Transcriptional Regulation of the Lantibiotic Structural Gene from *Bifidobacterium longum* DJO10A

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XIULAN LI

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Dedication

This thesis / project is dedicated to my love, Cheel Borjigin, and our first three years of life in United States of America.

Abstract

Bifidobacterium longum DJO10A produces a lantibiotic, but only during growth on agar media. A quantitative real-time PCR assay targeting its *lanA* gene was developed and revealed that the gene was up-regulated more than nine-fold on agar than in broth. A crude lantibiotic preparation revealed a broad spectrum of inhibition. Addition of this extract to broth cultures of *B. longum* DJO10A induced *lanA* gene expression in a dose-dependent and log-phase fashion. Sub-inoculation using > 10% of an induced broth culture maintained *lanA* expression. The inducer is most likely the lantibiotic itself, given that a ~ 3.2 kDa peak corresponding to the predicted size for the lantibiotic, was attached to the cell surface. A predicted repressor gene *lanRI* was codon optimized and expressed in vitro. Purification of this protein will further our understanding of its function and facilitate the development of strategies to enable production of this lantibiotic in broth.

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Literature Review

Contribution of the *Actinobacteria* to the Growing Diversity of Lantibiotics

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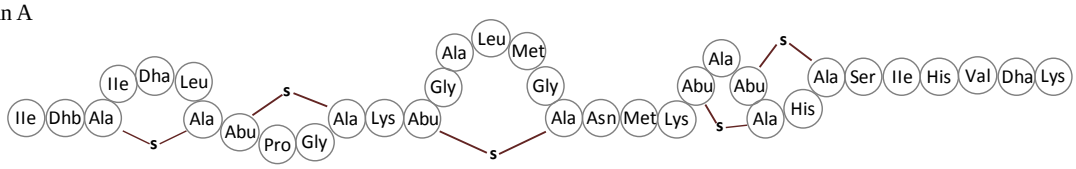
Introduction

Lantibiotics are gene-encoded, antimicrobial peptides that undergo posttranslational modifications and are only produced by some Gram-positive bacteria. While their antimicrobial activities are broader than other bacteriocins, they are primarily limited to effects on Gram-positive bacteria as the outer membrane of Gram-negative bacteria prevents their access to the cell membrane, which is their site of action. The designation "lantibiotic" is derived from "lanthionine containing antibiotic" (Schnell et al. 1988).

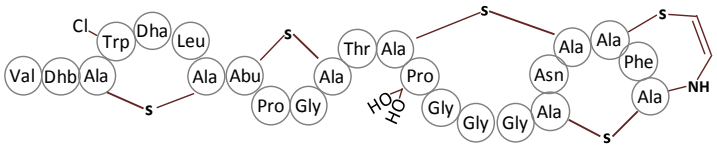
Lanthionines (See Fig. 1) are ring structures consisting of two alanine residues linked by a thioether bridge, which forms following the linkage of a cysteine residue to the unusual amino acid 2,3-didehydroalanine (Dha). When the unusual amino acid is 2,3-didehydrobutyrine (Dhb), the ring structure is referred to as methyllanthionine (MeLan). Other post translational modifications can also occur and include 1-α-aminobutyric acid, lysinoalanine, S-aminovinyl-D-cysteine (AviCys), 2-Oxobutyry, 2-Hydroxypropionyl, Dihydroxyproline, 2-Oxopropionyl, *erythro*-3-hydroxy-L-aspartic acid, chlorinated tryptophan and labionin (Lab). Currently, 76 lantibiotics have been described in the published literature and the vast majority of them are produced by members of the *Firmicute* phylum of bacteria. Nisin, which is produced by certain strains of *Lactococcus lactis,* was the first one to be characterized. It was initially observed as a non-acid antimicrobial in 1928 (Rogers and Whittier 1928) and was biochemically characterized in

FIG. 1. Molecular structure of nisin, illustrating characteristics of atypical lantibiotics, as well as lantibiotics from *Actinobacteria* that contain extra novel modifications. Labionin (Lab) is formed from two Dha and one cysteine linked consecutively (Sambeth and Sussmuth 2011).

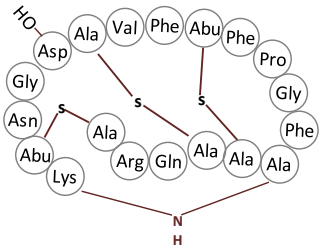
Nisin A



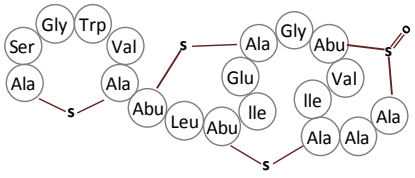
Microbisporicin A1



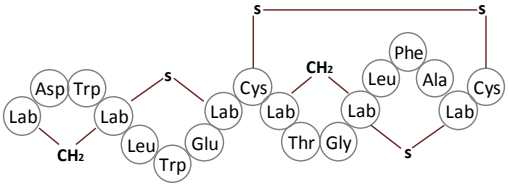
Cinnamycin



Actagardine



Labyrinthopeptin A2



the ensuing decades (Gross and Morell 1971). It has broad spectrum antimicrobial activity against most Gram-positive bacteria. Relatively few lantibiotics have been identified from the high G+C Gram-positive bacteria, classified in the phylum *Actinobacteria*. Those that have been characterized from this phylum have displayed novel modifications (Fig. 1), highlighting the potential for new lantibiotics with additional functionalities to be identified from other members of this phylum. Table 1 highlights the unique differences between lantibiotics from *Actinobacteria* and *Firmicutes*.

Characteristics of a typical lantibiotic gene cluster

The genes involved in lantibiotic biosysthesis are clustered and have been found on conjugative transposons (nisin), the chromosome of the host (subtilin, epilancin K7, etc.), or on plasmids (Pep5, lacticin 481, etc). Only nine lantibiotics from *Actinobacteria* have been genetically characterized and all are chromosomally located. Comparison of the gene clusters indicates the presence of a number of conserved genes believed to encode similar functions.

Figure 2 illustrates the gene clusters from the nine *Actinobacteria* lantibiotics. The structural gene designated as *lanA* encodes an unmodified prepropeptide, containing a C-terminal propeptide that undergoes post-translational modification prior to transport across the cell membrane and removal of the N- terminal leader peptide. This leader

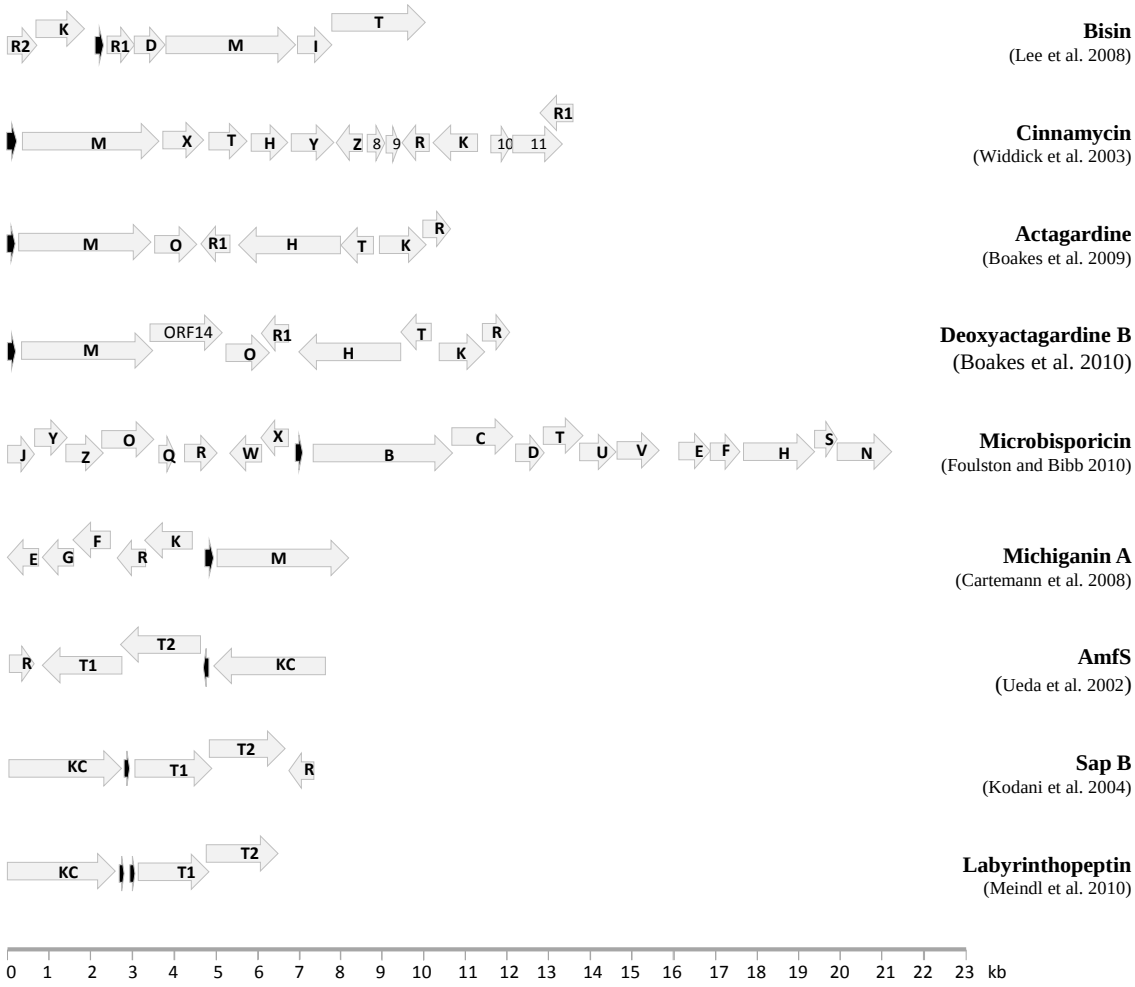
sequence for the 77 lantibiotic peptides currently characterized at the genetic level is between 20 and 59 amino acids (Supplementary Table 1). The LanA peptide is modified by both LanB and LanC enzymes or by a single LanM enzyme. Lan B is a dehydratase and is required for selective dehydration of Ser and Thr residues in the LanA propeptide to Dha and Dhb, respectively (Schnell et al. 1988; Sen et al. 1999). LanC (a cyclase enzyme) is responsible for forming the lanthionine structures by linking Dha and Dhb to cysteine residues via thiol groups generating *meso*-lanthionine (Lan) and methyllanthionine (MeLan), respectively (Meyer et al. 1995; Koponen et al. 2002). LanM is a bifunctional modification enzyme that fulfills the functions of both dehydration and cyclization (Xie et al. 2004). Sequence homologies at the C terminal of LanM has suggested its cyclase functions likely evolved from LanC, but it lacked sequence homologies with LanB (Siezen et al. 1996). We aligned the 30 available LanM sequences using Clustal Omega (Sievers et al. 2011) and it produces a consensus that confirms the C terminal homology to LanC and the lack of homology to LanB. However, this did show an N-terminal sequence homology to the DUF4135 family of proteins, which is functionally uncharacterized, but conserved among bacteria and archaea.

It is therefore likely that LanM evolved from a fusion of a related protein and LanC. LanP is a serine protease required for releasing the leader sequence during secretion from the cell (van der Meer et al. 1993) . LanT is an ATP-dependent (ABC) transporter that transports the prepropeptide to the cell membrane and in some cases also functions as the protease (Havarstein et al. 1995). The bifunctional LanT enzyme is

TABLE 1. Unique characteristics of lantibiotics from *Actinobacteria* and *Firmicute*

Lantibiotic characteristic	*Actinobacteria*	*Firmicute*
Novel modifications		
Chlorotryptophan	Microbisporicin	
Dihydroxyproline	Microbisporicin	
Labionin	Labyrinthopeptins	
2-Oxobutyryl		Pep5, lichenicidin α, lichenicidin β, lacticin 3147 β
Sulfoxide bond	Actagardine, ala(O)-actagardine	
2-Oxopropionyl		Lactocin S
2-Hydroxypropionyl		Epilancin 15X, epilancin 7X
Lysinoalanine	Cinnamycin, duramycin, Duramycin B and duramycin C	
AviMeCys		Mersacidin
N-ternimal acetylation		Paenibacillin
Erythro-3-hydroxy-L-aspartic acid	Cinnamycin, duramycin, Duramycin B and duramycin C	
No significant antimicrobial activity	SapB, SapT, AmfS	
Inhibitor of Phospholipase A2	Cinnamycin, duramycin, Duramycin B and duramycin C	
Angiotensin I converting enzyme inhibitor	Ancovenin	
Bacteriostatic mode of action		Nukacin ISK-1
Inhibition of Gram-negative bacteria	Microbisporicin, bisin	
Not autoregulated		Epidermin

FIG. 2. Gene clusters encoding lantibiotics produced by *Actinobacteria.* See text for description of genes important for lantibiotic production, immunity and regulation. The structural genes are indicated in *black.*



Bisin
(Lee et al. 2008)

Cinnamycin
(Widdick et al. 2003)

Actagardine
(Boakes et al. 2009)

Deoxyactagardine B
(Boakes et al. 2010)

Microbisporicin
(Foulston and Bibb 2010)

Michiganin A
(Cartemann et al. 2008)

AmfS
(Ueda et al. 2002)

Sap B
(Kodani et al. 2004)

Labyrinthopeptin
(Meindl et al. 2010)

Supplementary Table 1 Amino acid sequence of the 77 lantibiotic prepropeptides, listed according to increasing leader peptide size

Lantibiotics	Leader peptides	Propeptides
Labyrinthopeptins A2	MASILELQNLDVEHARGENR	SDWSLWECCSTGSLFACC
Labyrinthopeptins A1/A3	MASILELQDLEVERASSAAD	SNASVWECCSTGSWVPFTCC
AmfS	MALLDLQAMDTPAEDSFGELR	TGSQVSLLVCEYSSLSVVLCTP
SapB	MNLFDLQSMETPKEEAMGDVE	TGSRASLLLCGDSSLSITTCN
Variacin	MTNAFQALDEVTDAELDAILGG	GSGVIPTISHECHMNSFQFVFTCCS
Butyrivibriocin OR79	MNKELNALTNPIDEKELEQILGG	GNGVIKTISHECHMNTWQFIFTCCS
Nisin A	MSTKDFNLDLVSVSKKDSGASPR	ITSISLCTPGCKTGALMGCNMKTATCHCSIHVSK
Nisin Z	MSTKDFNLDLVSVSKKDSGASPR	ITSISLCTPGCKTGALMGCNMKTATCNCSIHVSK
Nisin Q	MSTKDFNLDLVSVSKTDSGASTR	ITSISLCTPGCKTGVLMGCNLKTATCNCSVHVSK
Nisin F	MSTKDFNLDLVSVSKKDSGASPR	ITSISLCTPGCKTGALMGCNMKTATCNCSVHVSK
Streptin	MNNTIKDFDLDLKTNKKDTATPY	VGSRYLCTPGSCWKLVCFTTTVK
Salivaricin D	MSTKDFNLDLVEVSKSNTGASAR	FTSHSLCTPGCITGVLMGCHIQSIGCNVHIHISK
Ruminococcin A	MRNDVLTLTNPMEEKELEQILGG	GNGVLKTISHECNMNTWQFLFTCC
Geobacillin I	MAKFDDFDLDIVVKKQDDVVQPN	VTSKSLCTPGCITGVLMCLTQNSCVSCNSCIRC
Nisin U	MNNEDFNLDLVTISKENNSGASPR	VTSKSLCTPGCKTGILMTCAIKTATCGCHFG
Subtilin	MSKFDDFDLDVVKVSKQDSKITPQ	WKSESLCTPGCVTGALQTCFLQTLTCNCKISK
Ericin S	MSKFDDFDLDVVKVSKQDSKITPQ	WKSESVCTPGCVTGVLQTCFLQTITCNCHISK
Ericin A	MSKFDDFDLDVVKVSKQDSKITPQ	VLSKSLCTPGCITGPLQTCYLCFPTFAKC
Entianin	MSKFDDFDLDVVKVSKQDSKITPQ	WKSESVCTPGCVTGLLQTCFLQTITCNCKISK
Epilancin K7	MNNSLFDLNLNKGVETQKSDLSPQ	SASVLKTSIKVSKKYCKGVTLTCGCNITGGK
Epilancin 15X	MKKELFDLNLNKDIEAQKSDLNPQ	SASIVKTTIKASKKLCRGFTLTCGCHFTGKK
Cytolysin L	MENLSVVPSFEELSVEEMEAIQGS	GDVQAETTPVCAVAATAAASSAACGWVGGGIFTGVTVVVSLKHC
SWLP1	MNKELFDLNLNKGVETQKSDLNPQS	ASVVKTTIKASKKLCKGATLTCGCNITGKK
Lacticin 481	MKEQNSFNLLQEVTESELDLILGAK	GGSGVIHTISHECNMNSWQFVFTCCS
Streptococcin A-F22	MEKNNEVINSIQEVSLEELDQIIGA	GKNGVFKTISHECHLNTWAFLATCCS
Streptococcin A-M49	MTKEHEIINSIQEVSLEELDQIIGA	GKNGVFKTISHECHLNTWAFLATCCS
Macedocin	MEKETTIIESIQEVSLEELDQIIGA	GKNGVFKTISHECHLNTWAFLATCCS
Bovicin HJ50	MMNATENQIFVETVSDQELEMLIGG	ADRGWIKTLTKDCPNVISSICAGTIITACKNCA
Thermophilin 1277	MMNATENQIFVETVSDQELEMLIGG	ADRGWIKTLTKDCPNVISSICAGTIITACKNCA
Mutacin K8-A3	MKQSNEMLELIQEVSLDELDQVIGG	MGKGAVGTISHECRYNSWAFLATCCS
BsaA2	MEKVLDLDVQVKANNNSNDSAGDER	ITSHSLCTPGCAKTGSFNSFCC
Mutacin II	MNKLNSNAVVSLNEVSDSELDTILGG	NRWWQGVVPTVSYECRMNSWQHVFTCC
Pep 5	MKNNKNLFDLEIKKETSQNTDELEPQ	TAGPAIRASVKQCQKTLKATRLFTVSCKGKNGCK
Epicidin 280	MENKKDLFDLEIKKDNMENNNELEAQ	SLGPAIKATRQVCPKATRFVTVSCKKSDCQ
Mutacin K8-A1	MKNTTNEMLELIQEVSLDELDQVIGG	MGKGAVGTISHECRYNSWAFLATCCS
Salivaricin A	MKNSKDILNNAIEEVSEKELMEVAGGKRG	SGWTATITDDCPNSVFVCC
Lacticin 3147 α	MNKNEIETQPVTWLEEVSDQNFDEDVFGA	CSTNTFSLSDYWGNNGAWCTLTHECMAWCK
BHT-A α	MKEIQKAGLQEELSILMDDANNLEQLTAG	IGTTVVNSTFSIVLGNKGYICTVTVECMRNCQ
Geobacillin II	MKGGIQMEKQEQTFVSKISEEELKKLAGG	YTEVSPQSTIVCVSLRICNWSLRFCPSFKVRCPM
Epidermin	MEAVKEKNDLFNLDVKVNAKESNDSGAEPR	IASKFICTPGCAKTGSFNSYCC
Gallidermin	MEAVKEKNELFDLDVKVNAKESNDSGAEPR	IASKFLCTPGCAKTGSFNSYCC

Staphylococcin T	MEAVKEKNELFDLDVKVNAKESNDSGAEPR IASKFLCTPGCAKTGSFNSYCC
Nukacin ISK-1	MENSKVMKDIEVANLLEEVQEDELNEVLGA KKKSGVIPTVSHDCHMNSFQFVFTCCS
Nukacin 3299	MENSKVMKDIEVANLLEEVQEDELNEVLGA KKKSGVIPTVSHDCHMNSFQFVFTCCS
Nukacin KQU-131	MENSKIMKDIEVANLLEEVQEDELNEVLGA KKKSGVIPTVSHDCHMNTFQFMFTCCS
Plantaricin W α	MKISKIEAQARKDFFKKIDTNSNLLNVNGA KCKWWNISCDLGNNGHVCTLSHECQVSCN
Smb α	MKEIQKAGLQEELSILMDDANNLEQLIAGI GTTVVNSTFSIVLGNKGYICTVTVECMRNCSK
Enterocin W α	MKKEELVGMAKEDFLNVICENDNKLENSGA KCPWWNLSCHLGNDGKICTYSHECTAGCNA
Salivaricin B	MAKQQMNLVEIEAMNSLQELTLEELDNVLGA GGGVIQTISHECRMNSWQFLFTCCS
Lactocin S	MKTEKKVLDELSLHASAKMGARDVESSMNAD STPVLASVAVSMELLPTASVLYSDVAGCFKYSAKHHC
Salivaricin 9	MKSTNNQSIAEIAAVNSLQEVSMEELDQIIGA GNGVVLTLTHECNLATWTKKLKCC
StapylococcinC55 α	MKSSFLEKDIEEQVTWFEEVSEQEFDDDIFGA CSTNTFSLSDYWGNKGNWCTATHECMSWCK
BHT-A β	MKSNLLKINNVTEVEKDMVTLIKDEDMELAGG STPACAIGVVGITVAVTGISTACTSRCINK
Smb β	MKSNLLKINNVTEMEKNMVTLIKDEDMELAGG STPACAIGVVGITVAVTGISTACTSRCINK
Planosporicin	MGISSPALPQNTADLFQLDLEIGVEQSLASPA ITSVSWCTPGCTSEGGGSGCSHCC
Enterocin W β	MTELNKRLQLKRDVSTENSLKKISNTDETHGG VTTSIPCTVMVSAAVCPTLVCSNKCGGRG
Pneumococcins A2	MKNDFVIGKSLKELSLEEMQLVYGGTDGADPR STIICSATLSFIASYLGSAQTRCGKDNKKK
Microbisporicin	MPADILETRTSETEDLLDLDLSIGVEEITAGPA VTSWSLCTPGCTSPGGGSNCSFCC
StapylococcinC55 β	MKNELGKFLEENELELGKFSESDMLEITDDEVYAA GTPLSLLGGAATGVIGYISNQTCPTTAC
Plantaricin W β	MTKTSRRKNAIANYLEPVDEKSINESFGAGDPEAR SGIPCTIGAAVAASIAVCPTTKCSKRCGKRKK
Lacticin 3147 β	MKEKNMKKNDTIELQLGKYLEDDMIELAEGDESHGG TTPATPAISILSAYISTNTCPTTKCTRAC
Cytolysin S	MLNKENQENYYSNKLELVGPSFEELSLEEMEAIQGS GDVQAETTPACFTIGLGVGALFSAKFC
Pneumococcins A1	MTNFNSNEKFCGKSLKSLSADEMSLIYGASDGAEPR WTPTPIILKSAAASSKVCISAAVSGIGGLVSYNNDCLG
Lichenicidin β	MKTMKNSAAREAFKGANHPAGMVSEEELKALVGGNDVNPE TTPATTSSWTCITAGVTVSASLCPTTKCTSRC
Mutacin 1140	MSNTQLLEVLGTETFDVQEDLFAFDTTDTTIVASNDDPDTR FKSWSLCTPGCARTGSFNSYCC
Mutacin III	MSNTQLLEVLGTETFDVQEDLFAFDTTDTTIVASNDDPDTR FKSWSLCTPGCARTGSFNSYCC
Mutacin I	MSNTQLLEVLGTETFDVQEDLFAFDTTDTTIVASNDDPDTR FSSLSLCSLGCTGVKNPSFNSYCC
Haloduracin α	MTNLLKEWKMPLERTHNNSNPAGDIFQELEDQDILAGVNGA CAWYNISCRLGNKGAYCTLTVECMPSCN
Haloduracin β	MVNSKDLRNPEFRKAQGLQFVDEVNEKELSSLAGSGDVHAQ TTWPCATVGVSVALCPTTKCTSQC
Lichenicidin α	MSKKEMILSWKNPMYRTESSYHPAGNILKELQEEEQHSIAGG TITLSTCAILSKPLGNNGYLCTVTKECMPSCN
Bisin	MSINEKSIVGESFEDLSAADMAMLTGRNDDGVAPASLSFAVS VLSVSFSACSVTVVTRLASCGNCK
Actagardine	MSALAIEKSWKDVDLRDGATSHPAGLGFGELTFEDLREDRTIYAA SSGWVCTLTIECGTVICAC
Deoxyactagardine B	MSAITVETTWKNTDLREDLTAHPAGLGFGELTFEDLREDRTIYAA SSGWVCTLTIECGTLVCAC
Michiganin A	MNDILETETPVMVSPRWDMLLDAGEDTSPSVQTQIDAEFRRVVSPYM SSSGWLCTLTIECGTIICACR
Mersacidin	MSQEAIIRSWKDPFSRENSTQNPAGNPFSELKEAQMDKLVGAGDMEAA CTFTLPGGGGVCTLTSECIC
Duramycin	MTASILQSVVDADFRAALIENPAAFGASTAVLPTPVEQQDQASLDFWTKDIAATEAFA CKQSCSFGPFTFVCDGNTK
Cinnamycin	MTASILQQSVVDADFRAALLENPAAFGASAAALPTPVEAQDQASLDFWTKDIAATEAFA CRQSCSFGPFTFVCDGNTK

specific for lantibiotics with a double glycine-type leader peptide. Interestingly, the nine genetically characterized lantibiotics produced by *Actinobacteria* have no LanP and rely on a bifunctional LanT. LanI is required for self-immunity and in some cases a LanFEG complex is needed for full immunity (Kuipers et al. 1993; Siegers and Entian 1995). A key feature of lantibiotic gene clusters is the presence of *lanRK* genes which encode a two component regulatory system, composed of a histidine kinase (LanK) and a response regulator (LanR) (Kuipers et al. 1995). This system detects the lantibiotic outside the cell, which induces/activates transcription of the lantibiotic gene cluster.

Classification of lantibiotics

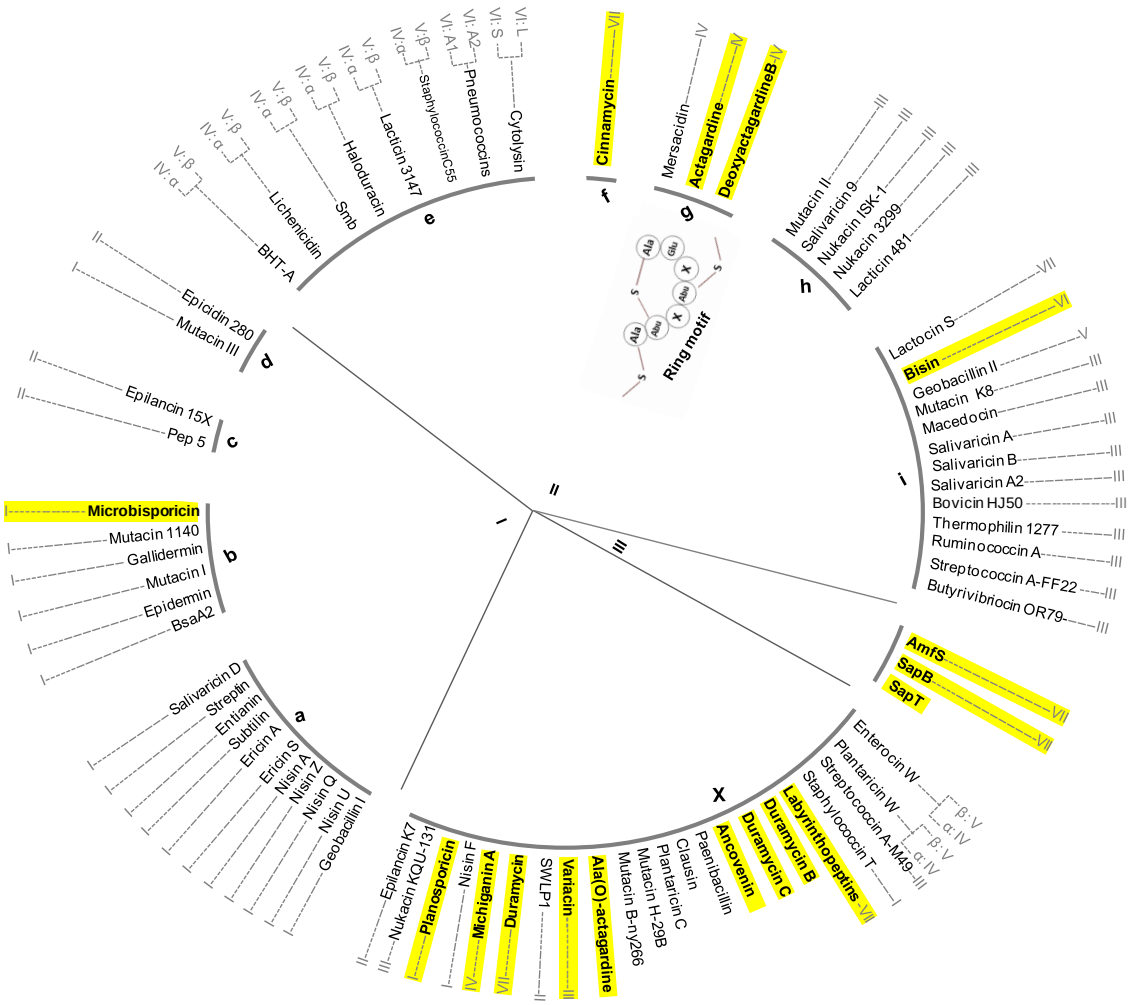
Structural similarities, charge and size of the active peptide were first used to classify lantibiotics into two groups, type A and type B, with the latter being smaller than 2.1 kDa, less positive charges and very similar ring structures (Jung 1991). Type A lantibiotics were further subdivided into type A-I and type A-II based primarily on their leader sequences, with type AI having a characteristic FNLDV consensus (Devos et al. 1995). More recently, Willey and van der Donk (2007) proposed a new classification for lantibiotics based on the organization of their gene clusters, leader peptide sequence, the mature peptide structure and antimicrobial activity. This resulted in three classes with class III solely reserved for lantibiotic-like compounds with no significant antimicrobial activity but likely to perform other functions for the cell. Class I lantibiotics are more linear in their structure and have a common "FNLD" motif in their leader sequences,

which also usually contains a proline at position - 2. They are also characterized by containing the LanB and LanC modifying enzymes and usually both a LanT and LanP. In contrast, class II lantibiotics contain the bifunctional LanM and LanT enzymes. There have been ten two-component lantibiotics identified to date and these are placed in Class II as each peptide is processed by a bifunctional LanM and a single bifunctional LanT is used for secretion and leader processing. Using this three class grouping, together with structural similarities, the 76 currently described lantibiotics in the published literature can be placed into eleven groups as illustrated in Fig. 3. Lantibiotics from *Actinobacteria* are present in all three classes. Not all of the listed lantibiotics are fully characterized and were grouped primarily based on the makeup of their modification enzymes. Twenty two lantibiotics could not currently be assigned to a class due to insufficient characterization. Among the two component lantibiotics (class II-e), each peptide is modified by a separate LanM, except for cytolysin which contains a single LanM for modifying both peptides.

Mode of action

The bactericidal activities of lantibiotics are primarily based on two mechanisms, pore formation and inhibition of peptidoglycan synthesis, with a few exceptions. The prototypic lantibiotic nisin is the most thoroughly studied lantibiotic exerting a dual mode of action: pore formation and inhibition of cell wall synthesis by binding to the lipid II precursor (Brotz et al. 1998b; Breukink et al. 1999). While many lantibiotics exhibit this

FIG. 3. Groupings of the 76 currently described lantibiotics, primarily based on the three class system of Willey and van der Donk (2007). The 11 groups are based on their structural similarity. *Class I-a* share an elongated structure and two similar N-terminal rings; *class I-b* also contains the conserved two similar N-terminal rings in addition to a LanD enzyme that catalyzes the formation of an AviCys residue (Kupke et al. 1992); *class I-c* is even more elongated, and contains 2-oxobutyryl or 2-hydroxypropionyl residues in their N-terminus; *class I-d* consists of two class I lantibiotics with characterized gene clusters but uncharacterized lantibiotic structures; *class II-e* consists of the genetically characterized two-peptide lantibiotics; *group II-f* consists of lantibiotic with a more globular or compact structure; *class II-g* is characterized by a compact globular structure as well as a conserved ring motif (this conserved ring is also present in plantaricin C and the alpha peptide of some two-peptide lantibiotics); *class II-h* consists of lantibiotics with linear N-termini and globular C-termini; *class II-i* consists of class II lantibiotics with characterized gene clusters but uncharacterized lantibiotic structures; group X consists of currently unclassified lantibiotics due to insufficient characterization. The *outer ring* indicates the phylogenetic grouping of each lantibiotic based on Fig. 4. Lantibiotics from Actinobacteria are in *bold* and *highlighted*.


I
II
III
IV
V
VI
VII
X
a
b
c
d
e
f
g
h
i
Salivaricin D
Streptin
Entianin
Subtilin
Ericin A
Ericin S
Nisin A
Nisin Z
Nisin Q
Nisin U
Geobacillin I
Microbisporicin
Mutacin 1140
Gallidermin
Mutacin I
Epidermin
BsaA2
Epilancin 15X
Pep 5
Epicidin 280
Mutacin III
BHT-A
Lichenicidin
Smb
Haloduracin
Lacticin3147
Staphylococcin C55
Pneumococcins
Cytolysin
Cinnamycin
Mersacidin
Actagardine
Deoxyactagardine B
Mutacin II
Salivaricin 9
Nukacin ISK-1
Nukacin 3299
Lacticin481
Ring motif
Lactocin S
Bisin
Geobacillin II
Mutacin K8
Macedocin
Salivaricin A
Salivaricin B
Salivaricin A2
Bovicin HJ50
Thermophilin 1277
Ruminococcin A
Streptococcin A-FF22
Butyrivibriocin OR79
AmfS
SapB
SapT
Enterocin W
Plantaricin W
Streptococcin A-M49
Staphylococcin T
Labyrinthopeptins
Duramycin C
Duramycin B
Ancovenin
Paenibacillin
Clausin
Plantaricin C
Mutacin B-ny266
Ala(O)-actagardine
Variacin
SWLP1
Duramycin
Michiganin A
Nisin F
Planosporicin
Nukacin KQU-131
Epilancin K7
α: IV
β: V
α: IV
β: V

dual mode of action, some only exhibit one of these mechanisms and in some cases different mechanisms have been proposed.

Pore formation

While pore formation has been demonstrated for many lantibiotics, its mechanism of action is most extensively studied for nisin. Nisin initially binds with the pyrophosphate moiety of lipid II using five hydrogen bonds. This interaction occurs specifically with its two N-terminal lanthionine rings (Fig. 1) (Hsu et al. 2004). This results in the elongated C-terminal of nisin inserting into the membrane in a perpendicular orientation, with a stoichiometry of eight nisin and four lipid II moieties that results in forming a stable pore (Hasper et al. 2004; van Heusden et al. 2002). This results in the outflow of ions and small cytoplasmic compounds, contributing to the dissipation of the membrane potential. Lipid II binding and subsequent pore formation also has been proposed for two-peptide lantibiotics. Studies with lacticin 3147-A1/A2 proposed a model whereby the A1 peptide binds with lipid II and then A2 binds forming a trivalent structure that initiates pore formation (Wiedemann et al. 2006; Oman and van der Donk 2009). More recently a similar mechanism has been demonstrated for the haloduracin two peptide lantibiotic (Oman et al. 2011). Some lantibiotics, such as streptococcin A-FF22 and pep5, can form unstable pores in membranes that are proposed to disipitate the membrane potential but not to release large molecules from the cell (Jack et al. 1994; Kordel et al. 1988). Other lantibiotics such as gallidermin and epidermin are shorter and their ability to form a functional pore is dependent on the membrane

thickness (Bonelli et al. 2006). While these two lantibiotics may not always utilize pores for their antimicrobial activities, they can still exert an efficient bactericidal effect by inhibiting peptidoglycan synthesis due to lipid II binding.

Outgrowth of bacterial spores, particularly from some species of *Clostridium* and *Bacillus*, is an important food safety concern. Nisin is an effective inhibitor of spore outgrowth and this is reflected in its approval by the Food and Drug Administration (FDA) in the US for use in processed cheese to prevent *C. botulinum* spore outgrowth, the sole approved use of purified nisin as a food ingredient in the US. Recently the mode of action for nisin mediated inhibition of spore outgrowth was investigated and it was found to also involve lipid II binding leading to pore formation (Gut et al. 2011). In this case the inhibition of peptidoglycan synthesis due to binding of nisin to lipid II, was not involved in the inhibition of outgrowth of the spore to a vegetative cell. This was concluded as the antibiotic vancomycin, which also binds lipid II, cannot form pores but does inhibit peptidoglycan synthesis and does not prevent spore outgrowth.

Lipid II binding mediated inhibition of peptidoglycan synthesis

While binding to lipid II is a precursor for pore formation by certain lantibiotics, it can also disrupt peptidoglycan synthesis. In the case of nisin-like lantibiotics with the conserved two N-terminal lanthionine rings, characteristic of class 1a and 1b (Fig. 3), this mechanism of action occurs by physically moving lipid II from its functional location in the cell wall (Hasper et al. 2006). This results in the accumulation of UDP-linked

peptidoglycan precursors in the cytoplasm as observed for microbisporicin and planosporicin (Castiglione et al. 2007; Castiglione et al. 2008). This mechanism is the sole antimicrobial mechanism for those lipid II binding lantibiotics that are too short to form effective pores, a primary characteristic of the class Ib lantibiotics in Fig. 3. This can still be a very effective bacteriocidal mechanism as evidenced by gallidermin that does not form pores in *Lactococcus lactis* subsp. *cremoris* HP but is still nearly ten times more potent than nisin (Bonelli et al. 2006).

Another ring motif (Fig. 3) that is conserved in all class IIg lantibiotics, as well as some class IIe and IIh, is also believed to be involved in lipid II binding (Szekat et al. 2003). This also inhibits peptidoglycan synthesis at the level of transglycosylation, but the precise mechanism of action is unclear (Brotz et al. 1997). No pore formation occurs with these lantibiotics due to their compact globular structure. It has also been shown that one of these class IIg lantibiotics, mersacidin, has significantly higher affinity for lipid II than lipid I, which only differs by the addition of GlcNAc, indicating that GlcNAc is part of the recognition motif of lipid II (Brotz et al. 1998a).

Other antimicrobial activities

Cinnamycin (Fig. 1) and duramycin are globular lantibiotics whose antimicrobial activities involve binding to phosphatidylethanolamine (PE), a component of the cell membrane. They have also been shown to have hemolytic activity (Choung et al. 1988). This binding to PE in the cell membrane is enhanced with the membrane curvature, with

smaller membrane bound vesicles having a higher affinity (Iwamoto et al. 2007). Binding results in physical changes to the membrane causing increased curvature and thus increasing the binding of the lantibiotics. However, the precise mechanism for cell death is not yet elucidated.

Nukacin ISK-1 is the only lantibiotic characterized to date, whose mode of action is bacteriostatic rather than bacteriocidal (Asaduzzaman et al. 2009). It does not affect membrane potential or form pores, but it was observed to reduce the width of the cell wall, causing incomplete septum formation, thus preventing active growth.

Regulation of Lantibiotic Biosynthesis

Lantibiotic production is tightly regulated and occurs mainly via a two component signal transduction mechanism. This typically consists of the trans-membrane histidine kinase (LanK) that senses the lantibiotic in the environment and phosphorylates an intracellular response regulator (LanR). This activates LanR to induce transcription of the lantibiotic gene cluster (Stock et al. 2000; Chatterjee et al. 2005). While extracellular lantibiotic functions as an auto-inducer for its production in the majority of cases that have been investigated, there are some exceptions such as epidermin production by *Staphylococcus epidermis*, which is not believed to be auto-induced (Kies et al. 2003).

Some lantibiotic gene clusters encode an extra regulator, demonstrating the potential for more complex regulation. These are primarily found for the lantibiotics produced from *Actinobacteria* (bisin, actagardine, deoxyactagardine and cinnamycin) (Fig. 2), while mersacidin is the only characterized lantibiotic from the *Firmicute* phylum to contain the extra regulator. In the case of mersacidin, the two component regulator (MrsR2/MrsK2) was shown to be required for transcription of the immunity (*lanFEG)* genes, but not mersacidin production, which required MrsR1 for induction (Guder et al. 2002), although a subsequent study by the same group questioned the accuracy of this model (Schmitz et al. 2006). In contrast, the two component regulator (LanR2/LanK) was found to be required for autoinduction of *lanA* transcription for bisin production by *Bifidobacterium longum*, whereas its LanR1 was proposed to be a transcriptional repressor of *lanA* (Lee et al. 2011). The extra CinR1 regulator in the cinnamycin gene cluster in *Streptomyces cinnamoneus* is proposed to be transcriptionally induced via its CinK/CinR two component regulatory system and it in turn induces transcription of *cinA* (Widdick et al. 2003).

The gene clusters of some lantibiotics only contain a single regulatory gene, such as mutacin II, epidermin and SapB, and these are proposed to be positively regulated by MutR, EpiQ and RamR, respectively (Chen et al. 1999; Peschel et al. 1997; Paolo et al. 2006). Other regulators can also influence production of these lantibiotics, such as the involvement of the quorum sensing regulator Agr (accessory gene regulator) in the regulation of the epidermin protease, EpiP (Kies et al. 2003). The regulation of

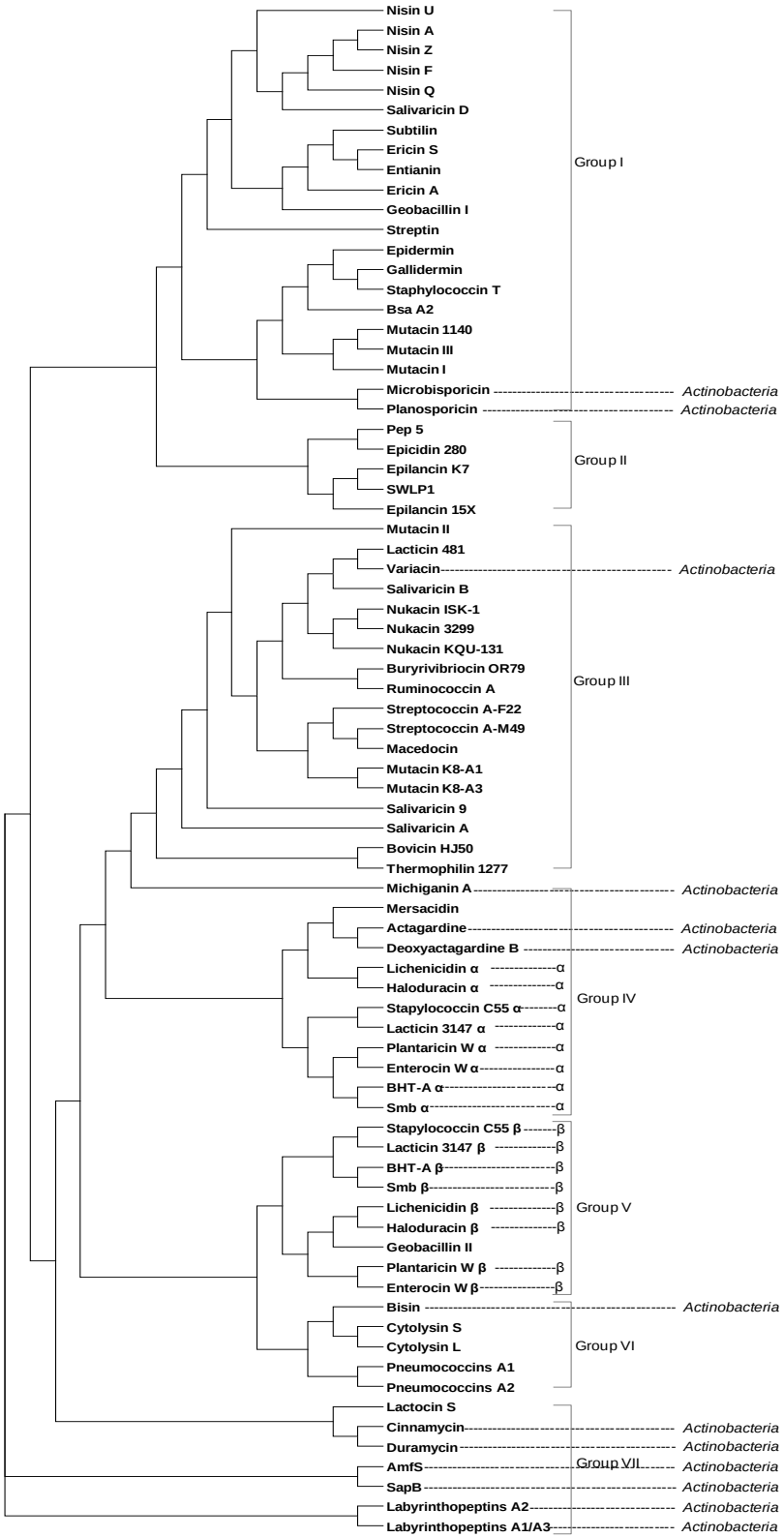
microbisporicin production by *Microbispora coralline* involves a MibR regulator that is activated by an unknown inducer, which in turn leads to low level microbisporicin production. This then interacts with MibW, thus releasing a sigma factor (σ^{MibX}) which leads to high level transcription of the gene cluster (Foulston and Bibb 2011). This is the only case to date where a dedicated sigma factor, encoded in the gene cluster, is required for its expression.

While lantibiotic production depends on dedicated regulatory systems, it can also be regulated in some instances by other environmental conditions. For example, nisin biosynthesis by *Lactococcus lactis* is normally fully dependent on autoinduction by nisin, but during growth in galactose (in the absence of a functional phosphotransferase system) transcription of *nisA* is induced by a component of the Leloir pathway (Chandrapati and O'Sullivan 1999, 2002). Ruminococcin A production by *Ruminococcus gnavus* requires the presence of trypsin to activate an extracellular peptide which is needed to signal the two component regulatory system (Gomez et al. 2002).

Evolution of lantibiotics

To understand the evolution of lantibiotic peptides, an unrooted phylogenetic tree was generated (Fig. 4) by aligning the primary amino acid sequences of the full pre-propeptides of the available 77 lantibiotic peptides (Supplementary Table 1) using Clustal W2 (Larkin et al. 2007), which was viewed using Mega 5 (Tamura et al. 2011).

FIG. 4. Unrooted phylogenetic tree of 77 characterized lantibiotic prepropeptides. The groupings are based on the presence of conserved motifs (see Fig. 5). Symbols α and β represent peptides of two component lantibiotics.


Nisin U
Nisin A
Nisin Z
Nisin F
Nisin Q
Salivaricin D
Subtilin
Ericin S
Entianin
Ericin A
Geobacillin I
Streptin
Epidermin
Gallidermin
Staphylococcin T
Bsa A2
Mutacin 1140
Mutacin III
Mutacin I
Microbisporicin
Planosporicin
Group I
Pep 5
Epicidin 280
Epilancin K7
SWLP1
Epilancin 15X
Group II
Mutacin II
Lacticin 481
Variacin
Salivaricin B
Nukacin ISK-1
Nukacin 3299
Nukacin KQU-131
Buryrivibriocin OR79
Ruminococcin A
Streptococcin A-F22
Streptococcin A-M49
Macedocin
Mutacin K8-A1
Mutacin K8-A3
Salivaricin 9
Salivaricin A
Bovicin HJ50
Thermophilin 1277
Group III
Actinobacteria
Michiganin A
Mersacidin
Actagardine
Deoxyactagardine B
Lichenicidin α
Haloduracin α
Stapylococcin C55 α
Lacticin 3147 α
Plantaricin W α
Enterocin W α
BHT-A α
Smb α
Group IV
Stapylococcin C55 β
Lacticin 3147 β
BHT-A β
Smb β
Lichenicidin β
Haloduracin β
Geobacillin II
Plantaricin W β
Enterocin W β
Group V
Bisin
Cytolysin S
Cytolysin L
Pneumococcins A1
Pneumococcins A2
Group VI
Lactocin S
Cinnamycin
Duramycin
AmfS
SapB
Labyrinthopeptins A2
Labyrinthopeptins A1/A3
Group VII

Interestingly, an analogous tree generated using just the propeptides gives a very similar tree in most cases, indicating coevolution of the leader peptides with their respective propeptides (Supplementary Fig. 1). However, there are some examples where this did not occur, such as bisin which branches very closely with to the cytolysins when including the leader peptide but branches quite differently when it is excluded. This therefore suggests a greater evolutionary relationship between bisin and cytolysin leader peptides than their propeptides.

This pre-propeptide phylogenetic tree can be divided into seven groups, each containing conserved regions as illustrated in Fig. 5. Group I contains a conserved SXSLCTPGC (X = any amino acid) motif, which corresponds to the conserved two N-terminal rings that are important for lipid II binding. Group II has a highly conserved M(/N)N(/K)K(/N)XLFDLN(/E)L(/I)N(/K)K motif in their leader peptides, which likely is involved in a modification or transport function, but its actual role is not currently understood. It also contains D(/E)LXP(/A)QS(/T)A(/L)S(/G),IK(/R)A(/V)S(/T)XK(/Q)X CXG(/K), TL(/V)T(/S)C and GC motifs that correspond with their three lanthionine rings. Group III contains a ELXXXXG motif in their leader peptides and this region, specifically the Leu residue of lacticin 481, was found to be important for dehydration of the propeptide (Patton et al. 2008). In addition, a conserved motif GXXXTXS(/T)XHE (/D)C in the propeptides of this group corresponds to the N-terminal first ring, which was recently associated with lipid I binding by Nukacin ISK-1 (Islam et al. 2012).

Supplementary Fig. 1 Phylogenetic tree of the 77 lantibiotic propeptides.

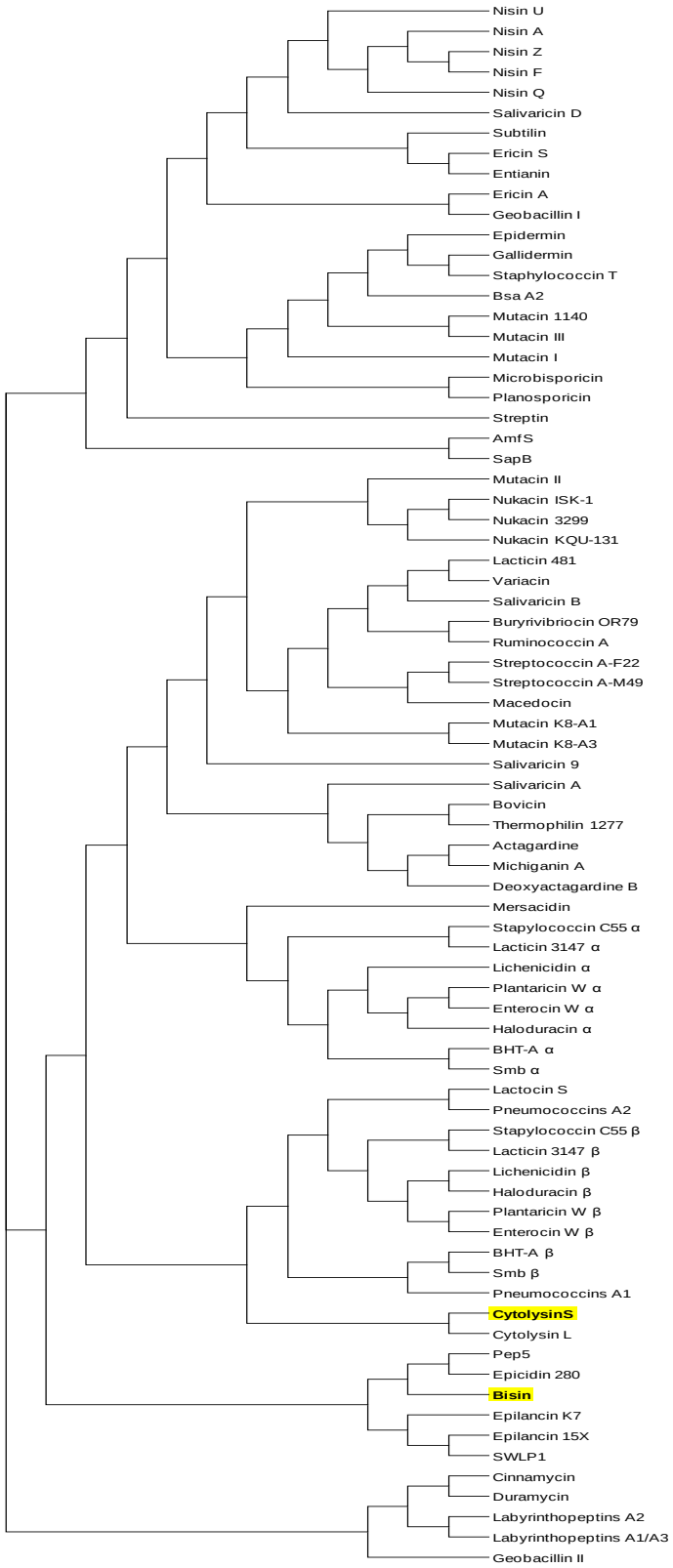
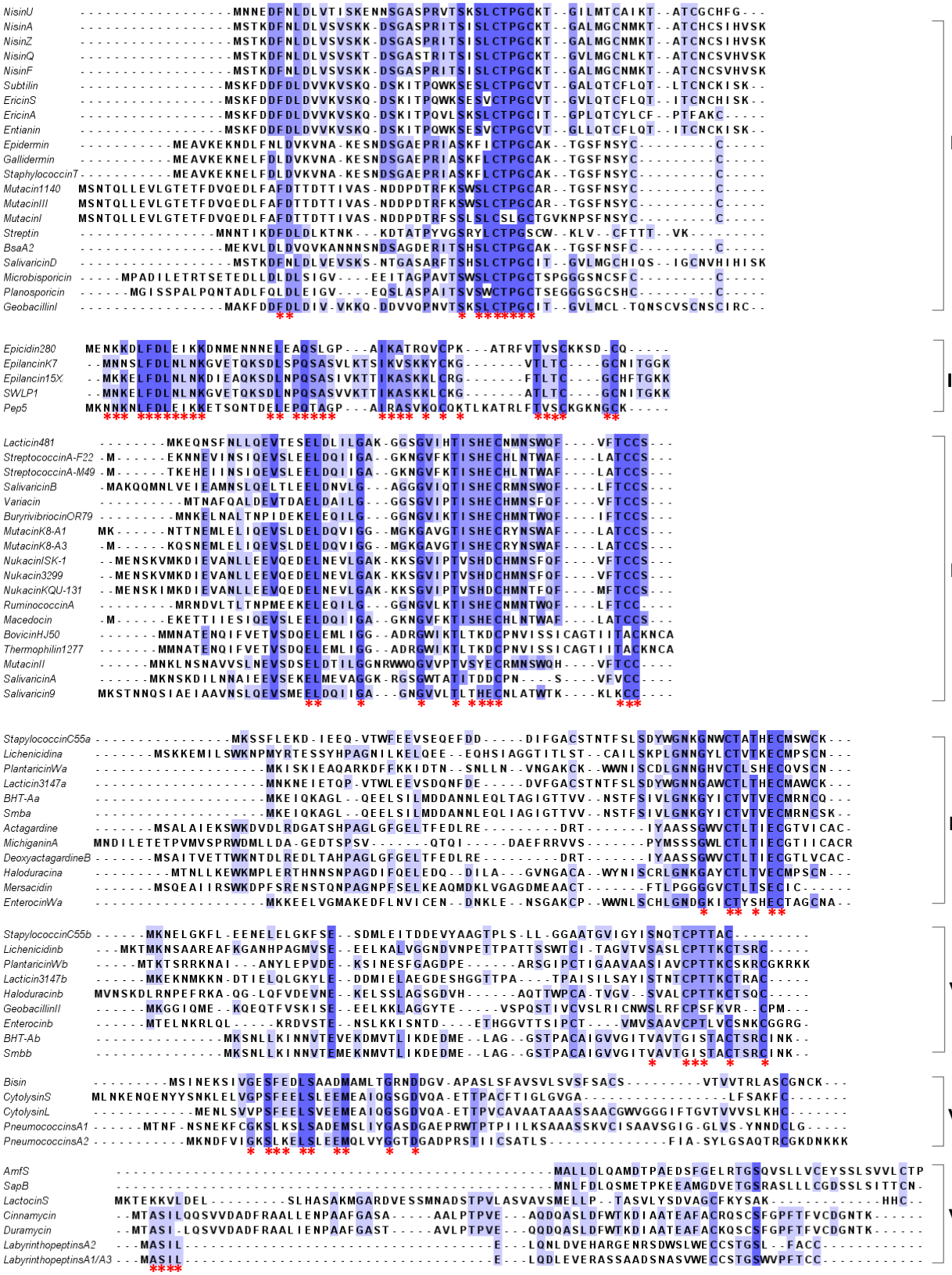

Nisin U
Nisin A
Nisin Z
Nisin F
Nisin Q
Salivaricin D
Subtilin
Ericin S
Entianin
Ericin A
Geobacillin I
Epidermin
Gallidermin
Staphylococcin T
Bsa A2
Mutacin 1140
Mutacin III
Mutacin I
Microbisporicin
Planosporicin
Streptin
AmfS
SapB
Mutacin II
Nukacin ISK-1
Nukacin 3299
Nukacin KQU-131
Lacticin 481
Variacin
Salivaricin B
Buryrivibriocin OR79
Ruminococcin A
Streptococcin A-F22
Streptococcin A-M49
Macedocin
Mutacin K8-A1
Mutacin K8-A3
Salivaricin 9
Salivaricin A
Bovicin
Thermophilin 1277
Actagardine
Michiganin A
Deoxyactagardine B
Mersacidin
Stapylococcin C55 α
Lacticin 3147 α
Lichenicidin α
Plantaricin W α
Enterocin W α
Haloduracin α
BHT-A α
Smb α
Lactocin S
Pneumococcins A2
Stapylococcin C55 β
Lacticin 3147 β
Lichenicidin β
Haloduracin β
Plantaricin W β
Enterocin W β
BHT-A β
Smb β
Pneumococcins A1
CytolysinS
Cytolysin L
Pep5
Epicidin 280
Bisin
Epilancin K7
Epilancin 15X
SWLP1
Cinnamycin
Duramycin
Labyrinthopeptins A2
Labyrinthopeptins A1/A3
Geobacillin II

FIG. 5. Sequence alignment of the 77 lantibiotic prepropeptides that are currently available. Conserved motifs are indicated with *stars*.


I
II
III
IV
V
VI
VII

Another conserved motif TCC is present in the C-terminus suggesting a likely importance of this region in the lantibiotic activity. Group IV contains a conserved motif of GXXCTXT(/S)XEC corresponding to the conserved ring motif (Fig. 3) believed to be a lipid II binding motif. Group V contains a conserved S(/V)XXXC(/G)P(/I)T(/S)XX C(/V)XXXC motif which corresponds to the three C-terminal rings. Group VI contains a conserved G(/V)XSF(L)E(K)XLSXXE(/D)MXXXXGXXD motif in its leader peptides, but no significant homology in the propeptides. Given this group contains bisin it substantiates the conserved nature of the leader peptides in this group, but not their propeptides as discussed above. Group VII does not contain any conserved motifs across the full group and may require further sub-grouping. The presence of an ASIL motif in cinamycin, duramycin, and labyrinthopeptins peptides substantiates this.

This phylogenetic grouping corresponds very nicely with the three classes in Fig. 3. All class I lantibiotics fall into the phylogenetic groups I and II. All of the two component lantibiotics (Class IIe) belong to the phylogenetic groups IV, V or VI. Given the presence of conserved motifs characteristic of these phylogenetic groups, and the association with the Fig. 3 classes, this may be a simpler, alternative approach for classifying lantibiotics rather than grouping based on the current comprehensive list of characteristics.

Lantibiotic Applications

Lantibiotics are mainly used as antibacterial food preservatives. While pure or semi-pure lantibiotic preparations are used in foods in many countries worldwide, only nisin has FDA approval in the US for use as a food ingredient in processed cheese only. Therefore unpurified fermentation products (such as dried fermentates) from lantibiotic producing food grade bacteria, such as the lactic acid bacteria, are incorporated in foods for their antimicrobial activities, as they are not subjected to new food ingredient approvals. In other cases lantibiotic producing starter cultures can be used in the production of foods, such as the use of lacticin 3147 or lacticin 481 producing *Lactococcus lactis* starter cultures in cheese for the specific purpose of inhibiting *Listeria monocytogenes* (McAuliffe et al. 1999; Rodriguez et al. 2001).

While these lantibiotic containing products afford good protection against Gram-positive bacteria, they are relatively ineffective against Gram-negative bacteria. As some strains of *E. coli* and *Salmonella* are very problematic for the food industry (currently being responsible for nearly half of all food recalls in the US), there is a big need for lantibiotics with broader antimicrobial activities. In this regard lantibiotics from the *Actinobacteria* are showing promise as they are incorporating greater diversity to lantibiotics such as microbisporicin which contains the unusual modifications of 5-chlorotryptophan and 3, 4-dihydroxyproline (Fig. 1). This also extended its antimicrobial activity to some Gram-negative bacteria, specifically *Moraxella catarrhalis*, *Neisseria*

spp., and *Haemophilus influenza* (Castiglione et al. 2008). While microbisporicin does not inhibit the *Enterobacteriaceae*, it does reveal the possibility of lantibiotics to inhibit Gram-negative bactaeria. More recently bisin, a lantibiotic produced by *Bifidobacterium longum* DJO10A showed a potential for inhibition of members of the *Enterobacteriaceae* (O'Sullivan and Lee 2011).

Lantibiotics also have potential for biomedical applications although their sensitivity to certain proteases does provide a hurdle for this application. Their prominent advantage is their activity against multi-drug resistant Gram-positive pathogens, such as methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant enterococci (VRE). This was demonstrated for mersacidin in vivo for eradicating MRSA from a mouse rhinitis model (Kruszewska et al. 2004). They also have potential to prevent and treat bovine mastitis, as demonstrated for lacticin 3147 in a teat dip application to cows (Klostermann et al. 2010). An analogous study using nisin for prevention of human staphylococcal mastitis showed a statistically positive effect, albeit the study participant numbers (8) were quite low (Fernandez et al. 2008). An oral bacterium, *Streptococcus salivarius* K12, which produces salivaricin B and salivaricin A2 has been shown to reduce bad breath when applied via lozenges following a mouth wash treatment (Burton et al. 2006).

While some lantibiotic applications have been experimentally demonstrated, there

are many other potential applications that may prove fruitful in the future. For example, gallidermin has been proposed as a treatment for acne, due to its effective antimicrobial activity against *Propionibacterium acnes* and its ability to be dermally absorbed (Manosroi et al. 2010). The class IIf lantibiotic cinnamycin and duramycin (Fig. 3) bind and inhibit the human enzyme phospholipase A2, which is a proinflammatory mediator (Marki et al. 1991). These may therefore have potential for use in nonsteroidal antiinflammatory drugs (NSAIDs). Another class X lantibiotic, ancovenin, has been shown to be an enzyme inhibitor, specifically for angiotensin I -converting enzyme (ACE) (Kido et al. 1983). ACE inhibitors are important pharmaceuticals particularly in the treatment of high blood pressure.

It is increasingly evident that lantibiotics have diverse functions and this diversity is particularly illustrated by the lantibiotics that have been characterized from *Actinobacteria*. It is likely that many more lantibiotics with novel functions remain to be discovered and the *Actinobacteria* will undoubtedly contribute even more novel structures and functions that may be of use in the future for both food and pharmaceutical applications.

Chapter II

Transcription Analysis of a Lantibiotic Gene Cluster from

Bifidobacterium longum DJO10A

Parts of this chapter were published in:

Lee, J. H., Li, X, and D. J. O'Sullivan. 2011. Transcription analysis of a lantibiotic gene cluster from *Bifidobacterium longum* DJO10A. Appl. Environ. Microbiol. 77:5879-5887

Introduction

Bifidobacteria are anaerobic and non-motile members of the *Actinobacteria*. They are also by far the most numerically dominant members of the *Actinobacteria* in the human gut as recently illustrated in a large scale metagenomic study of 124 humans (Qin et al., 2010). They are also often considered as beneficial bacteria for human intestinal health due to numerous potential health benefits attributed to them (Lee and O'Sullivan, 2010; O'Sullivan, 2001). While the majority of the LAB has been demonstrated to produce bacteriocins, including several lantibiotics, only two bacteriocins and no lantibiotics have previously been described for bifidobacteria. These are bifidin I produced by a strain of *B. longum* subsp. *infantis* and bifidocin B produced by a strain of *B. bifidum*, and while neither has been fully characterized, both are proposed to be class IIa bacteriocins based on N-terminal amino acid sequence analysis (Cheikhyoussef et al., 2010; Yildirim et al., 1999).

Recently, complete genome sequences for 27 bifidobacteria were reported and became available in GenBank database. Interestingly, the genome sequence analysis of *B. longum* DJO10A revealed a complete lantibiotic gene cluster (Lee et al., 2008) and a comparative genome analysis of the other bifidobacteria genomes showed the homologous remnants of part of this gene cluster in the genome of *B. longum* subsp. *infantis* ATCC 15697 and *B. angulatum* DSM 20098 (Lee and O'Sullivan, 2010). The 10.2 kb lantibiotic gene cluster of *B. longum* DJO10A encoded eight genes and in silico

analysis of the predicted protein sequences translated from these genes showed that the gene cluster has all potential genes required for lantibiotic production, as well as a dedicated two component system (*lanR2* and *lanK*), which is characteristic of lantibiotic gene clusters in other bacteria (Klaenhammer, 1993). In addition, there is a second predicted transcription regulator (*lanR1*), which is analogous to the mersacidin gene cluster from a *Bacillus* species (Altena et al., 2000) and some lantibiotic gene clusters from *Actinobacteria* (Fig. 1 of Chapter I).

However production of this lantibiotic by *B. longum* DJO10A could only be detected on agar (Lee et al., 2008), which was analogous to the streptin lantibiotic from *Streptococcus pyogenes* (Wescombe and Tagg, 2003). This therefore hampered efforts to purify and characterize this lantibiotic. Comparative microarray analysis revealed very different expression profiles of this gene cluster between agar and broth conditions. Interestingly, the lantibiotic production and immunity genes, *lanADMIT*, were highly up-regulated on agar but down-regulated in broth, which was consistent with the detection of the lantibiotic only on agar media (Lee et al., 2011). However, expression of the lantibiotic regulatory genes *lanR2, lanK* and *lanR1* was similar in both conditions, suggesting it should be possible to induce the gene cluster in broth media if sufficient lantibiotic was first added to the growth medium.

The objective of this chapter was to further understand the transcriptional regulation of the lantibiotic structural gene in *B. longum* DJO10A. A Real-time PCR

assay will be developed to quantitatively evaluate *lanA* gene expression. This will enable investigation of the autoinduction of the *lanA* gene. In addition, a predicted repressor gene *lanR1* will be further characterized using chemical and UV mutagenesis as well as its heterologous production in *E. coli*. This will provide an understanding of its expression in agar media, such that strategies can be designed to enable its production in broth media.

Materials and methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains, plasmids, and growth conditions used in this study are listed in Table 1. Anaerobic conditions were generated when needed using the BBL Anaerobic system (Cockeysville, MD) or the Bactron II Anaerobic/Environmental Chamber (Sheldon Manufacturing, Cornelius, OR).

Total RNA isolation

B. longum DJO10A was cultured in BLIM+Fe medium to an optical density (OD) at 600 nm of 1. To protect the integrity of the RNA, a 4 ml volume of RNAprotect solution (Qiagen, Valencia, CA) was directly added to 2 ml of bacterial culture and incubated at room temperature for 5 min after brief mixing. *B. longum* DJO10A grown on BLIM+Fe agar plates for 48 h were collected using 4 ml of RNAprotect in BLIM+Fe

TABLE 1. Bacterial strains, plasmids and growth conditions used in this study

Strain or plasmid	Characteristics	Culture media	Incubation conditions	Source or reference
Gram-positive bacteria				
Bifidobacterium longum subsp. *longum* DJO10A	Wild-type human isolate	MRS+L-Cys[a] BLIM+Fe[b]	37 ℃, Anaerobic	(Lee et al., 2008)
DJO10A-JH1	Lantibiotic gene cluster deletion mutant	MRS+L-Cys BLIM+Fe	37 ℃, Anaerobic	(Lee et al., 2008)
B. longum subsp. *infantis* ATCC 15702	Bioassay indicator strain	MRS+L-Cys	37 ℃, Anaerobic	ATCC[c]
B. adolescentis ATCC 15704	Bioassay indicator strain	MRS+L-Cys	37 ℃, Anaerobic	ATCC
B. bifidum ATCC 15696	Bioassay indicator strain	MRS+L-Cys	37 ℃, Anaerobic	ATCC
B. breve ATCC 15701	Bioassay indicator strain	MRS+L-Cys	37 ℃, Anaerobic	ATCC
B. animalis subsp. *lactis* S14	Bioassay indicator strain	MRS+L-Cys	37 ℃, Anaerobic	DCC[d]
Lactococcus lactis subsp. *lactis* LM0230	Bioassay indicator strain	M17G[e]	30 ℃, Anaerobic	DCC
L. lactis subsp. *lactis* C2	Bioassay indicator strain	M17G	30 ℃, Anaerobic	DCC
Enterococcus faecalis LG110	Bioassay indicator strain	M17G	37 ℃, Anaerobic	DCC
Streptococcus thermophilus ST403	Bioassay indicator strain	M17G	42 ℃, Anaerobic	DCC
Clostridium perfringens ATCC 13124	Bioassay indicator strain	RCM[f]	37 ℃, Anaerobic	ATCC
C. difficile S1	Bioassay indicator strain	RCM	37 ℃, Anaerobic	(Lee et al., 2008)
Staphylococcus aureus ATCC 29213	Bioassay indicator strain	MRS+L-Cys	37 ℃, Aerobic	ATCC
S. epidermidis ATCC 12228	Bioassay indicator strain	MRS+L-Cys	37 ℃, Aerobic	ATCC
Bacillus subtilis ATCC 21332	Bioassay indicator strain	LB[g]	37 ℃, Aerobic	ATCC
Micrococcus luteus ATCC 4698	Bioassay indicator strain	LB	37 ℃, Aerobic	ATCC
Gram-negative bacteria				
E. coli DH5α	Bioassay indicator strain	LB	37 ℃, Aerobic	Invitrogen
Serratia marcescens ATCC 25419	Bioassay indicator strain	LB	37 ℃, Aerobic	ATCC
Proteus vulgaris ATCC 13315	Bioassay indicator strain	LB	37 ℃, Aerobic	ATCC
E. coli ER2566	Host for in vivo expression			NEB[h]
Plasmids				
pTXB1	Expression vector			NEB
pMXB10	MBP[i] expression plasmid			NEB
DHFR	DHFR[j] expression plasmid			NEB

[a], MRS+L-Cys, de Man-Rogosa-Sharpe medium (Difco, Detroit, MI) supplemented with 0.05% L-cysteine HCl; [b], BLIM+$FeCl_3$, BLIM (Bifidobacteria-Low Iron-Medium) medium (Islam, 2006) (pH 7.0) consisting of 2.0% proteose peptone, 0.15% K_2HPO_4, 0.5% Glucose, 0.15% $MgSO_4$-$7H_2O$, and 100 mM Pipes supplemented with 0.05 mM $FeCl_3$ $6H_2O$; [c], ATCC, *American Type Culture Collection;* [d], DCC, Dairy Culture Collection, University of Minnesota; [e], M17G, M17 medium supplemented with 0.5% D-glucose; [f], RCM, Reinforced Clostridial Medium; [g], LB, Luria-Bertani medium; [h], New England Biolabs; [i], maltose binding protein; [j], dihydrofolate reductase.

broth until an OD600 nm of 1.0 and incubated at room temperature for 5 min. Bacterial cells were pelleted from RNAprotect solution by centrifugation at 3,400 rpm in a Beckman Coulter CH 3.8 swing-bucket rotor in a Beckman Coulter Allegra 6 Kr centrifuge, at 4 ℃ for 5 min and stored at - 80 ℃ until needed. Total RNAs were isolated and purified using the RiboPure-Bacteria Kit (Ambion, Austin, TX) according to the manufacturer's protocol.

Quantitative TaqMan real-time PCR

Two primers and one probe for TaqMan real-time PCR targeting the *lanA* gene were designed using the Primer Express software of the 7500 Real Time PCR System (Applied Biosystems, Carlsbad, California). The F-lanA forward primer (5'-TGAGAA GTCCATTGTCGGTGAA-3') and R-lanA reverse primer (5'-CGGTCAGCATCGCC ATATC-3') were used for PCR amplification of the *lanA* gene and the P-lanA probe was labeled with 6-FAM and 6-TAMRA (5'-/6-FAM/CCTTCGAGGATCTGTCGGCGGC/6-TAMSp/-3') was used for fluorescence detection during real-time PCR. All real-time PCRs were performed using a Roche LightCycler 480 (Roche Applied Science, Indianapolis, IN) with a TaqMan RNA-to-C_T *1-Step* Kit (Applied Biosystems) with the following conditions: 1 cycle of 48 ℃ for 15 min (reverse transcription), 1 cycle of 95 ℃ for 10 min (activation of AmpliTaq Gold DNA Polymerase), 45 cycles of 95 ℃ for 15 sec and 60 ℃ for 1 min (denaturation and annealing/extension). The real-time PCR mixture consisted of 12.5 µl of TaqMan RT-PCR mix (2X), 0.45 µl of F-lanA forward primer (50

µM), 0.45 µl of R-lanA reverse primer (50 µM), 0.625 µl of P-lanA TaqMan probe (10 µM), 0.625 µl of TaqMan RT enzyme mix (40X), 5 µl of RNA (20 ng/µl) and 5.35 µl of molecular grade water for a total volume of 25 µl. For absolute quantification of *lanA* gene expression, serially diluted template was used to generate a standard curve using the recommendations of the Roche LightCycler 480 real time PCR system. Collected data were analyzed statistically using the LightCycler 480 sw 1.5 software package.

Crude lantibiotic preparation from agar cultures of *B. longum* DJO10A

A fully grown broth culture of *B. longum* DJO10A in BLIM+Fe was spread plated onto agar plates consisting of 10 ml of BLIM+Fe or MRS+L-Cys media supplemented with 0.5% agar (Difco) and incubated anaerobically at 37 ℃ for 48 h. The former medium (BLIM+Fe) is a buffered medium and was used as a control as any bioactivity obtained would not be acid based. A total of 105 cultured agar plates were collected from each media, crushed and dispensed into three 1 L beakers per medium (~ 300 ml/beaker) and an equal volume of 95% methanol solution (w/v) was added. After stirring the mixture with magnetic bars at 4 ℃ for 24 h, the agar debris and cells were removed by centrifugation at 8,000 g at 4 ℃ for 20 min and approximately 1 L of the supernatant from each media was collected and filtered using a Corning 115 ml vacuum filter system with a 0.22 µm diameter filter (Corning, Lowell, MA). Methanol was removed by evaporation using a Savant SPD2010 SpeedVac concentrator (Thermo Scientific, Waltham, MA) using rotor RH 200-12 at 45 ℃ until completely dry by

dispensing 1.8 ml of the supernatant in 2.0 ml Eppendorf tubes. The dried pellets from each tube were resuspended in 30 µl of molecular grade water and transferred into a 50 ml falcon tube. The final volume of the collected lantibiotic from each media was 16.5 ml. Further purification of the lantibiotic solution was conducted by size fractionation using CentriPrep centrifugal filter units with 50, 30 and 10 kDa cut-off filters in sequence (Millipore, Billerica, MA).

Quantification and inhibitory spectrum analysis of crude lantibiotic preparation

The bioactivity of the crude lantibiotic preparation was assayed using standard well diffusion assays (Tagg, 1971). Arbitrary units (AU) were calculated based on the size of inhibitory zones against the lantibiotic sensitive strain, *B. longum* DJO10A-JH1. The lantibiotic was serially diluted (one in two) and the highest dilution that gave noticeable clear zone was defined as one arbitrary unit. Inhibitory analysis of various Gram-positive and Gram-negative bacteria as indicator strains was conducted using the same amounts of crude lantibiotic preparation (8 AU) using well diffusion assays. The diameter of inhibition zones was measured to quantify the amount of inhibition.

Production of the lantibiotic in broth using serial 10% sub-inoculations

B. longum DJO10A was inoculated from stock into BLIM+Fe broth and incubated at 37 ℃, overnight. A 1 ml aliquot of the overnight culture was sub-inoculated

into 12.9 ml of BLIM+Fe broth containing 120 AU of the crude lantibiotic and incubated for 15 h at 37 ℃. After reaching stationary phase, 4 ml of this culture was transferred into 8 ml of RNAprotect solution, incubated at room temperature for 5 min and centrifuged at 4 ℃ for 5 min at 2,641 g. The cell pellet was stored at - 80 ℃ for subsequent RNA analysis. The rest of the culture (9.9 ml) was sub-inoculated into 89.1 ml of BLIM+Fe broth (10% sub-inoculation), mixed thoroughly, and incubated at 37 ℃ for 6 h. After incubation, 5 ml of the culture was transferred into a test tube for OD measurement and another 4 ml of the culture was collected for RNA analysis as described above. The remaining 90 ml of the culture was sub-inoculated into 810 ml of BLIM+Fe broth, mixed thoroughly and incubated at 37 ℃ for 6 h. After incubation, samples were taken for OD and RNA analysis as described above and the remaining culture was inoculated (99 ml/bottle) into nine 1 L glass Kimax media bottles containing 891 ml of BLIM+Fe broth per bottle, mixed thoroughly and incubated at 37 ℃ for 6 h. The cells from each culture were harvested by centrifugation using nine 250 ml centrifuge bottles (Thermo Scientific) to collect pellets from each of the nine 1 L cultures at 8,000 g for 15 min at 4 ℃. The supernatant was stored in two 4 L flasks at 4 ℃ and the cell pellets were frozen at - 20 ℃.

MALDI-TOF MS analysis on cell pellets and supernatants

A 1 ml aliquot of supernatant and approximately 20 µl of cells that were scratched off of frozen cell pellets were treated with a ZipTip protocol from the center of Mass Spectrometry and Proteomics (University of Minnesota). Briefly, the samples were

acidified to pH 3.0 using 10% TFA (Trifluoroacetic acid) before being pipetted slowly through each ZipTipC18 (EMD Millipore, Billerica, MA) and washed with 0.1% TFA solution. They were then eluted with 1.3 µl of 60% ACN (Acetonitrile) and 0.1% TFA. A 0.6 µl aliquot of the eluted sample was deposited on an stainless steel MALDI target (Bruker Daltonics GmBH, Bremen, Germany) with 0.6 µl of a CCA matrix containing 50% cyano-4-hydroxycinnamic acid solution, 25% (v/v) ACN and 0.5% (v/v) TFA, and allowed to air-dry. The protein samples were analyzed in reflector mode.

Removal of attached lantibiotic from the cell surface

Three different procedures were used to remove the lantibiotic from the cell surface. The first procedure utilized boiling to remove the peptide from the cell surface. A pipette tip was used to scrape 0.12 g of cells from a frozen pellet and this was suspended in 1.08 ml of molecular grade water to generate a 10% solution. This was boiled for 10 min in two C-2170 micro centrifuge tubes (Denville Scientific Inc, South Plainfield, NJ) and one of the tubes was immediately centrifuged for 10 sec, at 16,100 g and the supernatant was collected immediately to prevent cooling. The other tube was placed on ice for 30 min, centrifuged and the supernatant was collected. Both supernatants were dried using a SpeedVac at 45 ℃ for 3 h. The powders were resuspended in 10 µl of molecular grade water and analyzed by the MALDI-TOF MS.

The second procedure utilized methanol to extract the peptide from the cell surface. A 1 g aliquot of the cell pellet was resuspended in 10 ml of 95% methanol and stirred with a magnetic bar at 4 ℃ overnight. The supernatant was collected by centrifugation at 5,000 g for 10 min and filtered using a 0.22 μm filter (Corning, Lowell, MA). The sample was dried with the SpeedVac at 45°C for 3 h and resuspended in 10 μl of molecular grade water prior to analysis by MALDI-TOF MS.

The third procedure utilized *n*-butanol to extract peptide from the cell surface (Garneau et al., 2003). A 0.23 g aliquot of cells was scraped from the cell pellet and resuspended in 2.07 ml of molecular grade water and extracted with 1.15 ml of *n*-butanol using orbital shaker (Model 4625, Lab line, India) at 1,100 rpm for 20 min at 37 ℃. The supernatants were collected by centrifugation at 16,100 g for 1 min. The sample was dried in a SpeedVac at 45°C for 4 h and resuspended in 10 μl of molecular grade water and analyzed by MALDI-TOF MS.

Mutagenesis

Three chemical mutagens were used for random mutagenesis using a modification of the procedure of Ibrahim and O'Sullivan (2000). The first mutagen used was *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG, TCI America, Portland, OR). *B. longum* DJO10A was grown overnight at 37 ℃ in 100 ml of BLIM+Fe broth. The fully grown culture was washed and resuspended in 10 ml of citrate buffer (pH 5.5).

Suspended cells were mixed with 0, 124, 166, 251, 316, 426 and 652 µl of MNNG (stock solution: 49 mg/ml in dimethyl sulfoxide) and incubated at 37 ℃ for 1.5 h with gentle agitation. A 1 ml aliquot of the treated and control samples (no mutagen added) was taken immediately after incubation with the mutagen, diluted appropriately, and plated onto MRS agar media to determine the number of viable cells remaining following mutagenesis (death rate). At the same time, 100 µl of the mutagenized culture and the control samples were spread on MRS agar plates with 10 µg of rifampicin/ml (optimized) to estimate the rate of mutagenesis (mutation frequency). The remainder of the mutagenized cultures were then mixed with 15% glycerol and stored at - 80 ℃ for subsequent screening.

The second mutagen used was ethyl methanesulfonate (EMS, Sigma). Cells were prepared as described above for MNNG mutagenesis, except the buffer used was a potassium phosphate buffer (pH 7.5) and the amounts of EMS used were 0, 50, 150, 250, 350 and 450 µl.

The third mutagen used was UV. A treatment of 0.5 mJ by UV Stratalinker 2400 (Stratagene, La Jolla, CA) was applied to ten open MRS agar plates that were previously seeded with 100 µl (concentrated from 1 ml) of a fully grown DJO10A culture. A 1 ml (concentrated from 10 ml) aliquot of the DJO10A was diluted appropriately and plated out on MRS agar plates for viable cell count measurements. All plates were incubated anaerobically at 37 ℃ for three days.

Screening of mutated cells

Two different procedures were used for screening of the mutated cells. The first procedure was negative screening, using *E. coli* ATCC 2159 as the indicator strain. The mutant pots were spread plated on MRS plates and incubated anaerobically at 37 ℃ for two days. Isolated colonies were picked and inoculated into 96-well microtiter plates containing 200 µl of BLIM+Fe broth plus 1.6 AU of crude lantibiotic (pH adjusted to 7.0) and incubated another two days anaerobically at 37 ℃. A 100 µl aliquot of culture from each well was transferred into new 96-well microtiter plates and mixed with 200 µl of LB broth containing 100 mM of PIPES, 15 mM of EDTA and 2% *E. coli*, and incubated, shaking overnight at 37 ℃. As a control, one well in each microtiter plate did not contain *E. coli*. Following incubation, plates were visually evaluated to see if any inhibition of cell growth occurred. When potential mutant occurred, they were retested with Bioscreen C (detailed in next paragraph) using *E. coli* as indicator strain.

The second procedure was a positive screening procedure using a co-culture of DJO10A and DJO10A-JH1. A 10 ml aliquot of each EMS and MNNG mutant pots (~ 10^5 cfu/ml) were inoculated separately with ~ equal amounts of DJO10A-JH1 (100 µl of 100 times diluted culture at OD_{600} = 1.005) in 90 ml of BLIM+Fe broth containing 864 AU of the crude lantibiotic, incubated anaerobically at 37 ℃ for 24 h. Meanwhile, 1 ml of each mutant pot and DJO10A-JH1 were diluted appropriately and plated out on BLIM+Fe plates for viable cell counts. A fresh 900 ml of BLIM+Fe broth was added to the co-

culture and incubated for another 24 h. A 10 ml aliquot of the culture was collected and pelleted for chromosomal DNA isolation and 100 ml of rest of the culture was sub-inoculated into 900 ml of fresh BLIM+Fe broth. Following incubation for 24 h, 10 ml of the culture was pelleted for DNA isolation. This 10% sub-inoculation and collection of samples for DNA isolation was repeated six more times. A 1 ml aliquot of the final sub-inoculation culture was diluted and plated out on MRS for isolated colonies. A total of 36 colonies were tested with *lanM* primers (F-lanM: 5'-CGCTATTACACCAGATACG-3', R-lanM: 5'-GGTAGACATACAGGTTCTCC-3') using PCR with the following conditions: 1 cycle of 96 ℃ for 3 min, 35 cycles of 96 ℃ for 30 sec and 60 ℃ for 30 sec and 72 ℃ for 45 sec, 1 cycle of 72 ℃ for 5 min. The PCR mixture consisted of 5 µl of *Taq* polymerase buffer (10X), 2.5 µl of F-lanM forward primer (20 µM), 2.5 µl of R-lanM reverse primer (20 µM), 1 µl of dNTP mixture (10 mM), 0.5 µl of Taq DNA polymerase (5U/µl), 37.5 µl of molecular grade water and 1 µl of DNA template for a total volume of 50 µl. Any DJO10A colonies that were detected were further evaluated using the Bioscreen C test using *E. coli* as the indicator strain, as described below.

Isolation of chromosomal DNA from bifidobacteria

The pelleted cells from 10 ml of the positive screening culture were washed with 600 µl of TES buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA and 0.1 M NaCl) and resuspended in 400 µl of TE buffer (10 mM Tris-HCl pH 8.0 and 1 mM EDTA), 50 µl of lysozyme solution (100 mg/ml in water) and 20 µl of mutanolysin (1000 U/ml) and

incubated at 37 ℃ for 1 h. Samples were then frozen for 10 min at - 80 ℃ and thawed for 10 min at 37 ℃. A fresh 200 µl of proteinase K mixture (140 µl of 0.5 M EDTA, 20 µl of proteinase K and 40 µl of 10% SDS) was added and incubated at 37 ℃ for 1 h. Samples were then extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) solution by vortexing for 10 sec, followed by centrifugation at 16,100 g for 5 min. The collected aquous phase was incubated at 37 ℃ for 15 min with 3 µl of RNase A (100 mg/ml). A second phenol extraction was conducted before precipitating the DNA with 10 µl of 5 M NaCl and two volumes of absolute ethanol, and pelleted at 16,100 g for 10 min. The pellet was washed with ice-cold 70% ethanol and pelleted again. The pellet was dissolved in 100 µl of TE buffer at 55 ℃ for 1 h.

Bioscreen C growth analysis of *E. coli*

Any potential mutants from positive and negative screenings were grown in 96-well microtiter plates containing 400 µl of BLIM+Fe broth plus 1.6 AU of crude lantibiotic (pH adjusted to 7.0) and incubated anaerobically for two days at 37 ℃. After incubation, a 100 µl aliquot of culture from each well was transferred into honeycomb 2 (Growth Curves USA, Piscataway, NJ) plates and mixed with 200 µl of LB broth containing 100 mM of PIPES, 15 mM of EDTA and 2% *E. coli*, and incubated in the Bioscreen C instrument (Growth Curves USA, Piscataway, NJ) at 37 ℃ with continuous shaking at medium speed, overnight. As a control, one well in the plate did not contain *E.*

coli. Optical density reading was performed at 600 nm, and data was collected every 30 min. All samples were performed in triplicate.

PCR amplification

All PCRs used in cloning were performed using a PTC-0200 DNA engine cycler (BioRad, Hercules, CA) and the Phusion® High-Fidelity PCR Kit (NEB). The reaction mixture (final volume, 20 µl) contained 4 µl of 5X phusion HF buffer, 0.4 µl of dNTP mixture (10 mM), 0.5 µl of forward primer (20 µM), 0.5 µl of reverse primer (20 µM), 1 µl of DNA template and 0.2 µl of Phusion DNA polymerase (2U/µl). The amplification conditions were as follows: 1 cycle of 98 ℃ for 30 sec, 35 cycles of 98 ℃ for 10 sec and X ℃ (3 ℃ above the lowest primer Tm) for 30 sec and 72 ℃ for 30 sec, 1 cycle of 72 ℃ for 5 min.

General molecular techniques

The PCR product was recovered from agarose gels using a Gel/PCR DNA Fragments Extraction Kit (IBI Scientific, Peosta, IA) following the manufacture's protocol. Restriction enzymes were purchased from NEB and used according to the manufacturer's guidelines. DNA ligations were performed using the QuickLink^{TM} DNA Ligation Kit (Sigma) with an optimized procedure. Briefly, the insert and vector were mixed with molecular grade water (final volume, 17 µl) and placed in a 90 ℃ beaker of

water and left to cool slowly until RT before 1 µl of T4 ligase and 2 µl of ligation buffer (10 X) were added into the tube and incubated at RT for 30 min.

Plasmid transformation into *E. coli*

A single colony of *E. coli* ER 2566 was grown in 5 ml of LB broth at 37 ℃ until an OD_{550} of 0.3. The culture was transferred into 100 ml of pre-warmed LB broth and incubated at 37 ℃ with vigorous aeration until an OD_{550} of 0.48. The cells were harvested using two 50 ml falcon tubes at 2,300 g for 10 min with a pre-chilled centrifuge. The following steps were conducted on ice. The pellet was resuspended (with brief vortexing) in 30 ml of ice-cold transformation buffer I (10 mM KOAc, 50 mM $MnCl_2$, 100 mM RbCl, 10 mM $CaCl_2$, 15% glycerol, pH 5.8 adjusted with HOAc, filter sterilized and stored at 4 ℃) and incubated on ice for 120 min. Cells were pelleted again at 2,300 g, for 5 min and resuspended gently in 4 ml of ice-cold transformation buffer II (10 mM NaMOPS (pH 7.0), 75 mM $CaCl_2$, 10 mM RbCl, 15% glycerol, filter sterilized and stored at 4 ℃). The resuspended competent cells were dispensed into several Eppendorf tubes (100 µl/tube) and stored at - 70 ℃ for further usage.

Plasmids were introduced into *E. coli* using heat-shock transformation. A 4 µl aliquot of plasmids was resuspended in each 100 µl of competent cells, and left on ice for 20 min, followed by heat shocking at 42 ℃ for 90 sec. The cells were returned to the ice for 90 sec, then mixed with 5 volumes of LB broth and incubated at 37 ℃, with gentle

shaking for 1 h. After incubation, 50-200 µl of the cultures were spread on LB plates with ampicillin (100 µg/ml), and incubated at 37°C overnight.

Plasmid isolation and purification

Plasmids were isolated using of *Zyppy*™ Plasmid Miniprep Kit **(**Zymo Research, Irvine, CA) with optimized procedures. Briefly, *E. coli* strains were inoculated from stock in 30 ml of LB Amp broth and incubated at 37 ℃ overnight with vigorous shaking. The cells were harvested at 6,000 g for 10 min at 4 ℃. The pellets were resuspended in 6 ml of molecular grade water and mixed with 1 ml of supplied 7 × lysis buffer and inverted several times until clear blue. Just after 2 min, the mixture was neutralized with 3.5 ml of the neutralization buffer by inverting several times forming a yellow precipitate. The supernatant was then collected following centrifugation at 8,000 g for 10 min at 4 ℃. Phenol extraction was conducted by adding one volume of phenol:chloroform:isoamyl alcohol (PCI:25:24:1) to the collected supernatant, and centrifuged at 10,000 g for 10 min at 4 ℃. After extraction, the upper phase was collected and mixed with 1/10 volume of 3M sodium acetate (pH 4.8) and one volume of isopropanol, mixed thoroughly and centrifuged at 11,400 g for 20 min at 4 ℃. The pellets were resuspended in 1 ml of the neutralization buffer and loaded onto the spin columns provided with the kit and centrifuged at 13,200 g for 30 sec. A 600 µl aliquot of the wash buffer was added onto the column left for 1 min and followed with 30 sec of centrifugation. The washing step was conducted twice. After washing, the columns were spun for another 30 sec at 13,200

g. The columns were transferred into new Eppendorf tubes and 100 µl of pre-warmed (60 ℃) nuclease free water was added, left for 2 min and centrifuged for 2 min. The eluted DNA was further purified with the Gel/PCR DNA Fragments Extraction Kit. The purified DNA was concentrated using the speedvac at 45 ℃ for 2 h and stored at - 20 ℃.

Plasmid miniprep

E. coli cells were inoculated into 5 ml of LB broth containing ampilicin and incubated overnight with vigorous shaking. The culture in each tube was transferred into Eppendorf tubes (filled) and pelleted at 13,200 g for 30 sec. The supernatant was decanted and centrifuged for 1 sec to enable all the medium to go to the bottom. The rest of the medium was aspirated with a P200 pipette tip. The cell pellet was resuspended by vortexing in 100 µl of TE buffer. A 200 µl aliquot of lysis solution (0.2 N NaOH, 1% SDS) was added into the tube and mixed by inverting 6-8 times. After 7-8 min, 150 µl of 5 M potassium acetate solution (pH 4.8) was added for neutralization. The supernatant was collected following centrifugation at 13,200 g, for 10 min and mixed with 2 volumes of - 20 ℃ pure ethanol and then centrifuge for 15 min. After centrifugation, the ethanol was poured off and immediately filled with 70% ethanol and decanted again. The tubes were centrifuged for 1 sec to enable all the liquid to go to the bottom, which then was aspirated using a P200 pipette tip. The plasmid pellets were dried at 37 ℃ for 10 min, and resuspended in 100 µl of TER (TE plus RNase 0.1mg/ml) and incubated at RT for 10 min.

In vivo expression

E. coli ER2566 strains containing expression plasmids were grown in 6 ml of LB medium containing ampicillin (100 μg/ml) until they reached mid-log phase (OD of 0.5), and then dispensed equally into two tubes, one of which was induced with isopropylthiogalactosid (IPTG, 0.4 mM) and the other one with no induction (negative control). After incubating at 37°C for 4 h, 200 μl of the culture was harvested by centrifugation at 13,200 g for 1 min. The pellets were resuspended in 15 μl of SDS buffer [(62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% glycerol, 0.01% (w/v) bromophenol blue, 13.3 mM DTT)] and boiled for 5 min. It was spun briefly for 5 sec and cooled to RT before loading into an SDS-PAGE gel as described below.

Protein analysis by SDS-PAGE gel

A 12% Tris-HCl SDS-PAGE ready gel was assembled in a Mini-PROTEAN 3 cell (BioRad). The electrophoresis buffer 1X consisted of 25 mM Tris, 192 mM Glycine and 0.1% SDS. An 8 μl aliquot of the precision plus protein all blue standard (BioRad) was used as the protein marker. Electrophoresis was initiated at 30 mA under constant current for 10 min, before increasing to 45 mA. The running time was 45 min. After electrophoresis, the gel was stained using protein staining solution (40% Methanol, 10% Acetic acid, 0.1% (w/v) Coomassie blue R-250) for 30 min, and destained using destaining solution (40% Methanol, 10% Acetic acid) for 2 h.

In vitro expression

The template DNA (250 ng/plasmid) was mixed with 10 µl of Solution A and 7.5 µl of Solution B (prepared from a master mix) and nuclease free water up to 25 µl as described in the manual of the PurExpress protein synthesis kit (NEB). A 2 µl (125 ng/µl) aliquot of DHFR plasmid (PurExpress protein synthesis kit, NEB) was used for *in vitro* expression positive control. The reaction mixtures were supplemented with 0.4 mM IPTG. The in vitro protein synthesis was conducted at 37°C, for 4 h. An 8 µl aliquot of the protein sample was mixed with 4 µl of SDS buffer (3X) and boiled for 5 min. It was spun briefly for 5 sec and cooled to RT before loading into an SDS-PAGE gel as described above.

Purification of LanR1 fused to intein-CBD (chitin binding domain)

A 10 µl aliquot of chitin beads (NEB) that were supplied in 20% ethanol, were transferred into an Eppendorf tube and washed twice with 500 µl of the column buffer [20 mM Tris-HCl (pH 8.5), 500 mM NaCl and 1 mM EDTA] before mixing with the sample. A 42 µl aliquot of the in vitro reaction product containing LanR1-intein-CBD was mixed with these pre-treated chitin beads and incubated at 4 ℃ for 1 h with gentle shaking. After binding, the beads were washed twice with 0.5 ml of the column buffer and resupended in 8 µl of molecular grade water and 4 µl of SDS buffer (3X) before

boiling for 5 min. Following centrifugation at 2,000 g for 10 sec and subsequent cooling to RT, the supernatant was loaded into an SDS-PAGE gel as described above.

Results

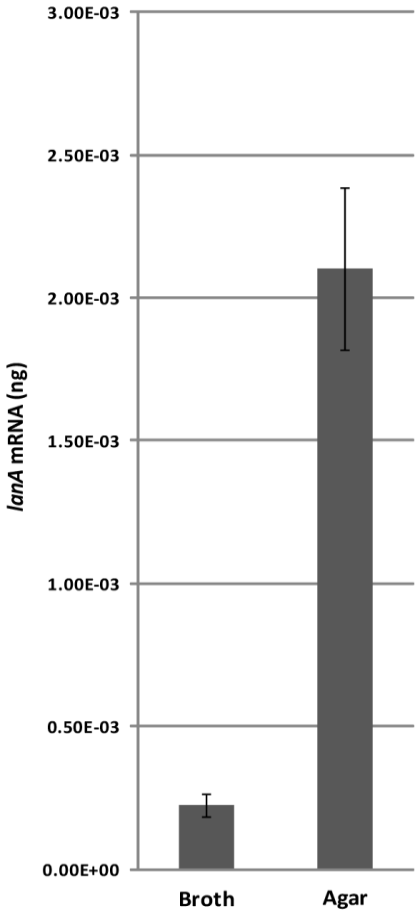
Real-time PCR analysis of the lantibiotic structural gene, *lanA*

To further investigate the different gene expression profiles of *lanA* in broth and agar cultures, a real-time PCR assay with a specific TaqMan probe targeting the *lanA* gene was developed. The quantitative real-time PCR analysis of total RNA samples from broth and agar cultures showed that the *lanA* gene was up-regulated more than nine times higher on agar, substantiating the induction of *lanA* during growth on agar media (Fig. 1).

Crude lantibiotic preparation and inhibitory spectrum analysis

A crude lantibiotic preparation was collected from *B. longum* DJO10A agar cultures by methanol extraction and size fractionation. While both MRS-cys and BLIM+Fe media were utilized for comparative purposes, better yields could be obtained from MRS-cys due to better growth of strain DJO10A. The amount of lantibiotic produced was low, but sufficient for MIC analysis. Typically from 105 cultured MRS-cys agar plates, 16.5 ml of crude lantibiotic could be produced at a concentration of 160 AU/ml. Interestingly, the antimicrobial spectrum analysis of this crude lantibiotic

FIG. 1. Quatitative real-time PCR analysis of *lanA* gene expression in broth and agar culture conditions. Error bars indicate standard deviations (n = 3).


3.00E-03
2.50E-03
2.00E-03
1.50E-03
1.00E-03
0.50E-03
0.00E+00
lanA mRNA (ng)
Broth
Agar

preparation with various indicator strains showed it had a broad spectrum of inhibition against various Gram-positive and some Gram-negative bacteria (Table 2) (Fig. 2). The lantibiotic-producing host, *B. longum* DJO10A, was not inhibited due to the predicted self-immunity function encoded by *lanI*. The complete loss of the lantibiotic gene cluster including this locus, in the mutant strain *B. longum* DJO10A-JH1 rendered it sensitive to this lantibiotic preparation (Lee et al., 2008), confirming that the inhibition was due to the lantibiotic and not acid.

Auto-induction of the *lanA* gene in broth cultures

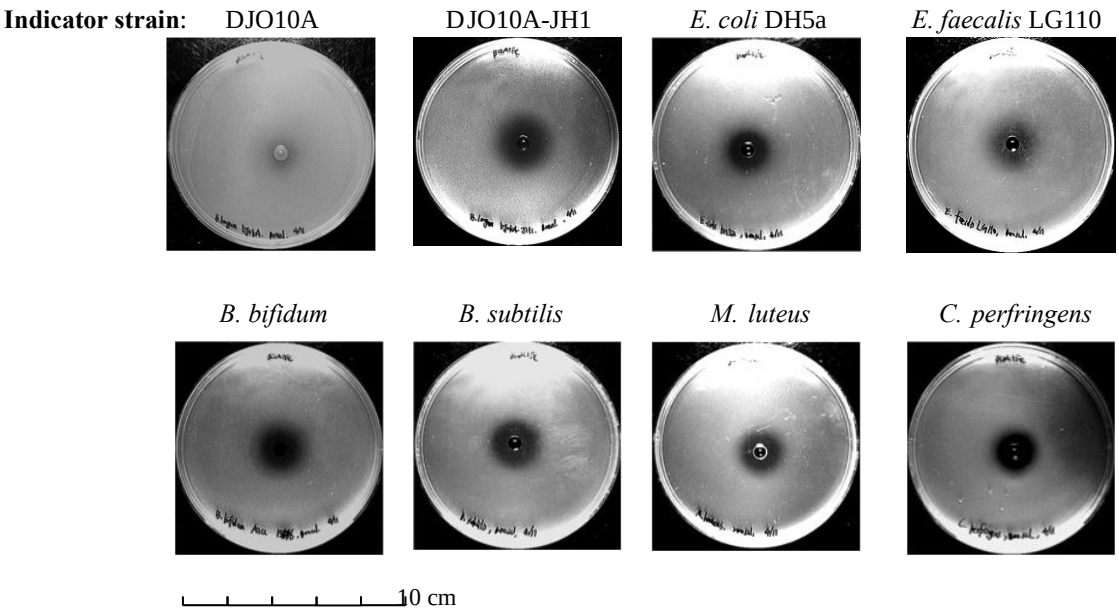
The comparative microarray analysis that was conducted by Ju-Hoon Lee (Lee et al., 2011) showed that the regulatory genes were expressed in broth cultures which suggested that the LanK sensor kinase moiety of the two-component LanRK system should be exposed to the outside of the cell and could respond to an external inducer signal if present. This could enable phosphorylation of the internal LanR to stimulate *lanA* transcription if sufficient levels of the inducer were added to the broth media. It is likely that the inducer (most likely the lantibiotic itself) is more concentrated in agar cultures, but is too dilute in broth cultures to initiate transcription of *lanA*. To investigate this hypothesis, a crude lantibiotic preparation from agar cultures of *B. longum* DJO10A, was added to broth media prior to inoculating with strain DJO10A to evaluate whether *lanA* transcription was induced. As the *lanI* gene is not expressed in broth cultures of *B. longum* DJO10A, it suggested it may be sensitive to the lantibiotic. To investigate this, a

TABLE 2. Antimicrobial spectrum analysis of crude lantibiotic preparation

Bacteria strains	Inhibition zone[a]
Gram-positive	
Bifidobacterium adolescentis ATCC 15704	+
B. breve ATCC 15701	+
B. longum subsp. *infantis* ATCC 15702	+
B. bifidum ATCC 15696	+++
B. animalis subsp. *lactis* S14	++
B. longum subsp. *longum* DJO10A-JH1	+++
B. longum subsp. *longum* DJO10A	-
Lactococcus lactis subsp. *lactis* LM0230	+
L. lactis subsp. *lactis* C2	+
Streptococcus thermophilus ST403	+
Enterococcus faecalis LG110	++
Micrococcus luteus	++
Clostridium perfringens	++
C. difficile	+
Staphylococcus epidermidis	+
S. aureus	++
Bacillus subtilis	+++
Gram-negative	
Serratia marcescens	+++
Proteus vulgaris	+
E. coli DH5a	++

[a], Diameter of inhibition zone: -, no inhibition; +, < 1.3 cm; ++, 1.3 to 1.7 cm; +++, > 1.7 cm.

FIG. 2. Bioassay of the crude lantibiotic against selected indicator strains.


Indicator strain:
DJO10A
DJO10A-JH1
E. coli DH5a
E. faecalis LG110
B. bifidum
B. subtilis
M. luteus
C. perfringens
10 cm

minimal inhibitory concentration (MIC) test of the crude lantibiotic preparation was conducted. This MIC test showed that 160 AU of this crude lantibiotic preparation was the maximum level that could be used without any growth inhibition of strain DJO10A. Therefore, to test the auto-induction potential of this crude lantibiotic preparation, amounts of up to 160 AU were added to broth media prior to monitoring for *lanA* gene expression. Interestingly, *lanA* gene expression was greatly increased in a dose-dependent fashion, confirming that increasing the external signal in broth cultures induces transcription of *lanA* (Fig. 3).

Kinetics of *lanA* transcription in broth cultures

To investigate the kinetics of *lanA* expression throughout the growth cycle, the optical density at 600 nm was monitored for a 50% sub-inoculated broth culture together with *lanA* expression. Interestingly, the expression of *lanA* was stimulated during the first hour and maintained at a high level throughout the full log phase, whereby expression levels reduced dramatically during stationary phase, with very little expression detected after 24 h of growth (Fig. 4). To evaluate if the induction could be maintained during subsequent sub-inoculations of the culture, it was sub-inoculated in BLIM+Fe media at various percentages and *lanA* expression monitored by quantitative real-time PCR. Sub-inoculations were conducted when the culture reached the early stationary phase of growth. While the 2.5 and 5% sub-inoculations showed a reduced level of *lanA* expression, those with 10% and higher revealed similar amounts of *lanA*

FIG. 3. Auto-induction of *lanA* gene expression in broth cultures with the crude lantibiotic preparation, using quantitative real-time PCR. Error bars indicate standard deviations (n = 3).

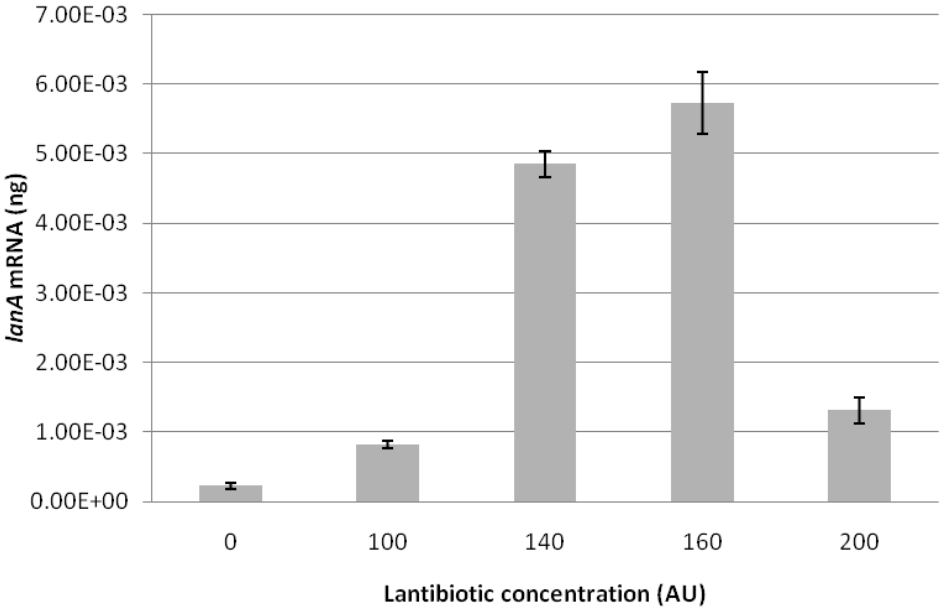
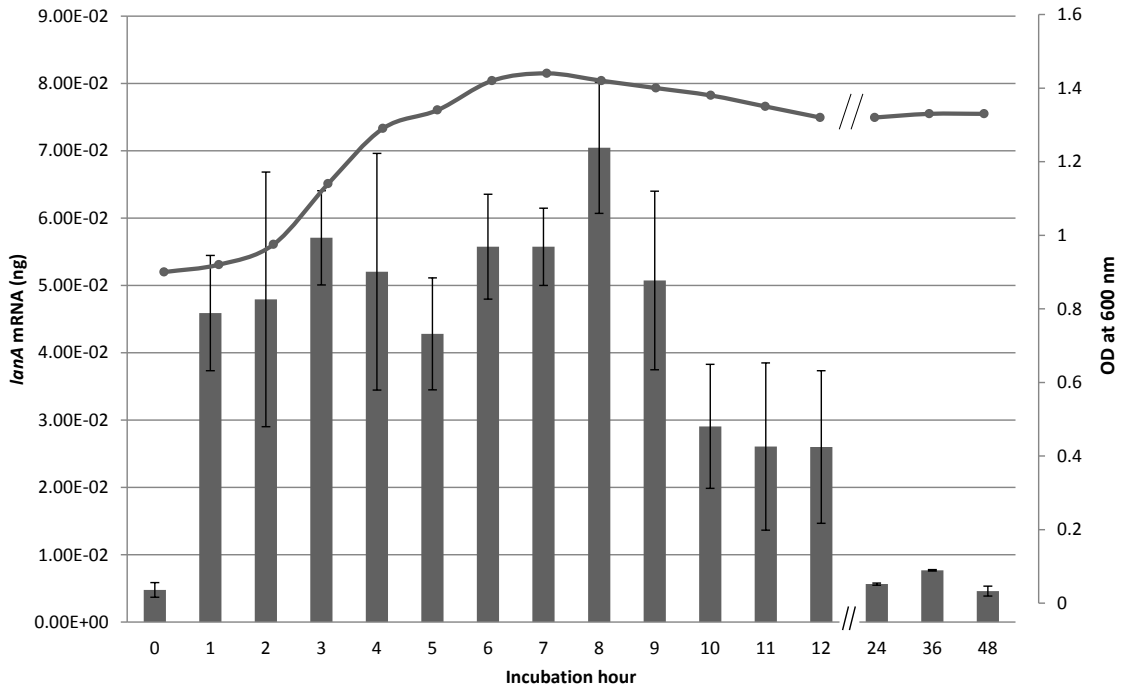

7.00E-03
6.00E-03
5.00E-03
4.00E-03
3.00E-03
2.00E-03
1.00E-03
0.00E+00
lanA mRNA (ng)
0
100
140
160
200
Lantibiotic concentration (AU)

FIG. 4. Growth curve analysis and quantification of *lanA* gene expression with quantitative real-time PCR during a broth culture following a 50% sub-inoculation with an auto-induced culture of *B. longum* DJO10A. Error bars indicate standard deviations (n = 3).


9.00E-02
8.00E-02
7.00E-02
6.00E-02
5.00E-02
4.00E-02
3.00E-02
2.00E-02
1.00E-02
0.00E+00
lanA mRNA (ng)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD at 600 nm
0 1 2 3 4 5 6 7 8 9 10 11 12 24 36 48
Incubation hour

expression compared to the parent culture (Fig. 5). Based on this result, a serial 10%-subinoculated culture was produced and assayed for lantibiotic activity using bioassays. However, no detectable activity was detected.

MALDI-TOF MS analysis of the auto-induced culture

To evaluate if any lantibiotic was present in the serial 10% sub-inoculated culture, a MALDI-TOF MS analysis of the cell pellet was conducted. This analysis of the cell pellet did show a typical lantibiotic profile consisting of a significant peak at the expected size of ~ 3.2 kDa corresponding to the predicted size for the lantibiotic (Fig. 6). This occurs due to the binding of the lantibiotic to LanK on the cell surface for signal transduction. To investigate if the peak represented the lantibiotic covalently attached to the cell, we removed the peptide from the cell surface using three different methods. All three methods, heating, *n*-butanol extraction and methanol extraction showed that the peptide could be removed from the cell surface with heating as the most efficient way (Fig. 7, 8 and 9).

Attempts to mutate *lanR1* using chemical and UV mutagenesis

To try to inactivate the *lanR1* gene of the lantibiotic gene cluster, 35 µl/ml of EMS and 0.6 mg/ml of MNNG were chosen for mutagenesis due to their acceptable mutation frequency and applicable amount of viable cells (Fig.10 and 11)(Table 3 and 4).

FIG. 5. Effect of sub-inoculation of an auto-induced broth culture of *B. longum* DJO10A on *lanA* gene expression. Error bars indicate standard deviations (n = 3).

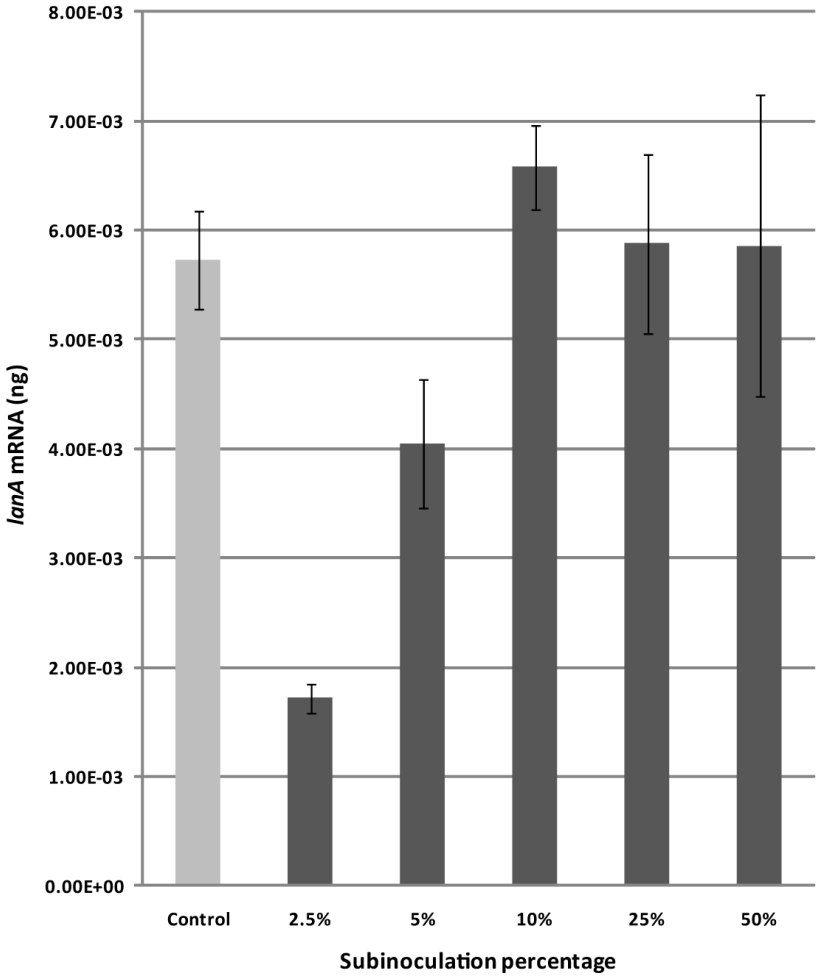

8.00E-03
7.00E-03
6.00E-03
5.00E-03
4.00E-03
3.00E-03
2.00E-03
1.00E-03
0.00E+00
lanA mRNA (ng)
Control
2.5%
5%
10%
25%
50%
Subinoculation percentage

FIG. 6. Peaks identified in the MALDI-TOF MS spectrum of the cell pellet from the serial 10% sub-inoculated sample. The mass (in Da) of the ions is shown on the x axis. The m/z value is the mass to charge ratio.

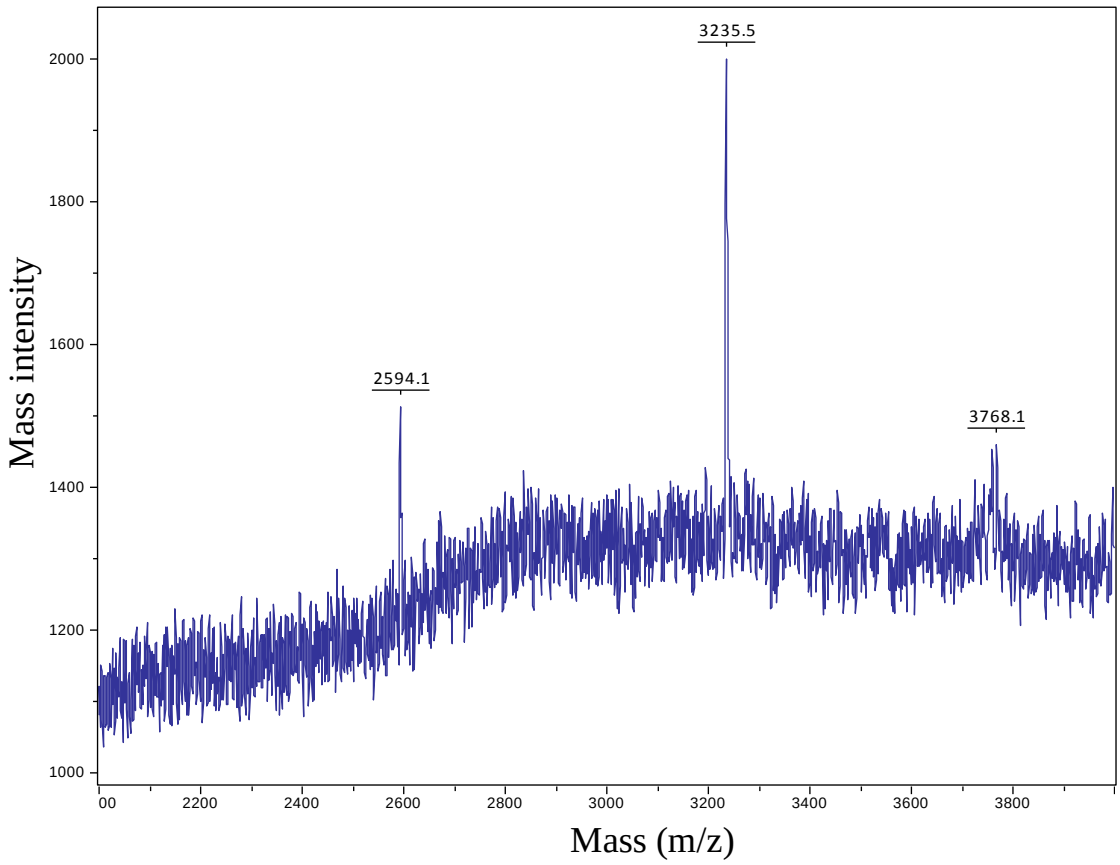
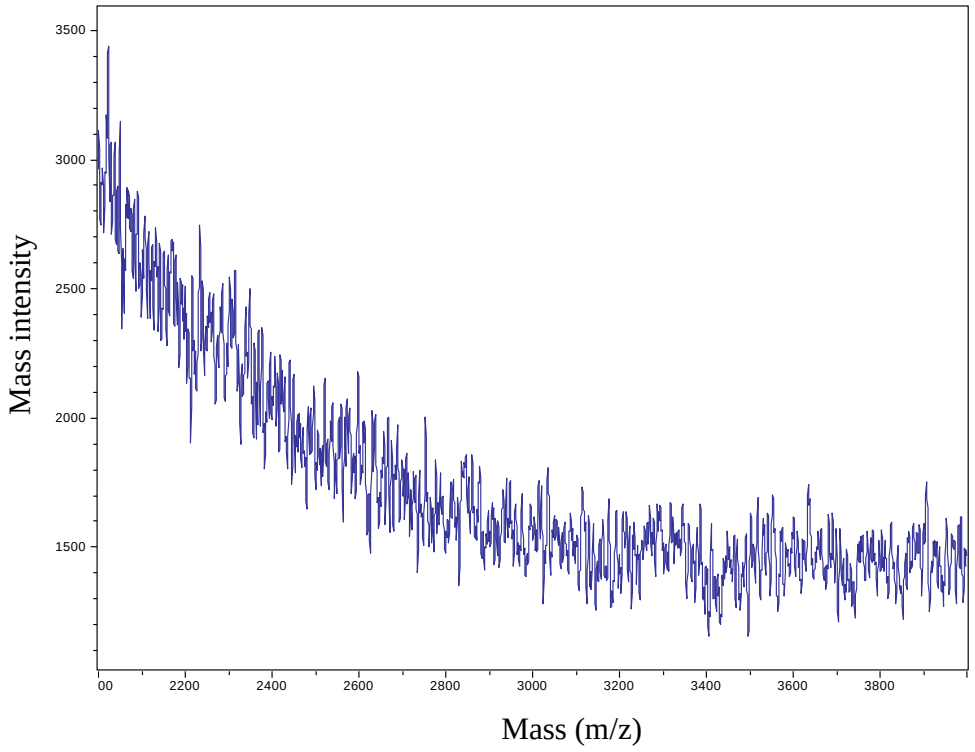

3235.5
2594.1
3768.1
Mass intensity
Mass (m/z)
2000
1800
1600
1400
1200
1000
00
2200
2400
2600
2800
3000
3200
3400
3600
3800

FIG. 7. Peaks identified in the MALDI-TOF MS spectrum using the boiling method. A, MALDI-TOF MS spectrum of the supernatant that was collected from boiling of the cell pellet followed by cooling; B, peaks identified in the MALDI-TOF MS spectrum of the supernatant that was collected just after boiling of the cell pellet without cooling down. The mass (in Da) of the ions is shown on the x axis. The m/z value is the mass to charge ratio.

A


Mass intensity
3500
3000
2500
2000
1500
00
2200
2400
2600
2800
3000
3200
3400
3600
3800
Mass (m/z)

B

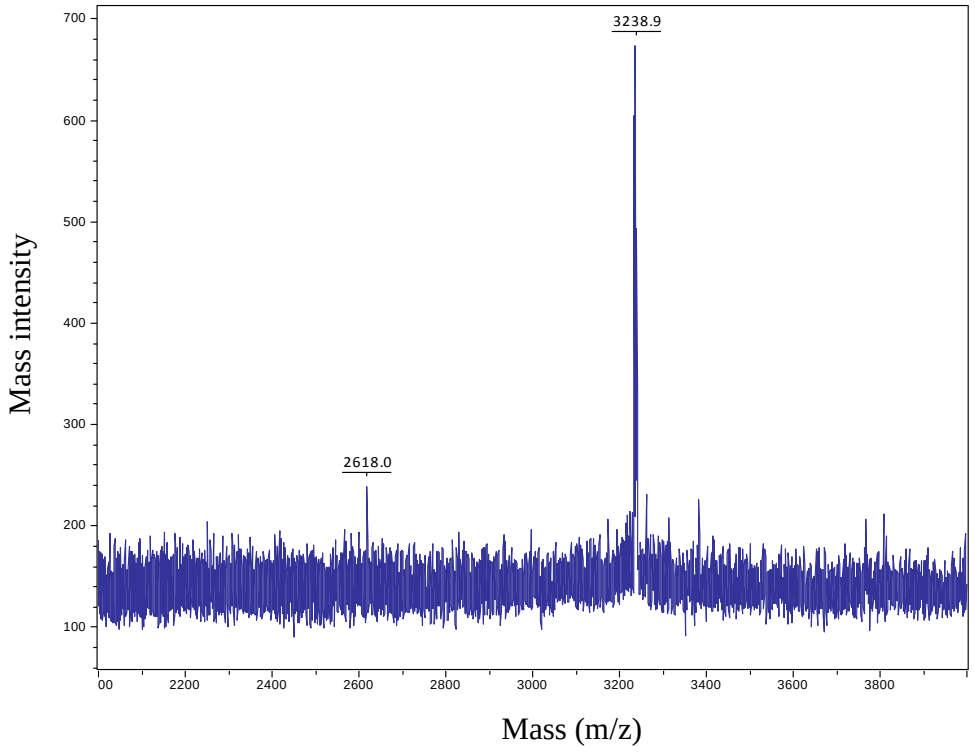

Mass intensity
700
600
500
400
300
200
100
3238.9
2618.0
00
2200
2400
2600
2800
3000
3200
3400
3600
3800
Mass (m/z)

FIG. 8. Peaks identified in the MALDI-TOF MS spectrum of an n-butanol extract from the cell pellet. The mass (in Da) of the ions is shown on the x axis. The m/z value is the mass to charge ratio.

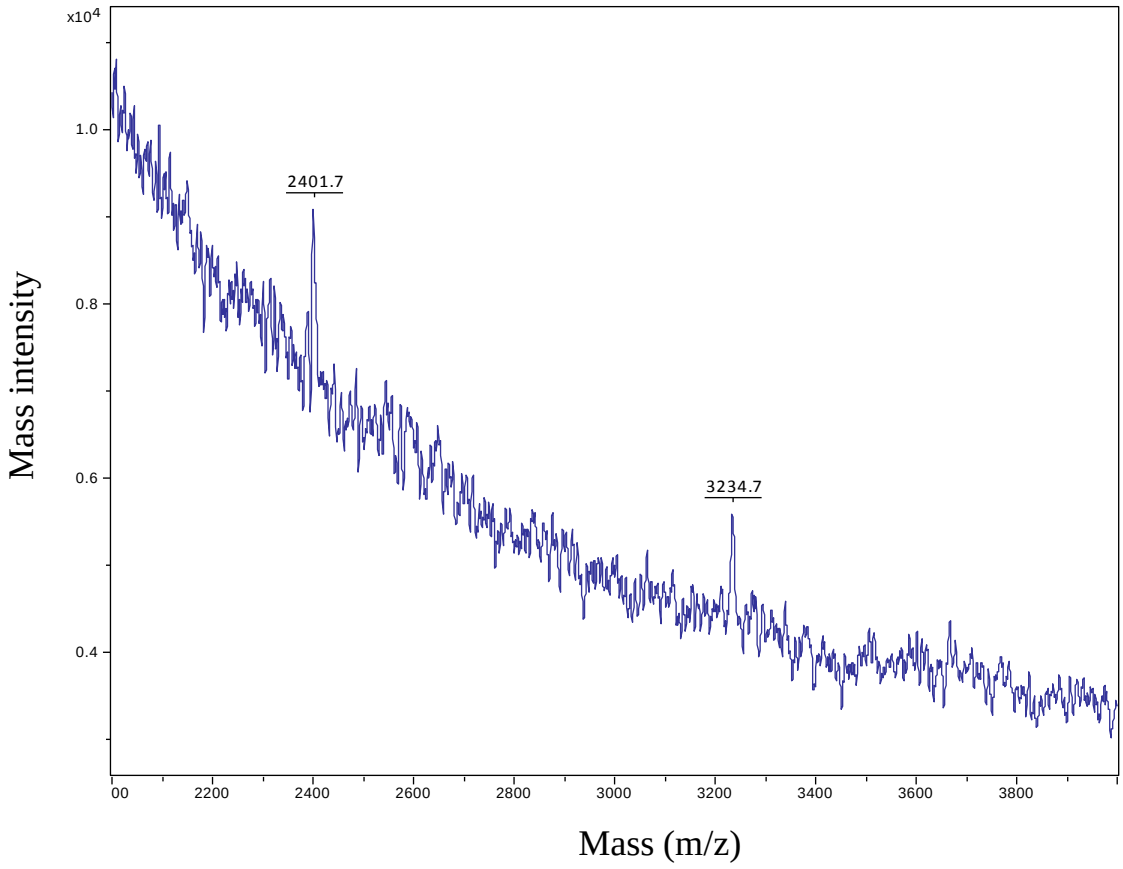

x10^4
1.0
0.8
0.6
0.4
2401.7
3234.7
00
2200
2400
2600
2800
3000
3200
3400
3600
3800
Mass intensity
Mass (m/z)

FIG. 9. Peaks identified in the MALDI-TOF MS spectrum of a methanol extract from the cell pellet. The mass (in Da) of the ions is shown on the x axis. The m/z value is the mass to charge ratio.

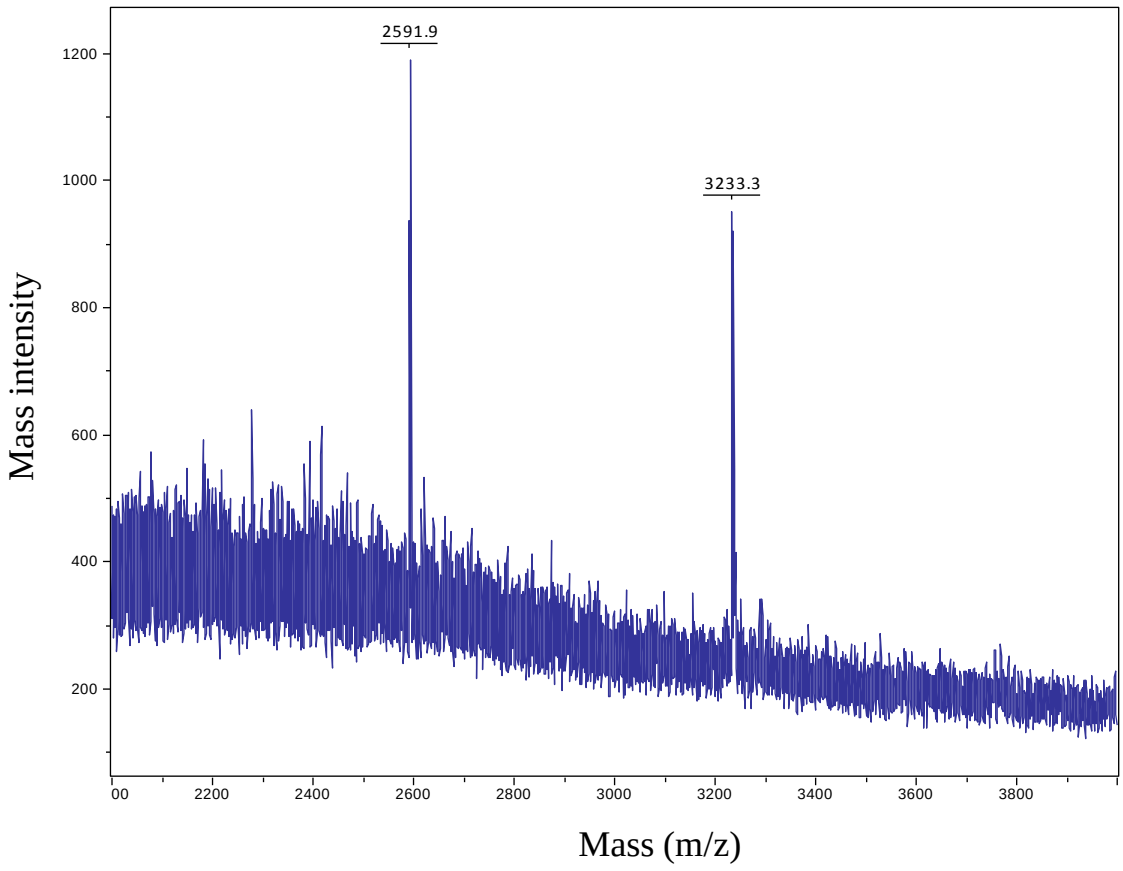

2591.9
3233.3
1200
1000
800
600
400
200
Mass intensity
00
2200
2400
2600
2800
3000
3200
3400
3600
3800
Mass (m/z)

FIG. 10. Viable cell count and mutation frequency of DJO10A after EMS mutagenesis.

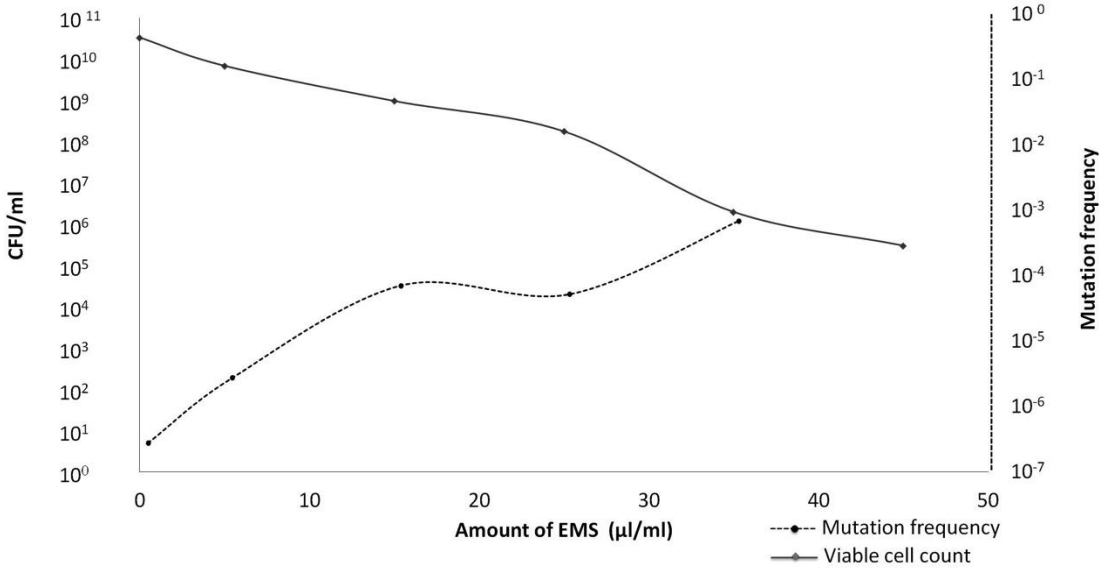


TABLE 3. Summary of EMS mutagenesis

Mutagen amount (µl/ml)	Viable cells (CFU/ml)	Death rate (%)	Mutation frequency [a]
0	3.40×10^{10}	0	2.60×10^{-7}
5	7.00×10^{9}	79.411765	2.60×10^{-6}
15	9.80×10^{8}	97.117647	6.80×10^{-5}
25	1.80×10^{8}	99.470588	5.00×10^{-5}
35	2.00×10^{6}	99.994118	6.60×10^{-4}
45	3.04×10^{5}	99.999106	---- [b]

[a], Resistance to rifampicin

[b], No growth on the plate

FIG. 11. Viable cell count and mutation frequency of DJO10A after MNNG mutagenesis.

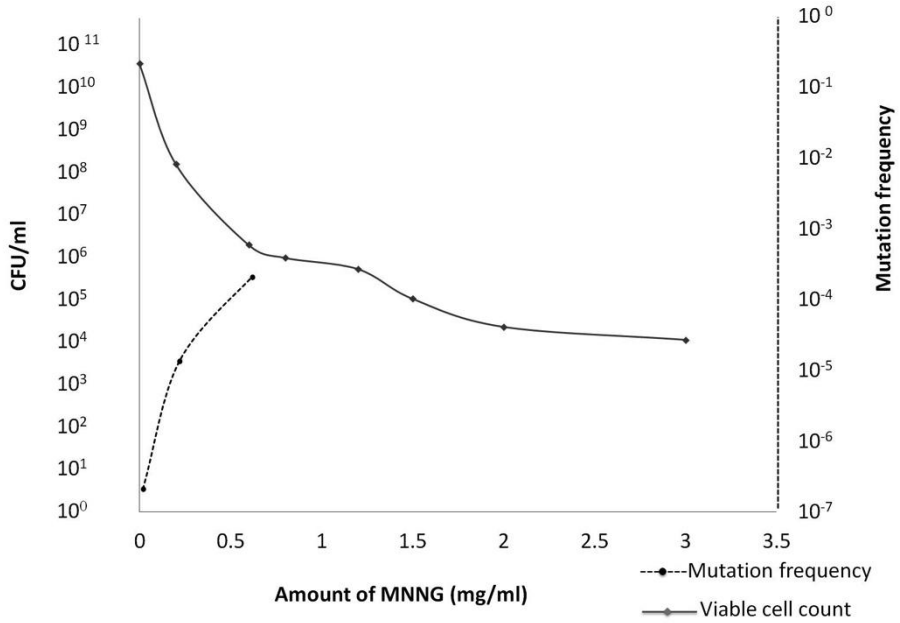


TABLE 4. Summary of MNNG mutagenesis

Mutagen amount (mg/ml)	Viable cells (CFU/ml)	Death rate (%)	Mutation frequency [a]
0	2.60×10^{10}	0	1.90×10^{-7}
0.2	1.20×10^{8}	99.953846	1.25×10^{-5}
0.6	1.60×10^{6}	99.999385	2.00×10^{-4}
0.8	8.00×10^{5}	99.999692	---- [b]
1.2	4.40×10^{5}	99.999831	---- [b]
1.5	9.00×10^{4}	99.999965	---- [b]
2.0	2.00×10^{4}	99.999992	---- [b]
3.0	1.00×10^{4}	99.999996	---- [b]

[a], Resistance to rifampicin

[b], No growth on the plate

A positive screening approach was initiated with 1.8×10^6 CFU of EMS mutated cells and 1.5×10^6 CFU of MNNG mutated cells co-cultured separately with 1.2×10^6 CFU of *B. longum* DJO10A-JH1. A potential *lanR1* mutant producing the lantibiotic will be dominant over *B. longum* DJO10A-JH1 in the co-culture, as *B. longum* DJO10A-JH1 lost the entire lantibiotic operon, including the immunity gene. To enrich for this potential mutant, each co-culture was 10% sub-inoculated eight successive times. In addition, a 10 ml aliquot of the culture was collected from each of the eight sub-cultures for chromosomal DNA isolation. Following standardization of the DNA amounts, PCR amplification targeting the *lanM* gene showed that the amount of *B. longum* DJO10A in the co-culture decreased during sub-inoculaitons, suggesting no *lanR1* mutant was present (Fig. 12). This was further confirmed by screening a total of 36 (20 colonies from the co-culture containing MNNG mutated cells and 16 colonies from the co-culture containing EMS mutated cells) isolated colonies from the final sub-inoculated co-culture with the *lanM* primer using PCR. Only 2 colonies from the co-culture containing EMS mutated cells were *B. longum* DJO10A and further analysis using the Bioscreen C assay indicated no lantibiotic production in broth. Using the negative screening approach, a total of 5.4×10^4 CFU of the mutated cells were screened and 33 potential mutants that were showing reduced growth of the indicator strain (*E. coli*) in the 96-well microtiter plates were analyzed using the Bioscreen C. All were negative using this screen, suggesting no *lanR1* mutant was present.

FIG. 12. Comparison of the standardized chromosomal DNA samples from the eight sub-cultures (A) and PCR amplification of each sample targeting the *lanM* gene (B). E1 to E8 and M1 to M8 represent the chromosomal DNA from the eight successive sub-cultures containing cells mutated with EMS and MNNG, respectively. M, 1 kb DNA marker; A, *B. longum* DJO10A; J, *B. longum* DJO10A-JH1.

Fig. 12A

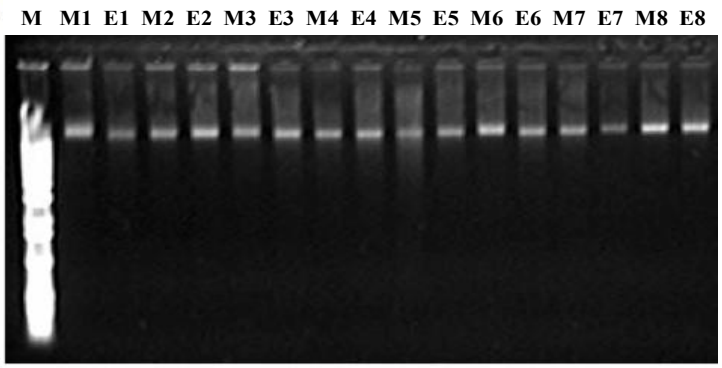
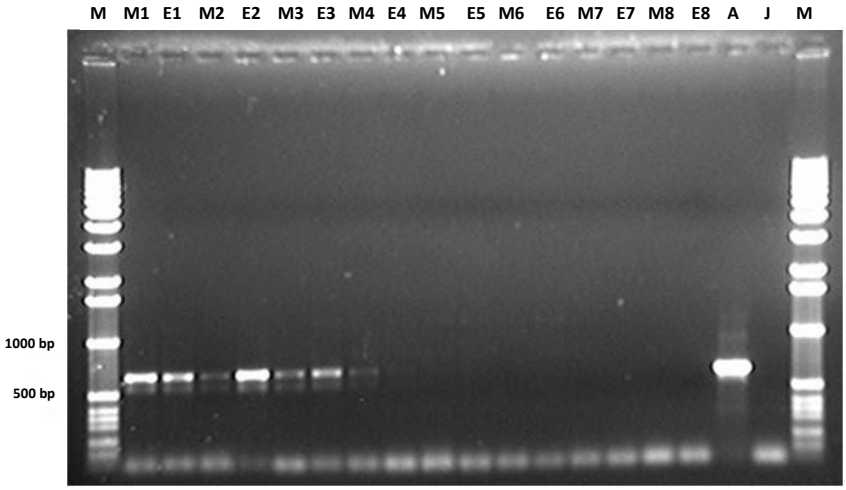


Fig. 12B



In vivo expression of *lanR1* in *E. coli*

The *lanR1* gene of *B. longum* DJO10A was previously cloned into a T7 expression system in *E. coli* by Ju-Hoon Lee, but no expression was detected in *E. coli.* Based on the codon differences between *E. coli* and this *B. longum* gene, it suggested that codon usage was preventing expression in *E. coli.* Therefore, a synthetic *lanR1* gene with an optimized *E. coli* codon usage was synthesized and cloned in pUC57 (432 to 1,088 bp) by GeneScript USA Inc (Piscataway, NJ) (Fig. 13). The *lanR1* synthetic gene was obtained from this vector by PCR using primers, lanR1-N-F forward 5'-GGTGGT**CATA TG**AAAGCGATTCTGTTCCGTG -3' (432 to 454 bp) and lanR1-S-R reverse 5'-GGTG GTT**GCTCTTCC**GCACAGTTCAATGTTCGTG -3' (1,073 to 1,088 bp) (restriction enzyme sites are shown in bold), introducing recognition sites for *Nde*I and *Sap*I, respectively.

Plasmid pTXB1 (NEB) was chosen as the expression vector for the synthetic *lanR1* gene, as it enabled fusion of the intein (modified *Mycobacterium xenopi gyrA* gene) -CBD (chitin binding domain) to the c-terminal of LanR1 to facilitate its purification with an affinity chitin-binding tag and subsequent cleavage of the intein when thiol agents, such as DTT (Dithiothreitol) are present. The PCR amplified *lanR1* fragment was digested with *Nde*I and *Sap*I and ligated into a similarly digested pTXB1 to construct pTXB1-lanR1 (Fig. 14). This resulted in fusing the T7 promoter directly upstream from the *lanR1*-intein-CBD gene (Fig. 15).

FIG. 13. Gene sequence of the codon-optimized *lanR1*.

Amino acid sequence of LanR1 from *Bifidobacterium longum* DJO10A

MKAILFRDCKACFQSFKNWGMVLIIMVTAIFVEMVASGAGILDPFTWAVFFGPFFIYSSCSVMVSILYQD
FRDGLFELYIQSGRSYWSYCWCKCLFPVILTVISVLLNLAFMRVLSSGFQITAGADDAIGALIVSVLGTIV
CSLGAMPMVYVSRNSDPTMAQLVLVLIAILLQLAYTLVVVNVMPLCLFSVGYVVLVVVAIGISTGVFSR
YFINTNIEL

Gene sequence of the codon-optimized *lanR1*

ATGAAAGCGATTCTGTTCCGTGATTGTAAAGCCTGTTTCCAGTCGTTCAAAAATTGGGGTATGGTGC
TGATTATTATGGTGACGGCAATTTTTGTTGAAATGGTCGCATCTGGCGCTGGCATTCTGGACCCGTT
CACCTGGGCAGTGTTTTTCGGTCCGTTTTTCATTTATAGCTCTTGTTCCGTCATGGTGTCAATCCTGT
ACCAGGATTTTCGTGACGGTCTGTTCGAACTGTATATTCAATCAGGCCGCTCGTATTGGAGCTACTG
CTGGTGTAAATGCCTGTTTCCGGTGATTCTGACGGTGATCTCAGTTCTGCTGAACCTGGCGTTTATG
CGTGTTCTGAGTTCCGGCTTCCAGATTACCGCAGGTGCAGATGACGCAATTGGTGCACTGATCGTTT
CGGTCCTGGGTACGATCGTGTGTAGCCTGGGCGCGATGCCGATGGTGTACGTTTCTCGCAACAGTG
ACCCGACGATGGCGCAGCTGGTCCTGGTGCTGATTGCAATCCTGCTGCAACTGGCTTACACCCTGG
TGGTGGTCAATGTCATGCCGCTGTGCCTGTTCTCCGTTGGCTATGTGGTTCTGGTCGTGGTTGCGAT
TGGCATTTCTACGGGCGTCTTCTCTCGCTATTTTATTAACACGAACATTGAACTG

FIG. 14. Construction of the LanR1 expression plasmid, pTXB1-lanR1.

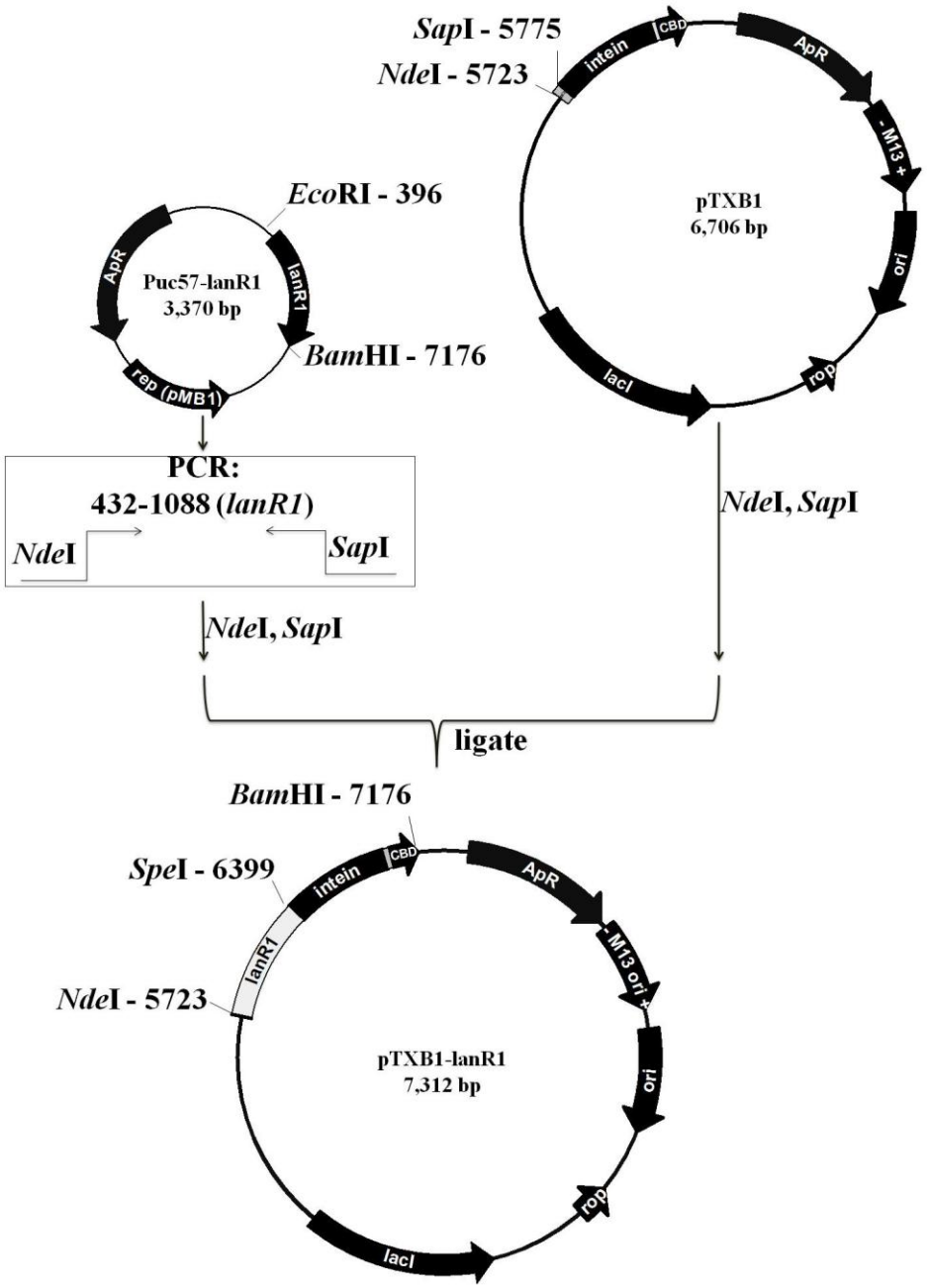
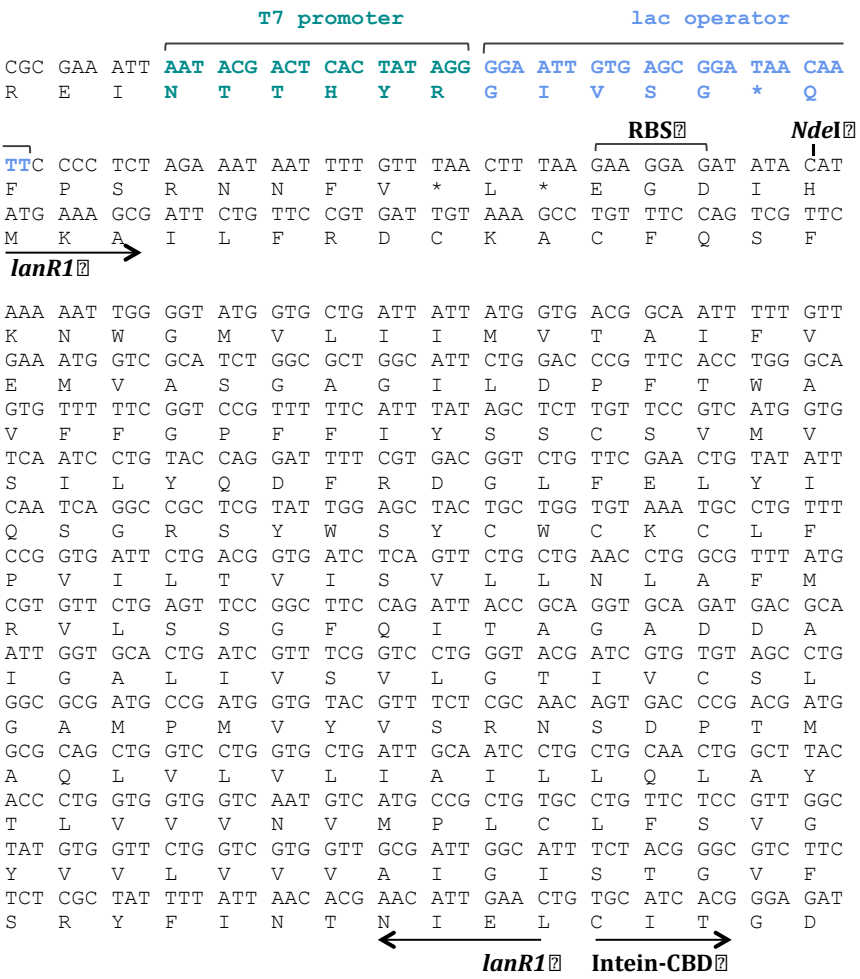

SapI - 5775
NdeI - 5723
intein
CBD
ApR
M13 +
pTXB1
6,706 bp
ori
rop
lacI
EcoRI - 396
ApR
Puc57-lanR1
3,370 bp
lanR1
BamHI - 7176
rep (pMB1)
PCR:
432-1088 (lanR1)
NdeI
SapI
NdeI, SapI
NdeI, SapI
ligate
BamHI - 7176
SpeI - 6399
intein
CBD
ApR
lanR1
M13 ori +
NdeI - 5723
pTXB1-lanR1
7,312 bp
ori
rop
lacI

FIG. 15. Sequence of the T7 promoter fused to *lanR1*-intein-CBD in pTXB1-lanR1.


T7 promoter
lac operator
RBS
NdeI
lanR1
lanR1
Intein-CBD

The fused *lanR1*-intein-CBD was expressed in *E. coli* using 0.4 mM IPTG for induction. *E. coli* ER2566(pMXB10), which encodes MBP (maltose binding protein) fused at the C terminal to intein-CBD, was used as a positive control. This ~ 71 kDa fusion protein was significantly overproduced after induction, demonstrating that the in vivo expression system was functional (Fig. 16). In addition, another band corresponding to the MBP (~ 43 kDa) was also observed in the same induced sample. However, there was no detectable difference between induced and un-induced cultures of *E. coli* ER2566 (pTXB1-lanR1) indicating either none or undetectable amounts of the LanR1 was produced.

In vitro expression of *lanR1*

The lack of *lanR1* expression in vivo may suggest that LanR1 might be toxic to the host. To investigate this possibility, a cell-free T7 expression system was used. As shown in Fig. 17, the control samples using the template DNA of DHFR and pMXB10, produced target bands demonstrating that the in vitro expression system was functional. A band corresponding to the MBP (~ 43 kDa) was also observed in the sample that was synthesized from pMXB10 and the amount is even more than the un-cleaved MBP-intein-CBD. Unfortunately, no band corresponding to LanR1 was observed.

FIG. 16. SDS-PAGE analysis of in vivo expression of MBP-intein-CBD and LanR1-intein-CBD in *E. coli* ER2566. Lane 1, *E. coli* ER2566(pMXB10) induced with 0.4 mM IPTG; lane 2, *E. coli* ER2566(pMXB10) un-induced; lane 3, *E. coli* ER2566(pTXB1-lanR1) induced with 0.4 mM IPTG; lane 4, *E. coli* ER2566(pTXB1-lanR1) un-induced; M, protein molecular weight marker. Arrows indicate the positions of MBP (~ 43 kDa) and MBP-intein-CBD (~ 71 kDa).

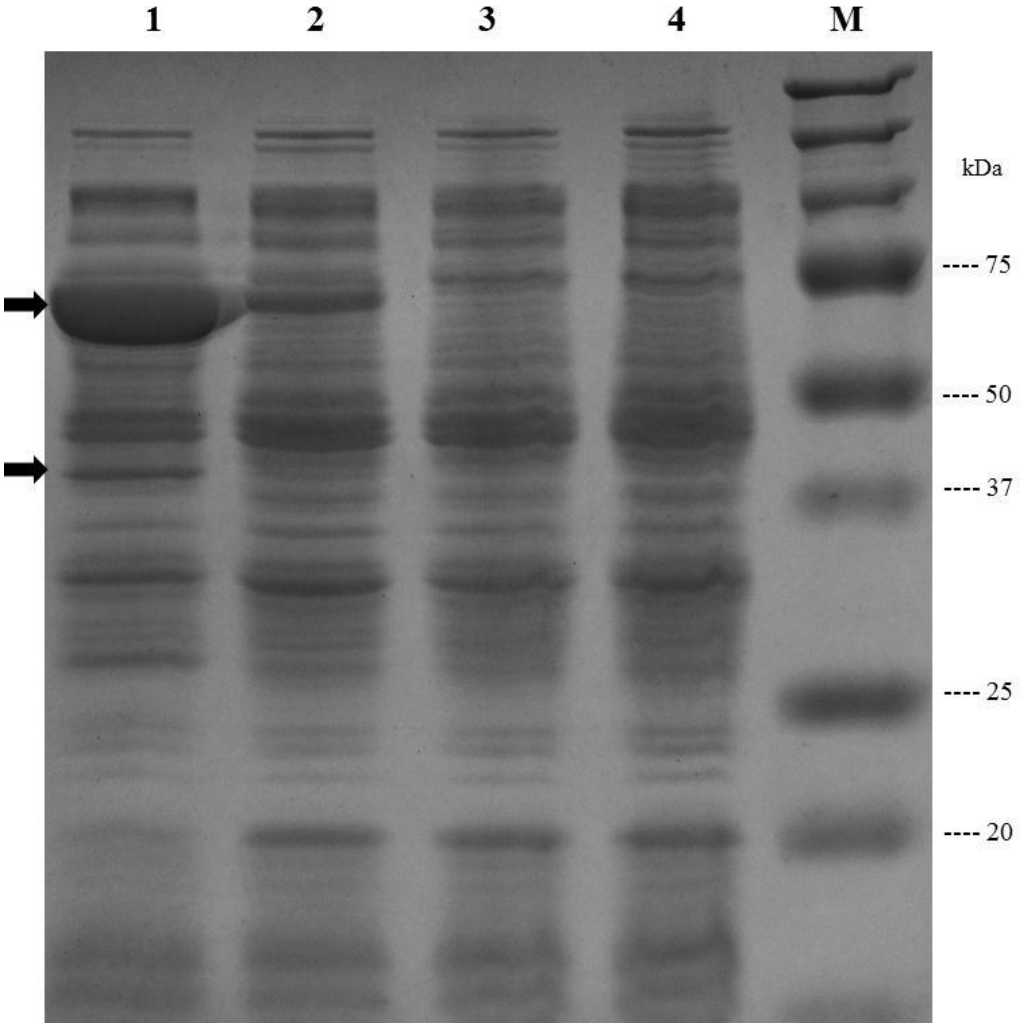
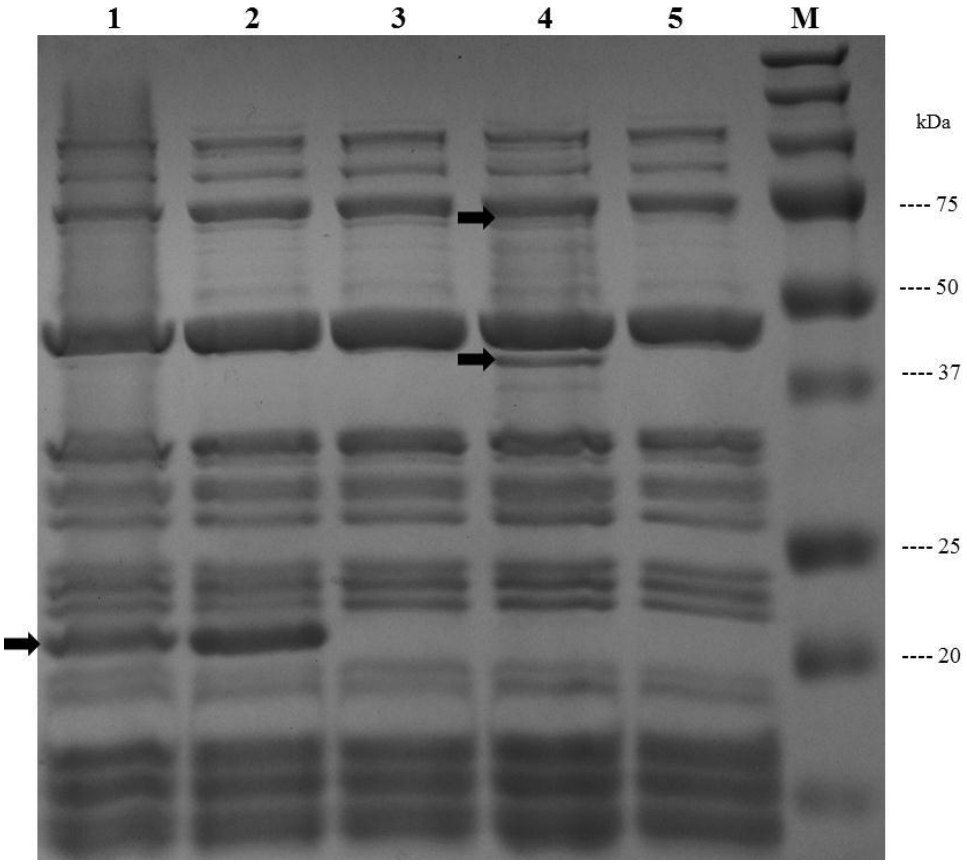

1
2
3
4
M
kDa
---- 75
---- 50
---- 37
---- 25
---- 20

FIG. 17. SDS-PAGE analysis of in vitro expression of MBP, MBP-intein-CBD, DHFR and LanR1-intein-CBD. Lane 1, pTXB1-lanR1 and DHFR plasmids; lane 2, DHFR plasmid; lane 3, pTXB1-lanR1 plasmid; lane 4, pMXB10 plasmid; lane 5, no template DNA; M, protein molecular weight marker. Arrows indicate the positions of DHFR (~ 18 kDa), MBP (~ 43 kDa) and MBP-intein-CBD (~ 71 kDa).


1
2
3
4
5
M
kDa
---- 75
---- 50
---- 37
---- 25
---- 20

Evaluation of possible LanR1 repression of the T7 promoter

Given that LanR1 is proposed to be a transcription repressor, it is conceivable that it may be able to bind lac operator. To evaluate this possibility, an in vitro expression of the DHFR and MBP positive control plasmids together with the pTXB1-lanR1 plasmid was conducted. As shown in Fig. 18, both were expressed with no obvious inhibition substantiating that LanR1 is not repressing the T7 expression system.

In vivo expression of a translational coupled MBP-*lanR1* in *E. coli*

The lack of LanR1 expression may suggest inefficient translation initiation of *lanR1* mRNA. To investigate this possibility, a translational coupling plasmid coupling the MBP and *lanR1* was constructed. The TAA-RBS (ribosomal binding site)- ATATAC AT-*lanR1* (region 5,707 to 6,423) was obtained from the vector pTXB1-lanR1 by PCR using primers, lanR1-E-F forward 5'-GGTGGT**GAATTC**TAAGAAGGAGATATACA TATG -3' (positions 5,707 to 5,727) and lanR1-Sp-R reverse 5'-CTCGCCCTCGGGTA GGGCA**ACTAGT** -3' (positions 6,399 to 6,423) (restriction enzyme sites are shown in bold, and the MBP stop codon is underlined), introducing a recognition site for *Eco*RI. The PCR amplified *lanR1* fragment was then digested with *Eco*RI and *Spe*I and ligated into a similarly digested pMXB10 to construct pMXB10-lanR1 (Fig. 19). This resulted in coupling the MBP directly upstream from the *lanR1*-intein-CBD gene with a stop codon and RBS (ribosome-binding site) in between (Fig. 20).

FIG. 18. SDS-PAGE analysis of in vitro expression of the mixed plasmids. Lane 1, pTXB1-lanR1, pMXB10 and DHFR plasmids; lane 2, pTXB1-lanR1 and pMXB10 plasmids; lane 3, pTXB1-lanR1 and DHFR plasmids; lane 4, DHFR plasmid; lane 5, no template DNA; lane 6, pMXB10 plasmid; M, protein molecular weight marker. Arrows indicate the positions of DHFR (~ 18 kDa), MBP (~ 43 kDa) and MBP-intein-CBD (~ 71 kDa).

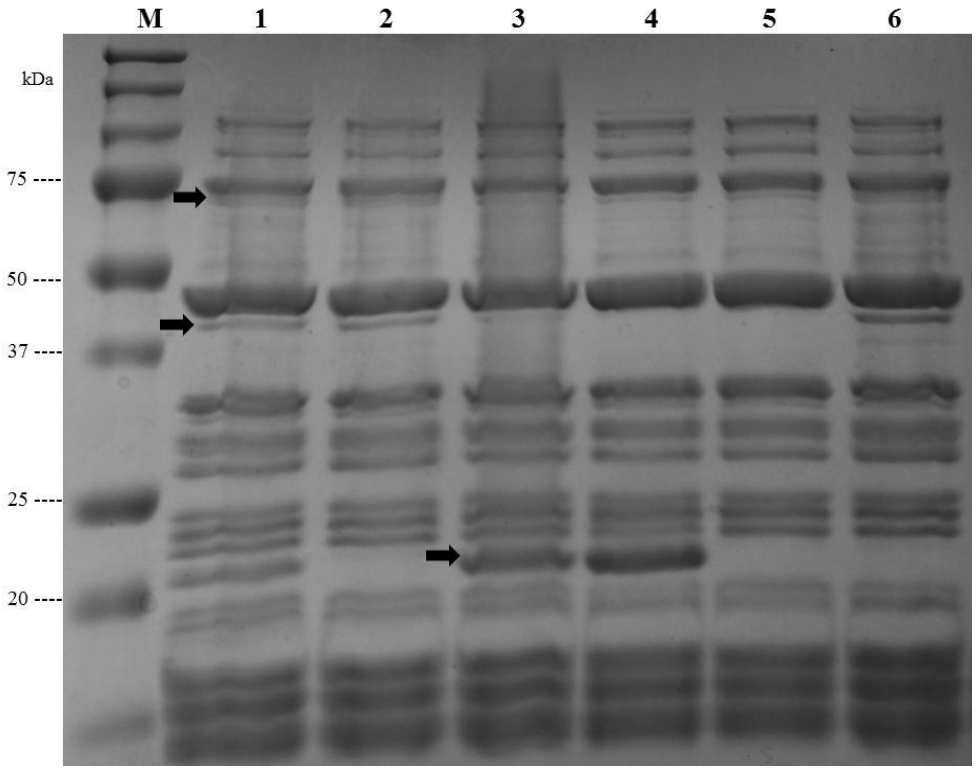

M
1
2
3
4
5
6
kDa
75 ----
50 ----
37 ----
25 ----
20 ----

FIG. 19. Construction of the translation coupled LanR1 expression plasmid, pMXB10-lanR1. The MBP stop codon is underlined and the ribosome-binding site (RBS) for *lanRI* (GAAGGAG) is underlined with dashes.

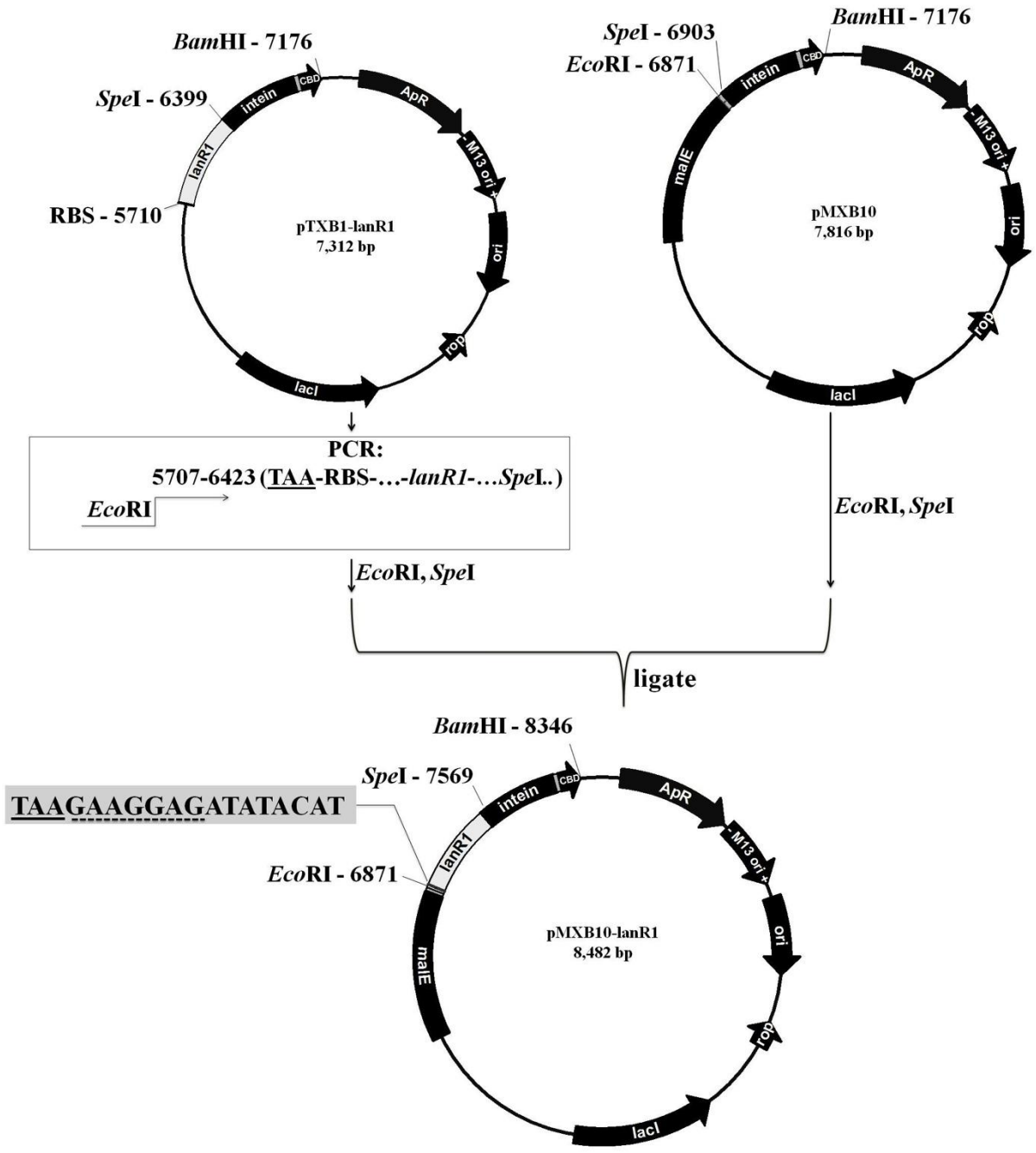
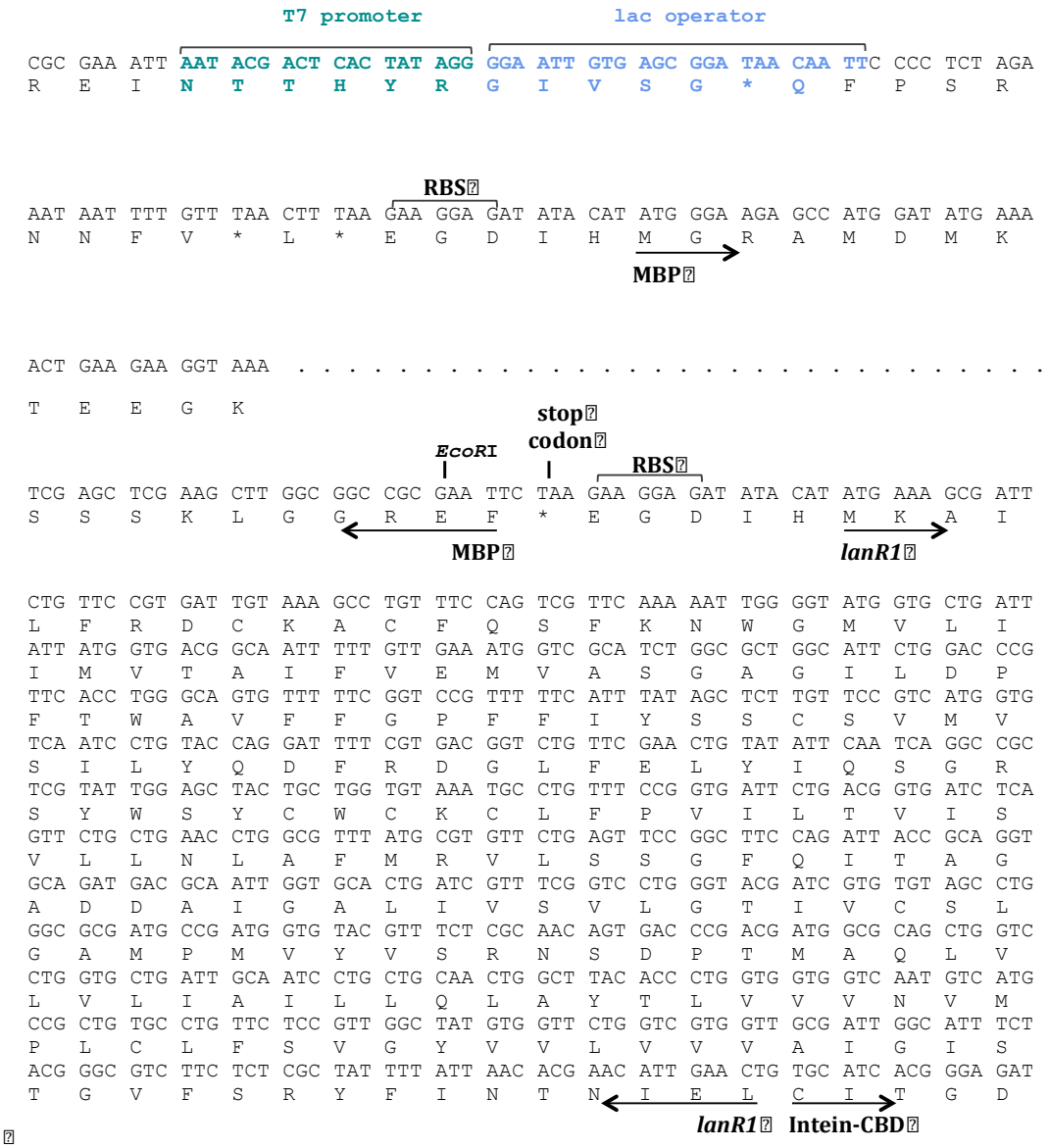

BamHI - 7176
SpeI - 6399
intein
CBD
ApR
M13 ori
lanR1
RBS - 5710
pTXB1-lanR1
7,312 bp
ori
rop
lacI
SpeI - 6903
EcoRI - 6871
BamHI - 7176
intein
CBD
ApR
malE
M13 ori
pMXB10
7,816 bp
ori
rop
lacI
PCR:
5707-6423 (TAA-RBS-…-lanR1-…SpeI..)
EcoRI
EcoRI, SpeI
EcoRI, SpeI
ligate
BamHI - 8346
SpeI - 7569
TAAGAAGGAGATATACAT
intein
CBD
ApR
M13 ori
lanR1
EcoRI - 6871
pMXB10-lanR1
8,482 bp
malE
ori
rop
lacI

FIG. 20. Sequence of the MBP coupled to *lanR1*-intein-CBD in pMXB10-lanR1. MCS represents multiple cloning sites.


T7 promoter
lac operator
CGC GAA ATT AAT ACG ACT CAC TAT AGG GGA ATT GTG AGC GGA TAA CAA TTC CCC TCT AGA
R E I N T T H Y R G I V S G * Q F P S R
RBS
AAT AAT TTT GTT TAA CTT TAA GAA GGA GAT ATA CAT ATG GGA AGA GCC ATG GAT ATG AAA
N N F V * L * E G D I H M G R A M D M K
MBP
ACT GAA GAA GGT AAA . . . . . .
T E E G K
stop codon
EcoRI
RBS
TCG AGC TCG AAG CTT GGC GGC CGC GAA TTC TAA GAA GGA GAT ATA CAT ATG AAA GCG ATT
S S S K L G G R E F * E G D I H M K A I
MBP
lanR1
CTG TTC CGT GAT TGT AAA GCC TGT TTC CAG TCG TTC AAA AAT TGG GGT ATG GTG CTG ATT
L F R D C K A C F Q S F K N W G M V L I
ATT ATG GTG ACG GCA ATT TTT GTT GAA ATG GTC GCA TCT GGC GCT GGC ATT CTG GAC CCG
I M V T A I F V E M V A S G A G I L D P
TTC ACC TGG GCA GTG TTT TTC GGT CCG TTT TTC ATT TAT AGC TCT TGT TCC GTC ATG GTG
F T W A V F F G P F F I Y S S C S V M V
TCA ATC CTG TAC CAG GAT TTT CGT GAC GGT CTG TTC GAA CTG TAT ATT CAA TCA GGC CGC
S I L Y Q D F R D G L F E L Y I Q S G R
TCG TAT TGG AGC TAC TGC TGG TGT AAA TGC CTG TTT CCG GTG ATT CTG ACG GTG ATC TCA
S Y W S Y C W C K C L F P V I L T V I S
GTT CTG CTG AAC CTG GCG TTT ATG CGT GTT CTG AGT TCC GGC TTC CAG ATT ACC GCA GGT
V L L N L A F M R V L S S G F Q I T A G
GCA GAT GAC GCA ATT GGT GCA CTG ATC GTT TCG GTC CTG GGT ACG ATC GTG TGT AGC CTG
A D D A I G A L I V S V L G T I V C S L
GGC GCG ATG CCG ATG GTG TAC GTT TCT CGC AAC AGT GAC CCG ACG ATG GCG CAG CTG GTC
G A M P M V Y V S R N S D P T M A Q L V
CTG GTG CTG ATT GCA ATC CTG CTG CAA CTG GCT TAC ACC CTG GTG GTG GTC AAT GTC ATG
L V L I A I L L Q L A Y T L V V V N V M
CCG CTG TGC CTG TTC TCC GTT GGC TAT GTG GTT CTG GTC GTG GTT GCG ATT GGC ATT TCT
P L C L F S V G Y V V L V V V A I G I S
ACG GGC GTC TTC TCT CGC TAT TTT ATT AAC ACG AAC ATT GAA CTG TGC ATC ACG GGA GAT
T G V F S R Y F I N T N I E L C I T G D
lanR1
Intein-CBD

The translation coupled *lanR1* gene in pMXB10-lanRI was expressed in *E. coli* using 0.4 mM IPTG for induction. Surprisingly, the SDS-PAGE analysis showed that the amount of the expressed MBP from *E. coli* ER2566(pMXB10-lanR1) was dramatically lower than the MBP-intein-CBD from *E. coli* ER2566(pMXB10) (Fig. 21). This suggested that LanR1 may have been expressed, but was toxic to the cell resulting in loss of the plasmid. Subsequent viable plate counts on LB and LB Amp plates after 4.0 hours of induction showed that all cells remained viable (1.3×10^8 cfu/ml), but ~ 91% had lost the plasmid, even though ampicillin (100 μg/ml) was present (Fig. 22). This loss of plasmid appeared to occur immediately following induction, as the viable cell counts on LB Amp plates remained constant through the induction period. This plasmid loss was not observed for the MBP-intein-CBD expression plasmid, pMXB10.

A microscopic examination of the *E. coli* ER2566(pMXB10-lanR1) and *E. coli* ER2566(pMXB10) cells after 4 h induction revealed elongation of cells overproducing the proteins (Fig. 23 A1 and B1). Such an effect was not observed in the un-induced control culture of *E. coli* ER2566(pMXB10-lanR1) (Fig. 23 A2). There are a few in the un-induced culture of *E. coli* ER2566(pMXB10) (Fig 23 B2), which is consistent with its leaky expression in vivo (Fig. 21). This suggested the elongated cells are responsible for the expression of the proteins, which is consistent with the small amount of coupled MBP that was expressed by *E. coli* ER2566(pMXB10-lanR1) after induction (Fig. 21). The loss of the pMXB10-lanR1 after induction in vivo suggested the coupled MBP and LanR1 should be expressed if a cell free system was used.

FIG. 21. SDS-PAGE analysis of in vivo expression of MBP, MBP-intein-CBD and translation coupled LanR1 in *E. coli* ER2566. Lane 1, *E. coli* ER2566(pMXB10-lanR1) un-induced; lane 2, *E. coli* ER2566(pMXB10-lanR1) induced with 0.4 mM IPTG; lane 3, *E. coli* ER2566(pMXB10) induced with 0.4 mM IPTG; lane 4, ER2566(pMXB10) un-induced. M, protein molecular weight marker. Arrows indicate the positions of MBP (~ 43 kDa) and MBP-intein-CBD (~ 71 kDa).

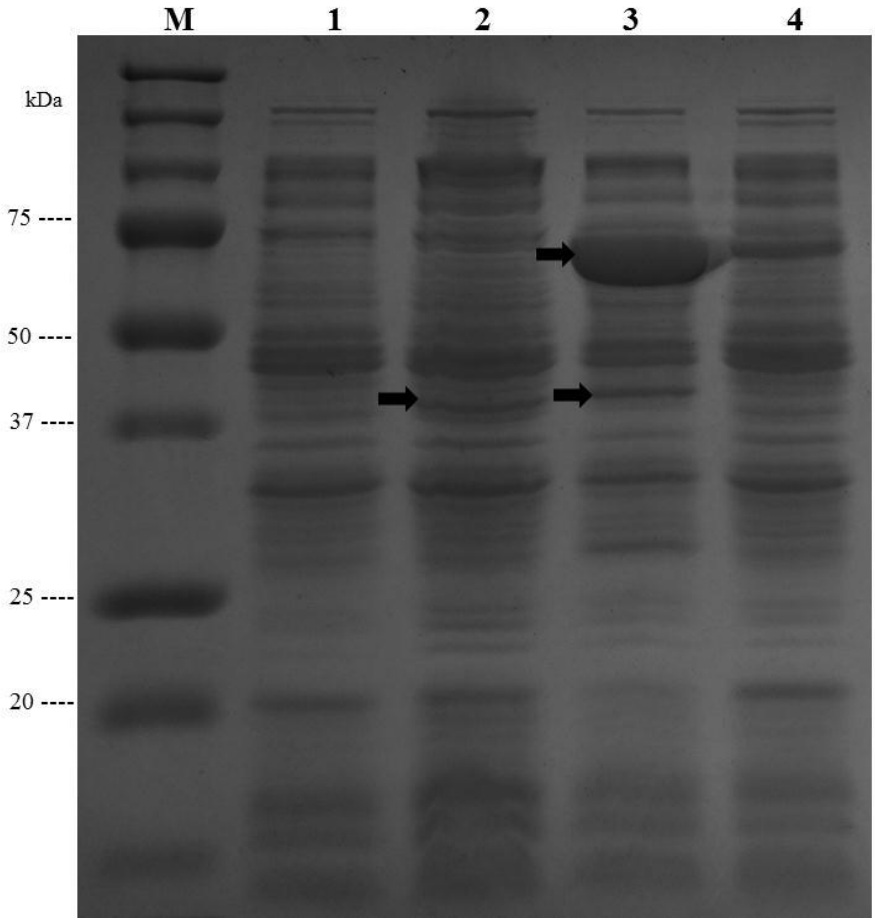

M
1
2
3
4
kDa
75 ----
50 ----
37 ----
25 ----
20 ----

FIG. 22. Viable cell counts of *E. coli* ER2566(pMXB10) and *E. coli* ER2566(pMXB10-lanR1) on LB Amp plates, before and after induction with 0.4 mM IPTG.

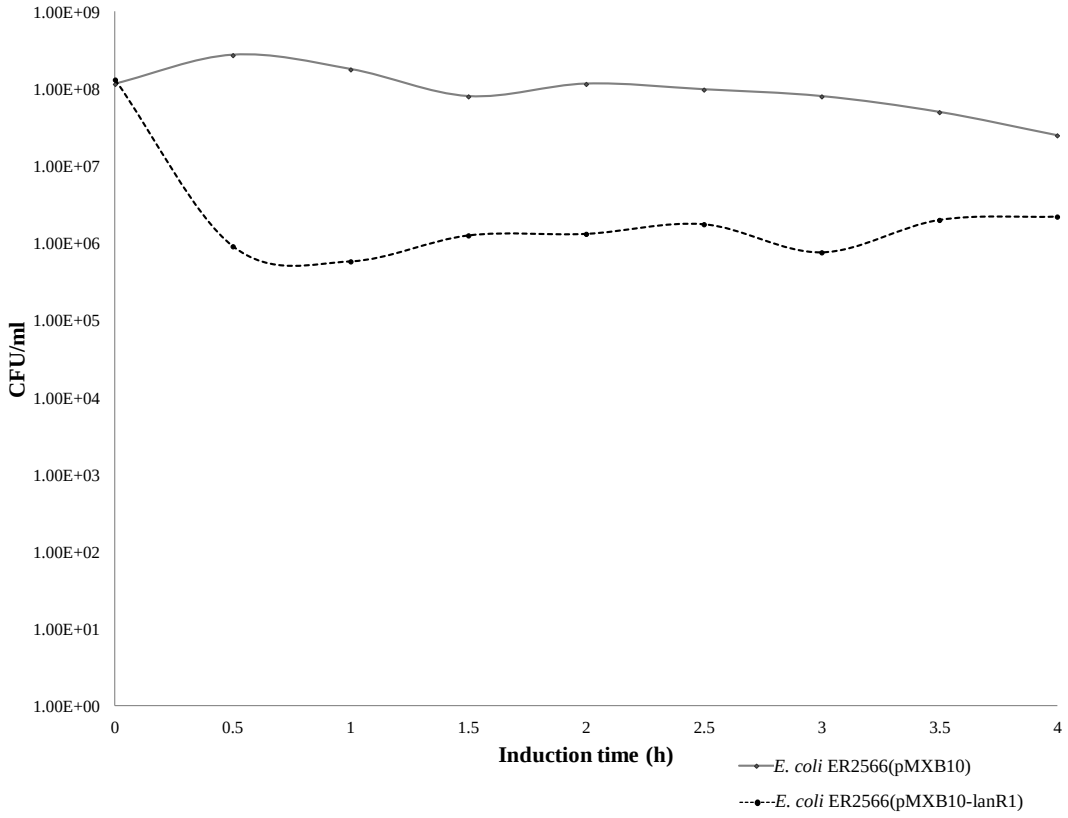
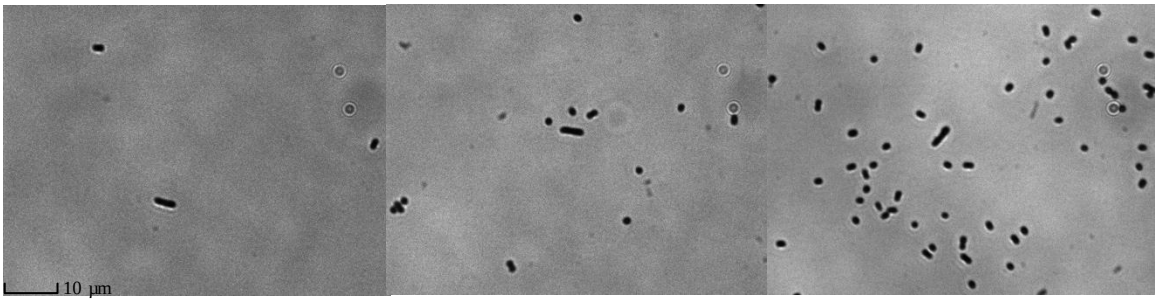
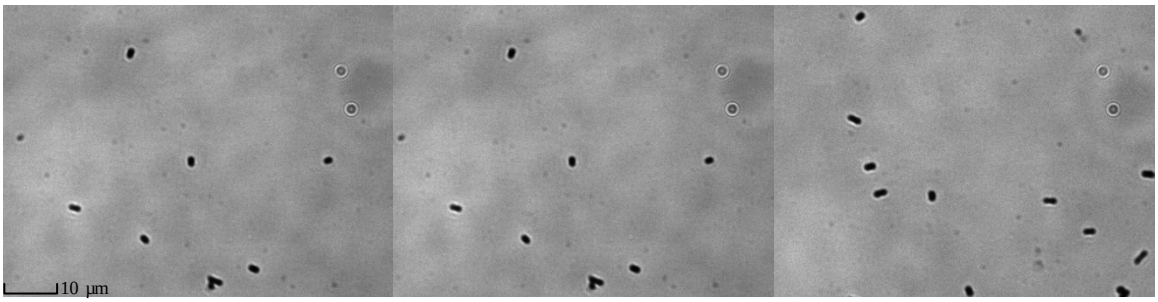

1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
CFU/ml
0
0.5
1
1.5
2
2.5
3
3.5
4
Induction time (h)
E. coli ER2566(pMXB10)
E. coli ER2566(pMXB10-lanR1)

FIG. 23. A1, morphology of *E. coli* ER2566(pMXB10-lanR1) cells after 4 h induction. A2, morphology of a control culture of *E. coli* ER2566(pMXB10-lanR1) after 4 h without induction. B1, morphology of *E. coli* ER2566(pMXB10) cells after 4 h induction. B2, morphology of a control culture of *E. coli* ER2566(pMXB10) after 4 h without induction.

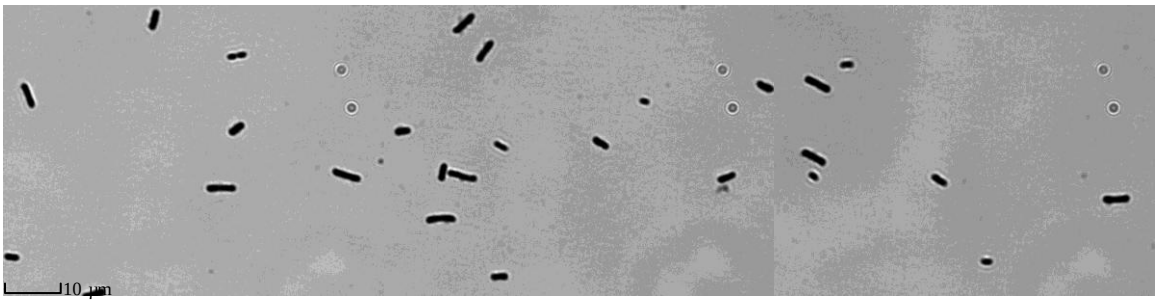
A1



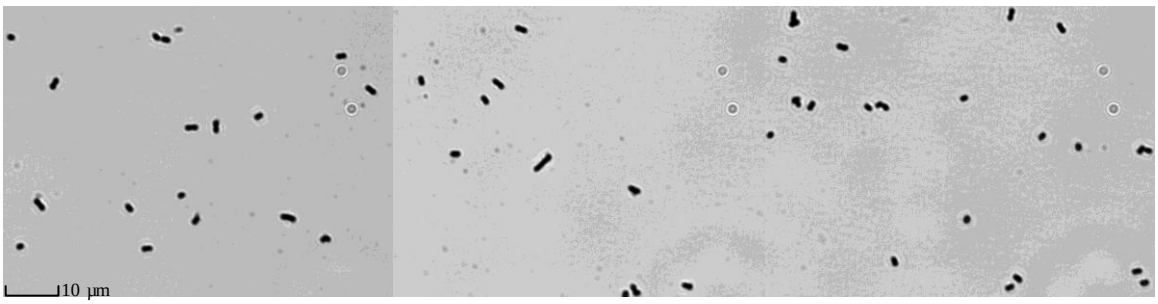
A2



B1



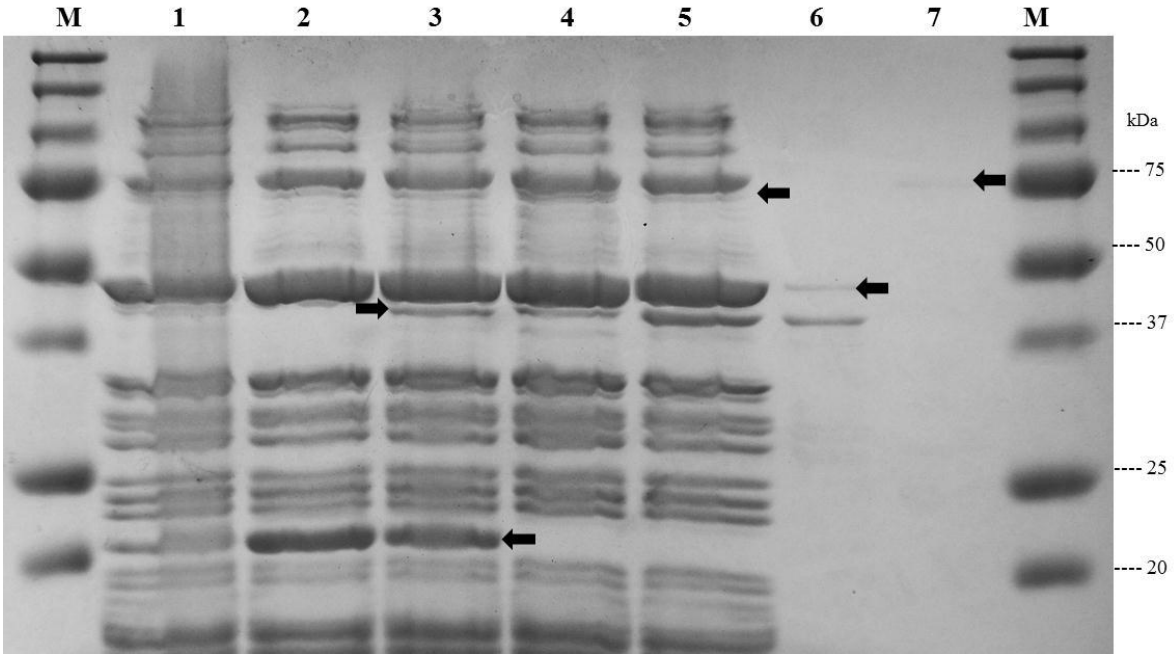
B2



In vitro expression of translational coupled *lanR1*

To overcome LanRI toxicity in *E. coli*, the proteins were expressed in vitro and SDS-PAGE analysis showed that the amount of the coupled MBP (~ 43 kDa) was not less than the MBP-intein- CBD (~ 71 kDa) (Fig. 24). This indicated the LanR1-intein-CBD should be expressed. However, the low level in vitro expression of LanR1-intein-CBD (~ 50 kDa) was not obvious as it would be masked by other *E. coli* proteins at this position on the gel. Therefore the LanR1-intein-CBD sample was concentrated using the chitin beads (NEB) that are designed to bind with proteins that have CBD (chitin binding domain) on their tail. As shown in Fig. 24, an MBP-intein-CBD band (~ 71 kDa) was observed from the control plasmid (pMXB10) substantiating the binding specificity of the beads. The LanRI-intein-CBD sample contained a predicted LanRI-intein-CBD protein band of ~ 50 kDa. Surprisingly, another band corresponding to the MBP (~ 43 kDa) was also present in the same lane, suggesting co-elution with the LanRI-intein-CBD protein.

FIG. 24. SDS-PAGE analysis of the in vitro expression of translation coupled LanR1. Lane 1, pMXB10 and DHFR plasmids; lane 2, DHFR plasmid; lane 3, pMXB10-lanR1 and DHFR plasmids; lane 4, pMXB10 plasmid; lane 5, pMXB10-lanR1 plasmid. Lane 6, 42 μl of the in vitro product of pMXB10-lanR1 that was bound to the chitin beads; lane 7, 17 μl of the in vitro product of pMXB10 that was bound to the chitin beads. M, protein molecular weight marker. Arrows indicate the positions of DHFR (~ 18 kDa), MBP (~ 43 kDa), MBP-intein-CBD (~ 71 kDa) and LanR1-intein-CBD (~ 50 kDa).


M
1
2
3
4
5
6
7
M
kDa
75
50
37
25
20

Discussion

The general organization of this lantibiotic gene cluster in *B. longum* DJO10A is similar to many other known lantibiotic gene clusters (Li and O'Sullivan, 2012), except it contains a second potential regulatory gene, *lanR1*, located just downstream from the *lanA* gene in this cluster. This organization is primarily found for the lantibiotics (such as cinnamycin, actagardine, deoxyactagardine) produced from *Actinobacteria*, and the mersacidin gene cluster in *Bacillus* sp. strain HIL Y-85,54728, where MrsR1 was determined to be the transcriptional activator required for *MrsA* transcription (Gudar et al., 2002). Interestingly, the *lanRK* system on the mersacidin gene cluster was not required for transcription of the lantibiotic production and modification genes, but rather the dedicated *lanFEG* immunity genes. Therefore, only this immunity cluster responded to signal transduction. This is not the case in the *B. longum* DJO10A lantibiotic gene cluster, as its *lanA* gene clearly responds to signal transduction from external crude lantibiotic preparation. However, it is unclear at this time if the inducer is the lantibiotic itself or another peptide. It was proposed that the additional predicted response regulator, LanR1, may be a repressor involved in down regulation of *lanA* transcription, given the long untranslated 5' region of the *lanA* mRNA (Lee et al., 2011). Two response regulators were also seen in the plantaricin bacteriocin gene cluster of *Lactobacillus plantarum* and while one was required for up-regulating transcription, the other was involved in down-regulating it (Diep et al., 2001), supporting this possible role for the *lanR1* gene in *B. longum* DJO10A.

The induction of *lanA* transcription in *B. longum* required a critical concentration of the external inducer (the crude lantibiotic preparation), as evidenced by the reduction in transcription when a culture was sub-inoculated at < 10% (Fig. 5). An analogous response was also seen for production of the bacteriocin piscicolin 126 by *Carnobacterium maltaromaticum*, where by sub-culturing cultures at 1% resulted in loss of bacteriocin production, but it could be restored by adding external bacteriocin to the culture (Gursky, 2006). This also indicated that in the case of piscicolin 126, the LanRK system was being expressed in broth even in the absence of external signal. This is also analogous to *B. longum* DJO10A, where a microarray analysis conducted by Ju-Hoon Lee demonstrated that the regulatory genes were being expressed in broth in the absence of external inducer, confirming that it was feasible to achieve signal transduction of this system in broth cultures (Lee et al., 2011). The induction was also in a dose-dependent fashion which is similar to many other *lanA* genes in other lantibiotic gene clusters, such as *salA* encoding salivaricin A and *nisA* encoding nisin (Kuipers et al., 1995; Upton et al., 2001).

Kinetics of *lanA* expression during growth in broth (following induction) revealed that this gene is highly expressed during log phase but decreases significantly in stationary phase, which is indicative of growth phase-dependent expression. This is analogous to subtilin production from *Bacillus subtilis*, whereby subtilin production decreased during stationary phase (Gutowski-Eckel et al., 1994) as well as expression of

its *lanA* gene (*spaB*), as measured using a *spaB* promoter fused to a *lacZ* reporter gene (Stein et al., 2002).

Induction of the *lanA* gene via signal transduction was likely initiated by interaction of the lantibiotic with LanK on the cell surface, given that the whole cell MALDI-TOF analysis on the serial 10% sub-inoculated culture revealed a peak (~ 3.2 kDa) corresponding to the predicted size for bisin. Such a mass spectrometry tool has been proposed as a rapid method for identification of bacteriocin /lantibiotic-producing bacteria (Stein, 2008). Furthermore, removal of the peptide from the cell surface using heat or organic solvents demonstrated the peptide was attached to the cell rather than an inherent component of the cell. Interestingly, another small peptide (~ 2.5 kDa) was also present on the cell surface that also was removed by heat and organic solvents. As the proteose peptone in the media does not contain peptides (Difco & BBL Manual, second edition, 2009), it is likely that the peptide (or other low MW compound) is produced from *B. longum* DJO10A. The only protein predicted to be excreted from the *B. longum* DJO10A curated proteins in the range of 2.5 kDa to 11 kDa is BLD_0138, a hemolysin-like peptide (11.2 kDa). However, analysis of this protein using SignalP 4.1 (Petersen et al., 2011) indicates it does not encode a signal sequence, eliminating it as a contender. It may therefore be a hypothetical protein or another small molecule that is post translationally synthesized. Heating was very efficient at removing the ~ 3.2 kDa peptide from the cell surface, with little extraction of the ~ 2.5 kDa peptide. Both peptides were very efficiently removed by methanol compared to butanol, suggesting both peptides are

more hydrophilic rather than hydrophobic. The ~ 3.2 kDa peak was shifted + 3 Da, - 1 Da and - 2 Da in the extracts from heating, butanol and methonal extraction, respectively. This indicates the level of protonation of the peptide, with different extraction procedures causing different levels of protonation (Hewavitharana et al., 2010). In general, the more denaturation of the peptide following extraction, the more access of basic residues, causes greater deprotonation (Lin and Dass, 2001). Interestingly, butanol extraction showed a -192 Da shift of the ~ 2.5 kDa peak, which cannot be explained by simple deprotonation. Butanol extraction was not shown to cause large MS shifts in peptides in other studies (Stein et al, 2002; Oscariz et al, 1999).

The attempts to mutate the potential repressor gene *lanR1* (or its predicted binding site upstream from *lanRI*) revealed no *lanR1* mutant out of ~ 3.4×10^6 CFU cells screened, indicating the low sensitivity of these screening procedures, which were developed based on the hypothesis that a *lanR1* mutant would produce the lantibiotic in broth. While the screen used in this study relied on visual observation of microbial growth in a microtiter plate and thus may be subject to human error, it may also suggest that production of the lantibiotic in broth may require more than just *lanR1* inactivation. Recent technological developments in bifidobacteria have now rendered targeted gene knockout a possibility in the near future. The conjugative gene transfer system developed by Wilfredo Dominguez in this lab showed efficient and reproducible gene transfer from *E. coli* to all *Bifidobacterium* species, including *B. longum* DJO10A (Dominguez and O'Sullivan, 2012). In addition, a homologous recombination approach using the

temperature-sensitive (Ts) plasmid constructed from the *Bifidobacterium-E. coli* shuttle vector pKKT427 showed efficient target gene knockout (Sakaguchi et al, 2012).

To purify the LanRI protein, the *lanR1* gene was expressed using a T7 expression system. However, in vivo expression of the T7-*lanRI* plasmids (pTXB1-lanR1 and pMXB10-lanR1) displayed toxicity for *E. coli*. Other regulatory proteins, such as the RegA repressors, have also displayed toxicity to *E. coli*, but could be expressed in vitro (Allen and Miller, 1999). This also suggested production of LanR1 should be feasible in vitro. The in vivo expression also revealed production of the MBP-intein-CBD, even un-induced with IPTG, indicating the T7 expression system could be leaky. An analogous leaky expression was also found when expressing SMAP using a similar T7 expression vector system in *E. coli* ER2566, which is the same *E. coli* strain used in our study (Morassutti et al., 2005).

The initial failure to detect LanR1 production in vitro, suggested that repression of the T7 expression system might have occurred. It was conceivable that LanRI, which is a predicted DNA binding protein, may be binding the T7 promoter or the lac operator, thus blocking expression. However, the in vitro expression of the DHFR and MBP control plasmids that were mixed with the pTXB1-lanR1 plasmid, revealed no obvious inhibition. The little variations among the protein products are due to the thiol agent (DTT) of the SDS buffer and in vitro reaction buffers, which can cleave the intein (Chong et al, 1997; Evans et al, 1998), and potential inhibitors (salts) in the plasmid templates.

Given there was no LanRI repression of the T7 expression system occurring, a translational coupling of *lanR1* to MBP was constructed in an attempt to improve LanRI expression. Translational coupling has been shown to enhance translation initiation and in the case of gene VII of the *E. coli* f1 phage was shown to be essential for its translation (Ivey-Hoyle and A. Steege, 1989). Expression of this coupled LanR1 in vivo showed a decrease of MBP production due to the loss of the plasmids, demonstrating toxicity of LanR1 to the host cell. Moreover, the cells that lost the plasmids were still alive after 4 h induction, even with ampicillin present in the media, suggesting the cells were not growing because ampicillin doesn't kill cells when they are not dividing (Roostalu et al, 2008). A microscopy examination of the sample overproducing MBP-intein-CBD showed elongation of the cells consistence with the production of the protein. This elongation has also been seen in the production of the fusion protein consisting of MBP and R-PE alpha apo-subunit after induction (Isailovic et al, 2006). Only a few cells of *E. coli* ER2566(pMXB10-lanR1) showed elongation after induction consistence with the number of cells that contained the plasmid, which suggested the elongation of the cells corresponded to protein production. To avoid the toxicity, the expression of the coupled LanR1 was conducted in vitro, but did not produce enough of LanR1 that could be seen on an SDS PAGE of total cell proteins.

The inefficient expression of the LanRI protein in vitro may have resulted in the protein being masked by other *E. coli* proteins. This was demonstrated by purification of the coupled LanR1-intein-CBD produced in vitro, which showed a protein band of the

expected size. Surprisingly, there was co-elution of another protein, corresponding to the MBP. It is less likely that MBP bound the chitin beads, as a cell extract containing MBP and MBP-intein (*Mth*)-CBD passed through the chitin beads and only the fusion protein bound to the beads (Evans et al, 1999). The interaction between MBP and LanR1 is more possible, given that MBP was shown to bind the Tar protein of *E. coli* when maltose is present (Zhang et al, 1999).

As this *lanA* promoter is significantly down regulated during growth in broth cultures, understanding its regulatory mechanisms provides the first step to achieving optimal production in broth cultures. Purification of the LanR1 protein will now facilitate the investigation of its role in expression of *lanR1*. Understanding this regulatory mechanism should facilitate strategies to be developed to enable production of this novel and potentially very broad spectrum lantibiotic in broth cultures to enable its further characterization as a novel antimicrobial agent.

Chapter III

Overall conclusions

Overall conclusions

Lantibiotics are prominent candidates for antimicrobial food preservatives and biomedical applications, due to their broad-spectrum range of inhibition against Gram-positive bacteria (including multi-drug resistant). Bisin represents the first lantibiotic reported from bifidobacteria (Lee et al., 2008). Moreover, it exhibited antimicrobial activity against some Gram-negative bacteria, including *Enterobacteriaceae* (O'Sullivan and Lee 2011). The production of this lantibiotic could only be detected on agar media, which hindered characterization of this lantibiotic. Understanding its transcription regulation can provide novel insights into how this lantibiotic is produced by bifidobacteria. This thesis focused on understanding the transcription analysis of the structural gene of bisin and heterologous expression of the extra regulator gene, *lanR1*.

An analogous lantibiotic, streptin from *Streptococcus pyogenes*, revealed a similar trend of production only on a solid surface, such as agar. The purification and characterization of this lantibiotic was achieved using a biphasic culture system, where by they hypothesized that the lantibiotic is more concentrated on agar, but too dilute in broth cultures (Wescombe and Tagg 2003).

Apart from this study, there is no study on regulation of bisin transcription. This study provides the first experimental evidence that the two component regulation system is functioning in the broth culture, and the signal molecule for bisin transcription is most likely the lantibiotic itself, due to the ~ 3.2 kDa peak present on the cell surface.

Moreover, turning on the system did not produce the lantibiotic in broth, indicating *lanR1* might be a repressor. The heterologous expression of LanR1 in this study would enable the investigation of the above hypothesis.

Purified LanR1 could be achieved by cleaving intein using thiol agents, which could be further used in electrophoretic mobility shift assays with the potential DNA binding region upstream of *lanA* (Lee et al., 2011). The exact binding sites could be mapped using DNA fingerprinting.

If *lanR1* is a repressor that is suppressing the expression of the lantibiotic gene cluster in broth cultures, exclusion of this gene should be considered in order to maximize the production. A target gene knockout approach that was discussed above could be applied in its original strain, *B. longum* DJO10A. Alternatively, a model plasmid containing the lantibiotic gene cluster except *lanR1* could be constructed and expressed in other bacteria, such as *Lactococcus lactis*, which are easy to work with.

To maximize the production of the lantibiotic in a broth culture, understanding the mechanism of the transcription regulation of its structural gene, and purification of the second regulator LanR1, play important roles in furthering this goal.

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