synthetase required for sporulation in *Bacillus subtilis*. J Mol Biol 232:468–483

Eustaquio AS, Luft T, Wang ZX, Gust B, Chater KF, Li SM, Heide L (2003) Novobiocin biosynthesis: inactivation of the putative regulatory gene *novE* and heterologous expression of genes involved in aminocoumarin ring formation. Arch Microbiol 180:25–32

Guo J, Frost JW (2002) Kanosamine biosynthesis: a likely source of the aminoshikimate pathway's nitrogen atom. J Am Chem Soc 124:10642–10643

He XM, Liu HW (2002) Formation of unusual sugars: mechanistic studies and biosynthetic applications. Annu Rev Biochem 71:701–754

Kato Y, Bai L, Xue Q, Revill WP, Yu TW, Floss HG (2002) Functional expression of genes involved in the biosynthesis of the novel polyketide chain extension unit, methoxymalonyl-acyl carrier protein, and engineered biosynthesis of 2-desmethyl-2-methoxy-6-deoxyerythronolide B. J Am Chem Soc 124:5268–5269

Kessler N, Schuhmann H, Morneweg S, Linne U, Marahiel MA (2004) The linear pentadecapeptide gramicidin is assembled by four multimodular nonribosomal peptide synthetases that comprise 16 modules with 56 catalytic domains. J Biol Chem 279:7413–7419

Kieser T, Mervyn JB, Buttner MJ, Chater KF, Hoopwood DA (2000) Practical Streptomyces genetics. John Innes Centre, Norwich

Kim C-G, Yu TW, Fryhle CB, Handa S, Floss HG (1998) 3-Amino-5-hydroxybenzoic acid synthase, the terminal enzyme in the formation of the precursor of the mC7N units in rifamycin and related antibiotics. J Biol Chem 13:6030–6040

Lamichhane J, Liou K, Lee HC, Kim CG, Sohng JK (2006) Functional characterization of ketoreductase (*rubN6*) and aminotransferase (*rubN4*) genes in the gene cluster of *Streptomyces achromogenes* var. *rubradiris*. Biotechnol Lett 28:545–553

Maharjan J, Liou K, Lee HC, Kim CG, Lee JJ, Yoo JC, Sohng JK (2003) Functional identificati-on of *rub52* gene involved in the biosynthesis of rubradirin. Biotechnol Lett 25:909–915

Marshall VP, McWethy SJ, Sirotti JM, Cialdella JI (1990) The effect of neutral resins on the fermentation production of rubradirin. J Ind Microbiol 5:283–287

Payton M, Mushtaq A, Yu TW, Wu LJ, Sinclair J, Sim E (2001) Eubacterial arylamine *N*-acetylt-ransferases— identification and comparison of 18 members of the protein family with conserved active site cysteine, histidine and aspartate residues. Microbiology 147:1137–1147

Rascher A, Hu Z, Viswanathan N, Schirmer A, Reid R, Nierman WC, Lewis M, Hutchinson CR (2003) Cloning and characterization of a gene cluster for geldanamycin production in *Streptomyces hygroscopicus* NRRL 3602. FEMS Microbiol Lett 218:223–230

Reusser F (1973) Rubradirin, an inhibitor of ribosomal polypeptide biosynthesis. Biochemistry 12:1136–1142

Reusser F (1979) Inhibition of ribosomal and RNA polymerase functions by rubradirin and its aglycone. J Antibiot (Tokyo) 32:1186–1192

Russer F, Bannister B, Tarpley WG, Althaus IW, Zapotocky BA (1988) Rubradirin derivatives for treatment of HIV infection. Patent Cooperation Treaty W08808707

Sambrook J, Russell DW (2001) Molecular cloning, a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Sohng JK, Oh TJ, Lee JJ, Kim CG (1997) Identification of a gene cluster of biosynthetic genes of rubradirin substructures in *S. achromogenes* var. *rubradiris* NRRL3061. Mol Cells 7:674–681

Staffa A, Zazopoulos E, Mercure S, Nowacki P (2001) Genetic locus for everninomicin biosynth-esis. Patent WO 0155180-A49

Sun Y, Zhou X, Dong H, Tu G, Wang M, Wang B, Deng Z (2003) A complete gene cluster from *Streptomyces nanchangensis* NS3226 encoding biosynthesis of the polyether ionophore nanchangmycin. Chem Biol 10:431–441

Tang L, Yoon YJ, Choi CY, Hutchinson CR (1998) Characterization of the enzymatic domains in the modular polyketide synthase involved in rifamycin B biosynthesis by *Amycolatopsis mediterranei*. Gene 216:255–265

Volchegursky Y, Hu Z, Katz L, McDaniel R (2000) Biosynthesis of the anti-parasitic agent megalomicin: transformation of erythromycin to megalomicin in *Saccharopolyspora erythraea*. Mol Microbiol 37:752–762

Wherli W (1977) Topics in current chemistry. Ansamycins. chemistry, biosynthesis and biological activity, vol 72. Berlin Heidelberg, pp 21–49

Yoo JC, Choi JY, Yun SC, Kim CG, Lee JJ, Kim SB, Sohng JK (2000) Sequence analysis and expression of *rubN2* as dTDP-Glucose 4,6-dehydratase gene cloned from *Streptomyces achromogenes* var. *rubradiris* NRRL 3061, rubradirin producer. J Biochem Mol Biol Biophys 4:313–321

Yu TW, Bai L, Clade D, Hoffmann D, Toelzer S, Trinh KQ, Xu J, Moss SJ, Leistner E, Floss HG (2002) The biosynthetic gene cluster of the maytansinoid antitumor agent ansamitocin from *Actinosynnema pretiosum*. Proc Natl Acad Sci USA 99:7968–7973