

# Bacteriocin (Mutacin) Production by *Streptococcus mutans* Genome Sequence Reference Strain UA159: Elucidation of the Antimicrobial Repertoire by Genetic Dissection

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***Streptococcus mutans* UA159, the genome sequence reference strain, exhibits nonlantibiotic mutacin activity. In this study, bioinformatic and mutational analyses were employed to demonstrate that the antimicrobial repertoire of strain UA159 includes mutacin IV (specified by the *nlm* locus) and a newly identified bacteriocin, mutacin V (encoded by SMU.1914c).**

Bacteriocins are antibacterial proteins produced by bacteria that kill or inhibit the growth of closely related strains, and their biogenesis is thought to modulate the growth of competitor organisms occupying the same microecological niche (12). The bacteriocins produced by the oral bacterium *Streptococcus mutans* are termed mutacins and are divided into two groups: (i) the lanthionine-containing (lantibiotic) mutacins (6, 11, 16, 19, 20) and (ii) the unmodified mutacins (2, 21). While most bacteriocin activities characterized to date consist of a single active polypeptide, several two-component lantibiotic and non-lantibiotic bacteriocins have also been described, and these are dependent upon the collaborative activity of two polypeptides to exert their full antimicrobial activity (7, 9, 14, 15, 17, 23). Although in most cases both peptides act synergistically to effect target cell death, exceptions have been noted. For example, in the case of thermophilin 13, ThmB (which has no apparent intrinsic inhibitory activity of its own) functions to enhance the antibacterial activity of ThmA (14).

Mutacin IV is a putative two-component bacteriocin that is composed of the peptides NlmA\* and NlmB\*, both of which are generated by the posttranslational removal of a signal peptide from their respective prepeptides NlmA and NlmB (encoded by *nlmA* and *nlmB*, respectively [21]). However, the contribution of each peptide to mutacin IV activity has not been definitively established, since NlmA\* and NlmB\* have not yet been separately purified (21). Although mutacin IV was originally characterized from *S. mutans* UA140, the *nlmAB* locus (SMU.150 and SMU.151 for *nlmA* and *nlmB*, respectively) was identified in the genome reference strain UA159, although its function has not been assigned (21). Despite previous reports that strain UA159 is nonbacteriocinogenic (1, 5), we have found that this strain (kindly provided by J. Novak [University of Alabama—Birmingham]) readily exhibits antimicrobial activity when tested using a deferred antagonism protocol (2) against a panel of 84 indicator bacteria consisting of 74 streptococcal strains (belonging to nine distinct species) of human (oral) and animal origin, eight strains of *Lactococcus*

*lactis*, and two strains of *Micrococcus luteus* (Table 1). On the other hand, *S. mutans* and *Streptococcus sobrinus* indicator strains were not inhibited by UA159 (Table 1), an observation consistent with previous findings (5; J. D. F. Hale and J. R. Tagg, unpublished data). The presence of the *nlmAB* locus and absence of any intact operons encoding lantibiotic mutacins in strain UA159 (1), together with its ability to inhibit the growth of three strains (*Streptococcus sanguinis* ATCC 10556, *Streptococcus oralis* ATCC 10557 and *Streptococcus gordonii* ATCC 10558) (Table 1) previously used as mutacin IV indicators (21), led us to speculate that the inhibitory activity of this strain is, at least in part, due to mutacin IV. Therefore, the initial aim of our investigation was to use genetic dissection to establish whether the *nlmAB* locus is functional in strain UA159 and, if so, to define the individual roles of NlmA\* and NlmB\* in mutacin IV activity.

Appropriate *nlm* mutants of *S. mutans* strain UA159 were generated by replacing either *nlmA*, *nlmB*, or the *nlmAB* locus with the erythromycin resistance determinant *ermAM* (3) using a PCR ligation mutagenesis strategy (13) (Fig. 1) with the following modifications: (i) inclusion of an additional PCR step to generate more of each mutagenic construct prior to transformation, (ii) the use of fetal calf serum in place of horse serum in the transformation medium, and (iii) selection of transformants on brain heart infusion agar (Becton Dickinson) containing 0.5% (wt/vol) yeast extract and 2.5 µg/ml erythromycin. All PCR primers (Invitrogen Corp., Auckland, New Zealand) used in this study are listed in Table 2. *ermAM*, which lacks a transcription terminator at its 3' end, was inserted in the same transcriptional orientation as the gene of interest (Fig. 1), thus precluding any polar effects. The presence of the desired mutations in selected erythromycin-resistant transformants was verified by sequencing of the PCR products generated using appropriate *nlm*- or *ermAM*-specific primers (Table 2) and by Southern hybridization experiments (22).

In order to determine the functions of NlmA\* and NlmB\* in mutacin IV activity, mutants of strain UA159 containing individually disrupted *nlmA* (UAΔNlmA) or *nlmB* (UAΔNlmB) were constructed (Fig. 1). When both mutants were assayed for activity against the panel of 84 mutacin-sensitive indicator bacteria, growth inhibition of 66 strains (comprising all

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TABLE 1. Inhibitory spectra of *S. mutans* UA159 and its mutants

Indicator bacteria (no. of strains tested) <sup>a</sup>	No. of indicator strains inhibited by:													
	UA159	UAΔNmT <sup>b</sup>	UAΔNmA	UAΔNmB	UAΔNmAB	UAA (281/283)	UAA423	UAA1892	UAA (1895/1896)	UAA (1905/1906)	UAAΔ1914	UAA (1914/NimAB)		
<i>Streptococcus constellatus</i> (10)	10	0	0	10	0	10	10	10	10	10	10	0		
<i>Streptococcus gordonii</i> (7)	7	0	0	7	0	7	7	7	7	7	7	0		
" <i>Streptococcus milleri</i> " (3)	3	0	0	3	0	3	3	3	3	3	3	0		
<i>Streptococcus pyogenes</i> (10)	10	0	0	10	0	10	10	10	10	10	10	0		
<i>Streptococcus salivarius</i> (10)	10	0	0	10	0	10	10	10	10	10	10	0		
<i>Streptococcus sanguinis</i> (5)	5	0	0	5	0	5	5	5	5	5	5	0		
<i>Streptococcus uberis</i> (10)	10	0	0	10	0	10	10	10	10	10	10	0		
<i>Streptococcus mitis</i> batch A (6)	6	0	0	6	0	6	6	6	6	6	6	0		
<i>Streptococcus oralis</i> batch A (5)	5	0	0	5	0	5	5	5	5	5	5	0		
<i>Lactococcus lactis</i> (8)	8	0	8	8	8	8	8	8	8	8	8	0		
<i>Micrococcus luteus</i> (2)	2	0	2	2	2	2	2	2	2	2	2	0		
<i>S. mitis</i> batch B (3)	3	0	3	3	3	3	3	3	3	3	3	0		
<i>S. oralis</i> batch B (2)	2	0	2	2	2	2	2	2	2	2	2	0		
<i>S. mitis</i> SK648 (1)	1	0	1	1	1	1	1	1	1	1	1	1		
<i>S. oralis</i> strains OB717 and OB718 (2)	2	0	2	2	2	2	2	2	2	2	2	2		
<i>S. mutans</i> (2)	0	NT <sup>c</sup>	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT		
<i>Streptococcus sobrinus</i> (1)	0	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT		

<sup>a</sup> *S. mitis* and *S. oralis* strains are divided into batches depending on their mutacin sensitivities (see text).<sup>b</sup> Strain UAΔNmT (10) was used as the null mutant (negative control) for all deferred antagonism assays.<sup>c</sup> NT, not tested.

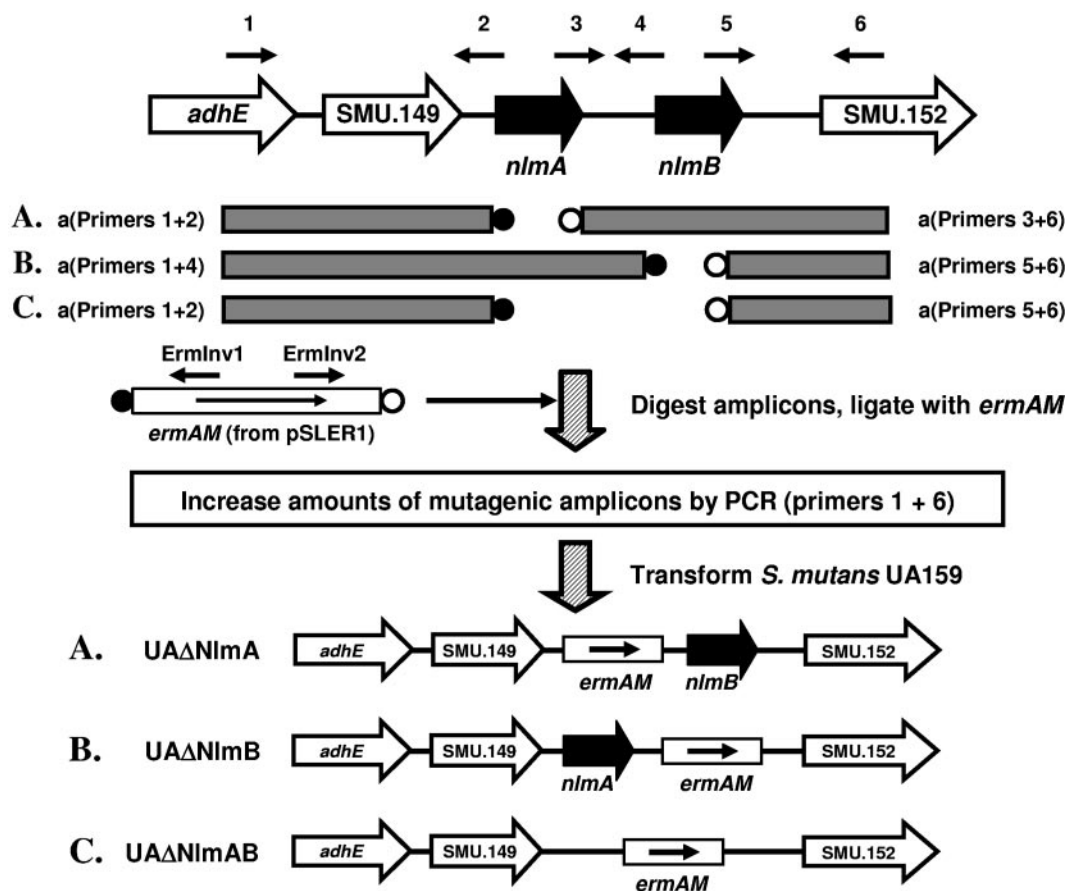


FIG. 1. Allelic replacement, by PCR ligation mutagenesis (13), of genes (*nlmAB*) encoding the peptides comprising mutacin IV. Other putative bacteriocin-encoding genes investigated in this study (see Fig. 2) were also inactivated using this strategy. For clarity, the loci are not drawn to scale. The PCR primers shown are numbered as follows: 1, NlmABUpF; 2, NlmAUpR; 3, NlmADwF; 4, NlmBUpR; 5, NlmBDwF; 6, NlmABDwR. The filled and unfilled circles refer to EcoRI and PstI restriction sites, respectively. The source of *ermAM* was pSLER1 (pSL1190 [4] containing *ermAM* cloned into the NdeI site; laboratory collection) and *ermAM* was always inserted in the same transcriptional orientation as the gene of interest. The locations of the ErmInv PCR primers used to confirm each mutation are also shown. *adhE*, putative alcohol-acetaldehyde dehydrogenase; *nlmA* and *nlmB*, prepeptides of NlmA\* and NlmB\*, respectively; SMU.149, putative transposase; SMU.152, hypothetical protein.

strains of *S. constellatus*, *S. gordonii*, *S. milleri*, *S. pyogenes*, *S. salivarius*, *S. sanguinis*, and *S. uberis*, as well as 6 [of 10] *S. mitis* strains and 5 [of 9] *S. oralis* strains) was dependent upon a functional *nlmA* gene (Table 1). In contrast, inactivation of *nlmB* did not appear to affect inhibitory activity against the same 66 strains (Table 1). The above observations were corroborated when selected strains were tested by a modification of the standard deferred antagonism assay (2) in which the number of producer cells inoculated was standardized (15- $\mu$ l drop inoculum of an 18-h Todd-Hewitt broth culture adjusted to an optical density at 600 nm of 0.02). No significant differences in the sizes of inhibitory zones were detected between wild-type UA159 and the *nlmB* mutant UAΔNlmB (Table 3). Taken collectively, our results demonstrate that the *nlm* locus is functional in *S. mutans* UA159 but also cast some doubt as to whether mutacin IV is a bona fide two-component bacteriocin. However, it is also possible that the range of indicator strains selected for this study may not have been sufficiently diverse to detect any effects conferred by NlmB\*.

Interestingly, growth of 18 of the 84 indicator strains (all

eight *L. lactis*, both *M. luteus*, four *S. mitis* and four *S. oralis*) did not appear to be affected by the individual inactivation of either *nlmA* or *nlmB* (Table 1). This raises the possibilities that either (i) each individual peptide possesses inhibitory activity against these strains or (ii) additional inhibitory agents (unrelated to mutacin IV) are produced by strain UA159. To resolve this unexpected finding, strain UAΔNlmAB was generated in which the entire *nlmAB* locus was deleted (Fig. 1). No loss of inhibitory activity was detected against any of these 18 indicator bacteria (Table 1), indicating that the antimicrobial repertoire of strain UA159 extends beyond what is attributable to mutacin IV.

We have recently reported that nonlantibiotic mutacin biogenesis in strain UA159 requires the ABC transporter NlmTE (10). Furthermore, a search of the UA159 genome sequence revealed several loci (Fig. 2) encoding hypothetical peptides, each possessing a double-glycine-type leader sequence similar to that of NlmA (10). This implies that these peptides could be exported by NlmTE and would therefore be candidates for the additional observed antibacterial activities. Nine potential mutacin-encoding genes (SMU.281, SMU.283, SMU.423,

TABLE 2. PCR primers used in this study

Primer	Nucleotide sequence (5'–3') <sup>a</sup>	Location of primer (nucleotides)
NlmABUpF	CCACGTCAGCCTTACATTGAAGAGATGAAGC	7676–7706
NlmAUpR	AAAAGAATTCCATCAAATGTTCAAATGCCTGTGTATCCA	8754–8783
NlmADwF <sup>b</sup>	AAAAGTGCAGGGCACTTGGGGACTCATTCGATCTCATTAAA	8927–8957
NlmBUpR <sup>b</sup>	AAAAGAATTCTTTAATTCATGGTATTAATTCCTCCATTCC	8967–8996
NlmBUpF	CGATCTGCAGGAGTTGGTGCAGGTTGGATCTGTAGTTTTTCC	9164–9194
NlmABDwR	GGCCCAACGAAAATCTTTGTGGAGATACG	9411–9440
Smu283UpF	TTCCTGTTGCTTATCTTGCTGGTTT	117–141
Smu283UpR	GCAGGAATTCCTCTCTGCTGTTTCAA	759–784
Smu283DwF	GGTGCTGCAGTTATTATTATGGCG	2259–2283
Smu283DwR	CCCATTATTACAAAACTGGAAGCAAAC	2798–2825
Smu423UpF	CAGAAGATGAGCGAATGAAGTGAGC	57–81
Smu423UpR	CATACCCCTAGAAATTCCTAAACCTG	1089–1114
Smu423DwF	CGGCTGCAGGAGGCTTGGTCTGG	1161–1182
Smu423DwR	AGCGGCATCGTAATCTATCGTCAT	2167–2190
Smu1892UpF	TGGTTTGGAGGCTCGTATTCGTC	3701–3723
Smu1892UpR	CGTTTCCAGAATTCAGTTTGTGTTTT	2978–3003
Smu1892DwF	GCAGCTGCAGGCTAATAAAAAATCTGAGTTGCTGTAA	2782–2807
Smu1892DwR	CCAAGGAATAGTAAAAATGGAATAAGG	2193–2219
Smu1895UpF	TCGCAAGTGAGCTAATGAATAATCCG	5582–5607
Smu1895UpR	ACCTAAAGCGCCTGTTTCAATTCGTA	4894–4918
Smu1895DwF	GTTGGTGCACCTGCAGGATCTTTTTAC	4622–4648
Smu1895DwR	CTTTTGACCGCTTGTGGAATGGC	3558–3581
Smu1905UpF	AACTTTTGGGTGCAGGTTCAGAA	1791–1813
Smu1905UpR	TCGGCTGAATTCCTCACTACATCC	747–769
Smu1905DwF	TGTGGGAAGTAGTATTATGATGG	347–370
Smu1905DwR	CGGCCAATATATAAGGTATAGCTGTTTTTC	9877–9905
Smu1914UpF	TAGTTTTATCTTCTCATCCACGACA	5797–5821
Smu1914UpR	GAAAGTGAATTCATTATCCATTACG	5243–5266
Smu1914DwF	CTTTGGGGCTATTGCTGCAGGAA	5094–5116
Smu1914DwR	CATGCTTTTCTATGCGGTCTATTGA	4029–4053
ErmInv1	CCAGTTCGCGTTAAATGCCCTTTACCTG	374–403
ErmInv2	CTTACCCGCCATACCACAGATGTTCCAGAT	784–813
KanF	GATAAACCCAGCGAATTCATTGAGGTGATAGGTAAG	1–36
KanR	TCGATACAAATTCCTCTGCAGGCGCTCTAGACCCCTATC	1451–1488

<sup>a</sup> The NlmX, Smu283x, Smu1892x/1895x (including Smu1905DwR), Smu1905x/1914x, ErmInv, and Kan primers were designed based on sequences with GenBank accession numbers AE014866, AE014877, AE015015, AE015016, Y00116, and V01547, respectively. Restriction sites for EcoRI (GAATTC), PstI (CTGCAG), or SpeI (ACTAGT) incorporated into the primer are underlined.

<sup>b</sup> The partners for the NlmADwF and NlmBUpR primers during PCR are NlmABDwR and NlmABUpF, respectively.

SMU.1892c, SMU.1895c, SMU.1896c, SMU.1905c, SMU.1906c, and SMU.1914c) (Fig. 2) were replaced with *ermAM*, either individually or in pairs, to yield the six mutants UAΔ(281/283), UAΔ423, UAΔ1892, UAΔ(1895/1896), UAΔ(1905/1906), and UAΔ1914, respectively. When tested against the 18 indicator strains that are not sensitive to mutacin IV, loss of inhibitory activity against all but three strains (*S. mitis* SK648, and *S. oralis* strains OB717 and OB718) was only observed with the

SMU.1914c mutant, UAΔ1914 (Table 1). As expected, a double-knockout mutant of UAΔ1914, strain UAΔ(1914/NlmAB), in which the *nlmAB* locus was replaced with the kanamycin resistance (600 μg/ml) determinant *aphA3* (24), exhibited the combined inhibitory spectra of both UAΔNlmAB and UAΔ1914 (Table 1). Thus, SMU.1914c appears to encode an additional novel mutacin. In order to maintain consistency in the nomenclature of mutacins, we propose that SMU.1914c be designated *nlmC* and its gene product be named mutacin V.

In conclusion, we have used bioinformatic and mutational analyses to show that *S. mutans* UA159 produces (i) a known bacteriocin, mutacin IV, which is a major contributor to the antimicrobial spectrum of the strain, and (ii) a newly identified antimicrobial agent, mutacin V, which is mainly active against nonstreptococcal targets. However, an additional inhibitory agent(s) active against certain *S. mitis* and *S. oralis* strains could not be identified using our existing experimental strategy. Furthermore, genetic dissection of the *nlmAB* locus does not support the hypothesis that mutacin IV is a two-component mutacin and thus the role, if any, of NlmB\* remains enigmatic. Clearly, *S. mutans* is a prodigious producer of mutacins, a

TABLE 3. Drop inoculum deferred antagonism assay for *S. mutans* UA159 and its *nlm* mutants

Indicator strain	Inhibitory zone diameter (mm) <sup>a</sup>			
	Wild-type	UAΔNlmA	UAΔNlmB	UAΔNlmAB
<i>S. gordonii</i> C219	22	0	21	0
<i>S. milleri</i> NCTC 10708	28	0	27	0
<i>S. mitis</i> OB714	24	0	24	0
<i>S. salivarius</i> 20P3	15	0	15	0
<i>S. uberis</i> ATCC 27958	21	0	20	0

<sup>a</sup> Aliquots (15 μl) of each producer strain were deposited as drop inocula (11-mm diameter) on the surface of the mutacin test medium (2).

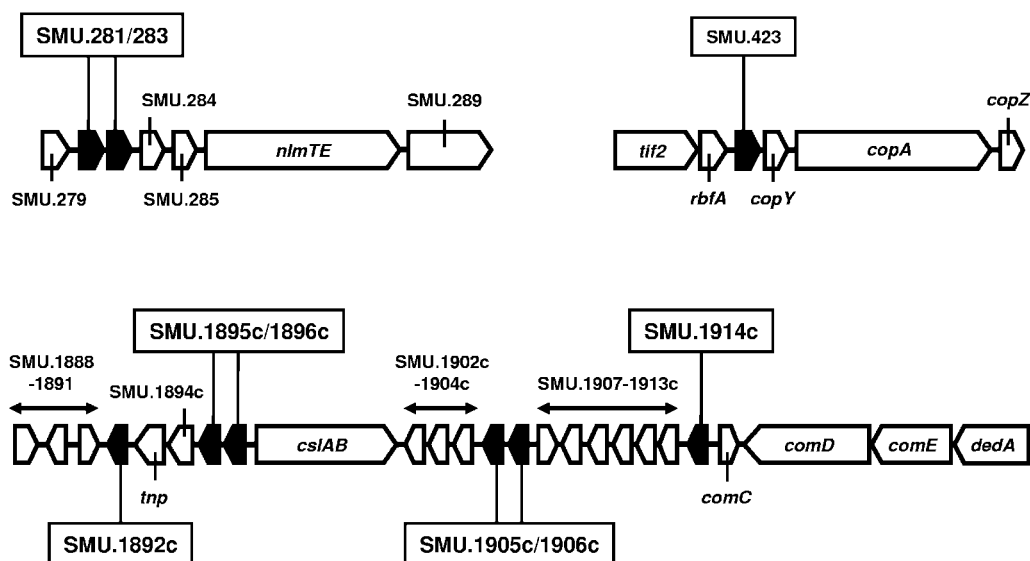


FIG. 2. Genomic organization of the open reading frames (ORFs; filled pentagons) encoding potential nonantibiotic mutacins investigated in this study. For simplicity, the loci are not drawn to scale. ORFs designated by their GenBank locus tags (e.g., SMU.283) encode hypothetical proteins. Note that SMU.282 does not exist. The translational orientation of each ORF is also indicated. *nlmTE*, ABC transporter required for export of nonantibiotic mutacins (10); *tif2*, translation initiation factor 2; *rbfA*, ribosome binding factor A; *copY*, putative transcriptional regulator; *copAZ*, components of a copper transport system; *tnp*, putative transposase of ISS<sub>SmuI</sub> (1); *csIAB*, ABC transport system required for natural transformation (10, 18); *comCDE*, signal transduction system essential for development of natural competence (8); *dedA*, putative membrane-associated protein.

deeper understanding of which may help elucidate how this oral pathogen establishes in the oral cavity.

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