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

Hiroyasu Onaka, Mizuho Nakaho, Keiko Hayashi, Yasuhiro Igarashi and Tamotsu Furumai

Biotechnology Research Center, Toyama Prefectural University, Imizu, Toyama 939-0398, Japan

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* (goadsoporin) genes involved in post-translational modification, immunity and transcriptional regulation. Although the gene organization is similar to typical bacteriocin biosynthetic gene clusters, each goadsoporin 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 goadsoporin immunity. Furthermore, four goadsoporin 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*, goadsoporin 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 sporation and/or secondary metabolite production at low concentrations of goadsoporin (Onaka et al., 2001). Currently, the target of the goadsoporin antibiotic activity is unknown.

A similar bioactivity to that of goadsoporin 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, goadsoporin is not a morphogenetic peptide for the strain TP-A0584, because goadsoporin did not induce the spore and pigment formation in TP-A0584 when it was added to the medium, and goadsoporin 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 goadsoporin have not yet been reported. The chemical structure of goadsoporin 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 goadsoporin
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 \textit{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.** \textit{Streptomyces} sp. TP-A0584 was used as the goadsporin production strain. \textit{S. lividans} TK23 served as a heterologous expression host. \textit{S. scabies} JCM 7914 was obtained from the Japan Collection of Microorganisms. \textit{E. coli} DH5α served as a host for subcloning in plasmid pUC19 and its derivatives. \textit{E. coli} XL1-Blue MR was used for the pTOYAMAcos cosmid libraries. \textit{E. coli} S17-1 was used for transconjugation (Mazodier et al., 1989). Growth conditions and manipulations for \textit{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 \textit{S. lividans} and \textit{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 \textit{E. coli} were performed as described by Sambrook & Russell (2001), and those in \textit{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. × 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 \textit{S. lividans} TK23 or \textit{S. scabies} JCM 7914 was overlaid onto a Bennett’s agar

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**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.
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′-GGC(G/C)AC(G/C)GTG(T/G)(A/T)(G/C)(G/C)AC(G/C)ATCCT(C/G)-TGC(A/T)(G/C)(G/C)(G/C)(G/C)(G/C)(CCTG(C/T)(A/T)(G/C)- (G/C)/(A/T)(G/C)(G/C)(G/C)(G/C)(G/C)TGGC-G3′. 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 BamHI digestion and cloned the corresponding DNA fragment into pUC19 to give pGSB1. The DNA sequence of the 1.3 kb BamHI fragment was determined and confirmed to have the goadsporin precursor sequence. Then, the 1.3 kb BamHI fragment was used as a probe for screening the cosmids library, which was constructed with Streptomyces sp. sp. TP-A0584 genomic DNA and a bi-functional cosmid, PTOYAmAcos (Onaka et al., 2003b). Chromosomal DNA was prepared and partially digested with Sau3A1 and DNA fragments greater than 30 kb were 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 pGSBC1 and pGSBC2.

**Construction of plasmids for heterologous expression.** (i) pGSB14k. pGSBC1 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. pGSBC1 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 pGSBC1 and cloned into the HindIII site of pGSB3k to construct pGSB16k.

(ii) pGSB20k. A 6 kb HindIII-ClaI fragment was prepared from pGSBC1 and subcloned into HindIII and Clal sites of pBluescript II KS−. 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) pGOdI. The godl gene was generated as a 1.2 kb DNA fragment by PCR, using pGSBC1 as the template and two oligonucleotide primers, 5′-GCGACCATATG-TTCGACA(C/T)CTCCGAT-3′ and 5′-ACGGAATTCTTTGCG-GATGTCGAAAAC-3′. The underlined bases indicate the Ndel and EcoRI restriction enzyme sites, for PCR. The PCR-generated godl fragment facilitates further subcloning, and was cloned into the Ndel and EcoRI sites of pTYM1ep to generate pGOdI. pTYM1ep, as well as pTYM18, is an actinomycetes integrating vector. pTYM1ep contains a TGI actinophage integration gene instead of the #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 godD.** 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/XbaI and XbaI/EcoRI sites of pK18mob (Schafer et al., 1994) to give pDGmob. 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′-GAGGAATTCGCCAGGACGTG-TGTGCGGGA-3′; godA1065DNXba, 5′-GGGTCAGACGCTGTCG-CCTAGTGTGACTA-3′; godA-D5-Xba, 5′-CTCTCTCAAGATATCGGC-GATTACACCGG-3′; godA-D5-78Hind, 5′-AGGAATTCTTTCG-GCGGAGCCAGGACGAA-3′. The underlined bases indicate the EcoRI, HindIII and XbaI restriction enzyme sites.

For the in-frame deletion of godB, a 1.8 kb Sphi fragment was prepared from pGSBC1 and cloned into the Sphi site of pK18mob to generate pDB1. A 3.4 kb BamHI fragment was prepared from pGSBC1 and cloned into pUC19 to generate pGS3, pGS3 was digested with KpnI, and the resulting 2.3 kb fragment was cloned into the KpnI site of pDB1 to generate pGgodB.

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′-ATCAAGCCTTGCGCTGCAATGGGCGGCGG-3′ and godI-DC, 5′-ACGGAATTCTTTGCGGATGTCGAAAAC-3′. Underlined bases indicate the EcoRI and HindIII 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 QuickChange site-directed mutagenesis kit (Stratagene) with pGSB1. The primers were as follows: TSS sense, 5′-CGCCGACCGTCA-GACGATCCTCGTGACGCTG-3′; TSS anti, 5′-CGCGACGACGACTCTCGTGACGCTG-3′; G10A sense, 5′-CATATGCGACGACGCGG-3′; G10A anti, 5′-CTCGAGCGACGACGCGG-3′; 5′-SSTG sense, 5′-GCGGACCGCCTCCGACGCGGCGG-3′; 5′-SSTG anti, 5′-GCGGACCGCCTCCGACGCGGCGG-3′. The point mutations are underlined. Following mutagenesis, the presence of the desired DNA sequences was confirmed. The mutated godA genes were digested with BamHI, and the resulting fragments were cloned into the BamHI site of pTYM19 to generate pTYM-TSS, pTYM-G10A, pTYM-S15T and pTYM-20K.

**RESULTS**

**Cloning of the goadsporin biosynthetic gene cluster.** It was assumed 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 BamHI, BglII, PstI, SacI and Sphi. Among the positive signals (>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 \textit{godA}, one of the genes present in the \textit{god} (goadsporin) cluster. To clone the entire set of genes in the goadsporin biosynthetic cluster, a cosmid library of genomic DNA from \textit{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, \textit{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.

\textit{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 \textit{Actinobacillus actinomycetemcomitans} (Lally \textit{et al}., 1991). LktB is required for the translocation and insertion of \textit{A. actinomycetemcomitans} leukotoxin (AaLtA) into the cell membrane (Lally \textit{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 \(<\text{GSSGSGKS}>\) 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 \(<\text{GSSGSGKS}>\) 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 \textit{gra-orf12}, which is involved in granaticin biosynthesis (44.4% identity, 60% similarity). Granaticin is a benzoiso- chromaquinone-type antibiotic produced by \textit{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 ffh, the signal recognition particle (SRP) in E. coli (44.6 % identity; 74-9 % similarity).

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 BamHI site of pTOYAMAcos. The other plasmids were constructed as described in Methods. The thick arrows indicate the extent and directions of the ORFs. Plasmid pGODI 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-harbouring pGSB14k and pGODI, 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.
The homologous region is partial.

SRP is a ribosomal protein that catalyses targeting of nascent secretory and membrane proteins to the protein translation 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 \( f_{ph} \) homologues in \( Strepomyces avermitilis \) 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).

### 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>
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<tr>
<td>godA</td>
<td>49</td>
<td>Goadsporin structure gene</td>
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<td>godB</td>
<td>550</td>
<td>lktB: leukotxin secretion ATP-binding protein</td>
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<tr>
<td>godC</td>
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<td>lktB</td>
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<td>735</td>
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<td>S. violaceoruber</td>
<td>T46517</td>
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<td></td>
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<tr>
<td>godG</td>
<td>229</td>
<td>Function unknown</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>godH</td>
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<td>Acetyltransferase (putative)</td>
<td>40-9</td>
<td>Mycobacterium tuberculosis</td>
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<td>godI</td>
<td>238</td>
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<td>S. hygroscopicus</td>
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</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 \( \mu \)g 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 \( \mu \)g 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
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 TS5 and SI5T 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).

Both photographs were taken after 5 days growth on Bennett’s agar media.

**DISCUSSION**

**Proposed overall biosynthetic pathway of goadsporin**

A proposed biosynthetic pathway for goadsporin is shown in Fig. 6. The 49 aa long \(\text{god}A\) 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. \(\text{god}D\) is similar to \(\text{gra-orf}34\), 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 lantibionine 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 \(\text{god}E\). 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* 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 \(\text{god}\) 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.

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**Fig. 4.** Effects of \(\text{god}I\) on goadsporin self-resistance. (a) \(S.\) lividans harbouring \(p\text{GODI}\) grow on a plate containing 30 \(\mu\)g goadsporin ml\(^{-1}\), whereas the wild-type strain (\(S.\) lividans) and \(S.\) lividans harbouring \(p\text{GSB14k}\) did not grow. (b) Strain TP-A0584 and its gene disruption mutants were inoculated onto a solid medium containing 7 \(\mu\)g goadsporin ml\(^{-1}\). TP-A0584, \(\Delta\text{god}A\) and \(\Delta\text{god}B\) grow on the goadsporin-containing plate, whereas \(\Delta\text{god}I\) grow did not grow. Both photographs were taken after 5 days growth on Bennett’s agar media.
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.
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 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. scabies, its activity for S. scabies was retained. S. scabies 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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Biosynthetic pathway of goadsporin


