The Specific Genes for Lantibiotic Mutacin II Biosynthesis in *Streptococcus mutans* T8 Are Clustered and Can Be Transferred En Bloc

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Mutacin II is a ribosomally synthesized peptide lantibiotic produced by group II *Streptococcus mutans*. DNA sequencing has revealed that the mutacin II biosynthetic gene cluster consists of seven specific open reading frames: a regulator (*mutR*), the prepromutacin structural gene (*mutA*), a modifying protein (*mutM*), an ABC transporter (*mutT*), and an immunity cluster (*mutFEG*). Transformations of a non-mutacin-producing strain, *S. mutans* UA159, and a mutacin I-producing strain, *S. mutans* UA140, with chromosomal DNA from *S. mutans* T8 with an *aphIII* marker inserted upstream of the mutacin II structural gene yielded transformants producing mutacin II and mutacins I and II, respectively.

Many bacteria produce antimicrobial peptides, termed bacteriocins. One group of bacteriocins is the lanthionine-containing lantibiotic group. The lantibiotics are ribosomally synthesized as prepeptides that undergo several posttranslational modification events (20, 21). The posttranslational modifications involve dehydration of the hydroxyl amino acids serine and threonine and addition of sulfur atoms of cysteines across the formed double bonds, leading to formation of the thioether amino acids lanthionine and methyllanthionine, respectively. Cleavage of the leader peptide liberates the active final product.

Mutacin II (4, 17) has been purified from a human isolate, *Streptococcus mutans* T8, and characterized (7, 15, 16). Mutacin II, a small thermostable peptide, has a molecular mass of 3,245 Da and exhibits bactericidal activity over a wide range of pHs against several gram-positive bacteria by inhibiting energy metabolism of sensitive cells (7). Mature mutacin II contains two lanthionine residues, one methyllanthionine, and one dihydro amino acid residue (16). Accordingly, mutacin II is a member of the lantibiotic family of antibiotics (21).

Two genes, *mutA* and *mutM*, encoding the prepromutacin and modification enzyme, respectively, have been cloned, sequenced, and characterized (29). The deduced primary sequence of mutacin II showed the presence of 27 amino acids in mature mutacin II, including three cysteine residues and four hydroxy amino acids (29). In this study, we provide evidence that all of the specific genes for mutacin II production are clustered and can be transferred as a unit. In addition, we identified the rest of the mutacin II biosynthetic genes in the cluster. Moreover, we constructed a strain capable of producing both mutacin I and mutacin II.

Transformation of non-mutacin-producing *S. mutans* UA159. In order to have a selection marker for the transformation process, we inserted the kanamycin resistance gene (*aphIII*) from *Streptococcus faecalis* (27) into the chromosomal DNA of *S. mutans* T8 at the *HpaI* site upstream of the mutacin II structural gene (*mutA*) by using a plasmid, pCBM6 (5). Plasmid pCBM6 was constructed in previous structure-activity

study (5). The insert in pCBM6 was linearized by restriction enzyme *XbaI* digestion and then transformed into competent *S. mutans* T8 by a previously published method (22). The kanamycin resistance gene (*aphIII*) was integrated into the chromosome via the mechanism of double-crossover recombination. The resultant transformants were mutacin positive and kanamycin resistant and were designated *S. mutans* T8kan. Chromosomal DNA from strain T8kan was isolated with the QIAGEN genomic-tip DNA isolation kit (QIAGEN, Santa Clarita, Calif.) and used to transform *S. mutans* UA159, which is naturally competent and non-mutacin producing (22). Randomly selected kanamycin-resistant transformants were tested for mutacin production by using a deferred-antagonism assay (4, 17). All transformants tested produced mutacin. As shown in Fig. 1, three randomly picked UA159 transformants, unlike the parental strain UA159, produced mutacin and inhibited the growth of the indicator strain, *Streptococcus sobrinus* OMZ176.

To further confirm the transfer of the mutacin II biosynthetic gene(s) into these transformants, Southern blot analysis was carried out with *mutA* and *mutM* as the probe. Chromosomal DNA isolated from the three randomly picked transformants was digested with the restriction enzyme *HindIII*. Restriction fragments were resolved on 0.65% agarose gels, transferred to a nylon membrane after denaturation and neutralization, and hybridized to the probe. The results (Fig. 2B) confirmed transfer of the biosynthetic genes, *mutA* and *mutM*, and a contiguous downstream region by competence transformation. Comparison of chromosomal DNA restriction patterns of the transformants and the parental strain UA159 (Fig. 2A) showed that all transformants were derivatives of the parental strain.

Biosynthesis of lantibiotics involves complex posttranslational modifications (12, 20). While Southern blot analysis indicated the transfer of the mutacin II biosynthetic genes *mutA* and *mutM* and the downstream region into these transformants, it was not obvious whether the active mutacins from these transformants were fully modified, as was the wild type from T8. To verify the correct processing of the *mutA* gene product in these transformants, we purified the mutacin from these transformants, as described previously (15), and analyzed it by electrospray ionization mass spectroscopy on a PE Sciex API III biomolecular mass analyzer (15). The results (Fig. 3)

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FIG. 1. Deferred-antagonism assay of mutacin phenotypes in selected *S. mutans* strains. 1, T8 wild type; 2 to 4, UA159 transformants; 5, UA159 wild type; 6, indicator strain *S. sobrinus* OMZ176. Mutacin production was indicated by the inhibition zones around the producing strains (strain T8 and UA159 transformants) in the lawn of *S. sobrinus* OMZ176. The absence of such an inhibition zone indicated a lack of mutacin production (parental strain UA159 and the negative control, *S. sobrinus* OMZ176).

showed that the mutacins from the *S. mutans* UA159 transformants and *S. mutans* T8 (15) were identical, indicating that the transferred genetic determinants were sufficient to catalyze the synthesis of fully modified lantibiotic molecules.

Transformation of mutacin I-producing *S. mutans* UA140.

The above results suggested that the entire mutacin biosynthetic locus was transferred into a non-mutacin-producing strain as a unit via competence transformation. It was not known, however, whether this locus could be also transferred into strains producing other mutacins. A group I strain, *S. mutans* UA140, which produces mutacin I (4), was transformed with the chromosomal DNA from strain T8kan. The resultant kanamycin-resistant transformants inhibited the growth of strain T8, as shown in Fig. 4A, suggesting that these transformants continued to produce mutacin I. Mutacin II production by these transformants was indicated by their ability to inhibit the growth of *Staphylococcus aureus* MSSA4, a strain susceptible only to mutacin II, in a deferred-antagonism assay (Fig. 4B). The Southern blot (Fig. 2B, lanes 7 to 9) indicated the successful transfer of the mutacin II biosynthetic operon, which was absent in the UA140 wild type (lane 6). The restriction patterns of the corresponding chromosomal DNA preparations (Fig. 2A, lanes 6 to 9) confirmed that these transformants were truly derivatives of UA140.

Identification and characterization of the mutacin II biosynthetic gene cluster. The above-mentioned results indicated that mutacin II-specific genes are likely to be clustered. To confirm this, up- and downstream fragments of the mutacin II structural gene (*mutA*) from *S. mutans* T8 were cloned by single-specific-primer PCR chromosomal walking (23, 24). The cloned fragments were sequenced by automated sequencing (Applied Biosystems 373A DNA sequencer), and the resultant sequence data were analyzed by using several programs in the

FIG. 2. Restriction and Southern blot analysis of *S. mutans* mutacin-producing and non-mutacin-producing strains and corresponding transformants. Chromosomal DNA was digested with *Hind*III, and the resultant fragments were resolved on a 0.65% agarose gel in Tris-borate-EDTA buffer and photographed under UV light (A). The gel was transferred to a nylon membrane and probed with part of the mutacin II biosynthetic operon (*mutAM*) (B). Lanes: 1, T8 wild type; 2, UA159 wild type; 3 to 5, UA159 transformants; 6, UA140 wild type; 7 to 9, UA140 transformants. DNA marker sizes are indicated on the left side of each panel, in kilobases.

GCG package (Genetics Computer Group, Madison, Wis.). The entire mutacin II biosynthetic gene cluster was determined.

This cluster consists of seven genes (Fig. 5) in the order *mutRAMTFEG*, based on BLAST similarity search. Southern blot analyses with an internal portion of each gene as a probe indicated that these seven genes exist only in mutacin II-producing strains (data not shown). In addition to the previously identified MutA and MutM (29), the products of five additional open reading frames (MutR, MutT, MutF, MutE, and MutG) were identified. The gene upstream of *mutA* was designated *mutR* because it encodes a protein with similarity (25% identity and 49% positives) to a positive transcriptional regulator, Rgg (25, 26), which regulates expression of glucosyltransferase G and influences the Spp phenotype of *Streptococcus gordonii* Challis. MutR is therefore proposed to be the regulator for the mutacin biosynthetic operon. Internal disruption of *mutR* resulted in mutants that did not produce any mutacin, due to abolished transcription of *mutA* (data not shown). This result further supported the proposed function of MutR as a regulator. Approximately 130 bp upstream of *mutR* resides a putative structural gene with a deduced amino acid sequence having similarity to transposases (*tra*) from lactococcal insertion elements (9, 28, 30) but lacking a translation start codon and a ribosomal binding site. PCR analyses indicated that this transposase does not exist in *S. mutans* UA140 (data not

FIG. 3. Electrospray ionization mass spectroscopic analysis of purified mutacin isolated from *S. mutans* UA159 transformants by a previously described method (15). The molecular mass indicated by multiple charged ions was calculated as $3,245.4 \pm 0.6$ Da, which is identical to that of mutacin II from *S. mutans* T8 (15).

shown). A 1.2-kb intergenic region and CTP synthetase (UTP-ammonium ligase) gene follows the *tra* gene.

The amino acid sequence deduced downstream of the *mutM* gene, MutT, exhibits a high degree of similarity with sequences of ATP-dependent transport proteins, especially LanT (38% identity and 59% positives) of lactacin 481 from *Lactococcus lactis* (18). Based on what is known about LanT, it is reasonable to propose that MutT has the dual function of cleaving the leader peptide and excreting mature mutacin. Insertion inactivation of *mutT* with a terminatorless antibiotic cassette completely abolished mutacin production (data not shown).

The products of the open reading frames after MutT were MutFEG. MutF showed its strongest sequence identities with LacF (44% identity and 65% positives) (19) and several other immunity proteins and ATP-binding membrane proteins. Thus, it is likely that MutF is involved in mutacin immunity. MutF is an ATP-binding protein without a transmembrane domain, and MutE and MutG are membrane proteins (data not shown) with homology to Cdd4, Cdd3, and Cdd2 from *Clostridium difficile* (2). As *cdd4*, *cdd3*, and *cdd2* do in *C. difficile*, *mutF*, *mutE*, and *mutG* code for an ABC transporter system, which is likely to be involved in mutacin immunity. Downstream of *mutG* was a 0.7-kb intergenic region followed by a fructose biphosphate adolase (*fba*) gene.

Genes for lantibiotic biosynthesis (8, 11, 14) can be found on plasmids (epidermin, Pep5, cytolysin, lactacin 481, lactocin S, and streptococcin A-FF22), on a conjugative transposon (nisin), or on the chromosome (subtilin, epilancin K7, salivaricin A, and variacin). Buchman et al. (3) suggested that many, if not all, lantibiotics probably evolved from a common ancestor, and

the ability to make lantibiotics has become dispersed among gram-positive bacteria by transfer of mobile genetic elements such as plasmids or transposons that encode lantibiotics. Although mutacin II biosynthesis genes are located on the chromosome, the presence of a silent transposase gene (*tra*) in the upstream region of the operon suggested that the ability to produce mutacin II in *S. mutans* T8 might be obtained from an ancestral transposon. It may be that, after entering a new host (for example, *S. mutans* T8), this ancestral transposon began to evolve to suit the needs of the new host by the usual means of spontaneous mutation and natural selection. Once stably situated within the host chromosome, the *tra* function, we hypothesize, may have become nonfunctional.

One way to identify the genes required for lantibiotic biosynthesis is to transfer the capacity for lantibiotic production from a producer strain to a nonproducer strain. Conversion of a non-bacteriocin-producing strain into a bacteriocin producer by transformation was reported for several lantibiotics, including subtilin (13), epidermin (1), nisin (10), and lactacin 481 (18). For subtilin, conversion was achieved by competence transformation from a natural producer (*Bacillus subtilis* ATCC 6633) into a nonproducing strain (*B. subtilis* 168), and a 40-kb region of chromosomal DNA was associated with conversion (13). For epidermin production, the transfer of an 8-kb DNA fragment containing six complete open reading frames into heterologous strains (*Staphylococcus carnosus* and *Staphylococcus xylosus*) was sufficient for antibacterial activity (1). The genes for nisin production are within a 68- to 70-kb transposon that can be transferred among strains of *L. lactis* by conjugative transposition (10). In the case of lactacin 481, two genes, *lcnA*

FIG. 4. Deferred-antagonism assay of the mutacin I and II phenotypes in selected *S. mutans* strains. (A) When *S. mutans* T8 was used as the indicator strain, the inhibition zones around *S. mutans* UA140 wild type (5) and its transformants (2 to 4) indicated mutacin I production in these strains, while the absence of such an inhibition zone around T8 wild type (1) indicated that mutacin I was not produced by this strain. (B) Mutacin II production by T8 wild type (1) and UA140 transformants (2 to 4) was indicated by their ability to inhibit growth of *S. aureus* MSSA4.

and *lcnM*, cloned on a plasmid vector were sufficient to convert a nonproducing strain into a lacticin-producing strain (18). Although *mutA* and *mutM* have high homology with *lcnA* and *lcnM*, this is not the case in the conversion of *S. mutans* UA159. Transforming only *mutA* and *mutM* is not sufficient to convert the wild-type strain UA159 into a mutacin-producing strain (unpublished results). The conversion of strain UA159 into a

FIG. 5. Genetic organization of the mutacin II biosynthetic gene cluster. The cluster consists of seven specific genes in the following order: regulator gene (*mutR*), structural gene (*mutA*), modifying enzyme gene (*mutM*), transporter and leader peptidase gene (*mutT*), and accessory self-protection genes (*mutFEG*). Upstream of *mutR* is a silent transposase gene (*tra*).

mutacin producer by integration of a fragment of T8 DNA does not conclusively prove that all of the genes required for mutacin production reside on the integrated DNA. The integrated DNA may merely provide specific genes required for mutacin II production. Another required regulator gene(s), such as the diacylglycerol kinase gene *dgc* (6), is already present in strain UA159.

In summary, we have identified the specific mutacin II biosynthetic gene cluster and successfully converted *S. mutans* UA159 into a mutacin II producer by competence transformation. In addition, we have successfully transformed a strain (UA140) producing another mutacin into a mutant strain with the ability to produce the resident mutacin I as well as the introduced mutacin II.

Nucleotide sequence accession numbers. The nucleotide sequences of *mutR*, *mutT*, and *mutFEG* have been deposited in the GenBank database under accession no. AF007761, AF026468, and AF082183, respectively.

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