

ORIGINAL ARTICLE

Prodigiosin biosynthesis gene cluster in the roseophilin producer *Streptomyces griseoviridis*

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Streptomyces griseoviridis 2464-S5 produces prodigiosin R1, a tripyrrole antibiotic, and roseophilin, a structurally related compound containing two pyrrole and one furan rings. A gene cluster for the biosynthesis of a prodigiosin was identified in *S. griseoviridis*. The cluster consisted of 24 open reading frames, including 21 genes (*rphD*–*rphZ*) homologous to prodigiosin biosynthesis genes in the *red* cluster in *Streptomyces coelicolor* A3(2). The expression of *rphN* in *S. coelicolor* lacking *redN* restored the production of prodigiosin.

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INTRODUCTION

Several tripyrrole antibiotics are known to belong to the prodigiosin family.^{1–4} *S. griseoviridis* 2464-S5 produces prodigiosin R1⁵ and roseophilin,⁶ a structurally related compound containing two pyrrole and one furan rings, as shown in Figure 1. Roseophilin is considered to be biosynthesized partially with the same pathway as prodigiosin R1. Prodigiosin-family biosynthesis genes have been reported to exist in the *pig* gene clusters in *Serratia marcescens* ATCC 274 and *Serratia* sp. ATCC 39006 (which make prodigiosin itself),⁷ in the *red* gene cluster in *S. coelicolor* A3(2) (which makes undecylprodiginine)⁸ and in the *hap* gene cluster in *Hahella chejuensis* KCTC 2396 (which makes prodigiosin itself).⁹ PCR amplification of *S. griseoviridis* genomic DNA has identified genes homologous to *redH*, *redM* and *redW*, involved in the biosynthesis of undecylprodiginine in *S. coelicolor* (Figure 2).⁵ We report herein the cloning and characterization of the *rph* gene cluster for prodigiosin biosynthesis from *S. griseoviridis* 2464-S5.

RESULTS

Amplification of prodigiosin biosynthesis genes from *S. griseoviridis*

In the biosynthesis of prodigiosin, the *redN/pigH* gene product is involved in the formation of the central pyrrole moiety (Figure 2). As roseophilin contains a furan ring in place of the central pyrrole ring in prodigiosins, *redN/pigH* might be characteristic of the prodigiosin biosynthesis. A pair of primers was designed from the conserved amino acid (aa) sequences of *redN/pigH* in the three prodigiosin producers, *S. coelicolor* A3(2), *Serratia marcescens* ATCC 274 and *Serratia* sp. ATCC 39006.^{7,8} PCR amplification of *S. griseoviridis* genomic DNA gave a *redN/pigH*-like gene fragment. Nucleotide sequencing and homology search showed that the fragment shared amino acid identity of 84% with *redN*.

The prodigiosin biosynthesis genes, *redM* and *redW*, encode L-prolyl-AMP ligase and L-prolyl-PCP dehydrogenase, respectively (Figure 2). These two enzymes have been reported to be commonly involved in the biosynthesis of pyrrole-containing antibiotics.¹⁰ PCR was carried out using several primers designed from the conserved amino acid sequences of L-prolyl-AMP ligase and L-prolyl-PCP dehydrogenase genes in the coumermycin A₁ producer, *Streptomyces rishiriensis* DSM 40489 (*proB* and *proA*),¹¹ in the pyrrolomycin producers, *Streptomyces vitaminophilum* ATCC 31673 (*pyr8* and *pyr7*) and *Streptomyces* sp. Strain UC 11065 (*dox8* and *dox7*)¹² and in the prodigiosin producer, *S. coelicolor* A3(2) (*redM* and *redW*). DNA sequencing of the PCR products identified single genes encoding L-prolyl-AMP ligase and L-prolyl-PCP dehydrogenase in *S. griseoviridis*.

Southern hybridization of genomic DNA with prodigiosin biosynthesis genes

Southern blot analysis was carried out on *S. griseoviridis* genomic DNA to identify *redH*-, *redM*-, *redN*- and *redW*-like genes. Digested genomic DNA with *Bam*HI in agarose gel was hybridized with the PCR products as probes. Each single band was detected by Southern hybridization as shown in Figure 3, suggesting that the strain contained a single set of genes for prodigiosin/roseophilin biosynthesis.

Cloning of a prodigiosin biosynthesis gene cluster from *S. griseoviridis*

A cosmid clone was selected by colony hybridization and Southern blot analysis from a cosmid library using the *redW*-like gene fragment as a probe. Nucleotide sequencing identified genes homologous to *redY*, *redX* and *redD*, as well as *redW*, in a *Sac*I 6.0-kbp fragment of the cosmid (Figure 4). Further Southern hybridization was carried out on

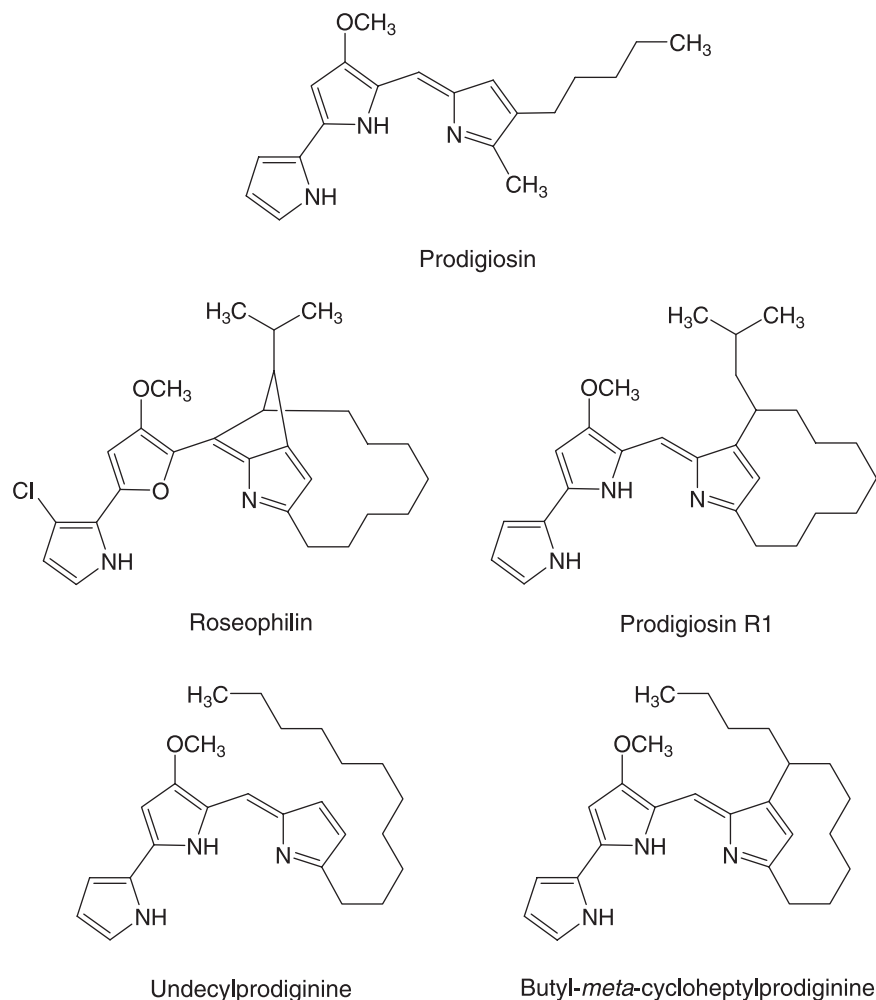


Figure 1 Structures of roseophilin and prodigiosins.

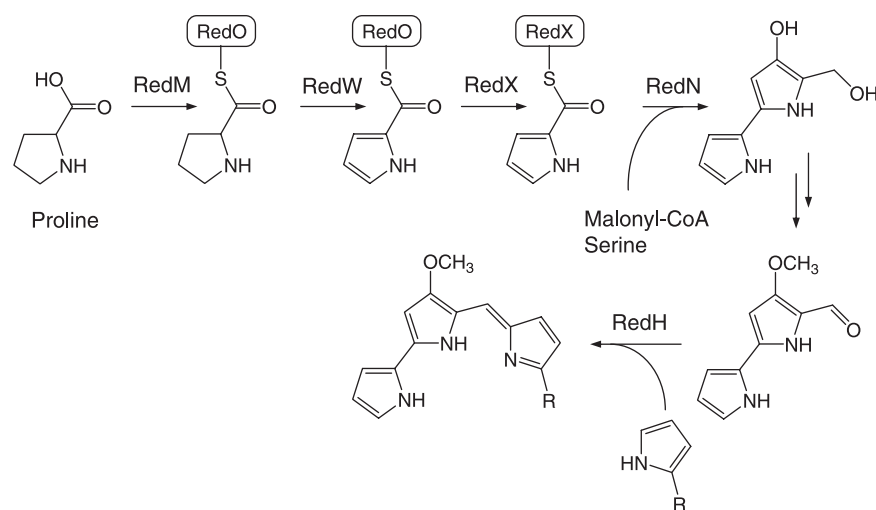


Figure 2 Proposed pathway of prodigiosin biosynthesis.

various cosmid fragments using the *redH*-, *redM*- and *redN*-like gene fragments as probes. Selected cosmid fragments were sequenced to identify a gene cluster containing 24 open reading frames (ORFs) as

shown in Figure 4. Homology search showed that 21 of the 24 ORFs were homologous to the *red* genes involved in the biosynthesis of undecylprodiginine and butyl-*meta*-cycloheptylprodiginine in

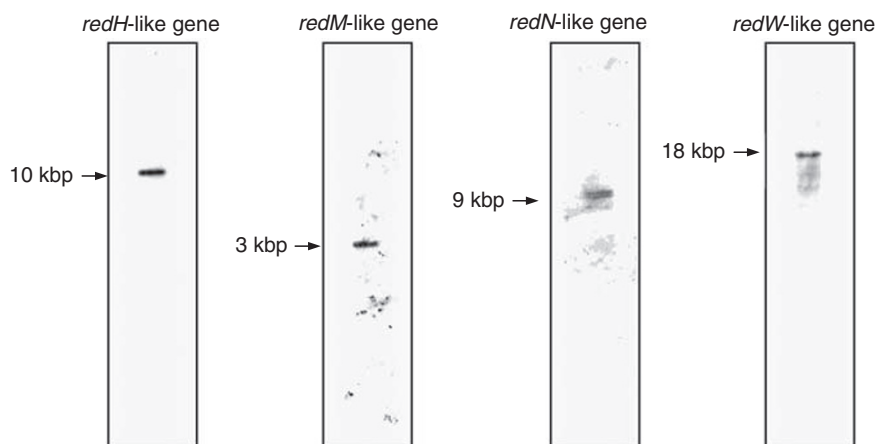


Figure 3 Southern blot analysis of *S. griseoviridis* genomic DNA.

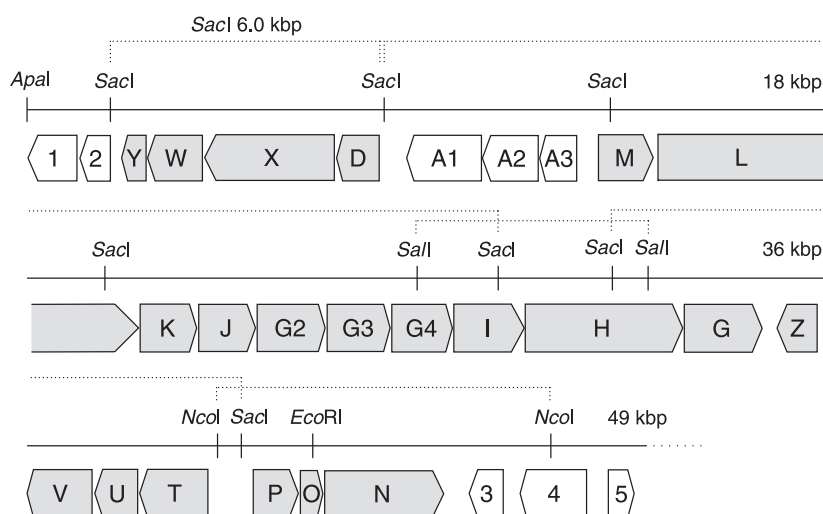


Figure 4 Prodigiosin biosynthesis gene cluster (*rph* cluster) from *S. griseoviridis*.

S. coelicolor A3(2),⁸ and the corresponding genes in *S. griseoviridis* were designated as *rphD*–*rphZ* (Table 1). In the cluster, sequence homology of *rphA1* and *rphA2* to ABC-transporter genes assigned them to self-resistance genes. A regulatory gene, *rphA3*, was also identified from sequence homology to a transcriptional regulator gene in *Mycobacterium ulcerans* (Table 1).

Expression of *rphN* in *S. coelicolor* lacking *redN*

To establish the function of the *rph* genes, we disrupted the *redN* gene in *S. coelicolor* A3(2). The disruption plasmid, pRedN-DIS, was constructed (Figure 5) and introduced into *S. coelicolor*. Three colorless colonies were selected on R2YE plates and the *redN* gene disruption was confirmed by PCR analysis using their genomic DNAs as templates.

The *rphN* expression vector, pWHM-*rphN*, was constructed and introduced into *S. coelicolor* lacking *redN* (*S. coelicolor* Δ *redN*). *S. coelicolor* Δ *redN*, harboring pWHM-*rphN* or an empty vector (pWHM860), and the wild-type strain were cultivated and the mycelial extracts were analyzed by HPLC. The *redN* disruptant expressing *rphN* produced undecylprodiginine, a major prodigiosin from the wild type (Figure 6), thereby showing that *rphN* can act as a prodigiosin biosynthesis gene in *S. coelicolor*.

DISCUSSION

The *rph* gene cluster consisted of 21 genes homologous to prodigiosin biosynthesis genes in *S. coelicolor*, two self-resistance genes and a regulatory gene. The putative functions and locations of *orf1* (DNA helicase) and *orf3* (RNA methyltransferase) suggested that *orf1*, *orf2*, *orf3*, *orf4* and *orf5* are excluded from the biosynthesis gene cluster (Table 1 and Figure 4). The *rph* cluster did not contain genes homologous to *redC*, *redE*, *redF*, *redQ*, *redR* or *redS* in *S. coelicolor* (Figure 7). Among them, *redQ* and *redR* have been reported to be involved in the biosynthesis of an alkyl side chain and not to be essential for the biosynthesis of undecylprodiginine.¹³ Although the *redF*-homologous gene, *pigN*, was involved in *O*-methylation, two *pigN* mutants reduced, but retained, prodigiosin productivity.¹⁴ No role has been assigned to *redS* in the prodigiosin pathway and only the 146 aa *N*-terminal region of PigB (671 aa) shows sequence similarity with RedS.⁷ In *Serratia* sp., PigB catalyzes the formation of 2-methyl-3-*n*-amylpyrrole (MAP), which is replaced by 2-undecylpyrrole in *S. coelicolor*.¹⁴ No role has been assigned to *redC* or to *redE*, and no homologous genes have been found in the *pig* cluster in *Serratia* sp.⁷ These findings suggest that these genes are non-essential for the biosynthesis of prodigiosins, and the *rph* gene cluster identified here contains all the genes for prodigiosin biosynthesis in

Table 1 Deduced functions of gene products in the *rph* cluster

Gene products	Homologous protein	Identity (%)
Orf1	ATP-dependent DNA helicase of <i>Streptomyces avermitilis</i>	77
Orf2	Uncharacterized protein of <i>S. avermitilis</i>	47
RphY	Hypothetical protein (RedY)	55
RphW	L-Prolyl-PCP dehydrogenase (RedW)	69
RphX	β -Ketomristoyl-ACP synthase (RedX)	47
RphD	Transcriptional regulator (RedD)	61
RphA1	ABC-transporter of <i>Mycobacterium ulcerans</i>	46
RphA2	ABC-transporter of <i>M. ulcerans</i>	57
RphA3	Transcriptional regulator of <i>M. ulcerans</i>	40
RphM	L-Prolyl-AMP ligase (RedM)	54
RphL	Polyketide synthase (RedL)	54
RphK	Oxidoreductase (RedK)	68
RphJ	Thioesterase (RedJ)	58
RphG2	Oxygenase (RedG)	51
RphG3	Oxygenase (RedG)	39
RphG4	Oxygenase (RedG)	44
RphI	O-methyltransferase (RedI)	59
RphH	Pyrrrole-condensing enzyme (RedH)	59
RphG	Oxygenase (RedG)	57
RphZ	Response regulator (RedZ)	50
RphV	Dehydrogenase (RedV)	42
RphU	Phosphopantetheinyl transferase (RedU)	46
RphT	Hypothetical protein (RedT)	63
RphP	3-Oxoacyl-ACP synthase (RedP)	58
RphO	Peptidyl carrier protein (RedO)	75
RphN	4-Hydroxy-2,2'-bipyrrrole-4-methanol synthase (RedN)	78
Orf3	RNA methyltransferase of <i>Brucella abortus</i>	31
Orf4	Uncharacterized protein of <i>Streptomyces coelicolor</i>	54
Orf5	Integral membrane protein of <i>S. coelicolor</i>	63

S. griseoviridis. Moreover, primers designed from *redS/PigB* and *redF/pigN* did not amplify *S. griseoviridis* genomic DNA (data not shown).

The *rph* cluster included four putative oxygenase genes (*rphG*, *rphG2*, *rphG3* and *rphG4*) homologous to *redG*, which was assumed to catalyze the cyclization of an alkyl side chain for the biosynthesis of butyl-*meta*-cycloheptylprodiginine.⁸ The same function is required by the high-sequence similarity of *rphG* and *redG* and their locations (Table 1 and Figure 7). The difference in the number and position of carbon-carbon bridges between prodigiosin R1 and roseophilin might be explained by the presence of multiple oxygenase genes.

The *rph* cluster did not contain biosynthesis genes characteristic of roseophilin. Such genes are involved in the furan ring formation and chlorination. The PCR and Southern hybridization analyses, however, detected single genes homologous to *redH*, *redM* and *redW*, which are considered to be common to both prodigiosin and roseophilin biosynthesis. These results suggested the existence of another gene cluster to complement the biosynthesis of roseophilin. Search for the second gene cluster for the roseophilin biosynthesis is in progress.

METHODS

Bacterial strains, plasmids and culture conditions

S. griseoviridis 2464-S5 and *S. coelicolor* A3(2) were used as sources of total DNA for the cloning experiment. Media and growth conditions for *S. griseoviridis* were described earlier.⁶ Prodigiosin production by *S. coelicolor* was assessed on R2YE plates.¹⁵ Yeast extract malt extract (YEME) liquid culture was used for genomic DNA isolation and for making protoplasts from *S. coelicolor*.¹⁵ *Escherichia coli* XL1-blue MRF⁺ and the plasmids, pGEM-5Z,

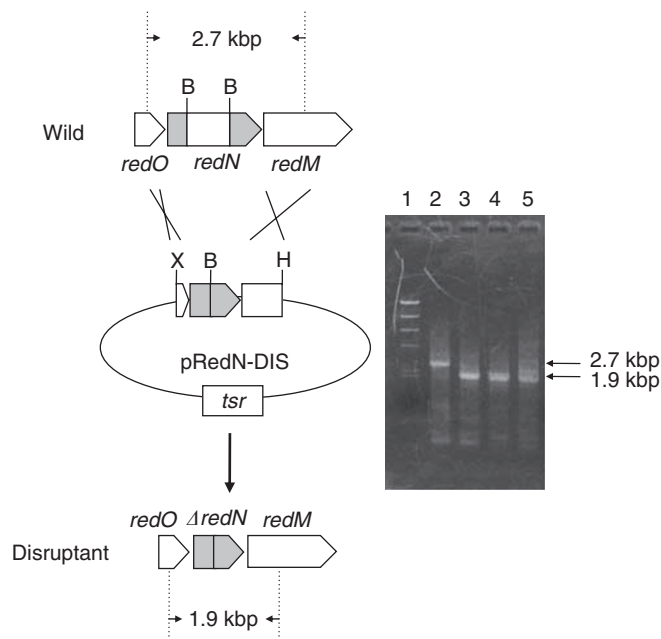


Figure 5 Disruption of the *redN* gene in *S. coelicolor*. Molecular marker (lane 1), PCR product from the wild-type strain (lane 2) and PCR products from the *redN* disruptants (lane 3–5). Amplified fragments are shown schematically. Arrows indicate primers used for PCR analysis. B: *Bgl*II site. X: *Xba*I site. H: *Hind*III site.

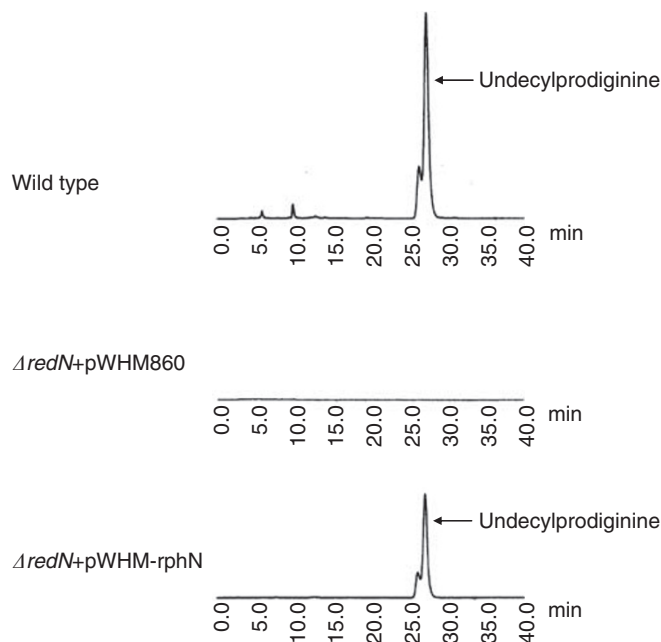


Figure 6 HPLC analysis of prodigiosins produced by the *redN* disruptant expressing *rphN*.

pGEM-7Z, pGEM-11Z, pUC118 and pUC119 were used for sequencing analyses.

DNA isolation and manipulation

All restriction enzymes, T4 DNA ligase and calf intestinal alkaline phosphatase were obtained from Nippon Gene (Toyama, Japan). Transformation of *E. coli*

with plasmid DNA by electroporation was carried out under standard conditions using a Gene Pulser II electroporation system (Bio-Rad, Hercules, CA, USA). Other general procedures were carried out as described by Sambrook *et al*.¹⁶

Amplification and cloning of prodigiosin biosynthesis genes

A *redN*-like gene fragment was amplified using *S. griseoviridis* genomic DNA and a pair of primers (5'-GAYGGSGTSTTYWSSATGCAYGG-3' and 5'-SGGRWASACSGTYTGRCA-3'). The reaction mixture consisted of 1× GC buffer I (TaKaRa, Kyoto, Japan), 0.4 mM dNTP (deoxyribonucleotide triphosphate) mixture, 2 μM of each primer, 0.5 ng μl⁻¹ genomic DNA and 0.05 U μl⁻¹ *LA Taq* DNA polymerase (TaKaRa). After heating at 95 °C for 4 min, PCR was carried out for 30 cycles (95 °C 1 min, 50 °C 30 s, 72 °C 45 s). After purification with QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), the PCR products were linked with pGEM-T Easy Vector (Promega, Madison, WI, USA) using a T4 DNA ligase. *E. coli* was transformed with the plasmid DNA and selected clones were used for sequencing analysis.

Three sets of primers (N1: 5'-CTSTACACSTCSGGSWSSACSGG-3'; C1: 5'-GTGCASACGTTSGTCTCSGTSGG-3'; C2: 5'-AGSRCGASGGACSGARTACCA-3'; C3: 5'-AGSRCGCTSGGSACSGARTACCA-3') were used to amplify 1-prolyl-AMP ligase gene fragments. Various pairs of primers (N1: 5'-TGGGAGMGRSCTGCCT-3', N2: 5'-CAGGCSRTSTCSCACMGSM-3', N3: 5'-CAGGCSRTSAGCCACMGSM-3'; C1: 5'-ATSTCSSWSGTSCCSGAGAADAT-3', C2: 5'-ATSTCSSWSGTSCCGCTGAADAT-3', C3: 5'-ATSTCSKTSGTSCCSGAGAADAT-3', C4: 5'-ATSTCSKTSGTSCCGCTGAADAT-3') were used for the amplification of 1-prolyl-PCP dehydrogenase gene fragments. PCR and cloning were carried out as described above.

Southern blot analysis of genomic DNA

Gene fragments homologous to *redH*, *redM* and *redW* have been amplified earlier by PCR from the roseophilin producer.⁵ DNA fragments used as probes were labeled using the AlkPhos Direct labeling kit (GE Healthcare, Piscataway, NJ, USA). *S. griseoviridis* genomic DNA was digested with *Bam*HI and run on a 0.7% agarose gel. The DNA was transferred to nylon membranes and fixed. After the membranes were incubated with probes at 65 °C for 16 h, they were washed and then developed using the ECF detection kit (GE Healthcare). Images were obtained with an FLA2000 image reader (Fujifilm, Tokyo, Japan).

Cloning of a prodigiosin biosynthesis gene cluster

A cosmid library was constructed from the roseophilin producer. Genomic DNA of *S. griseoviridis* 2464-S5 was partially digested with *Sau*3AI and ligated to the pWE15 cosmid vector (Stratagene, La Jolla, CA, USA). The ligated DNAs were packaged using Gigapack III XL Packaging Extract (Stratagene) and used to infect *E. coli* XL1-blue MRF. *E. coli* colonies obtained by transfection with the cosmids were then screened for the presence of a *redW*-like gene by colony hybridization, using the PCR product labeled with the AlkPhos Direct labeling kit (GE Healthcare) as a probe. Selected positive clones were subsequently analyzed by Southern hybridization with the same probe, and cosmid 31 was unambiguously found to contain the *redW*-like sequence. A *Sac*I

6.0-kbp fragment of the cosmid was sequenced to identify genes homologous to *redD*, *redX*, *redW* and *redY*. To isolate the biosynthesis gene cluster, cosmids were selected using *redM*-, *redH*- and *redN*-like genes as probes, and DNA sequencing was carried out using a *Sac*I 21.0-kbp fragment of cosmid16, a *Sall* 5.0-kbp fragment of cosmid4, a *Sac*I 8.0-kbp fragment of cosmid10 and a *Nco*I 7.0-kbp fragment of cosmid11 (Figure 4).

Gene disruption in *S. coelicolor*

The disruption plasmid, pWHM3-DIS, was based on pWHM3, an *E. coli*/*Streptomyces* shuttle vector (7.2 kbp) containing a thiostrepton-resistant gene.¹⁵ pWHM3 was digested with *Bcl*I and a 1.4-kbp fragment containing a part of the replication region in *Streptomyces* was removed. The remaining two *Bcl*I fragments (4.7 kbp and 1.1 kbp) were ligated to construct pWHM3-DIS.

The *redN* gene was disrupted by a double crossover event (Figure 5). A 2.7 kbp *Xba*I/*Hind*III fragment of *redN* contained both the upstream and downstream regions. The *redN* gene fragment was amplified by PCR with *S. coelicolor* genomic DNA and one set of primers with an additional restriction site (5'-TGCTCTAGATTTCGACCTCGTCCACTACCTGCAG-3' and 5'-ACC AAGCTTGTGCGCCGGGGTGAGGGAGGGCAG-3'). The PCR product and *Xba*I/*Hind*III-digested pGEM-11Z were ligated and introduced into *E. coli*. A plasmid carrying the appropriate DNA fragment was selected. After sequence confirmation, the plasmid was digested with *Bgl*II. A 0.7 kbp *Bgl*II DNA fragment in the *redN* gene was removed and the remaining 5.0-kbp fragment containing pGEM-11Z was self-ligated. A 2.0 kbp *Xba*I/*Hind*III fragment obtained from the plasmid was subcloned into the same site of pWHM3-DIS to give pRedN-DIS.

The disruption plasmid, pRedN-DIS, was introduced into *S. coelicolor*, and thiostrepton-resistant colonies were selected. After the selected colonies were cultivated for 3 days, an aliquot of the culture was plated on ATCC medium #5 plates to obtain spores. Diluted spores were plated on ATCC medium #5 plates and thiostrepton-sensitive colonies were selected. Genomic DNA was prepared from the transformant and subjected to PCR analysis with the same primers that were used to amplify a 2.7 kbp DNA fragment of the *redN* gene.

Expression of *rphN* in the *redN* disruptant

To express the *rphN* gene in the *redN* disruptant, *S. coelicolor* $\Delta redN$, the *rphN*-carrying plasmid, pWHM-rphN, was constructed. To obtain the entire *rphN* gene, PCR amplification was carried out under a standard condition with KOD-DNA polymerase (Toyobo, Osaka, Japan) using a pair of primers with an additional restriction site (5'-TGCTCTAGAATGACCCACATCATGACC-GACCGT-3' and 5'-ACCAAGCTTTCAGGCCGGATCGAGGGGGACGCC-3'). After sequence confirmation, the *Xba*I/*Hind*III DNA fragment was inserted into the same sites of pWHM860¹⁷ to give pWHM-rphN, in which the *rphN* gene was expressed under the control of the *ermE** promoter.

pWHM-rphN was introduced into *S. coelicolor* $\Delta redN$, and the transformants were cultivated at 27 °C for 6 days on a rotary shaker in 500-ml Erlenmeyer flasks containing 100 ml of production medium. The medium

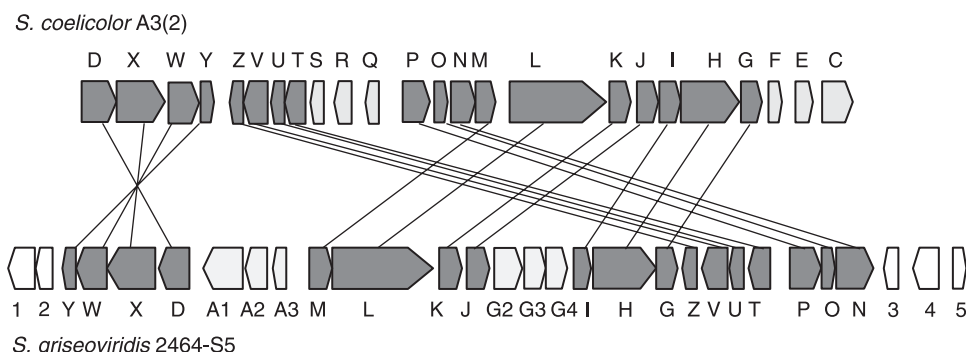


Figure 7 Comparison of the prodigiosin biosynthesis gene clusters between *S. coelicolor* (*red*) and *S. griseoviridis* (*rph*).

contained sucrose 103 g, glucose 10 g, yeast extract (Difco, Detroit, MI, USA) 5 g, Casamino acids (Difco) 0.1 g, sodium hydrogen L-glutamate monohydrate 3 g, K₂SO₄ 0.25 g, MgCl₂·6H₂O 10.12 g, NaNO₃ 0.06 g, TES (*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid) buffer 5.73 g, 0.5 % KH₂PO₄ 10 ml, 5 M CaCl₂ 4 ml, 1 M NaOH 7 ml and a trace element solution of 2 ml in 1000 ml distilled water (pH 7.2). The trace element solution consisted of ZnCl₂ 0.004%, FeCl₂·6H₂O 0.02%, CuCl₂·2H₂O 0.001%, MnCl₂·4H₂O 0.001%, Na₂B₄O₇·10H₂O 0.001% and (NH₄)₆Mo₇O₂₄·4H₂O 0.001%. The fermentation broth (100 ml) was centrifuged and mycelium was extracted with acetone. After evaporation, an aqueous concentrate was extracted with ethyl acetate. The extract was dissolved in methanol and the productivity of prodigiosins was examined by reversed-phase HPLC (Senshu Pak PEGASIL ODS 4.6 i.d.×250 mm, Senshu Scientific, Tokyo, Japan) with methanol–acetonitrile–trifluoroacetic acid–water (80:3:0.15:17) at a flow rate of 1.0 ml min⁻¹. The corresponding peak was detected at 530 nm.

Nucleotide sequence and accession number

DNA sequencing was carried out with an automatic DNA sequencer (3100 Genetic Analyzer, Applied Biosystems, Foster City, CA, USA). Nucleotide sequences reported here have been deposited in the GenBank, DDBJ and EMBL databases under accession number AB469822 (*rph* gene cluster).

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