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ORIGINAL ARTICLE

Organization of the biosynthetic genes encoding deoxyactagardine B (DAB), a new lantibiotic produced by *Actinoplanes liguriae* NCIMB41362

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Deoxyactagardine B (DAB) is a hitherto unknown type B lantibiotic, produced by *Actinoplanes liguriae* NCIMB41362. The mature peptide is 19 amino acids in length and structurally analogous to actagardine, differing by two amino acids (V15L and I16V) and the absence of a sulfoxide bond between residues 14 and 19. The biosynthetic genes encoding DAB are clustered, and in addition to the structural gene *ligA* include genes believed to encode for the proteins responsible for the modification, transport and regulation of DAB synthesis. Surprisingly, despite the presence of a gene that shares significant homology to the monooxygenase *garO* from the actagardine biosynthetic gene cluster, the oxidized form of DAB has not been detected. A *lanA* gene encoding the DAB peptide has been introduced into the plasmid pAGvarX and delivered into a strain of *Actinoplanes garbadinensis* lacking the structural gene for actagardine, *garA* (*A. garbadinensis* Δ*garA*). Expression of this gene in *A. garbadinensis* Δ*garA* resulted in the production of actagardine B, an oxidized form of DAB.
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INTRODUCTION

Actagardine and mersacidin are examples of type B lantibiotics that show antibacterial activity against a range of pathogenic bacteria. They adopt a globular-like conformation and inhibit peptidoglycan biosynthesis by binding to the cell wall biosynthetic intermediate lipid II. Interestingly, both actagardine and mersacidin are not antagonized by vancomycin, suggesting that their binding target differs from the current 'last resort drug'. The type A lantibiotics, which include the archetypal nisin[1] along with epidermin[2] and subtilin[3] exist as elongated, flexible molecules that are positively charged and act by forming pores in the cell membrane by binding to the cell wall component lipid II.[4] This group is often further divided into type AI and type AII based on the modification enzymes involved in their biosynthesis.

Lantibiotics are synthesized directly from the ribosome generating a pre-peptide that consists of a leader sequence, which is subsequently cleaved during the processing of the peptide to its mature form. This processing includes the introduction of lanthionine or methyllanthionine bridges formed through the dehydration of serine and threonine residues, respectively, followed by the conjugate addition of cysteine thiols. A single enzyme designated by the generic term LanM catalyzes these reactions amongst type B and type AII lantibiotics, whereas in the biosynthesis of type AI lantibiotics dehydration is mediated by LanB and LanC catalyzes the thioether crosslinking. Additional processing of lantibiotics to their mature form has also been described and includes; for example, oxidative decarboxylation of the C-terminal residue,[5] hydroxylation of an aspartate residue[6] and chlorination of the tryptophan.[7] In addition to a gene encoding the structural peptide and a *lanM*, the biosynthetic gene clusters encoding lantibiotics include a *lanT* gene that encodes a protein responsible for the transport of the lantibiotic from the cell. Genes encoding additional functions including immunity (*lanE, F* and *G*), regulation (*lanR*1, *R* and *K*) and proteolytic cleavage (*lanP*) have also been described.[8,9]

The ribosomal synthesis of lantibiotics potentially makes them more amenable to direct manipulation compared with metabolites that require the coordination of large multi-domain proteins such as polyketide synthases or non-ribosomal peptide synthetases. Together with the reported promising antibacterial activities of this class of compound, the lantibiotics now represent attractive precursors for generating variants with improved activity.[10,11] However, information regarding the organization of the biosynthetic genes of lantibiotics, particularly from G-C rich organisms, is still relatively limited. In this study, we have described the cloning and analysis of the biosynthetic gene cluster encoding deoxyactagardine B (DAB), a hitherto uncharacterized lantibiotic. The DAB cluster consists of structural, modifying and transport genes as well as a gene analogous to *garO* of the actagardine cluster. The gene *garO* is believed to encode a monooxygenase responsible for the oxidation of the sulfur atom bridging residues 14 and 19 to the sulfoxide, although the oxidized form of DAB has not been detected in fermentations of *Actinoplanes liguriae*.

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A variant generation system designed previously for engineering variants of actagardine has been used to investigate the possibility that DAB can be processed to the oxidized form.

MATERIALS AND METHODS

Strains and plasmids

A. liguriae NCIMB41362 and *Actinoplanes garbadinensis* Δ*garA*[12] were routinely maintained by growing on ABB13 medium[13] and incubating at 30 °C. *Escherichia coli* DH10B (Invitrogen, Paisley, UK) was used for the routine propagation of plasmids.

The cosmid SuperCos1 and *E. coli* XL1-Blue MR strain were obtained from Agilent Technologies (Santa Clara, CA, USA). The plasmids pUC19 and pLITMUS28 (both from New England Biolabs, Ipswich, MA, USA) were used for routine cloning. The plasmid pAGvarX used in the construction of the plasmid pAGvar6 has been described previously.[12]

The strain used to evaluate *in vitro* antimicrobial activity was *Micrococcus luteus* ATCC4698.

Identifying and cloning the DAB biosynthetic gene cluster from *A. liguriae* NCIMB41362

A cosmid library of genomic DNA isolated from *A. liguriae* NCIMB41362 was generated by cloning the Sau3AI partially digested DNA fragments (approximately 35–40 kb in size) into the cosmid SuperCos1 (Agilent Technologies) that was digested previously with BamHI and XbaI in accordance with the manufacturer's guidelines. This library was analyzed by colony blot hybridization using digoxigenin-labelled probes. The blotting and hybridization were performed in accordance with the manufacturer's guidelines (Roche Diagnostics, Basel, Switzerland). The membranes were washed post hybridization using 2×SSC (0.03 M sodium citrate, 0.3 M NaCl) including 0.1% v/v SDS for two periods of 5 min each at room temperature followed by two further periods of 15-min washes at 68 °C (hybridization temperature) using either 1×SSC including 0.1% SDS (for the membrane hybridized using SBDIG-1) or 0.1×SSC including 0.1% SDS (for the membrane hybridized using SBDIG-2). The probe, SBDIG-1, is an oligonucleotide designed from backtranslating the sequence for the actagardine A peptide, whereas SBDIG-2 is a 2.3 kb DNA fragment spanning the region encoding the *garA* gene from *A. garbadinensis*.[12] Eleven cosmids hybridized to both probes. DNA was isolated from each cosmid and sequenced using the primers T3 and T7 as well as characterized by restriction enzyme analysis. The cosmid CosAL02 was selected for complete sequencing.

The construction of plasmid pAGvar6

The oligonucleotides O/actV1F (5′-GTACACTGACGATCGAGTGCGGCACC CTCGTCTGCGCCTGCTGAC-3′) and O/actV2R (5′-CTAGGTCAGCAGGCG CAGACGAGGGTGCCGCACTCCATCGTCAGT-3′) were designed to anneal (100 μM of each oligonucleotide were mixed, then heated to 95 °C for 7 min followed by 65 °C for 2 min, and allowed to cool at room temperature) to one another generating a 45 bp fragment with cohesive ends. This fragment was then ligated to pAGvarX, digested previously with BsrGI and AvrII. Sequence analysis of clones transformed with this ligation mix identified a plasmid encoding the LigA peptide. This plasmid was designated pAGvar6.

Conjugation procedure

Intergeneric conjugation between *E. coli* and *A. garbadinensis* Δ*garA* was performed following the procedure described previously.[12] Exconjugants were transferred and patched out over an area approximately 1 cm^2 onto ABB13 medium containing nalidixic acid and apramycin, each at a final concentration of 50 μg ml^{-1}. These plates were incubated at 30 °C for 10–14 days before being used as inoculum for broth cultures.

Fermentation of *Actinoplanes sp.*

Agar plugs from *A. liguriae*, *A. garbadinensis* Δ*garA* and *A. garbadinensis* Δ*garA*/pAGvar6 exconjugants grown on ABB13 medium (supplemented with antibiotics as required) were used to inoculate 50-ml conical flasks containing 8 ml of actagardine A seed medium (AAS, soluble starch 10 g, glucose 10 g, bacto-peptone 5 g, dry corn steep liquor 1 g, yeast extract 2 g, reverse osmosis water 1 litre and pH adjusted to 7) with two glass beads and supplemented (exconjugants only) with nalidixic acid and apramycin, each to a final concentration of 50 μg ml^{-1}. The cultures were incubated at 30 °C, 250 r.p.m. for 72–96 h, after which 1.5 ml (3%) from each culture was used to inoculate a 250-ml conical flask containing 50 ml of GM1 (lab-lemco meat extract 4 g, peptone 4 g, NaCl 2.5 g, yeast extract 1 g, soyflour (Cargill, Minneapolis, MN, USA) 10 g, glucose 25 g, $CaCO_3$ 5 g, reverse osmosis water 1 litre and pH adjusted to 7.6). The cultures were incubated at 30 °C as described earlier. After 5 days of incubation, 1 ml of whole broth was removed from each culture and the cells were pelleted by centrifuging (IEC Micromax benchtop centrifuge, International Equipment Company, Needham Heights, MA, USA) at 12 000 r.p.m. The supernatants were decanted and analyzed by HPLC and liquid chromatography–MS.

Detection of DAB and its variants

HPLC analyses were performed using a Hewlett Packard 1050 series HPLC system (Agilent Technologies) with an Phenomenex Hyperclone BDS C18 4.6×150 mm, 5 μ column (Phenomenex, Torrance, CA, USA). The HPLC–MS analyses were performed on a HPLC system (as described) with an Agilent Zorbax SB-C18 4.6×150 mm, 5 μ column (Agilent Technologies) linked to a Micromass Platform LC operated with MassLynx version 3.5 software (Waters, Milford, MA, USA). The instrument methods for both HPLC and HPLC–MS are described in the Supplementary information.

NMR

NMR analyses were performed using a Bruker AV 500 spectrometer (Bruker Biospin Corp., Billerica, MA, USA) with a QN Z-gradient probe operating at 500.13 MHz (^{1}H, correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), nuclear overhauser effect spectroscopy (NOESY), heteronuclear single quantum correlation (HSQC) and heteronuclear multiple bond correlation (HMBC)) and 125.76 MHz (^{1}H decoupled and J-MOD-X ^{13}C). Spectra were processed using ACD (Advanced Chemistry Development, Toronto, ON, Canada) and TopSpin (Bruker Biospin Corp.) NMR processing software. The assignments for the protons and carbons are summarized in Supplementary Table S1.

Well diffusion assays

M. luteus ATCC4698 was inoculated from frozen stock into 10 ml of Mueller–Hinton broth (CM0405, Oxoid, Basingstoke, UK) and grown overnight at 30 °C and 200 r.p.m. 1 ml of this culture was used to inoculate 300 ml of molten Mueller–Hinton agar (Oxoid, CM0337) kept at 50 °C, which was then dispensed into petri dishes as 25-ml aliquots. Wells (6 mm in diameter) were made into the seeded agar and subsequently loaded with 50 μl of the samples. The bioassay plate was placed into a biological safety cabinet until the loaded samples diffused, at that point the plates were transferred to a 30 °C incubator and left for 48 h.

Software

Raw sequencing data were assembled using GAP (Genome assembly program version 4.10; Staden, Cambridge, UK), and the assembled sequences of CosAL02 and CosAG14 were compared by local alignment using Spin version 1.3 set with the following default values: score for match=1; score for transition=−1; score for transversion=−1; penalty for starting gap=6; penalty for each residues in gap=0.2.[14] Frameplot version 2.3.2[15] and BLAST (Basic Local Alignment Search Tool, National Centre for Biotechnology Information, Bethesda, MD, USA) were used to identify and annotate open reading frames (ORFs), while ClustalW (European Bioinformatics Institute, Hinxton, Cambridge, UK) was used to align sequences.

RESULTS

Analysis of the biosynthetic gene cluster encoding DAB

A. garbadinensis and *A. liguriae* have been described as producers of lantibiotics.[16,17] A cosmid library of genomic DNA isolated from *A. liguriae* NCIMB41362 was constructed and screened against a

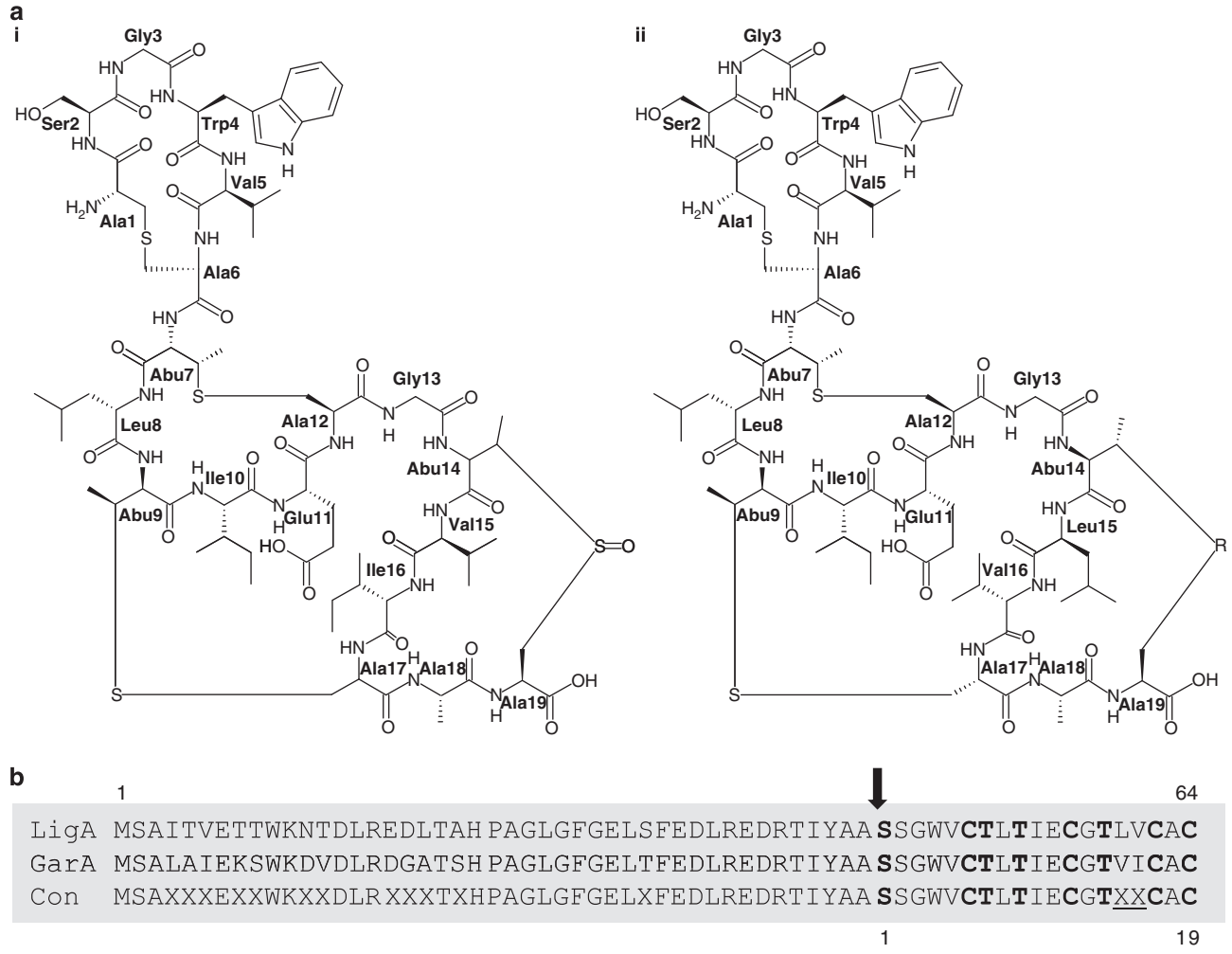

Figure 1 The structures and amino acid sequences of actagardine A and deoxyactagardine B (DAB) and the proposed structure of actagardine B. The structure of actagardine is shown in **a**(i) while the structure of DAB (R=S) and proposed structure of actagardine B (R=SO) are shown in **a**(ii). The amino acid sequence of the GarA and LigA prepeptides (1–64) together with the consensus (con) sequence are shown in (**b**). The arrow indicates the site of protease cleavage and release of the mature peptide (1–19) from the leader sequence. Residues involved in bridge formation are shown in bold while positions 15 and 16 of the mature peptides are underlined. Abu, 2-aminobutyric acid.

digoxygenin-labelled oligonucleotide and a DNA probe encoding the actagardine structural gene. The cosmid CosAL02 hybridized to both probes and was selected for complete sequencing. This cosmid contains a 40 402 bp fragment encoding one incomplete ORF at the 5′ end followed by 30 complete ORFs (this sequence has been deposited in the GenBank database with the accession number GQ372844). Translation of ORF12 identified a *lanA* gene, designated *ligA*, that encodes a 64-amino-acid protein representing a lantibiotic pre-peptide. The amino-acid sequence of the LigA peptide although identical in length to actagardine (19 amino acids) differs by two amino acids. LigA consists of a leucine and valine at positions 15 and 16, respectively, in place of the valine and isoleucine residues of actagardine (Figure 1). The product of the *ligA* gene identified in CosAL02 represents a novel lantibiotic as confirmed by mass spectrometry and NMR analyses of the DAB peptide isolated from *A. liguriae* NCIMB41362 fermentation broth (isolation from 2 l fermentations, mass spectroscopy data and NMR assignments are provided in the Supplementary information). This hitherto unknown lantibiotic has been designated DAB and together with the V15L and I16V mutations differs from actagardine by the absence of a sulfoxide moiety in the methyllanthionine bridge between residues 14 and 19. In accordance with the previously outlined recommendations regarding nomenclature,[18] we have designated actagardine[19] as "actagardine A" and assigned the suffix 'A' to variants thereof, while DAB and variants thereof are assigned the suffix "B".

The leader peptide of LigA is the same length (45 residues) as the leader of the actagardine A precursor GarA, and shares a 78% sequence identity. LigA also shows a double alanine proteolytic cleavage motif at positions −1 and −2 relative to the pre-peptide. This arrangement is consistent with those described for the leader peptides of actagardine A[12] as well as mersacidin[20] and staphylococcin C55β.[21] Partial cleavage at this motif is thought to result in the retention of the −1 alanine residue generating ala(0)-actagardine A in the fermentations of *A. garbadinensis*.[12] The arrangement of this putative proteolytic site is identical in DAB, so it is not surprising that the ala(0)-variant of DAB is routinely observed in fermentations of *A. liguriae* albeit at lower levels than DAB itself (approximately 5–10% of DAB levels).

The organization of the genes included in CosAL02 is shown in Figure 2, whereas the proposed function of the protein encoded

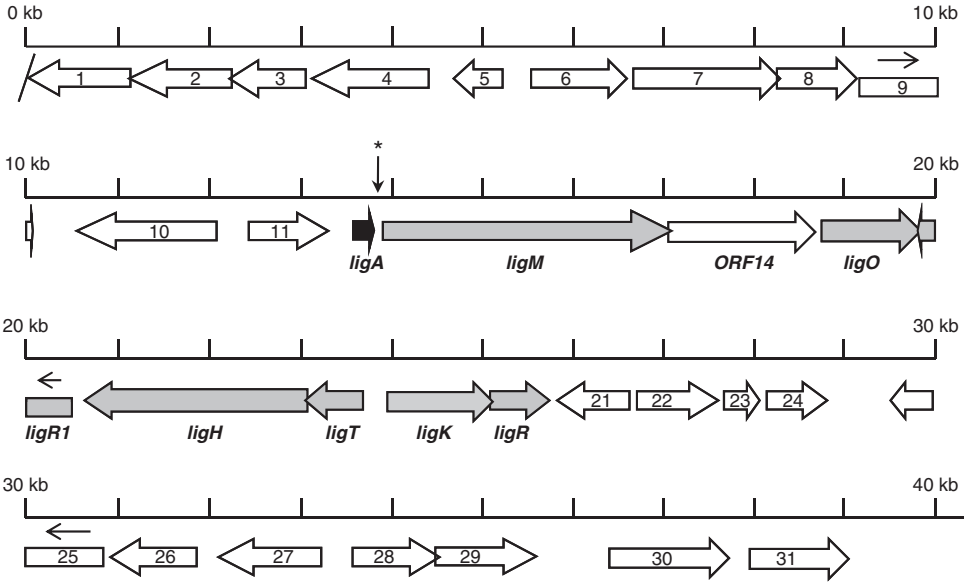

Figure 2 Map showing the gene organisation of the cosmid CosAL02 consisting of DNA from *Actinoplanes liguriae* NCIMB41362. The order and direction of transcription of all the open reading frames are shown. Genes believed to be involved in the biosynthesis of deoxyactagardine B are filled, with the structural gene *ligA* colored black. The approximate position of the putative stem loop downstream of *ligA* is indicated by an asterisk.

by each ORF based on analysis of sequence comparisons, is provided in Table 1. As reported for the biosynthetic gene clusters of actagardine A,[12] cinnamycin[6] and mersacidin,[20] the structural gene is followed by a putative stem-loop structure (ΔG, $-131.5\,kJ\,mol^{-1}$, beginning at 17 bp downstream from the stop codon and continuing for 32 bases). This loop is believed to act as a rho-independent transcription terminator. The eight genes that lie downstream from this loop represent ORFs 13–20, and share a similar arrangement and significant degree of homology to the actagardine A biosynthetic genes isolated from *A. garbadinensis* (encoded on the cosmid CosAG14)[12] as shown in Figure 3. The ORFs 13–20 have been assigned the following annotations based on the sequence alignments.

ligM (ORF13) lies adjacent to *ligA* and encodes a putative 1046-amino-acid protein that shares 70% sequence identity with GarM, and is 31% identical to MrsM from the mersacidin lantibiotic gene cluster.[20] The LigM protein is likely to catalyze the formation of the lanthionine bridges observed in the mature peptide.

Unlike the arrangement of the biosynthetic gene cluster reported for actagardine A wherein the gene encoding the monooxygenase *garO* lies downstream of *garM*,[12] this position is occupied by ORF14 in the DAB cluster. The putative protein of this gene shares a sequence identity of 51% with the putative ATP-binding cassette transporter of *Streptomyces coelicolor* A3(2) encoded by SCO3235.

ligO (ORF15) encodes a 347-amino-acid protein, which is highly homologous (sharing a sequence identity of 77%) to the monooxygenase (GarO) recently described in the actagardine A biosynthetic gene cluster.[12] An alignment of the putative peptide sequences of LigO with GarO is provided in Supplementary Figure S1.

ligR1 runs in the reverse orientation to *ligO* and encodes a putative 217-amino-acid protein with sequence identity ranging between 40–45% to response regulators containing an N-terminal CheY-like receiver domain and an helix-turn-helix DNA-binding domain. The conserved residues, Asp53 and Lys105, associated with receiver domains are present[22] together with key residues involved in helix-turn-helix DNA binding.[23] LigR1 shares a 64% sequence identity with GarR1 (ORF22) from the actagardine A gene cluster.

ligH likely encodes an 814-amino-acid protein that shares a 71% sequence identity with the ATP-binding cassette transporter permease, GarH, present in the actagardine A cluster. As with the arrangement of *cinH* of the cinnamycin biosynthetic genes, *ligH* seems to be translationally coupled to the downstream ORF designated *ligT*. The stop codon of *ligT* overlaps the start codon of *ligH* by 3 bp. The putative protein encoded by *ligT* is 240 amino acids in size and shares a 56% sequence identity with the probable ATP-binding cassette transporter, ATP-binding subunit encoded by SCO3089 of *S. coelicolor* A3(2).[24] The organization of the *ligH* and *ligT* genes together with their proposed products suggest that they are involved in the transport of DAB from the cell and/or have a role in self-immunity.

ORFs19 and 20 overlap by 3 bp and have been designated *ligK* and *ligR*, respectively. The putative products of these genes encode the two-component regulatory system made up of a histidine kinase (LanK) and response regulator (LanR). Analogous genes are present in the actagardine A cluster (*garK* and *garR*, respectively) as well as in the gene clusters of mersacidin, cinnamycin and michiganin A.[25] Studies by Guder *et al*.[26] have shown that MrsR2 and MrsK2 of the mersacidin cluster regulate the expression of genes (*mrsE, F* and *G*) involved in self-immunity. Although genes homologous to *mrsE*, *F* and *G* have not been identified amongst those described in this study, it remains conceivable that the products of *garK* and *garR* are involved in the regulation of a self-immunity mechanism.

Production of actagardine B in *A. garbadinensis* Δ*garA*

The putative peptide encoded by the gene *ligO*, is similar in size and shares a high degree of homology to the monooxygenase GarO (77% sequence identity), of the actagardine A biosynthetic gene cluster from *A. garbadinensis*. Previous studies in which the *garO* gene was deleted from *A. garbadinensis*, showed that the resulting strain (*A. garbadinensis* Δ*garO*) produced solely unoxidized actagardine A and suggest that GarO catalyzes the formation of the sulfoxide bond in actagardine A.[12] Given the proximity of *ligO* to the DAB structural gene and its similarity to *garO*, the putative protein encoded by *ligO* may be expected to catalyze an oxidation reaction equivalent to that observed for GarO

Table 1 Open reading frames identified in CosAL02. The lengths in amino acids of each protein are provided as the proteins with the closest degree of homology together with their accession numbers and E value

ORF	*Proposed function*	*Length*	*Protein with closest homology (accession number)*	E *value*
1[a]	Secretion system protein	375	Type II secretion system protein E, *Nocardioides* sp. JS614 (YP_924305.1).	4e−134
2	Response regulator	389	AAA ATPase, *Thermomonospora curvata* DSM 43183. (YP_003299464.1).	3e−69
3	Unknown	266	Hypothetical protein JNB_18858, *Janibacter* sp. HTCC2649. (ZP_00996974.1).	7e−15
4	Unknown	432	Hypothetical protein Tcur_1855, *T. curvata* DSM 43183. (YP_003299466.1).	8e−18
5	Response regulator ATP binding	136	PAS/PAC sensor hybrid histidine kinase, *Geobacter* sp. M18. (ZP_05312722.1).	3e−18
6	ABC sugar transporter	363	Sugar uptake ABC transporter periplasmic solute-binding protein *Streptomyces griseoflavus* Tu4000. (ZP_05543308).	7e−125
7	ABC sugar transporter ATP binding	501	Sugar uptake ABC transporter ATP-binding protein, *S. griseoflavus* Tu4000. (ZP_05543307).	0.0
8	ABC transport permease	319	Sugar uptake ABC transporter permease protein, *Streptomyces* sp AA4. (ZP_05477438).	1e−107
9	ABC transport permease	320	Putative sugar uptake ABC transporter permease protein, *Streptomyces griseus* subsp. *griseus* NBRC 13350. (YP_001828117).	4e−92
10	Metallopeptidase	486	Putative secreted aminopepetidase *Streptomyces scabiei* 87.22. (CBG69582).	8e−107
11	Transcriptional regulator	286	SaqI, *Micromonospora* sp. Tu 6368. (ACP19349).	2e−64
12	LigA, deoxyactagardine pre-peptide	64	Actagardine pre-peptide, *Actinoplanes garbadinensis* ATCC31049. (ACR33052).	1e−23
13	LigM, modification enzyme	1046	Actagardine modification enzyme, *A. garbadinensis* ATCC31049. (ACR33053).	0.0
14	ABC transporter	575	ABC transporter, *Streptomyces coelicolor* A3(2). (NP_627447).	4e−129
15	LigO, monooxygenase	347	Monooxygenase, *A. garbadinensis* ATCC31049. (ACR33054).	8e−135
16	LigR1, response regulator	217	Response regulator, *A. garbadinensis* ATCC31049. (ACR33055).	3e−68
17	LigH, transporter permease	814	ABC transporter associated permease, *A. garbadinensis* ATCC31049. (ACR33056).	0.0
18	LigT, ABC transporter	240	ABC transporter, *A. garbadinensis* ATCC31049. (ACR33057).	9e−103
19	LigK, response regulator kinase	362	Response regulator, *A. garbadinensis* ATCC31049. (ACR33058).	8e−104
20	LigR, response regulator sensor	218	Response regulator, *A. garbadinensis* ATCC31049. (ACR33059).	2e−79
21	Putative membrane protein	301	Hypothetical protein SgriT_32657, *S. griseoflavus* Tu4000. (ZP_05542899).	4e−80
22	α/β hydrolase-like protein	290	Hypothetical protein KRH_07570, *Kocuria rhizophila* DC2201. (YP_001854610).	2e−82
23	Transcriptional regulator	145	Transcriptional regulator, MarR family protein, *Burkholderia ubonensis* Bu. (ZP_02376166).	2e−23
24	Pyruvoyl-dependent arginine decarboxylase	175	Pyruvoyl-dependent arginine decarboxylase, *Beutenbergia cavernae* DSM 12333. (YP_002880881).	1e−22
25	Diaminopimelate decarboxylase	406	Diaminopimelate decarboxylase, *Rhodococcus erythropolis* SK121. (ZP_04387790).	8e−54
26	Kinase	309	Diacylglycerol kinase, *Micromonospora* sp. ATCC 39149. (ZP_04604204).	1e−111
27	Transcriptional regulator	367	Cell envelope transcriptional attenuator lytR, *Micromonospora* sp. ATCC 39149. (ZP_04605484).	1e−54
28	Glycosyl transferase	317	Glycosyl transferase family protein, *Salinispora tropica* CNB-440. (YP_001158908).	3e−102
29	Glycosyl transferase	369	Glycosyl transferase, group 1, *S. tropica* CNB-440. (YP_001158907).	1e−107
30	Dihydrolipoamide dehydrogenase	459	Dihydrolipoamide dehydrogenase, *Actinosynnema mirum* DSM 43827. (YP_003103801).	0.0
31	Putative membrane protein	348	Hypothetical protein RHA1_ro05161, *Rhodococcus jostii* RHA1. (YP_705100).	2e−21

Abbreviation: ABC transporter, ATP-binding cassette transporter.
[a]ORF1 is believed to be incomplete.

resulting in the production of actagardine B (the oxidized form of DAB). However, actagardine B has never been detected in fermentations of *A. liguriae*. This casts doubt over the proposed role of LigO or otherwise suggests that the gene is not expressed or the proposed product of this gene is nonfunctional. To investigate the possibility that the apparent lack of actagardine B production is a consequence of the differences in the amino-acid sequence between actagardine A and B, the plasmid pAGvarX[12] was modified to encode actagardine B.

The plasmid pAGvarX contains a modified version of the actagardine structural gene *garA*, which has been engineered to enable any of the amino acids encoded by *garA* to be replaced, deleted or inserted at any position with annealed oligonucleotides encoding the alternative gene sequence. Using pAGvarX as a backbone the plasmid pAGvar6, which encodes the DAB peptide, was constructed. This plasmid was introduced into *A. garbadinensis* Δ*garA*, a strain generated through the removal of the *garA* gene, but retaining the auxillary genes of the actagardine A biosynthetic gene cluster necessary to process the pre-peptide to the mature lantibiotic product. Apramycin resistant exconjugants were identified and grown in the GM1 production medium for 5 days. The supernatants were collected and analyzed by HPLC and liquid chromatography–MS in addition to being tested for antibacterial activity against *M. luteus*. The analyses of supernatants from strains of *A. garbadinensis* Δ*garA* transformed with the plasmid pAGvar6 (designated *A. garbadinensis* Δ*garA*::pAGvar6) confirmed the presence of a biologically active compound with a mass and retention time consistent to actagardine B, the oxidized form of DAB (Figure 4, well 3). This compound was absent in the fermentation broths of *A. garbadinensis* Δ*garA*, and no biological activity against the indicator strain was detected (Figure 4, well 2).

DISCUSSION

The lantibiotic DAB is a new type B lantibiotic, 19 amino acids in length, produced by the actinomycete *A. liguriae* NCIMB41362. It differs from actagardine A by two amino acids (V15L and I16V, actagardine A/DAB) and the omission of a sulfoxide moiety in the methyllanthionine bridge between residues 14 and 19. The organization of the biosynthetic genes believed to encode DAB is similar to the recently described gene cluster for actagardine A.[12] Both clusters consist of genes believed to encode for the modification of the mature peptide (*lanM*), pathway regulation (*lanR1*) including a two-compo-

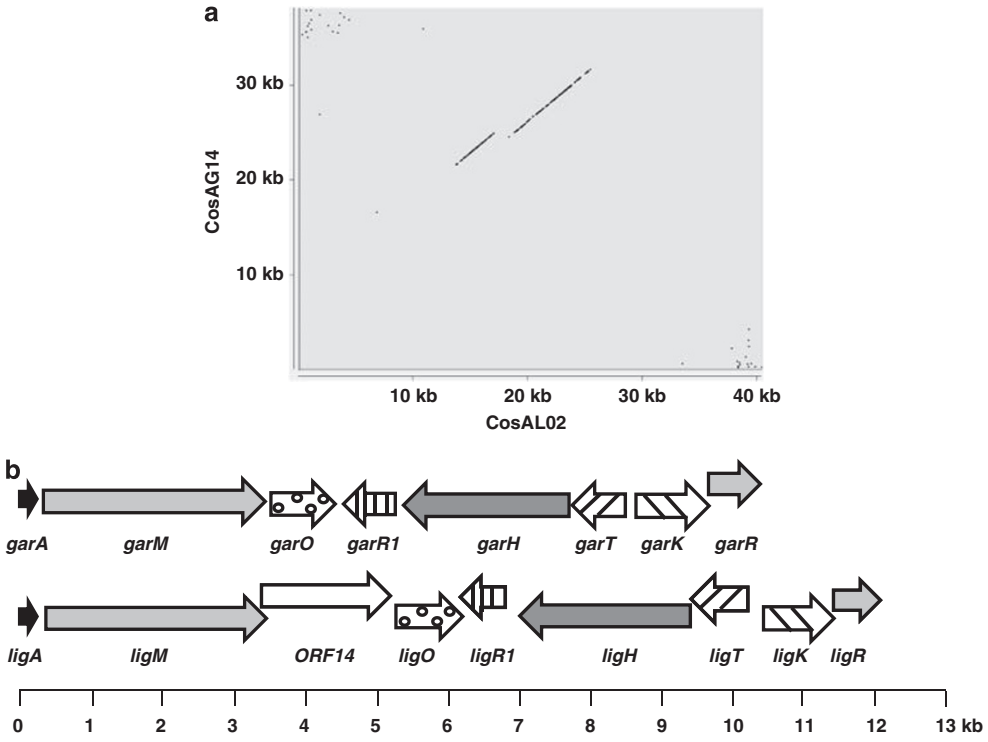

Figure 3 (**a**) Synteny plot of cosmids consisting of genomic DNA isolated from *Actinoplanes liguriae* NCIMB41362 (cosmid CosAL02, 40402 bp) and *Actinoplanes garbadinensis* ATCC31049 (cosmid CosAG14, 38168 bp). Aligned regions are colored black and correspond to the genes believed to encode for the biosynthesis of the respective lantibiotic (CosAG14: actagardine A; CosAL02: deoxyactagardine B). The gap between the alignments is consistent with the additional gene (ORF14) present within CosAL02. (**b**) Shows a relative alignment of the homologous regions proposed to encode the biosynthetic genes for actagardine A (prefix, *gar*) and DAB (prefix, *lig*) highlighted in the synteny plot.

nent regulation system (*lanR* and *K*), transport (*lanH* and *lanT*) as well as a monooxygenase (*lanO*) predicted to catalyze the oxidation of sulfur in the methyllanthionine bond between residues 14 and 19. However, there is no noticeable gene encoding a protease (*lanP*) responsible for the release of the mature peptide from its leader sequence. This is consistent with the arrangement described for the lantibiotic clusters of actagardine A,[12] cinnamycin[6] and michiganin A.[27] The apparent lack of a discrete protease or protease domain encoded amongst the genes identified in this work suggests that cleavage of the leader peptide is carried out by an enzyme encoded by a gene or genes elsewhere on the host's genomic DNA or alternatively occurs by a novel cleavage mechanism.

The organization and degree of similarity between the genes of the actagardine A biosynthetic cluster and DAB significantly decreases beyond the boundaries of their respective structural gene (*lanA*) and *lanR*, falling from around 76% (65% including ORF14) within this boundary to between 30–42% at the flanking regions. This clear decrease in the level of homology possibly signifies the boundary of the genes involved in the biosynthesis of the respective lantibiotic peptides, and suggests that the penicillin-binding protein identified in proximity to the structural gene of actagardine A is not required for self-immunity.[12] Interestingly, the most noticeable difference between the arrangement of the DAB biosynthetic genes and those for actagardine A is the presence of *ORF14*, an additional ORF located between *ligM* and *ligO*. Analysis of *ORF14* indicates that the start codon of this gene overlaps the 3′ region of *ligM* by 8 bp, and as such it is likely that these ORFs are translationally coupled. The putative protein encoded by *ORF14* is 575 amino acids in length and possesses many of the sequence motifs associated with transport proteins including a Walker A/P loop (from G370), a Q-loop (Q418), a Walker B aspartic acid (D498) and a conserved histidine residue (H530) denoting the H-motif.[28] The lack of an homologous gene amongst the actagardine A biosynthetic cluster suggests that the putative product encoded by this ORF may not be essential for the biosynthesis of DAB. The fact that *ORF14* seems to be translationally coupled to *ligM*, however, may suggest that its product is involved or contributes toward an alternative mechanism of transport or self-immunity that seems to be absent in the biosynthetic pathway of actagardine A. The importance of this gene and its possible role in the biosynthesis of DAB are the subject of continuing studies.

Despite the presence of a putative monooxygenase encoded by the *ligO* ORF, no products with masses corresponding to oxidized DAB were detected in fermentations of *A. liguriae*. This is somewhat surprising as LigO aligns closely to the monooxygenase of actagardine A, GarO, sharing a sequence identity of 77%. Previous studies have shown that GarO catalyzes the oxidation of deoxyactagardine A, resulting in the oxidation of the thioether linkage between residues 14 and 19 of the mature peptide.[12] Comparison of the peptide sequences of LigO and GarO shows that both proteins (Supplementary Figure S1) are similar in size and possess many of the residues conserved amongst bacterial monooxygenases, such as the well-characterized luciferase, LuxA peptide, from *Vibrio harveyi*[29] and MelF from *Mycobacterium marinum*, mel2.[30] The most striking difference between LigO and GarO is the presence of a region of eight residues toward the C-terminal of LigO, which are absent in GarO. These eight residues together with the proline and glycine that lie immediately upstream of this additional region are encoded by a prominent GC-rich stretch of 30 nucleotides. Sequence analysis of this region

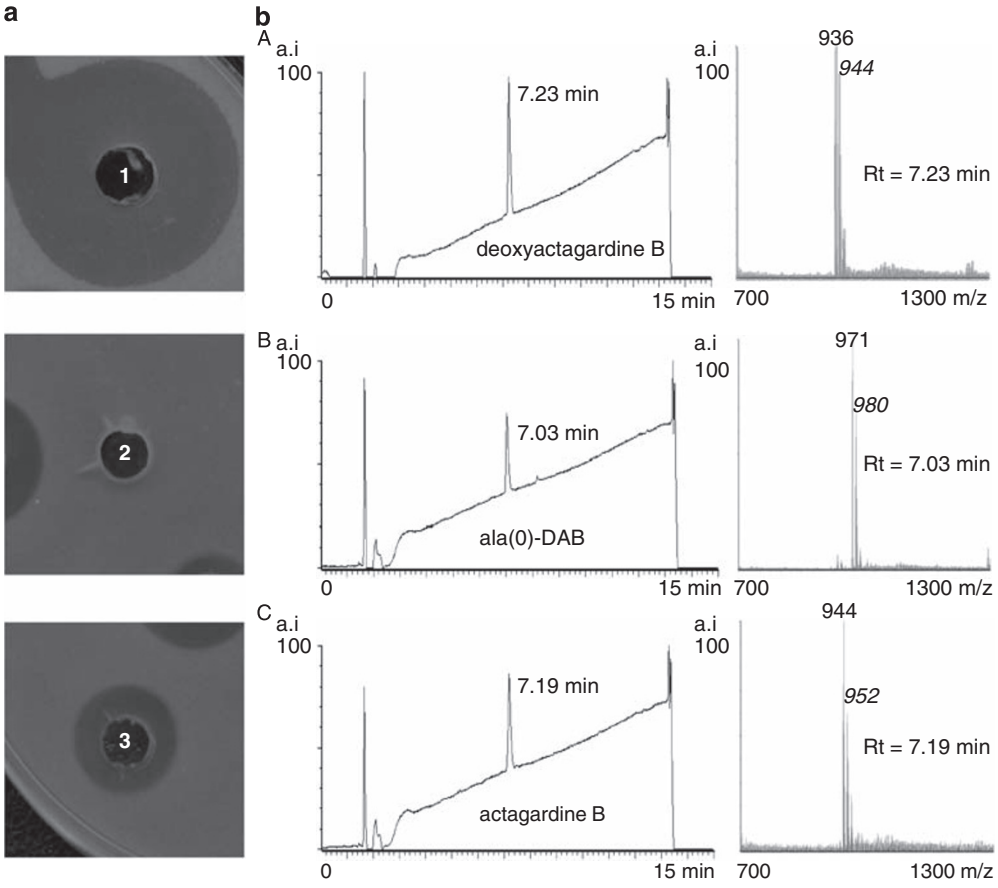

Figure 4 The analysis of supernatants from fermentations of *A. liguriae* and engineered strains of *A. garbadinensis* grown in the medium GM1 for 5 days. The wells in Mueller-Hinton agar seeded with *Micrococcus luteus* (**a**) were loaded with supernatants (50 μl) from fermentations of the following strains: (1) *A. liguriae*; (2) *A. garbadinensis* Δ*garA* and (3) *A. garbadinensis* Δ*garA*/pAGvar6. (**b**) HPLC and LC-MS traces of the purified active compounds detected in the supernatants of *A. liguriae* (A, B) and *A. garbadinensis* Δ*garA*/pAGvar6 (C). The major ions detected were the $[M+2H]^{+2}$ and the $[M+H+Na]^{+2}$ (italicized) ions. The mass spectrometry traces relate to the respective major HPLC peaks with the following retention times (Rt); DAB, 7.23 min; ala(0)-DAB, 7.03 min and actagardine B, 7.19 min. Total ion current spectra are provided in supplementary information (Supplementary Figure S2).

indicates that these residues could form a putative stem-loop structure (ΔG, $-16.10\,kcal\,mol^{-1}$). A stem-loop at this position may force premature termination and result in the formation of an inactive truncated protein. Unlike the arrangement in the actagardine A gene cluster, *ligO* overlaps the downstream ORF encoding LigR1. In addition, the positioning of the additional adjacent upstream ORF may represent an evolutionary artefact, the introduction of which has resulted in downstream polar effects that have somehow rendered *ligO* as not expressed or nonfunctional. However, no motifs indicative of an insertion element or elements flanking *ligO* have been identified to support this hypothesis.[31] Although the location and high degree of homology between LigO and GarO may suggest similar biosynthetic roles, in the absence of definitive biochemical data the possibility also remains that the putative enzyme encoded by *ligO* catalyzes an alternative reaction to the oxidation of DAB.

In contrast to the lack of detection of actagardine B from fermentations of *A. liguriae*, when an engineered version of *garA* that encodes actagardine B is expressed in the host *A. garbadinensis* Δ*garA* (*A. garbadinensis* Δ*garA*/pAGvar6) a compound with a mass corresponding to actagardine B was detected. Surprisingly, no compound corresponding to ala(0)-actagardine B (actagardine B including an additional alanine residue in position −1 relative to the mature peptide) was detected in the supernatant from fermentations of *A. garbadinensis* Δ*garA*/pAGvar6. The reasons for the seeming lack of ala(0)-actagardine B are unclear. It may be that ala(0)-actagardine B is produced, but is less stable and readily converted by a native protease, alternatively it could reflect the relative levels of production of actagardine B ($\sim 15\,mg\,l^{-1}$ compared with $\sim 200\,mg\,l^{-1}$ DAB). Nevertheless, the detection of actagardine B in *A. garbadinensis* Δ*garA*/pAGvar6 suggests that GarO together with the remaining processing enzymes of the actagardine A biosynthetic pathway are able to accept the LigA pre-peptide as a substrate. Although it is possible that the formation of a sulfoxide residue between positions 14 and 19 of LigA is because of an alternative monooxygenase within the host *A. garbadinensis* Δ*garA*, this does show that the LigA peptide is amenable to oxidation and highlights the flexibility of the actagardine A biosynthetic machinery in accepting alternative substrates. It also provides further validation of the use of the plasmid pAGvarX as a vehicle for the generation of lantibiotic variants based on the DAB peptide despite lower production levels as observed previously.[12] In contrast to the production levels of variants of the DAB peptide, the levels of DAB produced by *A. liguriae* are approximately fourfold higher ($200–250\,mg\,l^{-1}$) than those observed for actagardine A by *A. garbadinensis* ($50–80\,mg\,l^{-1}$).[12]

While work to delineate the minimal gene set required for the biosynthesis of DAB remains the subject of continuing study, the discovery of a new type B lantibiotic will hopefully serve to aid in a greater understanding of this important class of molecules.

CONFLICT OF INTEREST

All the authors are employees of Novacta Biosystems Ltd and the authors SB, JC and MD are shareholders of Novacta.

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