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. godD 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 sporeulation 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
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 Microorganisms. *E. coli* DH5α 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), 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 μm, 4-6 mm i.d. × 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).

**Goadsporin paper disc diffusion assay (GS disc assay).** Nutrient broth soft agar (3 ml) containing 10⁸ spores of *S. lividans* TK23 or *S. scabies* JCM 7914 was overlaid onto a Bennett’s agar.

![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.](image-url)
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′-GCCACACCTGCGTCCCGCATGTCGCGCGACGATCG-3′. The oligonucleotide probe was used to clone the goadsporin-structural-gene encoding DNA fragment from genomic DNA. The probes were ligated with BamHI purified by agarose gel electrophoresis. The fragments were ligated with BamHI-digested pTOYAMAcos and packaged into λ phage to give a genomic library of *Streptomyces* sp. TP-A0584. Two positive clones were isolated and renamed pGSB1 and pGSB2.

**Construction of plasmids for heterologous expression.** (i) pGSB14k. pGSB1C was digested with HindIII, and the resulting 14 kb fragment was cloned into the HindIII site of pTYM19 to generate pGSB14k. pTYM19 is an actinomycetes integrating vector. (ii) pGSB16k. pGSB1C was digested with XhoI, and the resulting 3 kb fragment was cloned into the SalI site of pUC19. The plasmid was digested with PstI and EcoRI, and the resulting 3 kb fragment was cloned into the PstI and EcoRI sites of pTYM19 to give pGSB3k. A 14 kb HindIII fragment was prepared from pGSB1C and cloned into the HindIII site of pGSB3k to construct pGSB16k. (iii) pGSB20k. A 6 kb HindIII–ClaI fragment was prepared from pGSB1C and subcloned into the HindIII and Clal sites of pBluescript II K5+. The plasmid was digested with KpnI and HindIII, and the resulting 6 kb fragment was cloned into the KpnI and HindIII sites of pTYM19 to generate pGSB6k. A 14 kb HindIII digested fragment was cloned into the HindIII site of pGSB6k to generate pGSB20k. (iv) pGODL. The gofl gene was generated as a 1.2 kb DNA fragment by PCR, using pGSB1C as the template and two oligonucleotide primers, 5′-GCCACACCTGCGTCCCGCATGTCGCGCGACGATCG-3′ and 5′-ACGGAATTCATCCTGCGAAGGAACGTTGGTCCCGCGGTGCGCGGCGGG-3′. The underlined bases indicate the Ndel and EcoRI restriction enzyme sites, for PCR. The PCR-generated gofl fragment facilitates further subcloning, and was cloned into the Ndel and EcoRI sites of pTYM1ep to generate pGODL. pTYM1ep, as well as pTYM18, is an actinomycetes integrating vector. pTYM1ep contains a TGI actinophage integration gene instead of the #231 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 godl.** 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 HindIII/Xbal and Xbal/EcoRI sites of pK18mob (Schafer et al., 1994) to give pGODa. The upstream region of godA was amplified by using pGSB2C as the template DNA, and the primers godA2937DNeco and godA1065DNXba. The downstream region of godA was amplified by using pGSB1C 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′-GAGGAATTCGCCAAGCGATGGTGTCGGCGA-3′; godA1065DNXba, 5′-GGCCCTAGACGCGTCGCTCTAGGGTGAATA-3′; godA-D5-Xba, 5′-CTCTCTAGACGCGTCGCTTCAGTACGGCGGCGGAGGCGG-3′; godA-D5-78Hind, 5′-AGGGACCTTCTTGCGCGAGACGAGAACG-3′. The underlined bases indicate the EcoRI, HindIII and Xbal restriction enzyme sites.

For the in-frame deletion of godI, a 1·8 kb Spfl fragment was prepared from pGSB1C and cloned into the Spfl site of pK18mob to generate pDB1. A 3·4 kb BamHI fragment was prepared from pGSB1C and cloned into pUC19 to generate pGS3. pGS3 was digested with Kpnl, and the resulting 2·3 kb fragment was cloned into the KpnI site of pDB1 to generate pGGodB.

For single crossover deletions of godl, a partial fragment of godl was PCR amplified with primers designed based on the internal regions of godl, and with pGSB1C as the template. The primer sequences were as follows: godl-DSN, 5′-ATCGAGCTTGGCGCCGTCGAGCTGCGGCG-3′ and godl-DC, 5′-ACGGGAATTCCTCGAGCTGCGGCGG-3′. Underlined bases indicate the EcoRI and HindIII restriction enzyme sites. The 987 bp amplified fragment was inserted into pK18mob to give pGGodl. 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: TSS sense, 5′-GCCCGACCTGACGATCCATTGCGCCTCGAGCTGCGGCGGC-3′; TSS anti, 5′-GGCCCTAGACGCGTCGCTCTAGGGTGAATA-3′. Southern blot hybridization was performed using this oligonucleotide as the probe against *Streptomyces* sp. TP-A0584 genomic DNA. Both the reactions generated 2 kb PCR products. The primer sequences are as follows: godA2937DNeco, 5′-GAGGAATTCGCCAAGCGATGGTGTCGGCGA-3′; godA1065DNXba, 5′-GGCCCTAGACGCGTCGCTCTAGGGTGAATA-3′; godA-D5-Xba, 5′-CTCTCTAGACGCGTCGCTTCAGTACGGCGGCGGAGGCGG-3′; godA-D5-78Hind, 5′-AGGGACCTTCTTGCGCGAGACGAGAACG-3′. The underlined bases indicate the EcoRI, HindIII and Xbal restriction enzyme sites.

**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 ATVSTILCSGTLSSAGCV 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 BamHI, BglII, PstI, SacI and Spfl. Among the positive signals (1·3, >23, >23, 4 and 1·6 kb, respectively), the 1·3 kb signal in the BamHI 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...
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 (goadsporin) 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).

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, 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.

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 benzoiso-chromatiinoimone-type antibiotic produced by Streptomyces
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 fhf, the signal recognition particle (SRP) in E. coli (44-6 % identity; 74-9 % similarity).
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 hfh homologues in *Streptomyces avermitilis* 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* genes products are not involved in goadsporin biosynthesis.

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>No. of amino acids</th>
<th>Homologous gene</th>
<th>% Identity of protein</th>
<th>Origin</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>godA</td>
<td>49</td>
<td>Goadsporin structure gene</td>
<td>22.7</td>
<td>A. actinomycetemcomitans</td>
<td>P23702</td>
</tr>
<tr>
<td>godB</td>
<td>550</td>
<td>lktR: leukotoxin secretion ATP-binding protein</td>
<td>18.7</td>
<td>A. actinomycetemcomitans</td>
<td>P23702</td>
</tr>
<tr>
<td>godC</td>
<td>557</td>
<td>lktB</td>
<td>22.7</td>
<td>A. actinomycetemcomitans</td>
<td>P23702</td>
</tr>
<tr>
<td>godD</td>
<td>735</td>
<td>gra-orf12 involved in granaticin biosynthesis</td>
<td>44.4</td>
<td>S. violaceoruber</td>
<td>T46517</td>
</tr>
<tr>
<td>godE</td>
<td>522</td>
<td>mcbC involved in microcin B17 biosynthesis</td>
<td>25.0*</td>
<td>E. coli</td>
<td>P23185</td>
</tr>
<tr>
<td>godF</td>
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<td>Function unknown</td>
<td>10.9</td>
<td>Mycobacterium tuberculosis</td>
<td>NP_215317</td>
</tr>
<tr>
<td>godG</td>
<td>229</td>
<td>Function unknown</td>
<td>40.9</td>
<td><em>E. coli</em></td>
<td>P07019</td>
</tr>
<tr>
<td>godH</td>
<td>222</td>
<td>Acetyltransferase (putative)</td>
<td>76.3</td>
<td><em>S. coelicolor</em> A3(2)</td>
<td>NP_631141</td>
</tr>
<tr>
<td>godI</td>
<td>518</td>
<td>ffr: signal recognition particle protein</td>
<td>44.6</td>
<td><em>E. coli</em></td>
<td>P07019</td>
</tr>
<tr>
<td>orf1</td>
<td>160</td>
<td>Transposase (putative)</td>
<td>93.8</td>
<td><em>S. avermitilis</em></td>
<td>NP_828729</td>
</tr>
<tr>
<td>orf2</td>
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<td>89.8</td>
<td><em>S. avermitilis</em></td>
<td>NP_828730</td>
</tr>
<tr>
<td>orf3</td>
<td>304</td>
<td>Transposase (putative)</td>
<td>76.3</td>
<td><em>S. coelicolor</em> A3(2)</td>
<td>NP_631141</td>
</tr>
<tr>
<td>orf4</td>
<td>43.8*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>orf5</td>
<td>73</td>
<td>Transposase (putative)</td>
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<td><em>S. avermitilis</em></td>
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<tr>
<td>orf6</td>
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<td>Transposase (putative)</td>
<td>42.5*</td>
<td><em>S. avermitilis</em></td>
<td>NP821430</td>
</tr>
</tbody>
</table>

*The homologous region is partial.*

**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-TSS, 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...
Interestingly, G10A retained the antibiotic activity against S. scabies whereas G10A and 20K lost the activity (Fig. 5b).

S. lividans production of pigments and sporulation 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 antibiotic activity against S. avermitilis, a potato scab pathogen strain (Fig. 5c).

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, ΔgodA and ΔgodB grow on the goadsporin-containing plate, whereas ΔgodA/ΔgodA did not grow. Both photographs were taken after 5 days growth on Bennett’s agar media.

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, ΔgodA and ΔgodB grow on the goadsporin-containing plate, whereas ΔgodA/ΔgodA 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 gene products are similar to those of some secondary metabolite biosynthetic enzymes. godD is similar to gra-orf34, which is responsible for gracetin 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 lantionine biosynthesis protein (YP_055572) and a putative lantibiotic biosynthesis protein (ZP_00237845) (< 25% partial identity).

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 lantionine 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 thiostrepton, 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.
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.

**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.
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 S15F 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. scabies, its activity for Streptomyces lividans was retained.

S. scabies is known to cause potato scab worldwide. The leader peptide is essential for the post-translational modification and characterization of the DNA-gyrase inhibitor microcin B17. Mol Microbiol 23, 161–168.

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