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Identification of *nlmTE*, the Locus Encoding the ABC Transport System Required for Export of Nonlantibiotic Mutacins in *Streptococcus mutans*

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Streptococcus mutans **UA159, the genome sequence reference strain, exhibits nonlantibiotic bacteriocin (mutacin) activity. In this study, we have combined bioinformatic and mutational analyses to identify the ABC transporter designated NlmTE, which is required for mutacin biogenesis in strain UA159 as well as in another mutacin producer, *S. mutans* N.**

The oral bacterium *Streptococcus mutans* produces small (<10-kDa), ribosomally synthesized antimicrobial peptides (bacteriocins) termed mutacins, which are generally divided into two categories: (i) the posttranslationally modified, lanthionine-containing (lantibiotic) mutacins (6, 11, 18, 20, 21) and (ii) the unmodified mutacins (3, 4, 9, 22). A general feature of the secretion leader sequences of many peptide bacteriocins produced by gram-positive bacteria (8, 10, 15), as well as some produced by gram-negative species (17), is a highly conserved double-glycine (GG) motif. This GG motif immediately precedes the site where specific proteolytic cleavage of the signal peptide occurs during export of the bacteriocin by ATP-binding cassette (ABC) transport systems (8, 10). The genes encoding the regulatory, biosynthetic, export, and immunity elements of the lantibiotic mutacins are entirely contained within multigene modules (6, 11, 18, 20, 21), similar to those of other lantibiotic systems (15, 16, 25). In contrast, the currently defined genetic loci for nonlantibiotic mutacins do not appear to contain any dedicated export- or immunity-associated genes juxtaposed with the mutacin structural genes (9, 22).

S. mutans UA159, the genome sequence reference strain, does not possess any genetic loci that encode lantibiotic mutacins (1). However, we have previously reported that strain UA159 produces the nonlantibiotic mutacin IV (22), as well as an additional, as yet unidentified, inhibitory agent(s) (J. D. F. Hale et al., submitted for publication). We report here the use of bioinformatic and mutational analyses to identify the locus encoding the ABC transport system responsible for mutacin processing and export in *S. mutans* strains UA159 and N (producer of the nonlantibiotic mutacin N) (3, 9).

Typical nonlantibiotic bacteriocin export systems consist of a ca. 700-amino-acid ABC transporter and a ca. 400-amino-acid accessory protein (8, 10). The ABC transporter component is characteristically composed of three domains: (i) an N-terminal peptidase domain, (ii) a membrane-spanning permease, and (iii) a C-terminal ATPase domain containing the characteristic Walker motifs (8, 10). When a BLAST (2) search of the *S. mutans* UA159 genome sequence was conducted by using the amino acid sequences of several known nonlantibiotic bacteriocin ABC exporters (e.g., PlnG from *Lactobacillus plantarum* [GenBank accession no. CAA64189] and CbnT from *Carnobacterium piscicola* [GenBank accession no. AAB81307]) as query sequences, the translated products of only two open reading frames were found to possess the characteristics described above (Fig. 1): SMU.286 (*comA*), encoding the 760-amino-acid ComA ABC transporter that plays a role in biofilm formation (27), and SMU.1881c (designated *orf763*), which specifies a 763-amino-acid protein with 73% identity (86% similarity) to ComA. While the gene immediately downstream of *comA* was *comB* (encoding the ABC transporter accessory protein), no *comB* counterpart adjacent to *orf763* was found (Fig. 1). Although not detected by the BLAST algorithm, a third ABC transporter/accessory protein-encoding locus, *cslAB* (SMU.1897 to SMU.1900 [Fig. 1]), has previously been reported to be essential for natural transformation in *S. mutans* (19).

In the present study, the *comA*, *comB*, *orf763*, *cslA*, and *cslB* genes of *S. mutans* strain UA159 were individually inactivated by allelic replacement with the erythromycin resistance determinant *ermAM* (5) by using the PCR ligation mutagenesis strategy described by Lau et al. (13), ultimately generating UAΔComA, UAΔComB, UAΔORF763, UAΔCslA, and UAΔCslB, respectively. The source of *ermAM* was pSLER1 (pSL1190 [Pharmacia] containing *ermAM* cloned into the NdeI site). *ermAM* was inserted in the same transcriptional orientation as the gene of interest, and the absence of a transcription terminator downstream of *ermAM* was expected to preclude any polar effects. All PCR primers (Invitrogen) used in this study are listed in Table 1. Transformants were selected on brain heart infusion agar (Becton Dickinson) supplemented with 0.5% (wt/vol) yeast extract containing 2.5 μg/ml erythromycin. The presence of the desired specific mutations was confirmed by Southern hybridization (23) and by sequencing of the PCR products generated by using the appropriate primer combinations (Table 1).

The various mutants chosen for further study were then tested for mutacin production by use of a standard deferred antagonism protocol (3) against a panel of 85 indicator bacte-

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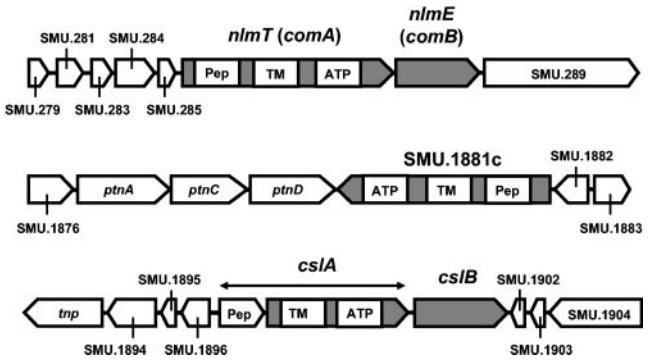

FIG. 1. Genomic organization of the open reading frames (symbolized by the filled pentagons) encoding the three putative ABC transport systems investigated in this study. The N-terminal peptidase (Pep), transmembrane permease (TM), and C-terminal ATP-binding (ATP) domains of the ABC transporter component are also shown. Note that the CslA ABC transporter appears to be the product of two open reading frames (SMU.1897 and SMU.1898). Genes encoding hypothetical proteins are identified by their GenBank locus tags, e.g., SMU.279. The translational orientation of each open reading frame is also indicated. *nlmTE*, ABC transporter required for export of nonlantibiotic mutacins (see the text); *ptnACD*, components of the mannose-specific phosphotransferase system; *tnp*, putative transposase of IS*Smu1* (1); *cslAB*, ABC transport system required for natural transformation (19).

ria (Table 2), 64 of which have previously been shown to be sensitive to mutacin IV, while the remaining 21 (comprising mainly nonstreptococcal strains, e.g., *Lactococcus lactis* and *Micrococcus luteus*) are inhibited by an additional, as yet unidentified inhibitory agent(s) produced by strain UA159 (Hale et al., submitted). Inactivation of either *comA* or *comB* resulted in complete abrogation of bacteriocin elaboration by *S. mutans* UA159, whereas deletion of *orf763*, *cslA*, or *cslB* had no discernible effect (Table 2), indicating that the ComAB ABC transporter alone was essential for the export of both mutacin IV and the additional inhibitory agent(s). Such a result was not unexpected, as a survey of mutacin-like prepeptides potentially encoded by the *S. mutans* UA159 genome reveals significant similarity between their secretion signal peptides and that of the NlmA peptide (22) of mutacin IV (Fig. 2).

The development of natural competence for genetic transformation in *S. mutans* is believed to be analogous to that in *Streptococcus pneumoniae* (7, 12). In *S. pneumoniae*, competence development occurs in two stages. The first (early) stage consists of a quorum-sensing signal transduction circuit, initiated by the binding of competence-stimulating peptide (CSP) to its cognate cell surface receptor ComD (a histidine kinase), which is then perpetuated by ComE, the global transcriptional regulator of competence (12). The second (late) stage involves the synthesis and assembly of the DNA uptake and processing machinery (12). The precursor of CSP, ComC, contains the GG motif in its signal peptide, and its export involves an ABC transport system which is designated ComAB in both *S. pneumoniae* and *Streptococcus gordonii* (12, 14). In the case of *S. mutans*, however, there appears to be some confusion in the nomenclature in that SMU.286/SMU.287 has been designated *comAB* (27) although *cslAB* has been previously shown to be essential for natural transformation (19). It is noteworthy that the role of *comAB* in natural competence in *S. mutans* has not been confirmed (27). Furthermore, the signal peptide of ComC differs from those of mutacin IV and other mutacin-like peptides (Fig. 2). In order to resolve this apparent discrepancy, we assessed the transformabilities of strains UA159, UAΔComA, UAΔComB, UAΔCslA, and UAΔCslB essentially as described by Petersen and Scheie (19) with minor modifications. Briefly,

TABLE 1. PCR primers used in this study

Primer	Nucleotide sequence (5′–3′)[a]	Location of primer (nucleotides)
ComAUpF	CAAAAATCATAGCAATAT	2410–2427
ComAUpR	CCCCCCGAATTCATAAATAACTTGTTTCAT	3378–3395
ComADwF	AAAACTGCAGATTATAACCTGTTTAATT	5641–5658
ComADwR	TTTGCTATTTTTCTTAGA	6457–6476
ComBUpF	TTTAGATGGTGATATTTCGTTTGA	4946–4969
ComBUpR	CCCCCCGAATTCTTTTTCTTTTCCTTCTCTTCTCG	6007–6029
ComBDwF	AAAACTGCAGAAAGGGGCAAACCGCTCGTCT	6315–6335
ComBDwR	TTTTGATTTGGTTGCCTGAAGC	7361–7382
ORF763UpF	GGTGAATTCACTCGTGTCTTGGGGTATTGGTGG	7551–7572
ORF763UpR	ACCTCTAGACATCTATGTAGTCAGGCTAGTGCTC	6965–6991
ORF763DwF	CCGTCGCTGCCATAATTTTCTGCAGTGTTAGTT	4883–4516
ORF763DwR	GGCTTCTCGAGCAATATCAGGAAACATCCTAGG	3681–3702
CslAUpF	CATAATCTAGAGACACCCTTATTTGTGAACGACC	4222–4255
CslAUpR	GGATGAATTCACTACCGTTTACGATATTGCTG	5277–5307
CslADwF	CCAAACTGCAGTCTGCCAGAGTGGCTAATAA	6816–6846
CslADwR	CAGTCTTGTCTGCCATGTGCAGCAGGTTAT	7461–7490
CslBUpR[b]	ATGATAGCGTCTTCGGTAGAATTCAGCG	7652–7679
CslBDwF	GCATCAGTGTCGTCTTACCCTGCCTCAG	8738–8765
CslBDwR	ACATCTGCTCTTAAAGATACTCCTATTGCC	9561–9590
ErmInv1	CCAGTTCGCGTTAAATGCCCTTTACCTG	374–403
ErmInv2	CTTACCCGCCATACCACAGATGTTCCAGAT	784–813

[a] The ComAx primers were adapted from those described by Yoshida and Kuramitsu (27). The ComBx, ORF763x, CslAx/Bx, and ErmInv primers were designed based on sequences with GenBank accession numbers AE014877, AE015014, AE015015, and Y00116, respectively. Restriction sites for EcoRI (GAATTC), PstI (CTGCAG), and XbaI (TCTAGA) incorporated into the primer are underlined.

[b] The partner for this primer during PCR was CslADwF.

TABLE 2. Inhibitory spectra of wild-type *S. mutans* strains UA159 and N and their mutants

Indicator bacterium[a]	No. of indicator strains inhibited by:							
	UA159	UAΔNlmT (UAΔComA)	UAΔNlmE (UAΔComB)	UAΔORF763	UAΔCslA	UAΔCslB	N	NΔNlmT
Lactococcus lactis (7)	7	0	0	7	7	7	7	0
Micrococcus luteus (1)	1	0	0	1	1	1	1	0
Streptococcus constellatus (10)	10	0	0	10	10	10	10	0
Streptococcus gordonii (7)	7	0	0	7	7	7	7	0
"Streptococcus milleri" (3)	3	0	0	3	3	3	3	0
Streptococcus mitis (12)	12	0	0	12	12	12	12	0
Streptococcus oralis (10)	10	0	0	10	10	10	10	0
Streptococcus pyogenes (10)	10	0	0	10	10	10	10	0
Streptococcus salivarius (10)	10	0	0	10	10	10	10	0
Streptococcus sanguinis (5)	5	0	0	5	5	5	5	0
Streptococcus uberis (10)	10	0	0	10	10	10	10	0
Streptococcus mutans (5)	0	NT[b]	NT	NT	NT	NT	5	0

[a] The numbers of strains tested are shown in parentheses.
[b] NT, not tested.

donor DNA (1 μg/ml) was added to cultures at an optical density at 600 nm of 0.15 to 0.20, and incubation was continued until the culture attained an optical density at 600 nm of 0.85 to 0.90, at which point 100-μl aliquots of appropriate dilutions were plated onto Todd-Hewitt agar containing 600 μg/ml kanamycin. The donor DNA was plasmid pFX-ErmKan, which consists of the replicative backbone of pFX3 (26) and the genetic determinants for erythromycin (*ermAM* [5]) and kanamycin (*aphA3* [24]) resistance.

As shown in Table 3, the transformation frequencies attained by the *comA* and *comB* mutants were comparable to that of the wild type. In contrast, dramatic reductions (>99%) in the transformabilities of both UAΔCslA and UAΔCslB were observed (Table 3), which corroborates previous findings obtained with *csl* mutants (19), suggesting that the *csl* locus is essential for competence in *S. mutans*. The addition of 500 ng/ml synthetic CSP (NH_2-SGSLSTFFRLFNRSFTQALGK-COOH; microcollections GmbH, Germany) boosted the transformation frequency of strain UAΔCslA by >30-fold (data not shown), further supporting a role for CslAB as the transport system responsible for CSP secretion (19). Due to the role of SMU.286 (*comA*) and SMU.287 (*comB*) in mutacin production but not in genetic transformation, we propose that SMU.286 and SMU.287 should be redesignated *nlmT* (*n*on*l*antibiotic *m*utacin *t*ransporter) and *nlmE* (*nlmT* accessory protein), respectively.

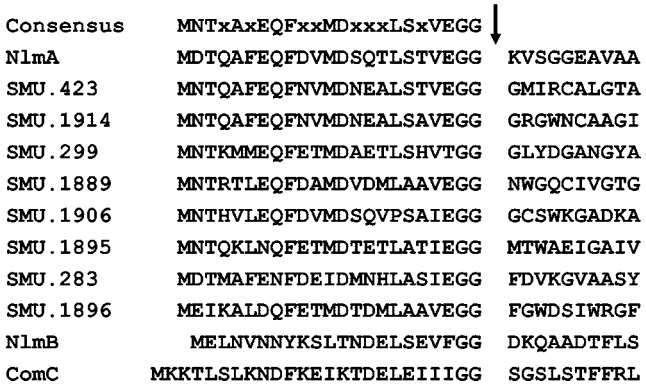

FIG. 2. Alignment of NlmA and NlmB (the prepeptides of the two-component nonlantibiotic mutacin IV [22]) with other putative prepeptides (in strain UA159) containing the double-glycine motif. BLAST similarities were obtained only when NlmA was used as the query sequence. The site where peptidase cleavage occurs during export is indicated by the inverted arrow. For simplicity, only the first 10 amino acids of each putative mature peptide are shown. ComC, the prepeptide which is processed to yield the competence-stimulating peptide, is included for additional comparison. The consensus sequence of the signal peptide displays only those amino acids present in at least five of the peptides listed.

In light of the findings described above, we decided to extend our study to inactivate (by allelic replacement with *ermAM*) the *nlmT* homologue in *S. mutans* N, a strain that produces the nonlantibiotic mutacin N (3, 4) and does not appear to contain any lantibiotic-associated genes (J. D. F. Hale, unpublished data). The inhibitory spectrum of mutacin N is distinctive compared to that of other mutacins in its ability to inhibit certain *S. mutans* strains (3, 4). The resulting *nlmT*-deficient mutant, NΔNlmT, failed to express any inhibitory activity (Table 2), indicating that the NlmTE ABC transporter is also required for nonlantibiotic mutacin export in *S. mutans* strain N.

In conclusion, we have determined that the *nlmTE* locus (SMU.286/SMU.287, previously designated *comAB*) encodes the ABC transporter required for nonlantibiotic mutacin biogenesis in *S. mutans* strains UA159 and N. Furthermore, we have resolved an apparent discrepancy in the nomenclature and function of two ABC-transporter-encoding loci in natural

TABLE 3. Transformation properties of *S. mutans* UA159 and its ABC transporter mutants

Strain	Transformation frequency (10^4 kanamycin-resistant CFU/ml)[a]
Wild-type UA159	1.5 (1.4–1.7)
UAΔNlmT (UAΔComA)	1.0 (0.95–1.1)
UAΔNlmE (UAΔComB)	1.4 (1.3–1.5)
UAΔCslA	0.015 (0.011–0.2)
UAΔCslB	0.014 (0.01–0.02)

[a] The average values (ranges) of three independent experiments are shown.

competence. While *cslAB* (19) is clearly involved in genetic transformation (possibly as the export mechanism for CSP), our results do not support a similar role for *comAB*.

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