Cloning and characterization of the goadsporin biosynthetic gene cluster from *Streptomyces* sp. TP-A0584

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The biosynthetic gene cluster of goadsporin, a polypeptide antibiotic containing thiazole and oxazole rings, was cloned from *Streptomyces* sp. TP-A0584. The cluster contains a structural gene, *godA*, and nine *god* (*goadsporin*) genes involved in post-translational modification, immunity and transcriptional regulation. Although the gene organization is similar to typical bacteriocin biosynthetic gene clusters, each goadsporin biosynthetic gene shows low homology to these genes. Goadsporin biosynthesis is initiated by the translation of *godA*, and the subsequent cyclization, dehydration and acetylation are probably catalysed by *godD*, *godE*, *godF*, *godG* and *godH* gene products. *godI* shows high similarity to the 54 kDa subunit of the signal recognition particle and plays an important role in goadsporin immunity. Furthermore, four goadsporin analogues were produced by site-directed mutagenesis of *godA*, suggesting that this biosynthesis machinery is used for the heterocyclization of peptides.

INTRODUCTION

Goadsporin, a secondary metabolite of *Streptomyces* sp. TP-A0584, is a 19 aa polypeptide containing four oxazole and two thiazole rings derived from serine, threonine or cysteine, and two molecules of dehydroalanine derived from serine (Fig. 1).

Goadsporin promotes secondary metabolism and morphogenesis at low concentrations and induces growth inhibition at high concentrations, in actinomycetes. For example, in *Streptomyces lividans*, goadsporin promotes the formation of red pigments and sporulation at a concentration of 1 µM and inhibits growth at > 1 µM. This activity is observed in a wide variety of actinomycetes, whereas no bioactivity is observed in other organisms. Among 42 tested actinomycetes strains, 36 strains showed an induction of sporulation and/or secondary metabolite production at low concentrations of goadsporin (Onaka *et al.*, 2001). Currently, the target of the goadsporin antibiotic activity is unknown.

A similar bioactivity to that of goadsporin is associated with SapB peptide. SapB is a morphogenetic peptide produced by *Streptomyces coelicolor* A3(2). It functions as a biological surfactant allowing the hyphae to grow upright. Recently Kodani *et al.* (2004) revealed that it is ribosomally

synthesized. However, goadsporin is not a morphogenetic peptide for the strain TP-A0584, because goadsporin did not induce the spore and pigment formation in TP-A0584 when it was added to the medium, and goadsporin is not released by TP-A0584 into the culture medium but accumulates in the cell (Onaka *et al.*, 2001).

Actinomycetes are well known for their production of a wide variety of polypeptide antibiotics. In particular, numerous nonribosomal peptides have been isolated from actinomycetes. Some of these peptides contain thiazole or oxazole rings (Roy *et al.*, 1999); however, structures related to goadsporin have not yet been reported. The chemical structure of goadsporin is related to that of microcin B17 (Yorgey *et al.*, 1994), a bacteriocin produced by *Escherichia coli*, rather than to those of the nonribosomal peptides isolated from actinomycetes. Microcin B17 is a glycine-rich linear 49 aa long polypeptide containing four oxazoles and four thiazoles with two sets of mixed tandem pairs in the sequence (Fig. 1). The peptide structure of microcin B17 is generated by ribosomes; hence, its peptide sequence is encoded in the structural gene, *mcbA*. In microcin B17 biosynthesis, the *mcbA* gene product is a 69 aa precursor polypeptide, and *mcbB*, *mcbC* and *mcbD* gene products form the microcin B17 synthetase complex, and catalyse the heterocyclization. In this case, cysteine and serine residues neighbouring glycine in the Gly-Cys, Gly-Ser, Gly-Ser-Cys, and Gly-Cys-Ser motifs are converted to thiazole, oxazole, oxazole–thiazole and thiazole–oxazole tandem structures, respectively (Li *et al.*, 1996). However, in goadsporin

Abbreviations: GS disc assay, goadsporin paper disc diffusion assay; SRP, signal recognition particle.

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goadsporin

T5S

Ala-MeOxa-Val-Deala-Oxa-Ile-Leu-Thia-Ser-Gly-Gly-MeOxa-Leu-Deala-Oxa-Ala-Gly-Thia-Val

G10A

Ala-MeOxa-Val-Deala-MeOxa-Ile-Leu-Thia-Ser-Ala-Gly-MeOxa-Leu-Deala-Oxa-Ala-Gly-Thia-Val

S15T

Ala-MeOxa-Val-Deala-MeOxa-Ile-Leu-Thia-Ser-Gly-Gly-MeOxa-Leu-Deala-MeOxa-Ala-Gly-Thia-Val

20K

Ala-MeOxa-Val-Deala-MeOxa-Ile-Leu-Thia-Ser-Gly-Gly-MeOxa-Leu-Deala-Oxa-Ala-Gly-Thia-Val-Lys

microcinB17

Fig. 1. Chemical structures of goadsporin, its derivatives and microcin B17. The unusual amino acids in goadsporin and its derivatives are abbreviated as follows: Oxa, oxazole; MeOxa, methyloxazole; Thia, thiazole; Deala, dehydroalanine. The replaced residues in the goadsporin derivatives are indicated by outline characters. The amino acid sequence of microcin B17 is indicated as a single letter code.

biosynthesis, cysteine, serine and threonine residues neighbouring glycine, serine, alanine or leucine are heterocyclized, and it is likely that the sequence specificity for heterocyclization of goadsporin is different from that of microcin B17.

In this paper, we describe the cloning and genetic analysis of the complete goadsporin biosynthetic gene cluster from *Streptomyces* sp. TP-A0584 and provide a basis for the construction of goadsporin analogue libraries using this biosynthetic machinery.

METHODS

Bacterial strains and growth conditions. *Streptomyces* sp. TP-A0584 was used as the goadsporin production strain. *S. lividans* TK23 served as a heterologous expression host. *Streptomyces scabies* JCM 7914 was obtained from the Japan Collection of Micro-organisms. *E. coli* DH5 α served as a host for subcloning in plasmid pUC19 and its derivatives. *E. coli* XL1-Blue MR was used for the pTOYAMAcos cosmid libraries. *E. coli* S17-1 was used for transconjugation (Mazodier *et al.*, 1989). Growth conditions and manipulations for *E. coli* were similar to those described by Sambrook & Russell (2001). A-3M was the production medium, and V-22 was the seed medium, for *S. lividans* and *Streptomyces* sp. TP-A0584 (Onaka *et al.*, 2001). Bennett's glucose agar, nutrient agar and mannitol soya flour agar (Onaka *et al.*, 2001) were used for transconjugation.

General recombinant DNA techniques. Restriction endonucleases, T4 DNA ligase and *Taq* polymerase were purchased from

New England Biolabs. PCR was carried out using a PTC-200 DNA Engine (MJ Research). Automatic DNA sequencing was carried out using a BigDye Terminator Cycle Sequencing Ready Reaction Kit and analysed on an ABI PRISM 310 DNA sequencer (Applied Biosystems). DNA manipulations in *E. coli* were performed as described by Sambrook & Russell (2001), and those in *Streptomyces* were performed as described by Kieser *et al.* (2000).

Sample preparation of goadsporin and its derivatives, and HPLC detection conditions. Detection of goadsporin was performed by HPLC analysis or a paper disc diffusion assay. Each strain was used to inoculate a 500 ml K-1 flask (K-techno) containing 100 ml V-22 medium. After incubation at 30 °C for 2 days on a rotary shaker at 200 r.p.m., 5 ml samples of the seed culture were transferred into 500 ml K-1 flasks containing 100 ml A-3M medium. Fermentation was carried out at 30 °C for 5 days on the same rotary shaker. Cell pellets from the whole culture broth were extracted with 100 ml *n*-butanol. After evaporation of the *n*-butanol, the residue was dissolved in methanol. HPLC analysis was performed with an HP1090 (Hewlett Packard) system using a C18 Rainin Microsorb column (3 μ , 4.6 mm i.d. $\times$ 100 mm length, Rainin Instrument). Acetonitrile, 0.15% KH₂PO₄ was used as elution buffer. The temperature was 40 °C, and the flow rate was 1.2 ml min⁻¹. Acetonitrile, 0.15% KH₂PO₄ (pH 3.5) was used as the solvent, and detection was performed at 254 nm. (Gradient diagrams are shown in Fig. 5a.) Goadsporin was identified based on retention time, UV spectrum and molecular mass. LC-MS spectra were obtained on an API165 machine (Applied Biosystems).

Goodspodin paper disc diffusion assay (GS disc assay). Nutrient broth soft agar (3 ml) containing 10^8 spores of *S. lividans* TK23 or *S. scabies* JCM 7914 was overlaid onto a Bennett's agar

plate. *n*-Butanol extracts, as described above, or purified samples were absorbed onto paper discs (diameter 10 mm), dried, settled on the plates and incubated at 30 °C. Growth inhibition, pigment production and/or cell differentiation around the paper disc was observed for 3 days.

Cloning of goadsporin biosynthetic genes. The oligonucleotide probe that was used for cloning the goadsporin-structural-gene encoding DNA fragment was designed according to the codon usage table for *Streptomyces*. The probe had the following sequence: 5'-GC(G/C)AC(G/C)GT(G/C)(A/T)(G/C)(G/C)AC(G/C)ATCCT(G/C)-TGC(A/T)(G/C)(G/C)GG(G/C)GG(G/C)AC(G/C)CT(G/C)(A/T)(G/C)-(G/C)(A/T)(G/C)(G/C)GC(G/C)GG(G/C)TGGCT-3'. Southern blot hybridization was performed using this oligonucleotide as the probe against *Streptomyces* sp. TP-A0584 chromosomal DNA, and we chose a 1.3 kb signal in the *Bam*HI digestion and cloned the corresponding DNA fragment into pUC19 to give pGSB1. The DNA sequence of the 1.3 kb *Bam*HI fragment was determined and confirmed to have the goadsporin precursor sequence. Then, the 1.3 kb *Bam*HI fragment was used as a probe for screening the cosmid library, which was constructed with *Streptomyces* sp. TP-A0584 genomic DNA and a bi-functional cosmid, pTOYAMAcos (Onaka *et al.*, 2003b). Chromosomal DNA was prepared and partially digested with *Sau*3AI, and DNA fragments greater than 30 kb were purified by agarose gel electrophoresis. The fragments were ligated with *Bam*HI-digested pTOYAMAcos and packaged into λ phage to give a genomic library of *Streptomyces* sp. TP-A0584. Two positive clones were isolated and renamed pGSBC1 and pGSBC2.

Construction of plasmids for heterologous expression. (i) pGSB14k. pGSBC1 was digested with *Hind*III, and the resulting 14 kb fragment was cloned into the *Hind*III site of pTYM19 to generate pGSB14k. pTYM19 is an actinomycetes-*E. coli* integrating vector (Onaka *et al.*, 2003b). (ii) pGSB16k. pGSBC1 was digested with *Xho*I, and the resulting 3 kb fragment was cloned into the *Sal*I site of pUC19. The plasmid was digested with *Pst*I and *Eco*RI, and the resulting 3 kb fragment was cloned into the *Pst*I and *Eco*RI sites of pTYM19 to give pGSB3k. A 14 kb *Hind*III fragment was prepared from pGSBC1 and cloned into the *Hind*III site of pGSB3k to construct pGSB16k. (iii) pGSB20k. A 6 kb *Hind*III-*Clal* fragment was prepared from pGSBC1 and subcloned into the *Hind*III and *Clal* sites of pBluescript II KS+. The plasmid was digested with *Kpn*I and *Hind*III, and the resulting 6 kb fragment was cloned into the *Kpn*I and *Hind*III sites of pTYM19 to generate pGSB6k. A 14 kb *Hind*III digested fragment was cloned into the *Hind*III site of pGSB6k to generate pGSB20k. (iv) pGODI. The *godI* gene was generated as a 1.2 kb DNA fragment by PCR, using pGSBC1 as the template and two oligonucleotide primers, 5'-GCGACCATATG-TTCGACACTCTCTCCGAT-3' and 5'-ACGGAATTCATCTTGCGA-GTGTCGAAGAAG-3'. The underlined bases indicate the *Nde*I and *Eco*RI restriction enzyme sites. The PCR-generated *godI* fragment facilitates further subcloning, and was cloned into the *Nde*I and *Eco*RI sites of pTYM1ep to generate pGODI. pTYM1ep, as well as pTYM18, is an actinomycetes integrating vector. pTYM1ep contains a TG1 actinophage integration gene instead of the ϕ C31 actinophage integration gene in pTYM18 (Onaka *et al.*, 2003b). In addition, pTYM1ep contains a constitutively active *ermE** promoter upstream of the multiple cloning sites (H. Onaka, unpublished work).

Construction of the strains for gene inactivation of *godA*, *godB* and *godI*. For the in-frame deletion of *godA*, the upstream and downstream regions of *godA* were PCR amplified, and the resulting fragments were cloned into the *Hind*III/*Xba*I and *Xba*I/*Eco*RI sites of pK18mob (Schafer *et al.*, 1994) to give pDgodA. The upstream region of *godA* was amplified by using pGSBC2 as the template DNA, and the primers godA2937DNEco and godA1065DNXba. The downstream region of *godA* was amplified by using pGSBC1 as the template DNA, and the primers godA-D5-Xba and godA-D5-78Hind.

Both the reactions generated 2 kb PCR products. The primer sequences are as follows: godA2937DNEco, 5'-GAGGAATTCGAGAGCGATGT-TGTCGGCGA-3'; godA1065DNXba, 5'-GCGTCTAGACGGTCGTCG-CCTAGGTGACTA-3'; godA-D5-Xba, 5'-CTCTCTAGACATATCGC-GATTACACGGCG-3'; godA-D5-78Hind, 5'-AGCAAGCTTTCTG-GCGGAGCCAGGAGCAAG-3'. The underlined bases indicate the *Eco*RI, *Hind*III and *Xba*I restriction enzyme sites.

For the in-frame deletion of *godB*, a 1.8 kb *Sph*I fragment was prepared from pGSBC1 and cloned into the *Sph*I site of pK18mob to generate pDB1. A 3.4 kb *Bam*HI fragment was prepared from pGSBC1 and cloned into pUC19 to generate pGS3. pGS3 was digested with *Kpn*I, and the resulting 2.3 kb fragment was cloned into the *Kpn*I site of pDB1 to generate pDgodB.

For single crossover deletions of *godI*, a partial fragment of *godI* was PCR amplified with primers designed based on the internal regions of *godI*, and with pGSBC1 as the template. The primer sequences are as follows: godI-DN, 5'-ATCAAGCTTGCCGGTCTGCAGGGTGCAGGC-3' and godI-DC, 5'-ACGGAATTCATCTGCGAGTGTGCAAGAAG-3'. Underlined bases indicate the *Eco*RI and *Hind*III restriction enzyme sites. The 987 bp amplified fragment was inserted into pK18mob to give pDgodI. The gene disruption procedure was as described by Onaka *et al.* (2003a).

Construction of goadsporin derivative expression vectors.

For *godA* mutagenesis, site-directed mutagenesis was carried out with QuikChange site-directed mutagenesis kit (Stratagene) with pGSB1. The primers were as follows: T5S sense, 5'-CGCCACCGTCA-GCAGCATCCTGTGCAGCG-3'; T5S anti, 5'-CGCTGCACAGGATG-CTGCTGACGGTGGCG-3'; G10A sense, 5'-CATCTGTGCAGCGCC-GGCACCTCAGC-3'; G10A anti, 5'-GCTGAGGGTGCCGGCGCTGC-ACAGGATG-3'; S15T sense, 5'-GGCGGCACCCCTCAGCACGGCCGG-CTGCCGTC-3'; S15T anti, 5'-GACGCAGCCGGCCGTGCTGAGGGTG-CCGCC-3'; 20K sense, 5'-GGCCGGCTGCGTCAAGTGATCGGTCG-TCG-3'; 20K anti, 5'-CGACGACCGATCACTTGACGCAGCCGGCC-3'.

The point mutations are underlined. Following mutagenesis, the presence of the desired DNA sequences was confirmed. The mutated *godA* genes were digested with *Bam*HI, and the resulting fragments were cloned into the *Bam*HI site of pTYM19 to generate pTYM-T5S, pTYM-G10A, pTYM-S15T and pTYM-20K.

RESULTS

Cloning of the goadsporin biosynthetic gene cluster

It was presumed that goadsporin is biosynthesized ribosomally since all its 19 constituent amino acids are common L-amino acids (Igarashi *et al.*, 2001). The dehydroalanine and oxazole rings were considered to be derived from serine, methyloxazole from threonine, and thiazole from cysteine. Thus, we predicted that the amino acid sequence for the goadsporin precursor is ATVSTILCSGGTLSSAGCV and synthesized an oligonucleotide based on this sequence. Southern blot hybridization was carried out using this as a probe with the *Streptomyces* sp. TP-A0584 chromosomal DNA digested with *Bam*HI, *Bgl*II, *Pst*I, *Sac*I and *Sph*I. Among the positive signals (1.3, >23, >23, 4 and 1.6 kb, respectively), the 1.3 kb signal in the *Bam*HI digest was selected, and the corresponding DNA fragment was cloned into pUC19 to generate pGSB1 (Fig. 2a). The nucleotide sequence of the 1.3 kb DNA fragment in pGSB1 contained a

Fig. 2. Detection of the goadsporin structural gene. (a) Southern blot hybridization using ^{32}P -labelled oligonucleotide DNA as the probe, and *Bam*HI-, *Bgl*II-, *Pst*I-, *Sac*I- and *Sph*I-digested chromosomal DNA of strain TP-A0584. (b) Nucleotide sequence of the *godA* region cloned in pGSB1, and the deduced amino acid sequence in single letter code. A putative ribosome-binding sequence is underlined. The precursor sequence of goadsporin is indicated by a broken line, and the site-directed mutation sites are indicated by arrows and replaced nucleotides. The post-translational modification residues are indicated as outline characters.

49 aa long ORF that contained the above-described amino acid sequence (Fig. 2b). We assumed that this ORF was the structural gene for goadsporin and named it *godA*, one of the genes present in the *god* (godsporin) cluster. To clone the entire set of genes in the goadsporin biosynthetic cluster, a cosmid library of genomic DNA from *Streptomyces* sp. TP-A0584 was screened by colony hybridization using pGSB1 as a probe. Two positive clones were obtained and designated pGSBC1 and pGSBC2 (Fig. 3a).

Heterologous production of goadsporin in *S. lividans*

S. lividans TK23, a surrogate host for heterologous expression, was transformed with both pGSBC1 and pGSBC2 clones. pTOYAMAcos cosmid vector was integrated into the specific chromosomal *attC* site in actinomycetes (Onaka *et al.*, 2003). Only the strain integrated with pGSBC1 produced goadsporin. Nucleotide sequencing of the pGSBC1 fragment revealed that *godA* was located at the end of the fragment, and 14 ORFs, spanning 20 kb, were located downstream of *godA*. pGSBC2 contains only the part of the cluster corresponding to the 5' end of *godE* and the genes upstream of it, as shown in Fig. 3(a). Next, three clones – pGSB14k, pGSB16k and pGSB20k – were constructed from pGSBC1 (Fig. 3a), and cells transformed with each construct were cultured in goadsporin production medium. After 5 days, the *n*-butanol extract of the mycelium was subjected to the GS disc assay (Fig. 3b). Only the extract from *S. lividans* harbouring pGSB20k was positive; thus, it was proved that the 20 kb region between *godA* and *orf5* was essential for goadsporin biosynthesis. Further analysis revealed that goadsporin production was detected in a pGSB14k and pGODI co-transformed strain (Fig. 3b and 5a). It was thus concluded that 10 genes – *godA*, *godB*, *godC*, *godD*, *godE*, *godF*, *godG*, *godH*, *godI* and *godR* – were responsible for goadsporin biosynthesis.

Characterization of the goadsporin biosynthetic gene cluster

Computer-aided BLAST analysis of the DNA sequence of the cloning region led to a tentative identification of the genes listed in Table 1.

The structural gene for goadsporin, *godA*, encodes a 49 aa propeptide of goadsporin. GodA contains a 30 aa long leader sequence at the N-terminal region, which has no homology to any known sequences.

godB encodes a protein composed of 550 aa. A BLAST search suggested that GodB is similar to LktB (22 % identity), a leukotoxin secretion ATP-binding protein, in *Actinobacillus actinomycetemcomitans* (Lally *et al.*, 1991). LktB is required for the translocation and insertion of *A. actinomycetemcomitans* leukotoxin (AaLta) into the cell membrane (Lally *et al.*, 1991). A hydropathy plot predicts the formation of six membrane-spanning helices within the membrane domain (amino acid residues 1–300). The ATP-binding motif (GSSGSGKS) is conserved in amino acid residues 375–382. The LktB N-terminal residues spanning from 1 to 125 contain the peptidase C39 domain, but the GodB N-terminal residues spanning from 1 to 96 show no homology to LktB.

GodC is a 577 aa protein that shows sequence similarity to members of the ABC transporter family, as well as to GodB. The ATP-binding motif, GPSGAGKT, is conserved at the N-terminus, amino acid positions 362–369. GodC shows sequence similarity to GodB over the entire sequence (20.0% identity). Both these proteins may be responsible for the translocation of goadsporin to the cell membrane.

GodD is a 735 aa protein that shows sequence similarity to *gra-orf12*, which is involved in granaticin biosynthesis (44.4 % identity, 60 % similarity). Granaticin is a benzoisochromaquinone-type antibiotic produced by *Streptomyces*

Fig. 3. Restriction map and subcloning of the cloned DNA fragment (a), and goadsporin production of each transformant (b). (a) Plasmid pGSBC1 contained the originally cloned 40 kb *Sau3AI* fragment at the *Bam*HI site of pTOYAMAcos. The other plasmids were constructed as described in Methods. The thick arrows indicate the extent and directions of the ORFs. Plasmid pGOD1 contains *godI* under the control of the *ermE** promoter (*ermE** pro) in the vector. GS pro., goadsporin production, which was detected using the GS disc assay. GS res., goadsporin resistance, which was determined on the basis of growth inhibition on Bennett's agar containing 30 µg goadsporin ml⁻¹. (b) A GS disc assay indicated that the culture extracts prepared from *S. lividans* harbouring pGSB20k, and co-harbours pGSB14k and pGOD1, produced goadsporin, whereas *S. lividans* harbouring pGSB16k did not. Goadsporin (5 µg) was absorbed onto the paper disc on the positive control plate (the panel at the right end of the figure). Goadsporin production is indicated by a zone of inhibition of *S. lividans* TK23 around the paper disc.

violaceoruber. The function of *gra-orf12* in granaticin biosynthesis is unknown (Ichinose *et al.*, 1998).

godE encodes a protein composed of 522 aa, which shows 25 % identity to McbC between amino acids 280 and 454 (Genilloud *et al.*, 1989). McbC forms a multimeric microcin B17 synthetase complex with McbB and McbD proteins, and it cyclizes four cysteine residues and four serine residues to thiazoles and oxazoles, respectively, in the microcin B17 propeptide.

godF and *godG* encode proteins composed of 867 and 229 aa, respectively. A BLAST search shows no significant similarities to known proteins.

godH encodes a putative 222 aa protein that shows sequence similarity to Rv0802c, a putative acetyltransferase (40.9 % identity). In goadsporin biosynthesis, GodH protein is believed to catalyse the acetylation of the N-terminal alanine.

godR encodes a 238 aa protein with sequence similarity to *brpA* (22.7 % identity) from the bialaphos biosynthetic gene cluster in *Streptomyces hygroscopicus* (Raibaud *et al.*, 1991). In particular, the helix–turn–helix DNA-binding motif is strongly conserved between amino acids 208–227 at the N-terminus (75 % similarity).

godI shows sequence similarity to *ffh*, the signal recognition particle (SRP) in *E. coli* (44.6 % identity; 74.9 % similarity).

Table 1. Deduced genes and their proposed functions in the *god* cluster.

Gene	No. of amino acids	Homologous gene	% Identity of protein	Origin	Accession no.
<i>godA</i>	49	Goadsporin structure gene			
<i>godB</i>	550	<i>lktB</i> : leukotoxin secretion ATP-binding protein	22.7	<i>A. actinomycetemcomitans</i>	P23702
<i>godC</i>	557	<i>lktB</i>	18.7	<i>A. actinomycetemcomitans</i>	P23702
<i>godD</i>	735	<i>gra-orf12</i> involved in granaticin biosynthesis	44.4	<i>S. violaceoruber</i>	T46517
<i>godE</i>	522	<i>mcbC</i> involved in microcin B17 biosynthesis	25.0*	<i>E. coli</i>	P23185
<i>godF</i>	867	Function unknown			
<i>godG</i>	229	Function unknown			
<i>godH</i>	222	Acetyltransferase (putative)	40.9	<i>Mycobacterium tuberculosis</i>	NP_215317
<i>godR</i>	238	<i>brpA</i> : regulator protein of bialaphos biosynthesis	22.7	<i>S. hygroscopicus</i>	Q01108
<i>orf1</i>	160	Transposase (putative)	93.8	<i>S. avermitilis</i>	NP_828729
<i>orf2</i>	147	Transposase (putative)	89.8	<i>S. avermitilis</i>	NP_828730
<i>orf3</i>	304	Transposase (putative)	76.3	<i>S. coelicolor</i> A3(2)	NP_631141
<i>godI</i>	518	<i>ffh</i> : signal recognition particle protein	44.6	<i>E. coli</i>	P07019
<i>orf5</i>	73	Transposase (putative)	43.8*	<i>S. avermitilis</i>	NP_821430
<i>orf6</i>	128	Transposase (putative)	42.5*	<i>S. avermitilis</i>	NP821430

*The homologous region is partial.

SRP is a ribosomal protein that catalyses targeting of nascent secretory and membrane proteins to the protein translocation apparatus of the cell (Luirink *et al.*, 1992). SRP homologues have been identified in all living cells; all these homologues have been analysed thus far. Hypothetical *ffh* homologues in *Streptomyces avermitilis* and *S. coelicolor* A3(2) are also highly conserved with *godI* (76.6% and 75.9% identity, respectively).

The DNA sequences of *orf1*, *orf2*, *orf3*, *orf4* and *orf5* show high similarity to putative transposase genes, assigned by the genome project for *S. coelicolor* A3(2) and *S. avermitilis*. These five *orf* gene products are not involved in goadsporin biosynthesis.

Inactivation of *godA*, *godB* and *godI*

For disruption of the chromosomal genes, insertional inactivation via a double crossover was used with the derivatives of pK18mob and non-replicating *E. coli* plasmids (pDgodA and pDgodB), and via a single crossover (pDgodI). *godA* and *godB* were disrupted by in-frame deletion because insertional inactivation of *godA* or *godB* would be expected to have a polar effect on the transcription of genes downstream from *godA* or *godB* (Fig. 3). Finally, three mutants were independently isolated for *godA* and *godB*, and were further characterized. However, a *godI* disruptant could not be isolated, suggesting that *godI* either is essential for cell growth, or is a goadsporin self-resistance gene. We then constructed the *godA* and *godI* double-disruption mutants, which were isolated and further characterized. HPLC analysis of the fermentation extracts revealed that goadsporin was not produced in either disruptant and their mutations did not affect cell differentiation in TP-A0584 on Bennett's media (data not shown).

The SRP homologue, *godI*, is the goadsporin self-resistance gene

pGSBC1- or pGSB20k-transformed *S. lividans* exhibits resistance to goadsporin in the GS disc assay (Fig. 3a), suggesting that these plasmids contain a goadsporin immunity gene. On the other hand, pGSB14k- and pGSB16k-transformed *S. lividans*, both of which lack *godI*, were sensitive to goadsporin. *godI* was then cloned downstream of the constitutive *ermE** promoter and was used to transform *S. lividans* using an integrating vector. The resulting transformant, which has *godI* integrated into the chromosomal DNA and expresses it constitutively, grew on a plate containing 30 µg goadsporin ml⁻¹, whereas a *S. lividans* TK23 and pGSB14k-transformed strain could not (Fig. 4a). Furthermore, the *godI/godA* disruptant could not grow on the plate containing 7 µg goadsporin ml⁻¹, whereas the *godA* disruptant, *godB* disruptant and the wild-type strain grew on the same plate (Fig. 4b).

Biosynthesis of goadsporin analogues by *godA* mutants

Goadsporin analogues were produced using the goadsporin biosynthetic machinery. The amino acid sequence of *godA* was changed by site-directed mutagenesis (Fig. 2b), and four analogues were isolated from the recombinant gene strains. The mutated *godA* genes were cloned into the pTYM19 integration vector to generate pTYM-T5S, pTYM-G10A, pTYM-S15T and pTYM-20K. These plasmids were introduced into the *godA* disruptant and integrated into the TP-A0584 chromosomal DNA. The transformants were cultured at 30 °C for 5 days, and *n*-butanol extracts of the fermentation broth were analysed by LC-MS. In each extract, the production of new derivatives was detected as a

Fig. 4. Effects of *godI* on goadsporin self-resistance. (a) *S. lividans* harbouring pGODI grow on a plate containing 30 µg goadsporin ml⁻¹, whereas the wild-type strain (*S. lividans*) and *S. lividans* harbouring pGSB14k did not grow. (b) Strain TP-A0584 and its gene disruption mutants were inoculated onto a solid medium containing 7 µg goadsporin ml⁻¹. TP-A0584, $\Delta godI$, $\Delta godA$ and $\Delta godB$ grow on the goadsporin-containing plate, whereas $\Delta godI\Delta godA$ did not grow. Both photographs were taken after 5 days growth on Bennett's agar media.

peak at 13.7, 15.8, 19.9 and 12.1 min (Fig. 5a). These peaks showed UV-visible spectra identical to those of goadsporin and the molecular ion $[M+H]^+$ at m/z 1597.6, 1625.7, 1625.7 and 1739.8, respectively. These values were in accordance with those calculated for the structures shown in Fig. 1. In the GS disc assay, derivatives T5S and S15T retained the activity of the parent compound, namely the production of pigments and sporulation in *S. lividans*, whereas G10A and 20K lost the activity (Fig. 5b). Interestingly, G10A retained the antibiotic activity against *S. scabies*, a potato scab pathogen strain (Fig. 5c).

DISCUSSION

Proposed overall biosynthetic pathway of goadsporin

A proposed biosynthetic pathway for goadsporin is shown in Fig. 6. The 49 aa long *godA* polypeptide is ribosomally produced, and it is processed by putative goadsporin synthetases, GodD, GodE, GodF and GodG. These enzymes transform the amino acids at positions 2, 5, 8, 12, 15 and 18, from the N-terminus, into heterocycles, and the serine

residues at positions 4 and 14 into dehydroalanines, to generate progoadsporin. Proteolysis of progoadsporin is then catalysed by a peptidase, GodB or GodC. Finally, GodH catalyses the N-acetylation of the N-terminus to produce goadsporin.

It is uncertain whether these gene products catalyse post-translational modification; however, subcloning experiments revealed that these gene products are necessary for goadsporin production (Fig. 3a); furthermore, the amino acid sequences of these genes products are similar to those of some secondary metabolite biosynthetic enzymes. *godD* is similar to *gra-orf34*, which is responsible for granaticin biosynthesis in *S. violaceoruber* Tu22. GodE is similar to McbC, a microcin B17 biosynthetic enzyme. GodF and GodG also show a slight similarity to a putative lanthionine biosynthesis protein (YP_055572) and a putative lantibiotic biosynthesis protein (ZP_00237845) (<25 % partial identity).

GodB or GodC is believed to be involved in the processing of the N-terminal leader since some ABC transporters involved in bacteriocin biosynthesis digest the leader sequence at the double glycine residue (Havarstein *et al.*, 1995). In goadsporin biosynthesis, the processing site is predicted to be at the double alanine residues at positions -1 and 1. The processing site is different from typical signal peptidase cleavage sites. Therefore, *godB* and *godC* could not be characterized as encoding peptidases based on the deduced amino acid sequence homology in comparison with typical bacteriocin peptidases.

Goadsporin is structurally related to microcin B17 produced by *E. coli*. Genetic analysis has shown that microcin B17 is produced ribosomally, and its subsequent post-translational modification generates the thiazole and oxazole rings. Although the biosynthetic pathway of goadsporin and microcin B17 appear similar, the genes involved in their biosynthesis have no similarity except for *godE*. These findings suggest that the enzymes responsible for heterocyclization in goadsporin and microcin B17 have evolved independently. Recently, Widdick *et al.* (2003) reported on the bacteriocin cinnamycin biosynthetic gene cluster from *Streptomyces cinnamoneus cinnamoneus* DSM40005. Cinnamycin is a lantibiotic that contains lanthionine bridges derived by the post-translational modification of amino acid residues. The biosynthetic genes are not similar to *god* genes. This means that goadsporin and cinnamycin have also evolved independently. The G + C content of the *Streptomyces* genus is generally high. For example, the G + C content of *S. avermitilis* is 70.7 mol% and that of *S. coelicolor* A3(2) is 72.1 mol%. However, the G + C content of the goadsporin biosynthetic gene cluster is 65.7 mol%. In addition, the location of the goadsporin biosynthetic gene cluster in the genome is between some transposase-encoding genes. It is likely that the horizontal transfer of the cluster occurred from another genus that does not have a high G + C content.

Fig. 5. HPLC analysis (a), and biological activity of goadsporin and selected goadsporin analogues (b, c). (a) HPLC conditions and sample preparation are described in Methods. The elution was with a linear gradient as indicated on the right-hand scale in the bottom chart. The elution peaks of each derivative are indicated by black arrow heads, and the values on the arrow heads indicate their retention time. The elution peaks with white arrow heads at 18.5 min in T5S, G10A, S15T, 20K and pGSB14k+pGODI indicate the peak of thiostreptone, which was present in recombinant strain cultures. (b, c) GS disc assays with the goadsporin analogues indicating the response against *S. lividans* TK23 and *S. scabies* JCM7914. Growth inhibition is indicated by a clear zone of inhibition of *S. lividans* TK23 (b) or *S. scabies* JCM7914 (c) around the paper disc.

Fig. 6. The proposed overall organization of the goadsporin biosynthesis gene cluster in *Streptomyces* sp. TP-A0584. (1) GodB and GodC deliver GodA to the cell membrane. (2) GodD, GodE, GodF and GodG catalyse post-translational modification of GodA (cyclization, dehydration). (3) GodB, GodC or some peptidase digests the leader peptide of GodA, and the N-terminus of goadsporin is acetylated by GodH. (4) GodI binds to the goadsporin and anchors it to the cell membrane.

Immunity to goadsporin in *Streptomyces* sp. TP-A0584

S. lividans harbouring *godI* is resistant to goadsporin, and the *godI* disruptant is sensitive to goadsporin. These results suggest that *godI* is involved in the goadsporin immunity system of the producing strain. *godI* has some similarity to *ffh*, a component of the SRP. The SRP recognizes the signal peptide of secretory or membrane proteins, and promotes their delivery to the cytoplasmic membrane. Generally, in bacteria, SRP forms a complex with Ffh and 4·5S RNA. Interestingly, a sequence that shows 91·6 % identity to 4·5S RNA in *S. lividans* (Palacin *et al.*, 2003) is present 235 bp upstream from *godI* in the genome of strain TP-A0584 (Fig. 3a). Although pGODI plasmids did not contain the 4·5S RNA, it therefore seems reasonable that GodI forms a complex with *S. lividans* 4·5S RNA. One speculative mechanism for goadsporin resistance is shown in Fig. 6. GodI may bind to mature goadsporin, and the SRP receptor, which is located in the membrane, binds to GodI, and anchors goadsporin to the membrane.

In most bacteriocin biosynthesis, ABC transporters are responsible for the immunity. In goadsporin biosynthesis, *godB* and *godC*, ABC transporter homologues, are not responsible for the immunity because *S. lividans* transformed with pGSB14k exhibits no immunity for goadsporin. pGSB14k contains *godB* and *godC*, but not *godI*. Also the

godB disruptant maintains goadsporin immunity. These results suggest that *godI* is the gene responsible for immunity. GodB and GodC are probably responsible for goadsporin delivery to the cell membrane. In addition to the ABC transporter system, other immunity systems are recognized in bacteriocin producers. They consist of self-resistant proteins that encode 50–250 amino acids. These self-resistant proteins are of a wide variety and show no significant homology to known proteins. The functions of most immunity proteins are not yet clear (Sonomoto & Sashihara, 2001). Recently Tran & Jacoby (2002) revealed that the microcin B17 immunity gene, *mcbG*, is similar to the gene encoding the quinolone resistance protein, *qnr*, which was isolated from a multiresistance plasmid. They tested the ability of Qnr to reverse the inhibition of gyrase activity by quinolones *in vitro*. However, *mcbG* is not similar to *godI* or *ffh*.

SRP is a ubiquitous ribonucleoprotein particle, and one of its components, Ffh, is the only protein component present in all SRPs; hence, it plays an essential role in signal peptide and SRP receptor binding (Nagai *et al.*, 2003). In strain TP-A0584, *godI*, an *ffh* homologue, is responsible for goadsporin immunity. To the best of our knowledge, this is the first report finding that an *ffh* homologue possesses another function besides translocation of secretory or membrane proteins.

Production of goadsporin analogues by site-directed mutagenesis of *godA*

The versatility of the goadsporin biosynthesis machinery has been indicated by the production of goadsporin analogues. For example, G10A is an analogue in which the 10th glycine is replaced with alanine. In the S15T analogue, the oxazole at the 15th position is changed to methyloxazole. In the T5S analogue, the methyl group of the 5th methyloxazole is substituted with hydrogen. 20K is an analogue having an additional lysine residue at the carboxyl end (Fig. 1). In microcin B17 biosynthesis, the N-terminal leader sequence of pre-microcin B17 is essential for its *in vivo* post-translational modification to pro-microcin B17 (Madison *et al.*, 1997). It has been suggested that the leader is recognized as a binding site by post-translational modification enzymes, McbB, McbC and McbD. *godA* also contains a 30 aa leader peptide at the N-terminus, the sequence of which is not similar to the secretion signal sequence; therefore, the post-translational modification enzymes might recognize it as a binding site. A wide variety of goadsporin analogues could be produced by this method, and the goadsporin biosynthesis machinery can also be used for the heterocyclization of oligopeptides. Although G10A lost its activity against *S. lividans*, its activity for *S. scabiei* was retained. *S. scabiei* is known to cause potato scab worldwide. We have demonstrated that the goadsporin antibiotic spectrum could be changed by amino acid replacement. This approach will enable the application of the analogues as agricultural chemicals against potato scab.

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REFERENCES

- Genilloud, O., Moreno, F. & Kolter, R. (1989). DNA sequence, products, and transcriptional pattern of the genes involved in production of the DNA replication inhibitor microcin B17. *J Bacteriol* **171**, 1126–1135.
- Havarstein, L. S., Diep, D. B. & Nes, I. F. (1995). A family of bacteriocin ABC transporters carry out proteolytic processing of their substrates concomitant with export. *Mol Microbiol* **16**, 229–240.
- Ichinose, K., Bedford, D. J., Tornus, D., Bechthold, A., Bibb, M. J., Revill, W. P., Floss, H. G. & Hopwood, D. A. (1998). The granaticin biosynthetic gene cluster of *Streptomyces violaceoruber* Tu22: sequence analysis and expression in a heterologous host. *Chem Biol* **5**, 647–659.
- Igarashi, Y., Kan, Y., Fujii, K., Fujita, T., Harada, K., Naoki, H., Tabata, H., Onaka, H. & Furumai, T. (2001). Goadsporin, chemical substance which promotes secondary metabolism and morphogenesis in streptomycetes II. structure determination. *J Antibiot* **54**, 1045–1053.
- Kieser, T., Bibb, M. J., Buttner, M. J., Chater, K. F. & Hopwood, D. A. (2000). *Practical Streptomyces Genetics*. Norwich, UK: The John Innes Foundation.
- Kodani, S., Hudson, M. E., Durrant, M. C., Buttner, M. J., Nodwell, J. R. & Willey, J. M. (2004). The SapB morphogen is a lantibiotic-like peptide derived from the product of the developmental gene *ramS* in *Streptomyces coelicolor*. *Proc Natl Acad Sci U S A* **101**, 11448–11453.
- Lally, E. T., Golub, E. E., Kieba, I. R., Taichman, N. S., Decker, S., Berthold, P., Gibson, C. W., Demuth, D. R. & Rosenbloom, J. (1991). Structure and function of the B and D genes of the *Actinobacillus actinomycetemcomitans* leukotoxin complex. *Microb Pathog* **11**, 111–121.
- Li, Y. M., Milne, J. C., Madison, L. L., Kolter, R. & Walsh, C. T. (1996). From peptide precursors to oxazole and thiazole-containing peptide antibiotics: microcin B17 synthase. *Science* **274**, 1188–1193.
- Luirink, J., High, S., Wood, H., Giner, A., Tollervey, D. & Dobberstein, B. (1992). Signal-sequence recognition by an *Escherichia coli* ribonucleoprotein complex. *Nature* **359**, 741–743.
- Madison, L. L., Vivas, E. I., Li, Y.-M., Walsh, C. T. & Kolter, R. (1997). The leader peptide is essential for the post-translational modification of the DNA-gyrase inhibitor microcin B17. *Mol Microbiol* **23**, 161–168.
- Mazodier, P., Petter, R. & Thompson, C. (1989). Intergeneric conjugation between *Escherichia coli* and *Streptomyces* species. *J Bacteriol* **171**, 3583–3585.
- Nagai, K., Oubridge, C., Kuglstatter, A., Menichelli, E., Isel, C. & Jovine, L. (2003). Structure, function and evolution of the signal recognition particle. *EMBO J* **22**, 3479–3485.
- Onaka, H., Tabata, H., Igarashi, Y., Sato, Y. & Furumai, T. (2001). Goadsporin, a chemical substance which promotes secondary metabolism and morphogenesis in streptomycetes. I. Purification and characterization. *J Antibiot* **54**, 1036–1044.
- Onaka, H., Taniguchi, S., Igarashi, Y. & Furumai, T. (2003a). Characterization of the biosynthetic gene cluster of rebeccamycin from *Lechevalieria aerocolonigenes* ATCC 39243. *Biosci Biotechnol Biochem* **67**, 127–138.
- Onaka, H., Taniguchi, S., Ikeda, H., Igarashi, Y. & Furumai, T. (2003b). pTOYAMacos, pTYM18, and pTYM19, actinomycete-*Escherichia coli* integrating vectors for heterologous gene expression. *J Antibiot* **56**, 950–956.
- Palacin, A., de la Fuente, R., Valle, I., Rivas, L. A. & Mellado, R. P. (2003). *Streptomyces lividans* contains a minimal functional signal recognition particle that is involved in protein secretion. *Microbiology* **149**, 2435–2442.
- Raibaud, A., Zalacain, M., Holt, T. G., Tizard, R. & Thompson, C. J. (1991). Nucleotide sequence analysis reveals linked N-acetyl hydrolase, thioesterase, transport, and regulatory genes encoded by the bialaphos biosynthetic gene cluster of *Streptomyces hygroscopicus*. *J Bacteriol* **173**, 4454–4463.
- Roy, R. S., Gehring, A. M., Milne, J. C., Belshaw, P. J. & Walsh, C. T. (1999). Thiazole and oxazoles peptides: biosynthesis and molecular machinery. *Nat Prod Rep* **16**, 249–263.
- Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schafer, A., Tauch, A., Jager, W., Kalinowski, J., Thierbach, G. & Puhler, A. (1994). Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* **145**, 69–73.

Sonomoto, K. & Sashihara, T. (2001). Structure and mode of action of bacteriocins produced by lactic acid bacteria. *Tanpakushitsu Kakusan Koso* **46**, 323–332.

Tran, J. H. & Jacoby, G. A. (2002). Mechanism of plasmid-mediated quinolone resistance. *Proc Natl Acad Sci U S A* **99**, 5638–5642.

Widdick, D. A., Dodd, H. M., Barraille, P., White, J., Stein, T. H., Chater, K. F., Gasson, M. J. & Bibb, M. J. (2003). Cinnamycin

biosynthetic gene cluster from *Streptomyces cinnamoneus* DSM 40005. *Proc Natl Acad Sci U S A* **100**, 4316–432.

Yorgey, P., Lee, J., Kördel, J., Vivas, E., Warner, P., Jebaratnam, D. & Kolter, R. (1994). Posttranslational modifications in microcin B17 define an additional class of DNA gyrase inhibitor. *Proc Natl Acad Sci U S A* **91**, 4519–4523.