Biosynthesis and Transport of the Lantibiotic Mutacin 1140 Produced by Streptococcus mutans

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ABSTRACT
Lantibiotics are ribosomally synthesized peptide antibiotics composed of an N-terminal leader peptide that is cleaved to yield the active antibacterial peptide. Significant advancements in molecular tools that promote the study of lantibiotic biosynthesis can be used in Streptococcus mutans. Herein, we further our understanding of leader peptide sequence and core peptide structural requirements for the biosynthesis and transport of the lantibiotic mutacin 1140. Our study on mutacin 1140 biosynthesis shows a dedicated secondary cleavage site within the leader peptide and the dependency of transport on core peptide posttranslational modifications (PTMs). The secondary cleavage site on the leader peptide is found at the −9 position, and secondary cleavage occurs before the core peptide is transported out of the cell. The coordinated cleavage at the −9 position was absent in a lanT deletion strain, suggesting that the core peptide interaction with the LanT transporter enables uniform cleavage at the −9 position. Following transport, the LanP protease was found to be tolerant to a wide variety of amino acid substitutions at the primary leader peptide cleavage site, with the exception of arginine at the −1 position. Several leader and core peptide mutations produced core peptide variants that had intermediate stages of PTM enzyme modifications, supporting the concept that PTM enzyme modifications, secondary cleavage, and transport are occurring in a highly coordinated fashion.

IMPORTANCE
Mutacin 1140 belongs to the class I lantibiotic family of ribosomally synthesized and posttranslationally modified peptides (RiPPs). The biosynthesis of mutacin 1140 is a highly efficient process which does not lead to a discernible level of production of partially modified core peptide variants. The products isolated from an extensive mutagenesis study on the leader and core peptides of mutacin 1140 show that the posttranslational modifications (PTMs) on the core peptide occur under a highly coordinated dynamic process. PTMs are dictated by the distance of the core peptide modifiable residues from PTM enzyme active sites. The formation of lanthionine rings aids in the formation of successive PTMs, as was observed in a peptide variant lacking a C-terminal decarboxylation.

Lantibiotics are a class of ribosomally synthesized peptide antibiotics produced by Gram-positive bacteria, such as Lactococcus lactis and Streptococcus mutans (1, 2). Lantibiotics are characterized by the presence of posttranslational modifications (PTMs), such as dehydrated residues and lanthionine rings. The modified residues 2,3-didehydroalanine (Dha) and 2,3-didehydrobutyryl (Dhb) are formed from dehydration of serines and threonines, respectively. The cyclization between a cysteine and either a Dha or a Dbh forms a lanthionine or a methyllanthionine ring, respectively. The biosynthetic gene cluster contains all the genes necessary to produce a lantibiotic. The lan operon for class I lantibiotics contains genes encoding the lantibiotic peptide (lanA); the dehydratase (lanB), responsible for the dehydration of serine and threonine residues; the cyclase (lanC), responsible for the stereospecific formation of the thioether linkages; and an ABC-like transporter (lanT) for the export of the modified peptide. The mutacin 1140 operon also contains an additional gene called lanD, which is essential for the C-terminal decarboxylation of the core peptide, forming an aminovinyl-cysteine residue (3). Lantibiotics contain a leader peptide sequence that is cleaved by a dedicated protease called LanP following posttranslational modifications (4, 5). The substrate specificity of LanP varies among lantibiotics (6). In most lantibiotic systems, LanP is believed to be associated with the extracellular leaflet of the membrane and cell wall (7). Therefore, cleavage does not occur until mutacin 1140 is transported out of the cell by LanT.

The lantibiotic mutacin 1140, produced by S. mutans JH1140, has been shown to have a mechanism of action similar to that of another class I lantibiotic called nisin. Mutacin 1140 and nisin have structurally similar rings A and B (Fig. 1A), and both nisin and mutacin 1140 bind and abduct lipid II (8–13). Mutacin 1140 also has been shown to be bactericidal to pathogenic bacteria, such as methicillin-resistant Staphylococcus aureus (MRSA), with no significant development of resistance (14). Mutacin 1140 is one of a small group of peptides that is currently under preclinical development for the treatment of Gram-positive bacterial infections (15). Many studies have revealed that lantibiotics are structurally diverse and have differences in activity from that of nisin. For instance, mutacin 1140 is a class I lantibiotic like nisin. However, the core peptide of mutacin 1140 is 12 amino acids shorter than
that of nisin. Mutacin 1140 has the same ability to bind to lipid II, but it does not form bacterial membrane pores like nisin does (12, 13).

The leader peptide sequences of lantibiotics belonging to the same class generally share structural motifs related to their function. The mutacin 1140 leader peptide sequence is unique among class I lantibiotics in that its primary sequence has little similarity to that of other class I lantibiotics (Fig. 1B). However, the structure of the leader peptide of mutacin 1140 is similar to that of the leader peptide of mutacins produced by other 

![Diagram of Lantibiotic Structural Elements](image)

**FIG 1** Lantibiotic structural elements. (A) Covalent structures for nisin and mutacin 1140, with the lanthionine rings labeled from the N terminus to the C terminus. (B) Leader peptide sequence alignments of structurally related class I lantibiotics, i.e., nisin produced by Lactococcus lactis, subtilin produced by Bacillus subtilis, epidermin produced by Staphylococcus epidermidis, gallidermin produced by Staphylococcus gallinarum, Pep5 produced by Staphylococcus epidermidis, epilancin K7 produced by Staphylococcus epidermidis, mutacin Ny266 produced by S. mutans Ny266, mutacin III produced by S. mutans UA787, mutacin 1140 produced by S. mutans JH1140, and, for comparison, lacticin 481 (a class II lantibiotic) produced by Lactococcus lactis (16, 18, 47–53). In the sequences, the F(N/D)LD box and C-terminal proline in class I lantibiotics are underlined and in bold, respectively. The EV and EL motifs and the GA cleavage site of the class II lantibiotic lacticin 481 are in bold. (C) Secondary structure prediction for the mutacin 1140 leader peptide using SOPMA. h, alpha helix; e, extended strand; c, random coil; t, beta turn. Alpha-helical regions are underlined, while random coils in the leader peptide sequence are in bold.

The common motif found in class I lantibiotics is the F(N/D)LD box (Fig. 1B), which some studies show is important for the maturation of the core peptide antibiotic (19, 20, 23). Class II lantibiotics contain a GG or GA which is used as a cleavage site for the removal of the leader peptide from the core peptide and contain an upstream EV and/or EL conserved sequence motif (24, 25). Amino acid substitutions in the conserved regions within the leader peptide have shown that the composition of these common motifs is important for the maturation of the core peptide, while a vast majority of the leader peptide does not have any amino acid sequence specificity (19, 20, 26, 27). Solution nuclear magnetic resonance studies have shown that the leader peptide sequence lacks secondary structure (28), while the cocrystal structure of NisB in complex with the substrate peptide NisA shows an antiparallel β strand (29). It is likely that the leader peptide remains unstructured until it binds to the PTM enzymes. However, additional studies are needed to verify this assumption. Experimental studies suggest that the leader peptide is important for interacting with the dehydratase LanB, the cyclase LanC, and the transporter.
Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. The cloning strain Escherichia coli DH5α (Invitrogen, Carlsbad, CA) was cultured at 37°C on Luria-Bertani (LB) broth or agar. THyex broth (30 g/liter Todd-Hewitt broth, 3 g/liter yeast extract, 15 g/liter Bacto agar [Becton, Dickinson and Company, Franklin Lakes, NJ]), and top agar medium (30 g/liter Todd-Hewitt broth, 3 g/liter yeast extract), THyex agar medium (30 g/liter Todd-Hewitt broth, 3 g/liter yeast extract, 7.5 g/liter Bacto agar [Becton, Dickinson and Company]) were used to culture the wild-type strain JH1140. Micrococcus luteus (ATCC 55676) was obtained from Oragenics Inc. Relevant characteristic(s) Reference or source

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M. luteus ATCC 10760 | Indicator strain in the different antagonism assays | 35 (strain) |
E. coli DH5α | pCR2.1-TOPO | Intermediate cloning host | Invitrogen |

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and media.** The bacterial strains and plasmids used in this study are listed in Table 1. The cloning strain Escherichia coli DH5α (Invitrogen, Carlsbad, CA) was cultured at 37°C on Luria-Bertani (LB) broth or agar. THyex broth (30 g/liter Todd-Hewitt broth, 3 g/liter yeast extract), THyex agar medium (30 g/liter Todd-Hewitt broth, 3 g/liter yeast extract, 15 g/liter Bacto agar [Becton, Dickinson and Company, Franklin Lakes, NJ]), and top agar medium (30 g/liter Todd-Hewitt broth, 3 g/liter yeast extract, 7.5 g/liter Bacto agar [Becton, Dickinson and Company]) were used to culture the wild-type strain JH1140. Micrococcus luteus (ATCC 55676) was obtained from Oragenics Inc.

**Mutagenesis**

**leader peptide.** The S. mutans genome database and lan gene cluster (GenBank/EMBL accession number AF051560) were used to design primers for the mutagenesis and sequencing work. pIFDC2 (32) is an in-frame-deletion (IFD) cassette vector which uses a highly expressed constitutive promoter to drive the expression of a synthetic operon containing both a positive selection marker (ermAM) and a negative selection marker (−pheS) (33). Fragments consisting of ~500 bp of DNA upstream of lanA (primers MutA-UpF and MutA-UpR-IDH) and ~500 bp of DNA downstream of lanA (primers MutA-DnF-erm and MutA-DnR) were amplified using PCR. These DNA fragments were attached to the 5′ and 3′ ends of the IFDC2 cassette, respectively. Transformation of this PCR-amplified product with S. mutans JH1140 ATCC 55676 or S. mutans ΔlanP generated S. mutans strain ΔlanA/IFDC2 and S. mutans strain ΔlanP/IFDC2 ΔlanP (Table 1). The S. mutans natural competence pathway was used for transforming PCR and plasmid products. Natural competence can be activated using a competence-stimulating peptide (CSP) (34). An overnight culture of either S. mutans strain ΔlanA/IFDC2 or S. mutans strain ΔlanA/IFDC2 ΔlanP was diluted to an optical density at 600 nm (OD₆₀₀) of 0.1 and grown to an OD₆₀₀ of 0.25 before the addition of 2 μl of 10 μg/ml
CSP to 200 μl of bacterial suspension. After 30 min of incubation, 1 μl of the PCR product of the IFDC2 cassette spanned by upstream and downstream DNAs from the structural gene (lanA) was added to the cells. After 4 to 5 h of incubation, 50 μl of solution was plated on THyx plates containing 15 μg/ml of erythromycin. Colonies that grew in the presence of erythromycin were sequenced to confirm that the IFDC2 cassette replaced the lanA gene. This strain was used for subsequent transformations of plasmids containing leader peptide mutations. Leader peptide mutations were introduced into the lanA gene by 2-step PCR. The mutations were then inserted into the pCR2.1-TOPO vector according to the protocol provided by the manufacturer (Invitrogen). The sequences of the transformants approximately 300 bp upstream and downstream from lanA were determined by the use of primers MutAsegF and MutAsegR. S. mutans ΔlanA/IFDC2 was transformed by the same protocol described above with 1 μl of cloned pCR2.1-TOPO vector. The transformants of the leader peptide mutants were plated on THyx plates with 4 mg/ml of chlorophenylalanine. Colonies growing in the presence of chlorophenylalanine represent those that have lost the IFDC2 cassette and have an insertion of lanA with the expected mutation. Colonies from these plates were identified on THyx and THyx with erythromycin to remove false-positive colonies from the screen. Mutants were further confirmed by sequencing.

Deferred antagonism assay. The deferred antagonism assay was performed as previously reported (35). S. mutans wild-type and mutant strains were grown overnight in liquid culture. The next morning, the culture was diluted to an OD600 of 0.1 and allowed to grow to mid-logarithmic phase. The culture was then diluted to an OD600 of 0.05 before 2 μl of the bacterial suspension was spotted on fresh prewarmed THyx plates. Duplicates of triplicate spots were tested for each strain, with the wild-type and ΔlanA strains serving as positive and negative controls, respectively. The plates were incubated for 18 h at 37°C in a candle jar. On the next day, the bacterial colonies were heat killed at 65°C for 1.5 h and then cooled to 37°C. M. luteus from a fresh overnight plate was used to inoculate prewarmed THyx broth and grown to mid-logarithmic phase at 37°C. The culture was then diluted to an OD600 of 0.2 and diluted 25-fold in prewarmed (42°C) top agar. Five milliliters of top agar containing the bacterial suspension was then poured onto each plate with heat-killed bacteria, and the plate was incubated overnight at 37°C. Trypsin was used to remove the leader peptide from the mutant 1140 core peptide by adding it to the top agar at a concentration of 5 μg/ml. The area for each zone of inhibition was calculated and compared to wild-type areas of inhibition. The Student’s t test was used to determine whether the differences in the activities of each mutant strain with respect to the activity of the wild-type strain were statistically significant. The activities of the purified variants were determined using the same conditions for overlaying the indicator strain described above, in which 5 μl of the extracted variants was spotted on prewarmed THyx plates after being overlaid with M. luteus.

Isolation of mutacin 1140 leader peptide variants. Mutacin 1140 and variants of mutacin 1140 were isolated as previously reported (18). A modified THyx medium was used as the fermentation medium for inoculation. The medium contained 30 g/liter Todd-Hewitt broth, 3 g/liter yeast extract, 1 g/liter NaHPO4, 0.2 g/liter Na2HPO4, 0.7 g/liter MgSO4, 0.005 g/liter FeSO4, 0.005 g/liter MnSO4, and 0.3% agar. Five hundred millilitres of the semisolid fermentation medium was placed in a 1-liter glass beaker and stab inoculated using an inoculating needle. The inoculum was placed at 37°C for 72 h and then immediately frozen at −80°C. The medium was then thawed in a 55°C water bath for 1 h. The inoculum was then placed in 250-ml centrifuge bottles and centrifuged at 20,000 × g for 30 min. The collected supernatant was pooled and mixed with chloroform at a 1:1 ratio, and the mixture was shaken vigorously. The mixture was centrifuged again at 20,000 × g for 30 min. The phase between the aqueous and chloroform layers was collected and allowed to dry overnight. The dried precipitate was resuspended in 35% acetonitrile and tested by deferred antagonism assay for activity. The crude extract was run on either a semiprep C18 column (octydecyl silane [C18]; particle size, 5 μm; 4.6 by 250 mm; Agilent Zorbax) or an analytical column, as previously reported (35). 2-Mercaptoethanol (BME) modification of mutacin 1140 peptide variants was done as previously reported (13, 36). The lack of PTM cycle activity would prevent lanthionine ring formation and result in the presence of cysteine thioles that can be selectively labeled. 1-Cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) and tris(2-carboxyethyl)phosphine (TCEP) were used to determine whether the isolated products had undergone all of the PTM cycle modifications (37). We followed the procedure previously reported by Kluskens et al. (38) with slight modification. We used 0.1 M hydrochloric acid to dissolve the CDAP. As a positive control, we used an extended analog of the chemotactic peptide recep (LRGGGCGCPTVGCYGGG–NH2) (39). The purified products were confirmed by mass on a Shimadzu matrix-assisted laser desorption ionization mass spectrometer (MALDI-MS) in both the linear and reflection modes.

RESULTS

LanP cleavage site. We have previously reported that substitutions at the +1 position of the core peptide lead to an increase in bioactivity or do not significantly reduce bioactivity (35). This suggests that the presence of an F residue at position +1 [F(+1)] is not essential for cleavage by LanP. Alignments of the cleavage site of class I lantibiotics show a basic amino acid, such as arginine, at the −1 position of the cleavage site and a conserved proline at the −2 or −4 position (Fig. 1B). Mutations were made to determine whether there is a recognition motif for LanP upstream of the cleavage site. Secondary structure analysis using the self-optimized prediction method with alignment (SOPMA) (40) predicts that the N-terminal end of the leader peptide (the region from positions −32 to −39) is an alpha helix, while the C-terminal end (the region from positions −1 to −8) is a random coil (Fig. 1C). Interestingly, the region from positions −9 to −20 of the mutacin 1140 leader peptide is primarily predicted to be an extended strand, which is in agreement with the secondary structure of the nisin leader peptide in the cocystal structure of NisA and NisB (29). Given the lack of predicted secondary structure at the C-terminal end of the leader peptide, LanP protease recognition would presumably be dependent on structural elements upstream or downstream of the primary cleavage site. First, alanine substitutions were made to determine the importance of the amino acids from positions −7 to −1 of the leader peptide (Fig. 2A and B). The T-to-A substitution at position −2 [T(−2)A] resulted in a slight reduction in activity, while the other alanine substitutions from residues −7 to −3 resulted in no statistically significant decrease in activity. The small change or the lack of a statistically significantly reproducible reduction in activity indicates that the region upstream of the cleavage site does not have a strict motif for proteolytic activity. Interestingly, the substitution of the proline for an alanine at the −4 position did not result in a decrease in bioactivity. The presence of a proline near the cleavage site is conserved among other class I lantibiotics, and the lack of any change in bioactivity with the alanine substitution from residues −7 to −3 resulted in no statistically significant decrease in activity. The small change or the lack of a statistically significantly reproducible reduction in activity indicates that the region upstream of the cleavage site does not have a strict motif for proteolytic activity. Interestingly, the substitution of the proline for an alanine at the −4 position did not result in a decrease in bioactivity.
None of the substitutions in the *lanP* deletion strain resulted in activity against the *M. luteus* indicator strain, but activity was restored with the addition of trypsin to the top agar. Removal of the leader peptide using trypsin in a *lanP*-deficient strain is possible, given the presence of an Arg at the −1 position of the leader peptide and the core peptide resistance to trypsin cleavage at the Lys2 and Arg13 positions. Interestingly, the bioactivity was significantly greater in the *lanP* strain when trypsin was used to remove the leader peptide. Reversed-phase high-pressure liquid chromatography (RP-HPLC) of the lantibiotic extracted from the *lanP* strain yielded at least a 2-fold increase in the amount of purified product (Fig. 3). The histidine substitution of residues at positions 7 to 2 resulted in an 85% reduction in activity but did not completely remove all activity. This observation suggests that LanP is still able to process the removal of the leader peptide containing 6 histidine substitutions, albeit at a much lower efficiency. Furthermore, product could not be isolated from the culture broth, suggesting that biosynthesis or transport of the product is drastically reduced with histidine substitutions of residues at positions −7 to 2. A substitution of histidine at the −1 position of the leader peptide was evaluated. A histidine side chain has a pK of 6.0 and would be predominantly neutral at the physiological pH of the medium. We introduced this mutation to determine whether the positive charge within the −1 position was essential for activity and figured that this idea could be tested by changing the pH of the growth medium. Testing of medium with a lower pH was not needed since there was no significant change in the bioactivity of the construct with the R(−1)H mutation in the medium with the neutral pH. However, we cannot rule out the possible need for a positive charge at the −1 position, given that the lactic acid production by *S. mutans* could lower the pH around the bacterial colony, enabling the histidine to be charged. These data show that the LanP protease is tolerant to a wide variety of amino acid substitutions upstream of the primary leader peptide cleavage site and that the R residue at position −1 is not essential for the removal of the leader peptide.

**Discovery of an additional leader peptide cleavage site.** MALDI-MS of purified products obtained from the culture broth of the *S. mutans* Δ*lanP* and R(−1)A strains showed that it contained single products with masses of 3,165 Da and 3,079 Da,

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**FIG 2** Identification of structural elements within the mutacin 1140 leader peptide that are important for bioactivity. (A) Covalent structure representation of the mutations made on the leader peptide. (B to D) The bioactivities of the leader and core peptide mutants were measured as the percent difference in the zone of inhibition between the wild-type and the mutant strains. The Δ*lanA* strain was used as a negative control for bioactivity in all experiments. The change in activity was measured for mutations near the LanP cleavage site (B), mutations within the newly discovered cleavage site (C), and mutations in the transporter *lanT* and core peptide regions responsible for ring formation (D). For each mutation, the bioactivity was compared to the activity of wild-type strain *S. mutans* JH1140. All the activities were determined in the absence of trypsin in the top agar of the overlays. The Student t test was used for statistical analysis, and the asterisks signify statistical significance (*P* < 0.05).
The absence of LanP produces partially cleaved leader peptide products (41, 42), with the full-length leader peptide being the main structural state. It has been shown that the biosynthesis of nisin in the truncated leader peptide keeps mutacin 1140 in an inactive structural state. The absence of any inhibitory activity of these products also demonstrates that the peptide for cleavage presumably occurs upstream of the P(−4) position. We have previously shown that a serine engineered at the +1 position does not undergo dehydration (35), suggesting that the distance from either the LanP or the new cleavage site is important for a dehydration modification. We engineered a P(−4)A F(1)S mutant to determine if the proline substitution would enable dehydration at the +1 position. This finding suggests that the P(−4)A substitution did not promote dehydration at the +1 position and that the length from the +1 position appears to be important for the dehydratase activity. Interestingly, the double mutation resulted in an ∼35% reduction in activity, whereas the P(−4)A and F(1)S mutations individually resulted in no loss in activity. There was no cyanylation of free thiols by CDAP in the P(−4)A F(1)S mutant, suggesting that all lanthionine rings were formed by the cyclase.

Given that a deletion of S(−8) shifted the cleavage site downstream to N(−7) and D(−6), we decided to investigate a larger deletion downstream of S(−8). The mutant with the deletion of positions −7 to 2 [the Δ(−7–2) mutant] was engineered in the wild-type strain and in the lanP deletion strains. The deferred antagonism assay for each of these strains showed that they had no bioactivity (Fig. 2A and C and 4A). Products were isolated from the culture liquor of these mutants and were characterized to have two predominant masses of 1,936 and 2,508 Da. The 2,508-Da product corresponds to the mutacin 1140 peptide that has undergone all of the posttranslational modifications with a short leader peptide of S(6)A (Table 2 and Fig. 4Band C). This mass corresponds to a truncated mutacin 1140 peptide that has been cleaved between S(+3) and Trp(+4). Furthermore, the mass of the product corresponds to a loss of dehydration on a serine or a threonine residue. We have previously shown that a serine engineered at the +1 position or the P(−4)A F(1)S strain does not undergo dehydration (35), suggesting that the distance from either the LanP or the new cleavage site is important for a dehydration modification. Therefore, a loss of dehydration in the truncated core peptide product presumably occurs at S(+5).

The observation of the 2,508-Da peptide corresponds to a fully modified core peptide that has two additional amino acids (S and position of neighboring amino acids by introducing a turn in the peptide structure. A proline mutation at S(−8) shifted the cleavage site to I(−11) or T(−13), as indicated by the masses of the isolated products (Table 2). Interestingly, N(−7)P had a major product with a cleavage site at I(−11) but also had multiple minor products with cleavage sites ranging from T(−13) to D(−6). P(−4)A had only a single product that had no shift in the cleavage site between A(−9) and S(−8) (Table 2). The structural region of the peptide at P(−4) is not important for the alignment of the peptide to be cleaved at the new secondary cleavage site. The structural region for aligning the peptide for cleavage presumably occurs upstream of the P(−4) position. We have previously shown that a serine engineered at the +1 position does not undergo dehydration (35), suggesting that the distance from either the LanP or the new cleavage site is important for a dehydration modification. We engineered a P(−4)A F(1)S mutant to determine if the proline substitution would enable dehydration at the +1 position. This finding suggests that the P(−4)A substitution did not promote dehydration at the +1 position and that the length from the +1 position appears to be important for the dehydratase activity. Interestingly, the double mutation resulted in an ∼35% reduction in activity, whereas the P(−4)A and F(1)S mutations individually resulted in no loss in activity. There was no cyanylation of free thiols by CDAP in the P(−4)A F(1)S mutant, suggesting that all lanthionine rings were formed by the cyclase.

FIG 3 HPLC chromatograms of crude extracts obtained from modified THyex medium inoculated with wild-type S. mutans H1140 (A) and S. mutans ΔlanP (B). There was a more than 2-fold increase in the amount of product isolated from the S. mutans ΔlanP strain. AU, absorbance units.

respectively (Table 2). The peptide masses indicate the attachment of the amino acids at positions −8 to −1 instead of the whole 41-amino-acid leader peptide to the fully modified lantibiotic, as expected in the absence of LanP activity. This observation suggests that an additional cleavage site exists internally within the leader peptide between residues A(−9) and S(−8). The absence of any inhibitory activity of these products also demonstrates that the truncated leader peptide keeps mutacin 1140 in an inactive state. It has been shown that the biosynthesis of nisin in the absence of LanP produces partially cleaved leader peptide products (41, 42), with the full-length leader peptide being the main product (43). Unlike nisin, only a single truncated leader peptide product is observed for the mutacin 1140 leader peptide. To further understand the importance of the new cleavage site, point mutations were made at the new cleavage site in the S. mutans ΔlanP strain. This was done in order to prevent cleavage at the primary cleavage site, R(−1). The residue at S(−8) or A(−9) was replaced by either an alanine or a phenylalanine (Fig. 2A and C). The switch to a smaller or bulkier side chain did not affect secondary cleavage activity, nor did it affect the bioactivity of the mutant strains in the deferred antagonism assay when trypsin was added (Fig. 2C). The masses of the isolated mutant products were determined, and they still indicated the attachment of the 8-amino-acid leader peptide (Table 2). Deletion of S(−8) yielded two products with masses of 3,063 Da and 2,948 Da, which correspond to partial cleavage at N(−7) and D(−6), respectively. To further determine whether the cleavage is absolutely necessary, proline mutations were made at either S(−8) or N(−7). Proline mutations alter the
TABLE 2 MALDI-MS data for isolated products from \textit{S. mutans} \textsuperscript{a}

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<td>\textit{S. mutans} \textit{Δ}(−8) \textit{Δ}lanP</td>
<td>2,963.32 ± 1, 3,077.42 ± 1</td>
<td>SADDPAADTR</td>
</tr>
<tr>
<td>\textit{S. mutans} \textit{S}(−8)P \textit{Δ}lanP</td>
<td>3,457.90 ± 1, 3,660.12 ± 1</td>
<td>SADDPAADTR</td>
</tr>
<tr>
<td>\textit{S. mutans} \textit{N}(−7)P \textit{Δ}lanP</td>
<td>3,430.74 ± 1, 3,632.75 ± 1, 3,317.66 ± 1, 3,218.59 ± 1, 3,147.51 ± 1, 2,963.42 ± 1</td>
<td>SADDPAADTR</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Some mutant strains produced more than one product. The product masses are listed in the order of the highest to the lowest relative peak intensity. Some mutant products were not detectable (ND), and this information is indicated.

R) from the leader peptide, while the 1,936-Da peptide corresponds to a variant that has been cleaved at S(+3) and is lacking a dehydrated Dha or Dhb residue. Given the observation of two distinct cleavage sites in the (Δ(−7−2)) mutant, there is a possibility that the 2,508-Da peptide fragment has been cleaved at both A(−3) and S(+3) but is being held together by the thiocysteine linkage in ring A. The lack of a single dehydration on the 1,936 Da fragment enabled us to test this assumption by 2-mercaptoethanol (BME) derivatization of the two products. The lanthionine rings, the unsaturated amino acids Dha and Dhb, and the aminovinylcysteine residues are susceptible to the addition of BME. The 2,508- and 1,936-Da peptides have seven and six modifiable positions, respectively. If there is no proteolytic cleavage between S(+3) and Trp(+4), derivatization of both the 2,508-Da and the 1,936-Da peptides should increase by ~78 Da for each addition of BME. If there is a proteolytic cleavage between S(+3) and Trp(+4) of the 2,508-Da peptide, derivatization would provide a mass profile similar to that of the derivatized 1,936-Da peptide. The latter scheme was observed following BME derivatization (Fig. 4D). Given the absence of dehydration and the presence of a free thiol in the 1,936-Da peptide product, there would be an approximately 16-Da lower mass for each addition of BME to the 2,508-Da peptide product. The mass difference of 16 Da is attributed to the lack of dehydration within the 1,936-Da fragment and the loss of 2 Da from the formation of a disulfide linkage between BME and the free thiol on the cysteine within the 1,936-Da fragment. The addition of two to six BMEs was observed by MALDI-MS (Fig. 4D).

\textbf{Transport of mutacin 1140.} Mutations were engineered to disrupt the formation of ring A, ring B, ring C, ring D, and combined rings AB and CD (Fig. 2A and D). Alanyl residues were substituted for the cysteines in rings A, B, and C. Given that the C-terminal cysteine is decarboxylated by LanD, an alanine was substituted for the serine in ring D. Three distinct peptides were isolated from the culture medium for the ring A mutant (Table 2). A 2,250-Da peptide corresponds to the core peptide that has not undergone dehydration on one of its serine or threonine residues. The 2,232-Da peptide corresponds to the core peptide that has undergone dehydration on all of its serine or threonine residues. A truncated core peptide with a mass of 1,702 Da corresponds to mutacin 1140 with the loss of FKSW from the N-terminal end. The mass of the major peptide identified in the ring B mutant was 2,250 Da, followed by a peptide with a mass of 2,232 Da, corresponding to the lack of a single dehydration within the core pep-
tide and a core peptide that has undergone all dehydrations, respectively (Table 2). There was an additional peptide with a mass of 2,278 Da. This corresponds to a core peptide that has undergone all dehydrations and lacks a C-terminal decarboxylation. The ring C mutant had only one peptide product with a mass of 2,232 Da, which is the mass of the core peptide that has undergone all dehydrations (Table 2). There was no discernible product from the ring D mutant for which to obtain a mass (Fig. 5). The formation of ring D appears to be absolutely necessary for transport. Ring A, ring B, and ring C mutants yielded approximately 60%, 90%, and 30% of the product, respectively, compared to the amount of the product from the wild-type JH1140 strain on the basis of the peak volume measurements determined by RP-HPLC chromatograms. The ring A and B mutant yielded levels similar to those of the ring A mutant, and the ring C and D mutant had no measurable product, as was the case for the ring D mutant. The ring A, ring D, ring A and B, and ring C and D mutants had no inhibitory activity (Fig. 2D). The ring B and ring C mutants had approximately 65% and 35% reductions in activity, respectively.

The bioactivity of the S. mutans ΔlanT strain was evaluated, and it was found to be active in our deferred antagonism assay. The lack of the mutacin 1140 transporter resulted in only an approximately 20% reduction in activity. The product isolated from this strain had the predicted 2,265-Da mass, suggesting that it had undergone all of the posttranslational modifications. These results show that there is an alternative transporter within S. mutans JH1140 that can transport mutacin 1140. Given the importance of the ring D region within the core peptide for transport (Fig. 5), the alternative transporter in S. mutans JH1140 must also depend on the ring D region for transport. A strain with the double deletion of lanP and lanT was generated to evaluate the nature of the leader peptide. Several peptide products that correspond to the core pep-

![FIG 4](image)

Characterization of the leader peptide Δ(−7–2) mutant. (A) Overlay assay using M. luteus as an indicator strain. From the top left to the bottom right, spotting of 5 μl of purified mutacin 1140 (10 μg/ml) in acetonitrile-water (1:1), spotting of 5 μl of acetonitrile (ACN)-water (1:1; negative control), deferred antagonism assay of S. mutans Δ(−7–2), deferred antagonism assay of S. mutans ΔlanPΔ(−7–2), spotting of 5 μl of the product from S. mutans Δ(−7–2) digested with trypsin, and spotting of 5 μl of the product from S. mutans ΔlanPΔ(−7–2) digested with trypsin. (B) MALDI-MS data for the product from S. mutans Δ(−7–2). (C) MALDI-MS data for the product from S. mutans ΔlanPΔ(−7–2). (D) MALDI-MS data for the BME derivatization of the products isolated from the S. mutans Δ(−7–2) strain.

![FIG 5](image)

Transport efficiencies of mutacin 1140 core peptide variants. Alanine substitutions were made to disrupt ring A [C(7)A], ring B [C(11)A], ring C [C(21)A], ring D [S(19)A], rings A and B [C(7)A C(11)A], and rings C and D [S(19)A C(21)A]. Three independent extractions of each mutant strain were characterized by RP-HPLC at 220 nm. The peak volumes of the variants of the mutacin 1140 fractions were compared to the peak volume of wild-type strain JH1140 and are presented as the percentage of the product relative to the amount for the wild-type strain. The Student t test was used for statistical analysis, and the asterisks signify statistical significance (P < 0.05).
ptide containing a leader peptide of 2 to 10 amino acids were isolated (Table 2). The variation in peptide length is similar to that observed with our peptides with proline mutations within the secondary cleavage site.

**DISCUSSION**

In this study, we investigated the role of structural regions within the leader peptide and core peptide in biosynthesis. The mutacin 1140 leader peptide sequence is different and longer than the leader peptide sequences of other lantibiotics, while the core peptide sequence is similar to the core peptide sequences of nisin, epidermin, and gallidermin. A better understanding of the biosynthesis of all lantibiotics will allow us to advance the use of the PTM enzymes for protein chemistry applications and the design of novel peptide-based therapeutics. We show that secondary cleavage, PTM enzyme modifications, and transport are highly coordinated activities. Terminal core peptide ring formations are essential for transport, and the coordinated cleavage at the −9 position is dependent upon the presence of the LanT transporter. LanP tolerates amino acid substitutions surrounding the primary cleavage site at position −1.

A proline usually denotes a site of importance, given that it confers breaks in secondary structure and causes turns in peptides or proteins. The P(−4)A mutation resulted in no significant loss of bioactivity. This observation suggests that there is no secondary structure within this region of the leader peptide and that this region is not important for PTM enzyme activity. Furthermore, there was no significant loss of bioactivity or notable interference with the activities of the LanB, LanC, LanD, and LanP PTM enzymes with the removal of the proline [P(−4)A] or the insertion of prolines [S(−8)P or N(−7)P]. We observed a slight statistically significant loss of activity only with the T(−2)A substitution. However, the bioactivity and mass of the isolated T(−2)A product show that it has undergone all dehydrations and decarboxylation. Secondary structure predictions suggest that the C-terminal end of the leader peptide is a random coil. The findings of our mutational study also suggest that this region is functionally unstructured. Terminal core peptide ring formations are essential for transport, and the coordinated cleavage at the −9 position is dependent upon the presence of the LanT transporter. LanP tolerates amino acid substitutions surrounding the primary cleavage site at position −1.

One possibility that we are exploring is whether the removal of the leader peptide from the core peptide has an inhibitory role in the production of mutacin 1140 or whether the leader peptide bound to core peptide is an activator of mutacin 1140 production.

The lack of specificity for both the upstream residues at positions −7 to 2 and the F at position +1, near the cleavage site, indicates that the protease does not require the recognition of a specific amino acid sequence near the cleavage site of the leader peptide. The specificity of cleavage by the LanP protease is unknown. It has been suggested that the specificity can come from structural elements within the core peptide or regions farther upstream of the core peptide or the protease recognizes the tertiary structure of the truncated leader peptide and core peptide. The latter explanation is unlikely, given that the P(−4)A substitution did not result in any loss of activity and that this region is predicted to be unstructured. Furthermore, the recognition of a region upstream of S(−8) is not likely, given that the leader peptide is only 8 amino acids and given the presence of the secondary cleavage site. Core peptide recognition by NisP for the cleavage of the leader peptide has been suggested for nisin (7, 22, 45), and given the current results, core peptide structure is important for the specificity of mutacin 1140 leader peptide cleavage by LanP. Additional studies will need to be done to determine whether LanP can recognize unmodified core peptide or whether PTM enzyme modifications are important for the recognition. The inability of the LanP protease to process the 2,508-Da product’s SR leader peptide when there was a cleavage at S(+3) of ring A suggests that the opening of ring A prevented LanP cleavage. This is also supported by the observation of a TR leader peptide variant in the lanT and lanP deletion strain that was not present in the lanT deletion strain, suggesting that the short TR sequence was removed by LanP. Therefore, the short leader peptide appears to be recognized by LanP. Furthermore, the major product in the ring A mutant was a truncated fragment (1,699 Da) of the leader peptide. These data suggest that an intact ring A is important for the specificity of cleavage by LanP, or they suggest that the ring A mutant product is susceptible to proteolytic cleavage by another protease. It is possible for LanP to function in coordination with the transporter LanT and that this interaction coordinates protease specificity. However, we have shown that LanP is efficient at processing the removal of truncated leader peptides in the LanT deletion strain.

The deletion of positions −7 to 2 resulted in cleavage within the core peptide S(+3) and cleavage at A(−3). This cleavage led to the formation of two peptide products with masses of 1,936 and 2,508 Da. The presence of the 2,508-Da fragment, which was also cleaved after S(+3) and held together by a thiourea linkage, suggests that dehydration, cyclization, and secondary cleavage function in a highly coordinated fashion. In our proposed model, the formation of the terminal lanthionine rings C and D stabilizes an interaction with the transporter (LanT), and this interaction coordinates secondary cleavage at the −9 position by an unidentified protease or proteolytic domain. There are two possible scenarios for the formation of the 1,936- and 2,508-Da products. The first scenario would involve a highly mobile leader peptide moving back and forth from LanB to LanC, enabling the coordination of dehydration and ring formation before coordination of the secondary cleavage that would yield the 1,936- and 2,508-Da prod-
ucts. A more simplified scenario would involve the binding of the leader peptide at a single position in which the core peptide can be acted upon by the PTM enzymes. This scenario would explain the formation of ring A, followed by cleavage after S(+3) and a subsequent lower-frequency cleavage after the −3 position that would free the peptide for transport. The mutant with the deletion from positions −7 to 2 presumably has a lower rate of dehydration at S(+3), which prevents cyclase activity. Without the formation of ring A, the peptide is cleaved and transported, yielding the 1,936-Da fragment. If ring A forms, the peptide is still cleaved at the +3 position within the ring but is not free to be transported until an additional cleavage occurs after the −3 position. These data support the view that secondary cleavage is a component integral to the biosynthesis of mutacin 1140 and that the PTM activity involving dehydration, cyclization, secondary cleavage, and transport is a dynamic process acting on the core peptide in a highly coordinated fashion.

Secondary cleavage of the leader peptide was observed in the lanT and lanP deletion strain, albeit the cleavage appeared to be disordered and led to the formation of a number of peptide products. This suggests that the transporter is involved in coordinating the secondary cleavage site. The isolation of a 1,936-Da product also suggests that transport of the core peptide is not directly dependent on the leader peptide. Given that cleavage at the secondary site is independent of LanT, it is logical to assume that the presence of the 1,936-Da product is recognized and the product is transported without an attached leader peptide.

The fact that cleavage still occurred regardless of the mutations made in the leader peptide suggests that the new cleavage site on the leader peptide is an integral component within the biosynthetic pathway of mutacin 1140. The new cleavage site appears to rely more on the length of the leader peptide sequence than the actual amino acid composition, with the exception of the proline insertions. It is possible that cleavage aids in the release of the modified peptide from a PTM enzyme before transport or having a truncated leader peptide results in a more energy-efficient approach for transporting the modified core peptide. Deletion of lanT resulted in the production of a fully modified product, but in the background of the lanT and lanP deletion strain, the ordered cleavage between A(−9) and S(−8) was not observed. This is similar to the random cleavage observed in our mutants with A(−9)P and S(−8)P mutations. These mutations may interfere with the efficiency of transport of the modified product, leaving it susceptible to cleavage by cytoplasmic proteases before being transported. Furthermore, we observed a dependency on ring D formation for the isolation of any product from the mutacin 1140 biosynthesis system. We believe that the interaction of the core peptide with the transporter coordinates the ordered cleavage at A(−9) and the absence of LanT leads to a random secondary cleavage within the leader peptide. S. mutans JH1140 contains an alternative transporter that efficiently transports these products out of the cell. A lantibiotic transporter in nonlantibiotic-producing strain S. mutans GS-5 has recently been reported (46). In this study, the authors demonstrated that the transporter confers protection against two-component lantibiotics similar to haloduracin and Smb. It is possible that lantibiotic transporters are more widespread in S. mutans, given their environmental exposure to lantibiotic and nonlantibiotic bacteriocins.

To date, the biosynthesis of only a few lantibiotics has been studied. Even though these studies have enriched our understanding of the PTM enzymes, we have barely scratched the surface of understanding how these enzymes coordinate their activities toward the synthesis of a fully functional lantibiotic without the production of a significant amount of side products. Studies with the mutacin 1140 biosynthesis system and other lantibiotic biosynthesis systems will promote our understanding of this dynamic process. The structural variants isolated from our mutagenesis study support a simplified model for mutacin 1140 biosynthesis, a model in which the leader peptide binds to one locus that coordinates the core peptide PTMs. PTMs on the core peptide would occur under a dynamic process, dictated by the distance of modifiable residues from PTM enzyme active sites and core peptide structural elements. The products isolated from our mutagenesis study do not support the current consensus within the field for class I lantibiotic biosynthesis, in which the leader peptide shuttles the core peptide to each PTM enzyme and transporter. For instance, the presence of products that were lacking dehydration or C-terminal decarboxylation following disruption of ring A or ring B formation supports the coordinated activities of PTM enzymes following successive steps of core peptide modifications. Furthermore, a dedicated secondary cleavage site at A(−9) is present. The ΔS(−8) or Δ(−7–2) truncation mutation resulted in a concomitant cleavage toward or within the core peptide, suggesting that the secondary cleavage is coordinated by the binding of the leader peptide upstream of the secondary cleavage site. Furthermore, the truncation of the C-terminal portion of the leader peptide also affected the rate of dehydration at S(+3) or cyclase activity, preventing ring A formation. These results further support the idea that the leader peptide is bound at one location and that the length of the C-terminal portion of the leader peptide is important for promoting an efficient interaction of the core peptide with the PTM enzymes.

The study provides a functional model for the biosynthesis of mutacin 1140 and other related lantibiotics. In this model, the PTM enzymes and transporter function in a highly coordinated fashion, while the binding of the leader peptide to one of the PTM enzymes facilitates the core peptide’s interaction with the PTM enzymes and transporter. Following the secondary cleavage of the leader peptide at A(−9), facilitated by interaction of the modified core peptide with the LanT transporter, the remaining portion of the leader peptide is released from the PTM enzyme and the core peptide with the truncated leader peptide is exported. Further studies of the mutacin 1140 biosynthetic pathway are under way to determine whether this model will satisfy all of our experimental observations.

ACKNOWLEDGMENTS

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REFERENCES


