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Overproduction of Wild-Type and Bioengineered Derivatives of the Lantibiotic Lacticin 3147

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Lacticin 3147 is a broad-spectrum two-peptide lantibiotic whose genetic determinants are located on two divergent operons on the lactococcal plasmid pMRC01. Here we introduce each of 14 subclones, containing different combinations of lacticin 3147 genes, into MG1363 (pMRC01) and determine that a number of them can facilitate overproduction of the lantibiotic. Based on these studies it is apparent that while the provision of additional copies of genes encoding the biosynthetic/production machinery and the regulator LtnR is a requirement for high-level overproduction, the presence of additional copies of the structural genes (i.e., *ltnA1A2*) is not.

Lantibiotics are posttranslationally modified antimicrobial peptides produced by gram-positive bacteria (5, 9). The enormous chemotherapeutic potential of these peptides stems from the fact that a number of lantibiotics have been found to be active at nanomolar concentrations (3, 23), can inhibit multidrug-resistant pathogens (4, 13, 18), and are among a rare group of antibacterial compounds that target the lipid II component of the bacterial cell wall (2, 3). However, the study and application of lantibiotics are often compromised by limited production of these peptides by the native producer. This problem is particularly notable when working with bioengineered derivatives (8). While the optimization of fermentation processes is one option, genetic strategies offer an alternative approach which has already proved successful in the case of nisin and subtilin (6, 15). These studies demonstrated that the provision of additional copies of the regulatory (*RK*) or immunity [(*I*)*FEG*] genes resulted in 1.5- to 1.7-fold-greater yields of the relevant lantibiotic (6, 15). Furthermore, deletion of the *Bacillus subtilis* general regulator AbrB brought about an extraordinary sixfold enhancement in subtilin yields, although unfortunately this was in a less active, succinylated form (15). Here we describe a genetic system that facilitates significant overproduction of both wild-type and bioengineered derivatives of the broad-spectrum, two-peptide lantibiotic lacticin 3147 through the provision of additional copies of the genes responsible for production of, and immunity to, this antimicrobial. Through the use of 14 different subclones we also established that although the provision of additional copies of the biosynthetic/transport genes and of the regulator LtnR was required for high-level overproduction, the presence of additional copies of the two structural genes (*ltnA1A2*) was not.

Creation of constructs to facilitate lacticin 3147 overproduction. The genes responsible for production of and immunity to lacticin 3147 span a 12.6-kb region on the 60.2-kb plasmid pMRC01, originally identified in *Lactococcus lactis* DPC3147 (Fig. 1) (28). These genes are present on two operons, i.e., *ltnRIFE*—an immunity operon including genes for a regulator (*ltnR*), an immunity protein (*ltnI*), and an ABC transporter (*ltnFE*)—and *ltnA1A2M1TM2J*—a biosynthetic operon with genes for the two structural peptides (*ltnA1A2*) and for proteins involved in posttranslational modification (*ltnM1M2J*) and transport/processing (*ltnT*) (10, 12, 20–22, 27). It has already been reported that a subclone containing all 10 genes on a high-copy-number vector (pOM02) does not lead to higher production of lacticin 3147 than that observed from the low-copy-number parent plasmid pMRC01 (20). In this study, we sought to determine whether the introduction of the cloned region into a background containing the parent plasmid would lead to improved production. To this end, pOM02 was introduced (17) into *L. lactis* MG1363(pMRC01). Once it was established that the colocalization of pMRC01 and pOM02 in MG1363 did not impact the copy number of either plasmid (E. O'Connor, unpublished data), the antimicrobial activities of this strain and of MG1363 (pMRC01) were compared using a method described previously (28). This involved serial dilution of cell-free supernatant, from antibiotic-free overnight cultures (4×10^8/ml), in one-fourth-strength Ringer's solution and the addition of 50-μl volumes of these dilutions to 4.6-mm wells bored in agar plates seeded with approximately 10^6 cells of overnight-grown indicator bacteria (*Lactococcus lactis* HP)/ml. After overnight incubation of the plate, activity was determined and expressed as arbitrary units/ml (AU/ml), i.e., the reciprocal of the highest dilution which gave a definite zone multiplied by the conversion factor (i.e., 20 when 50 μl was used). For simplicity, data are presented as relative activity, i.e., activity relative to that of MG1363(pMRC01). It was found that the activity of the MG1363(pMRC01, pOM02) derivative was dramatically higher (400% activity) than that of the strains containing either plasmid alone (100% activity) (Fig. 1). To determine more specifically which of the genes introduced into pOM02 were responsible for this overproduction, we created a set of different subclones that were introduced into

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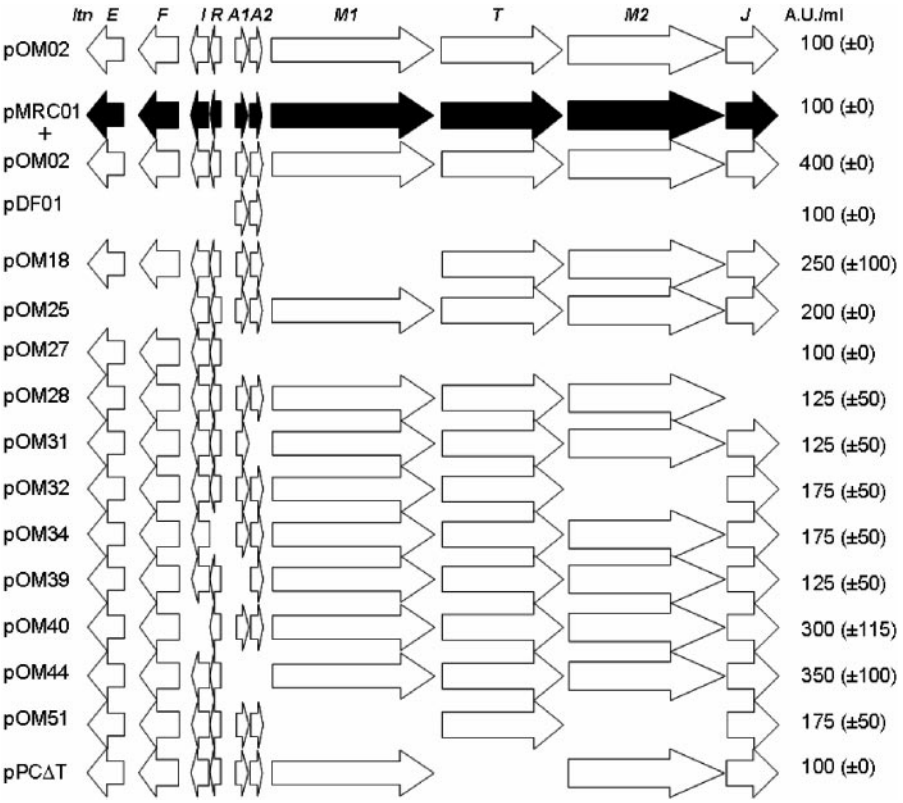

FIG. 1. Cell-free supernatant activity (relative bioactivity) of lacticin 3147-producing strains as a percentage of that produced by MG1363(pMRC01) (black arrows). The *ltn* genes present in the pCI372 derivatives (pDF, pOM, and pPC) introduced into MG1363(pMRC01) are illustrated. The data presented are the averages of quadruplicate experiments (± standard deviations).

MG1363(pMRC01) (Table 1). PCR amplification was carried out with the Expand High-Fidelity Polymerase system (Roche) or KOD (EMD Biosciences), and restriction enzymes and T4 DNA ligase were purchased from Roche. The sequences of primers are shown in Table 2. Some of these subclones have been described previously (20, 21), while others were made during the course of this study (Table 1; Fig. 1). The plasmids were initially created in *Escherichia coli* DH5α or XL1 and subsequently introduced into *L. lactis* MG1363 (pMRC01) by electroporation. Transformants were selected on M17 agar (Oxoid) with 0.5% glucose (GM17) and 5 μg/ml chloramphenicol.

Agar well diffusion assays were again performed on all strains. It was apparent that, with the exceptions of pOM27 (which lacks all biosynthetic genes), pPCΔT (which lacks *ltnT*), and pDF01 (carrying structural genes only), all of the constructs facilitated lacticin 3147 overproduction at least to some extent. While the addition of pOM02 containing all 10 genes resulted in the highest levels of antimicrobial activity, the strain containing pMRC01 and pOM44 (which contains all *ltn* genes except *ltnA1A2*) also displayed high relative bioactivity (350%; Fig. 1). Although percent overproduction was lower in all other cases, the results generated gave an insight into the relative importance of each of the genes in contributing to overproduction. The assays revealed that even in the absence of additional copies of *ltnFE* (pOM25) and *ltnI* (pOM40), respective two- and threefold increases in activity were apparent. Although it has yet to be established definitively that LtnFE contribute to lacticin 3147 immunity, it may be that an inability to tolerate increased levels of lacticin 3147 limits the ability of these strains to overproduce the lantibiotic as efficiently as MG1363(pMRC01, pOM02). The absence of *ltnR* (pOM34), a negative regulator of the *ltnRIFE* operon (22), resulted in more limited overproduction (175%). It has previously been established that MG1363(pOM34) exhibits activity which is approximately 50% of that of MG1363(pOM02) (22). The cause of this reduced activity has yet to be ascertained. When individual genes involved in biosynthesis/transport were absent from the newly introduced subclones, it was apparent that the presence of *ltnT* (pPCΔT; 100%) and *ltnJ* (pOM28; 125%) was important. Based on homologies with other LanT enzymes, LtnT is responsible for the transport of the lacticin 3147 peptides from the cell and the concomitant removal of the leaders. Although it has been established that LctT is dispensable for lacticin 481 production (26, 29), the inactivation of *mutT* abolishes mutacin II production (7). LtnJ is required for the presence of D-alanine residues in LtnA1 and LtnA2, and an MG1363(pMRC01Δ*ltnJ*) mutant has previously been shown to exhibit little antimicrobial activity (10). Overproduction was limited to between 175 and 250% as a consequence of not introducing additional copies of *ltnM1* and/or *ltnM2* (pOM18, pOM32, and pOM51). LtnM1 and LtnM2 are required for the production of fully modified LtnA1 and LtnA2, respectively

TABLE 1. Plasmids used in this study

Plasmid	Description and/or source (reference)
pMRC01	60.2-kb natural lactococcal plasmid containing *ltnEFIRA1A2M1TM2J* (12)
pMRC01S7G	Derivative of pMRC01 generated by site-directed mutagenesis (10)
pCI372	High-copy-number *E. coli-Lactococcus* cloning vector (14)
pOM02	pCI372-*ltnEFIRA1A2M1TM2J* (21)
pOM18	Equivalent to pOM02Δ*ltnM1* (20)
pOM31	Equivalent to pOM02Δ*ltnA2* (20)
pOM32	Equivalent to pOM02Δ*ltnM2* (20)
pOM39	Equivalent to pOM02Δ*ltnA1* (20)
pOM11	6-kb fragment from pOM02 (primers 5145 and 5146) cloned into BamHI/SalI-digested pCI372
pOM27	pCI372-*ltnEFIR*; 4-kb PCR product (primers 5144 and 5558) cloned into KpnI/BamHI-digested pOM11
pOM25	Equivalent to pOM02Δ*ltnFE*; 4.3-kb product (primers 5144 and 5556) cloned into SacI/BamHI site of pOM11
pOM28	Equivalent to pOM02Δ*ltnJ*; 4.7-kb product [primers LtnX(f) and LtnX(r)] cloned into BamHI/SalI-digested pOM02
pOM34	Equivalent to pOM02Δ*ltnR*; 2.4-kb product [primers FP(OP)372 and 5557] cloned into SacI/KpnI-digested pOM27
pOM38	4-kb product (primers 6163 and 5144) cloned into KpnI/BamHI-digested pOM11
pOM40	Equivalent to pOM02Δ*ltnI*; 2-kb product [primers FP(OP)372 and 6164] cloned into SacI/KpnI-digested pOM38
pOM44	Equivalent to pOM02Δ*ltnA1A2*; replacement of a 3.5-kb SacI/KpnI fragment from pOM31 with a 2.9-kb SacI/KpnI fragment from pOM39
pOM51	Equivalent to pOM02Δ*ltnM1M2*; replacement of a BamHI/SacI fragment from pOM18 with the corresponding region from pOM32
pPCM2J	4.7-kb product (primers ΔT for and pOM02rev) cloned into BamHI/SalI-digested pCI372
pPCΔT	Equivalent to pOM02Δ*ltnT*; 6-kb product [primers 5144 and FP(OP)372] cloned into BamHI/SacI-digested pPCM2J
pDF01	pCI372-*ltnA1A2*; 1-kb product (primers A1soeA and A2soeD) cloned into PstI/EcoRI-digested pCI372

(20). Previously an attenuator that impacts on the levels of *ltnM1TM2J* transcript was identified. Its presence undoubtedly impacts the levels of biosynthetic/transport proteins and may explain why the provision of additional copies of the corresponding set of genes is beneficial. In contrast, the presence of additional copies of the structural genes is not essential for overproduction. Curiously, while the benefits of adding pOM44 (Δ*ltnA1A2*) were great, these benefits were almost eliminated when only one of the structural genes was missing (pOM31/39; both 125%). It may be that, as a consequence of an uneven balance of substrate, one of the enzymes that is involved in the modification/production of both peptides, i.e., LtnT or LtnJ, functions less efficiently. Taken in combination these results indicate that while the presence of additional copies of the biosynthetic/transport machinery and LtnR is required for high-level overproduction, there is no single bottleneck that can be relieved by the addition of a particular gene product.

TABLE 2. Oligonucleotides used in this study

Primer	Sequence (5′ to 3′)[a]
5144	GATGGATCCATTATTATTACTGTTT
5145	ATGGATCCATCAGTAGGTAGAACTA
5146	GCTGTCGACTTAAGTATAGGGCAAT
LtnX(f)	ATGGATCCATCAGTAGGTAGAACTA
LtnX(r)	GCTGTCGACAAATTAACCACCCAAA
FP(OP)372	AAAGAGCTCTGCGAATAACATCAAGGGAA
5144	GATGGATCCATTATTATTACTGTTT
5556	AGAGCTCTTTTTTTCTTATTTGA
5558	AGGTACCCAGAGTTACTAATAGAA
6163	AAGGTACCTGAGTAAAAAAGAGA
6164	TTGGTACCGGATTTATTGTCTTTT
A1soeA	AACTGCAGTTATATATTTGCGGC
A2soeD	ACGAATTCTCTTACAGAGTT
pOM02rev	AGCTGTCGACTTAAGTATAGGGCAAT
ΔT for	TTGGATCCTGGGTTCAAATTACC

[a] Restriction sites in oligonucleotides are underlined.

The observation that overproduction can be achieved in the absence of the wild-type structural genes from pOM44 presented the possibility that this construct could facilitate the overproduction of bioengineered LtnA1 and LtnA2 derivatives. To test this theory, pOM44 was introduced into a strain containing a pMRC01 derivative in which the serine at position 7 in LtnA1 (normally converted to a D-alanine in the mature peptide) is replaced with an L-glycine (10). It has previously been established that although the $A1_S7_G$ peptide has only 50% of the relative activity of the parent peptide, its further characterization was hampered by a 94% decrease in its production (10). It was apparent from cell-free supernatant assays that the activity associated with supernatant from the strain containing pOM44 was increased twofold (40 versus 20 AU/ml), a small but significant increase.

Quantification of lacticin 3147 overproduction. While the antimicrobial assays described above allowed us to determine the relative benefits of adding different constructs, the relationship between peptide concentration and antimicrobial activity as ascertained by the well diffusion assay is not always linear (11). To more accurately quantify the production of LtnA1 and -A2 from the strains of greatest interest, the lacticin 3147 peptides were purified from the surface of selected strains and quantified by quantitative reverse-phase high-pressure liquid chromatography (RP-HPLC) as described previously (10) (Fig. 2). The purification of LtnA1 and LtnA2 in this way results in greater peptide yields and overcomes the problems associated with contaminating peptides from culture media, which hamper the quantification of the lacticin 3147 peptides obtained from cell-free supernatant. Relative to the yield from MG1363 containing pMRC01 alone, the yield of LtnA1 from strains also containing pOM44 or pOM02 was 1.45- and 2.74-fold greater, respectively, while the yields for LtnA2 were 1.48- and 6.62-fold greater, respectively. Mass analysis (matrix-assisted laser desorption ionization–time of flight mass spectroscopy) using an AXIMA-CFRplus mass spectrometer (Shi-

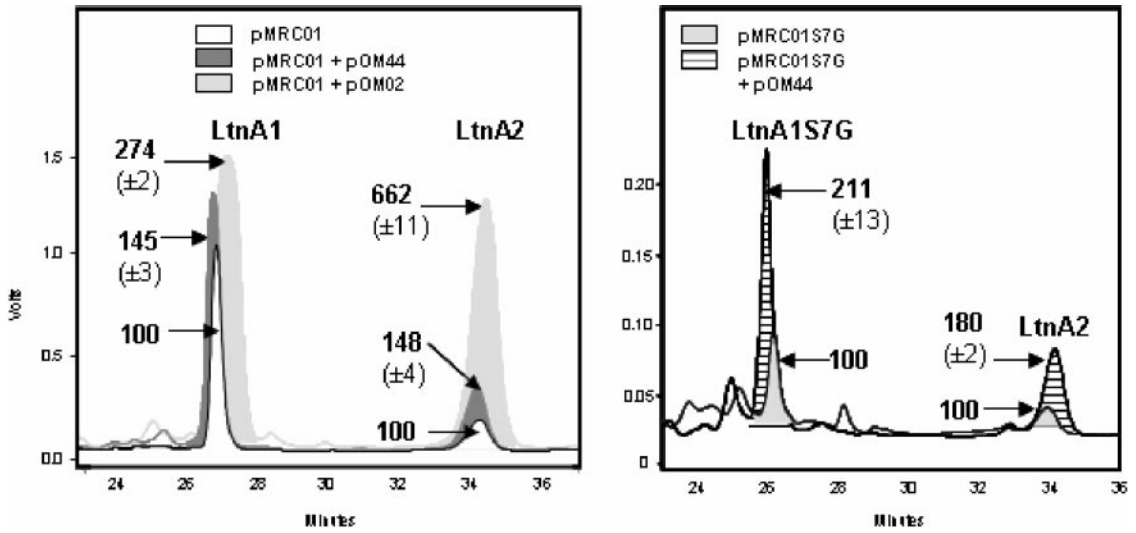

FIG. 2. Quantitative RP-HPLC of wild-type lacticin 3147 peptides purified from MG1363 containing pMRC01, pMRC01 plus pOM44, and pMRC01 plus pOM02 (left) and A1S7G peptide purified from LtnA1Ser7Gly and LtnA1Ser7Gly containing pOM44 (right). Values refer to peak size as a percentage of the relevant control (average of duplicate purification experiments).

madzu Biotech, Manchester, United Kingdom) demonstrated that the masses of LtnA1 (3,305 kDa) and LtnA2 (2,847 kDa) were as expected in all cases (data not shown), indicating the absence of unexpected modifications. It is curious that pOM02 led to differential overproduction of LtnA1 and LtnA2. In MG1363(pMRC01) and MG1363(pMRC01, pOM44), the ratios of LtnA1 to LtnA2 are similar in that there would appear to be significantly more LtnA1 than LtnA2 present. This trend was also apparent when the corresponding peptides were purified from the related two-peptide lantibiotics staphylococcin C55 and plantaricin W (16, 24). The reason for this is not apparent, but it is surprising given that these peptides function in a 1:1 ratio. However, the fact that this trend exists may explain the differential overproduction of the two peptides in MG1363(pMRC01, pOM02), in that because the levels of LtnA2 being produced by the parental strain are relatively low, there is simply more room for improvement. Conversely, as the quantities of LtnA1 that are produced by MG1363(pMRC01) are relatively high, it may be that the MG1363(pMRC01, pOM02) strain is producing the maximum amount of LtnA1 that the cell can either facilitate or tolerate.

RP-HPLC analysis also revealed that the yield of $A1_S7_G$ from the strain possessing pOM44 was more than twofold greater than that from the strain lacking the construct. The masses of the peptides produced were also as expected (A1S7G, 3,291 kDa; LtnA2, 2,847 kDa). It should be noted that despite being more difficult to quantify accurately, the trends with respect to peak sizes from cell-free supernatant preparations mirrored those shown for cell preparations in all cases (data not shown).

Concluding remarks. With respect to the contributions of individual components to lacticin 3147 overproduction, it has been demonstrated that one of the requirements for overproduction of both peptides of the lantibiotic lacticin 3147 is the introduction of additional copies of biosynthetic and transport genes into an existing lacticin producer. This suggests that one of the rate-limiting steps for lacticin 3147 production in the pMRC01-containing host is the abundance of modification and transport proteins but not substrate availability (i.e., unmodified peptides). These observations again raise questions about the role of the stem-loop attenuator that impacts transcription of the genes in question. The presence of a stem-loop structure between the structural gene and the downstream biosynthetic genes is a feature of a number of other lantibiotics (1, 19, 25). It has been established that in addition to allowing partial read-through to downstream synthetic genes, the stem-loop structure in Pep5 is also required for mRNA stability (25). It may be that further investigations are needed to determine whether mutagenesis of such structures in a way that improves read-through without impacting mRNA stability could facilitate lantibiotic overproduction. pOM34 (Δ*ltnR*) is the only construct that contains all of the biosynthetic/transport genes and yet fails to improve the activity of MG1363 by at least 200%. To date, analysis of LtnR has focused on its role as a negative regulator of immunity gene expression. In light of these results, further analysis to determine how it directly or indirectly contributes to lacticin 3147 production would appear to also be merited.

This study represents the first occasion on which non-nisin-like antibiotics have been overproduced by genetic means. The extent to which overproduction occurs, i.e., 2.74-fold for LtnA1 and 6.62-fold for LtnA2, is greater than what has been observed for wild-type nisin or subtilin peptides. In fact, LtnA2 overproduction is also more dramatic than the *abrB* deletion-associated production of succinylated subtilin. Significantly, although deletion of *abrB* is an option available only to subtilin-associated applications, the subcloning of entire operons in a manner analogous to that described here for lacticin 3147 is, with the increasing availability of polymerases suited to the amplification of large products with high fidelity, a realistic option when endeavoring to overproduce other lantibiotics. As many of these peptides are of great fundamental interest and show great potential with respect to either food or clinical applications, this is particularly worthwhile. The development

of the related strategy to facilitate the overproduction of bioengineered derivatives is also significant, as the low-level production of many bioengineered lantibiotics can present a major hurdle to their further characterization and utilization. While a number of new strategies involving either in vitro or heterologous production are being investigated, it remains to be seen whether they will be successful in producing large quantities of correctly modified peptide. The overproduction of a bioengineered lantibiotic for the first time is thus a welcome event.

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