Genetic basis for mutacin N and of its relationship to mutacin I

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Background & objectives: The mutans streptococci (MS) are a group of 7 species of dental caries-associated bacteria of which Streptococcus mutans and Streptococcus sobrinus are the most important in humans. Many MS produce bacteriocin-like inhibitory substances (BLIS), some of which have been characterised as small peptides capable of inhibiting the growth of closely-related species. These peptides have most commonly been referred to as mutacins. S. mutans strains N and UA140 appear to have closely similar BLIS activities. Both produce mutacins that seem to target the same species of bacteria. On closer analysis however, these two strains have been shown to produce distinctly different mutacins, known as mutacin N and mutacin I respectively. In the present study the mutacin N structural gene (mutN) was cloned and compared with the mutacin I structural gene (mutA).

Methods: Cloning and sequencing of S. mutans N was done. The distribution of mutN using DNA from 216 streptococcal strains was determined by dot blotting.

Results: Mut N was cloned and sequenced from an 1800 bp Bam HI/Eco RI fragment. PCR with the mutN primers mutNF and mutNR on the four mutN-positive strains identified identical bands to S. mutans N. The location of mutN differs significantly from that of mutA in that it is directly upstream of comC, a gene encoding a putative competence stimulating factor.

Interpretation & conclusion: The close upstream proximity of mutN to comC suggests a link between mutacin N production and competence development. Further studies need to be done to detect competence-related genes in S. mutans strain N.

Key words: Bacteriocin - like inhibitory substance - mutacin - Streptococcus mutans

Bacteriocins are typically defined as small ribosomally synthesised post-translationally modified proteinaceous molecules that generally inhibit the growth of closely related bacteria. Bacteriocin-like inhibitory substances (BLIS) produced by the mutans streptococci (MS) have been referred to as mutacins\(^1\). Some of the strongly inhibitory mutacin-producing Streptococcus mutans have recently been categorised into four groups (A-D) according to their cross-immunity characteristics and their spectra of inhibitory activity\(^2\). This study\(^2\) further established that the different bacteriocin activities of S. mutans reflect distinct phylogenetic lineages. It is becoming evident however that some strains that appear to have similar spectra of inhibitory activity produce quite different bacteriocins. For example, in a previous study it was shown that S. mutans N produced mutacin N,\(^3\) a 4806 Da peptide having partial homology with the b component of the two-peptide bacteriocin mutacin IV\(^4\). The aim of the present study was to clone the mutacin N structural gene and to identify other genes that may be implicated in mutacin N production.

Material & Methods

Bacterial strains and culture media: S. mutans N was originally isolated from a supragingival plaque specimen from an adult volunteer. The 216 streptococci screened for the distribution of mutN included 106 isolates of S. pyogenes, 82 S. salivarius, 14 S. mutans, 12 S. uberis, one S. dysgalactiae and one S. rattus.
from J.R. Tagg’s collection. All strains were kept frozen in skim milk at -70°C. *S. mutans* N was routinely grown in Todd Hewitt Broth (THB) (Difco, USA).

**Cloning and sequencing of mutN:** *S. mutans* N was grown in 20 ml THB for 18 h at 37°C in 5 per cent CO₂ air. The chromosomal DNA was extracted using the DNeasy Tissue Kit (Qiagen Ltd., Crawley, England) according to the manufacturer’s instructions, with the following modifications. The THB overnight cultures were pelleted by centrifugation at 1155xg and washed twice in 10 mM Tris-EDTA buffer pH. The cells were resuspended in 140 µl lysis buffer containing 40 µl 50 mg/ml lysozyme and incubated for 30 min at 37°C. DNA was digested with *Bam*HI and *Eco*RI (Roche Diagnostics) and separated on a 1 per cent agarose (Roche Diagnostics) TAE buffer gel. Southern hybridisation was carried out according to the manufacturer’s instructions [Hybond™-N+ (Amersham Pharmacia Biotech Inc)].

The degenerate oligopeptide probe MutNC (5’-GCWTATGGWGCGCAARGG-3’) was based on residues 12-18 (N-AYGAAKG-C) of the mutacin N amino acid sequence. MutNC was end-labelled with [³²P]ATP using T4 polynucleotide kinase and used to screen Southern hybridisations using established methodology. Competent *Escherichia coli* DH5a cells were prepared using the protocol described by Sheng et al. Ligated pUC19 containing the mutN insert was electroporated into strain DH5a. Briefly, 3 µl of DNA was mixed with 40 µl of DH5a cells, thawed on ice and placed in a chilled 0.1 cm electrode gap Pulsar™ cuvette (Bio-Rad Laboratories, California, USA) and left for 1 min. A single pulse was applied (1.5 kV, 25 µF, 200 W). Following electroporation, the cells were resuspended in 1 ml of 2YT broth and incubated at 30°C with shaking for 1 h. Aliquots (50 µl) of the cell suspensions were then plated onto Luria broth (LB) agar plates containing 50 µg/ml ampicillin and X-gal.

**DNA sequencing:** Sequencing was carried out at the Centre for Gene Research at the University of Otago using a Perkin-Elmer ABI 377A sequencer. 

**Inverse PCR:** DNA sequence outside of the cloned contig was obtained by inverse PCR. Briefly, approximately 5 µg of *S. mutans* N chromosomal DNA was digested for 4 h at 37°C with SpeI restriction enzyme. 5 µl of this was then used for a ligation reaction using T4 ligase [2 µl T4 buffer, 1 µl T4 ligase (Roche Diagnostics Ltd., Lewes, England) and 12 µl MQ water] for 18 h at 12°C. 1 µl of this preparation was used in a PCR reaction using primers NinvF (5’-G CGT GCG CGC AAG TAC C-3’) and NinvR (5’-G AAATGCTAT CTT TAC TTA C-3’) designed to the cloned contig. The PCR was carried out using Taq polymerase (Roche Diagnostics Ltd., Lewes, England) for 30 cycles, with denaturation at 95°C for 2 min, annealing at 50°C for 30 sec and extension at 68°C for 10 min.

**Evaluation of the distribution of mutN in streptococci:** The distribution of mutN using DNA from 216 streptococcal strains of six species was determined by dot blotting. The DNA was derived by a method based on that of Upton et al., but with use of only a single phenol-chloroform extraction step. 5 µl of each DNA sample was applied to a nylon membrane [Hybond™-N+ (Amersham Pharmacia Biotech Inc)] via a vacuum manifold followed by 100 µl of 2 x SSC [0.3 M NaCl, 0.03 M sodium citrate (pH 7)]. Denaturation of the DNA was by exposure to two x 2 min washes of 0.4 M NaOH followed by two x 2 min washes with 1 M Tris HCl. The membrane was then exposed to UV light for approximately 5 min and probed with a digoxigenin-dUTP (Roche Diagnostics Ltd., Lewes, England)-labelled mutN probe, derived using PCR primers mutNF (5’-CGT CAG CTC AAC ATC T-3’) and mutNR (5’-G T A C T C T G C A C C T A G-3’). The PCR was carried out using Taq polymerase (Roche Diagnostics Ltd., Lewes, England) for 28 cycles, with denaturation at 95°C for 2 min, annealing at 55°C for 30 sec and extension at 72°C for 30 sec.

**Deferred antagonism test:** The P-type BLIS fingerprint was as described by Tagg et al. but *S. mutans* was grown at 37°C in air with 5 per cent CO₂.

**Results**

**Cloning and sequencing of mutN:** An 1800 bp *Bam*HI/*Eco*RI fragment containing mutN was identified (Fig.1),
cloned and sequenced. A further 900 bp was sequenced following inverse PCR. Analysis of this sequence revealed four open reading frames (ORF) (Fig.2). In addition to mutN, there were ORF having homology to comC, a transposase gene (orfA) and a bacteriocin-like peptide gene (blpI). The translated product of mutN indicated a 79 amino acid precursor peptide having a double glycine cleavage site preceding the mutacin N propeptide. The predicted mutacin N leader sequence is MNVEENMSYNDTREDLSQTTIGG and the predicted mutacin N propeptide is SRQADTFLS GAYGAKGVTA CASTGV YVVPATLVCVG V VAGLNIAFP. Underlined amino acids indicate differences in the predicted sequence on the basis of the nucleotide sequence when compared with the sequence previously obtained by protein sequencing3. Directly preceding mutN was an ORF with homology to the bacteriocin-like peptide gene blpI from Streptococcus pneumoniae strain KNR.7/8710.

**Evaluation of the distribution of mutN in streptococci:**
The distribution of mutN was investigated using a probe generated by PCR using the mutN primers mutNF and mutNR. Probing was carried out using a dot-blot method with DNA preparations from 216 strains of streptococci. Of these, 4 of 14 S. mutans hybridised with the mutN probe. None of the 106 S. pyogenes, 82 S. salivarius, 12 S. uberis, or the single representative strains of S. dysgalactiae and S. rattus were positive for mutN. The mutN-positive strains were shown to produce a similar P-type pattern of 777 against nine standard indicators9. In addition, these mutN-positive strains produced an identical inhibitory BLIS pattern against an extended set of indicator strains (data not shown). MutN-positive strains were resistant to each other when cross-tested by deferred antagonism indicating they have cross-immunity and thus may be producing similar bacteriocins. PCR with the mutN primers mutNF and mutNR on the four mutN-positive strains identified identical bands to S. mutans N.

**Discussion**

In this study the mutacin N structural gene was cloned and sequenced. MutN was sequenced from an 1800 bp BamHI/EcoRI fragment. The derived sequence established mutacin N to comprise a propeptide of 50 amino acids, one more than previously obtained by Edman N-terminal sequencing3. A 29-amino acid leader sequence with a typical double glycine cleavage site was identified upstream of mutN. Immediately adjacent to mutN is an ORF with homology to a bacteriocin-like peptide encoded by blpI10. Further upstream was an ORF identical to comC, a gene involved in competence development in S. mutans strain BM71. One further ORF was identified downstream of mutN using inverse PCR which was found to have homology with a
S. agalactiae transposase (orfA). In an initial screen mutN was shown to be present in four strains of S. mutans.

The MS are known to frequently produce mutacins, a number of which have been well characterised including: mutacin N³, mutacin I¹⁴, mutacin II¹², mutacin III¹³, mutacin IV¹ and B-Ny266¹⁴. S. mutans N has been shown to produce a similar BLIS activity spectrum to that of the mutacin I producer S. mutans UA140. Mutacin I has been identified as a 2364 Da lantibiotic, a class of bacteriocins characterised by the presence of lanthionine residues and modified amino acids such as 2, 3-didehydroalanine and 2, 3-didehydrobutyrine. MutA, the gene encoding mutacin I, is located within an operon containing 14 genes involved in the processing, transport of the lantibiotic and the self immunity of the producing cell¹¹.

Mutacin N is clearly a different molecule to mutacin I, despite the two producer strains showing identical inhibitory spectra. Mutacin N differs markedly from mutacin I in that it is not a lantibiotic. The reason for the apparent close similarity in the activity spectra remains to be determined.

The discovery of a bacteriocin-like ORF with homology to blpI was unexpected. Several two-component bacteriocins¹⁵,¹⁶ and lantibiotics¹⁷,¹⁸ have been reported but to date only one strain of S. mutans has been found to produce two bacteriocins. BlpI is located adjacent to another bacteriocin-like ORF, blpJ in S. pneumoniae strain KNR7/87. The two peptides encoded by blpI and blpJ are thought to comprise a two-component bacteriocin with homology to thermophilin 13, a two-component bacteriocin from Streptococcus thermophilus strain Sfi13¹⁵.

Competence refers to the ability of a bacterial cell to take up foreign exogenous DNA. ComC, comD and comE respectively encode a quorum sensing peptide, histidine kinase and a response regulator. Their involvement in the establishment of competence in S. mutans has been shown¹⁹. ComC is found in the opposite orientation to comD and comE in S. mutans¹⁹. The close upstream proximity of mutN to comC suggests that there may be a link between mutacin N production and competence development. A link between bacteriocin production and competence has already been shown in Streptococcus gordonii (Heng, personal communication). Further studies are required to detect the presence and activity of competence-related genes in S. mutans strain N.

In this study the mutN gene has been cloned and sequenced. Homology of adjacent ORFs indicate that mutN may be part of a two-component bacteriocin system and also that there may be some association with competence development. The fact that these two S. mutans strains produce markedly different bacteriocins, yet have closely similar inhibitory spectra, requires further investigation.

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