Mode of action of nisin on Escherichia coli

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Mode of action of nisin on *Escherichia coli*.

(running title: Nisin mode of action)

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ABSTRACT

Nisin is a class I polycyclic bacteriocin, produced by the bacterium *Lactococcus lactis*, and is used extensively as a food additive to inhibit the growth of foodborne Gram-positive bacteria. Nisin also inhibits growth of Gram-negative bacteria when combined with membrane-disrupting chelators such as citric acid. To gain insight into nisin’s mode of action, we analysed chemical-genetic interactions and identified nisin-sensitive *Escherichia coli* strains in the Keio library of knockout mutants. The most sensitive mutants fell into two main groups. The first group accords with the previously proposed mode of action based on studies with Gram-positive bacteria where nisin interacts with factors involved in cell wall/membrane/envelope biogenesis. We identify an additional, novel mode of action for nisin based on the second group of sensitive mutants involving cell cycle and DNA replication, recombination and repair. Further analyses supported these two distinct modes of action.
INTRODUCTION

Nisin is a class I bacteriocin that is used extensively as a food additive to inhibit the growth of food spoilage, food poisoning and pathogenic Gram-positive bacteria (Coma et al. 2001; Sobrino-Lopez and Belloso-Martin 2008; LeBel et al. 2013; Tong et al. 2014; Chai et al. 2015). Nisin has additional important roles in the nutraceutical industry, as a veterinary medicine (anti-mastitis application against Staphylococcus aureus), and in human medicine (treatment for dermatitis, dental caries, respiratory and vaginal infections) (Cotter et al. 2005; Arauz et al. 2009). Nisin is a 34 amino-acid polycyclic amphiphilic and cationic peptide. Like other class I bacteriocins, nisin is post-translationally modified to generate the lanthionine rings that are functionally important for antibacterial activity. Nisin is encountered as multiple variants in different bacterial species (O’Connor et al. 2015). For example, nisin A and nisin Z, among the best characterized, possess a similar structure but differ by a single amino acid residue at position 27, being a histidine in nisin A and an asparagine in nisin Z (Cheigh and Pyun 2005).

The antimicrobial mode of action of nisin has been studied in Gram-positive bacteria where it appears to depolarize the cytoplasmic cell membrane through its high affinity for the cell wall precursor lipid II (undecaprenyl-pyrophosphoryl-MurNAc-(pentapeptide)-GlcNAc). Nisin recruits lipid II molecules to form stable channels in the cytoplasmic membrane, with 8 nisin and 4 lipid II molecules/channel. Formation of these channels results in leakage of cellular contents, efflux of ions that dissipate the cell’s proton motive force, and depletion of intracellular ATP, which further disrupts the cell membrane and ATP-dependent biochemical processes. Nisin binding to lipid II also prevents peptidoglycan synthesis and thus disrupts cell
wall biogenesis and functions (Hasper et al. 2004; Wiedemann et al. 2004; Bauer and Dicks 2005; Christ et al. 2007).

In contrast, Gram-negative bacteria tend to be resistant to nisin due to the outer membrane (OM) that impedes the movement of large hydrophobic molecules such as nisin into the cytoplasmic cell membrane. Metal chelators can destabilize and permeabilize the OM by binding to Ca$^{2+}$ and Mg$^{2+}$ and allowing nisin to reach its target in the cytoplasmic cell membrane. Previous studies have demonstrated that a combination of nisin and EDTA, disodium pyrophosphate, sodium hydrogen orthophosphate, citric acid, or lactic acid can efficiently inhibit growth of Gram-negative bacteria (Boziaris and Adams 1999; Alakomi et al. 2000). Despite studies on the primary molecular targets of nisin in Gram-positive bacteria, the mode of action of this bacteriocin on Gram-negative bacteria has not been investigated.

In this study, we carried out a high-throughput chemical-genetic interaction analysis, using nisin in combination with citric acid (pH 5) and the Keio set of *E. coli* knockout mutants. The Keio library comprises a set of approximately 3900 *E. coli* K-12 strains, each with a non-essential gene knocked out (Baba et al. 2006). Previously, knockout and deletion libraries such as this have enabled stimulus-response studies at a genome-wide level and have provided information on gene-gene and gene-drug interactions, as well as gene function discovery (Babu et al. 2009; Brochado and Typas 2013; Kumar et al. 2016). From our analysis of the genetic profile of mutant strains that were highly sensitive to nisin we obtained molecular mechanistic insights into nisin mode of action. As expected from previous work with Gram-positive bacteria, functional clustering of the phenotypic analysis showed that nisin interacts with factors involved in cell membrane transport systems and cell wall biosynthesis. In addition,
strains with disruptions in genes involved in DNA-replication-repair were also very sensitive to nisin, and we further investigated this potentially novel mode of action by nisin.

MATERIALS AND METHODS

Chemicals

Nisin A (2.5 % purity, 1000 IU/mg) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA) and prepared as a 20 mg/mL stock solution in 0.02 N HCl. Nisin was sterilized before use by heating at 80 °C for 10 min. Citric acid (Bioshop, Burlington, ON, Canada) was prepared as a 200 mM stock solution adjusted to pH 5.0 ± 0.02 with NaOH and sterilized by passage through 0.2 µm membrane before use. LB (Miller) medium (1% peptone, 0.5% yeast extract and 1% NaCl) was from Bioshop. NaOH, HCl (37%), ethanol (95%), ampicillin, ciprofloxacin and kanamycin were from Sigma (Oakville, ON, Canada). Propidium iodide and RNAase were purchased from Life Technologies (Burlington, ON, Canada).

Strains and growth conditions

The Keio strain collection of about 3900 Escherichia coli knockout mutants was provided by the National BioResource Project (National Institute of Genetics of Japan). Keio strains were grown at 30 °C on LB agar (2% agar) containing 30 µg/mL of kanamycin, and stored long term in LB broth + kanamycin with glycerol (16% v/v) at -80 °C. Additional E. coli strains used in this study (Table 1) were grown in LB broth at 37 °C with constant agitation (150-200 rpm) unless otherwise specified.

Nisin and Nisin-Citric Acid sensitivity tests

Drop-out tests were performed to estimate suitable citric acid and nisin concentrations for chemical-genetic profiling experiments. For drop-out tests, an overnight culture of E. coli
JCM1649 was grown in LB at 37 °C and diluted to adjust cell density to 10⁵ cells/mL. This cell suspension was distributed into culture tubes containing different concentrations of nisin (0, 0.25, 0.5 and 1.0 mg/mL) and citric acid (0, 10 and 20 mM, pH 5.0) to test for growth inhibition. All cultures were incubated for 3 hours at 37 °C. After incubation, 10-fold serial dilutions (10⁻³ to 10⁻⁸) of each culture were prepared and 10 µl of each dilution was spotted onto LB agar plates and incubated for 16-24 hours at 37 °C.

Microdilution analyses were used to evaluate sensitivity of *E. coli* strains to various inhibitors. For microdilution analyses, 1:1 serial dilutions of nisin (combined with 20 mM citric acid, pH 5.0), citric acid carrier solution with no nisin, and novobiocin (positive control) were done in a 96-well microtitration plate containing LB broth, according to standardized protocols (NCCLS 2013). An overnight culture of the *E. coli* was diluted to 10⁴ cells/mL and 100 µl of cell suspension was added to each well of the microtitre plate. Inhibitory effect was assessed after 16-24 hr incubation (37 °C) based on optical density measurements at 600 nm (OD₆₀₀) with a Cytation 5 cell imaging multi-mode reader (BioTek, Winooski, VT, USA). Inhibition percent (I%) was calculated using the formula:

\[ I\% = 100 - \left( \frac{Abs_{exp} - Abs_{blank}}{Abs_{carrier} - Abs_{carrier\ blank}} \right) \times 100. \]

MIC₉₀ and MIC₅₀ were defined as the lowest concentrations of nisin that caused, respectively, a 90% or 50% growth inhibition. Each concentration was tested three or more times and mean % inhibition values (±SE) were calculated.

**High throughput chemical-genetic profiling**

To identify partial inhibitory conditions for Keio knockout mutant strains, we used the drop-out assay results as a guide, considering that slightly higher concentrations of inhibitors are often needed in agar medium compared to liquid medium. Therefore, we tested nisin
concentrations between 1.2 and 2.0 mg/mL in combination with 10 and 20 mM citric acid (pH 5) using LB + 2% agar medium (nisin-CA medium). CA-control medium did not contain nisin but was otherwise identical to the nisin-CA medium. Colonies from two randomly chosen plates from the Keio set were inoculated onto the nisin-CA and CA-control media using a 384-floating replicator. The plates were incubated at 30 °C for 16 hours and the colony sizes were visually examined. The conditions (20 mM citric acid and 1.56 mg/ml nisin) at which approximately 5-10% of the strains showed a colony size reduction of between 0 to 50% compared to CA-control medium was selected for experiments with the complete Keio mutant set.

The Keio collection of about 3900 knockout mutants on 24 plates was simultaneously inoculated by pinning onto experimental (with nisin-CA) and control (with CA) plates, incubated at 30 °C for 16-24 hours and then digital images were acquired and analyzed by growth detector software (Memarian et al. 2007). Sensitive mutants identified by digital analysis were verified by visual examination. Four replica experiments with the entire Keio set were done. From each experiment, the percent inhibition of each mutant strain was calculated based on colony size measurements on control and experimental plates:

\[
\text{Colony size reduction } \% = \left(\frac{\text{Area}_{\text{control}} - \text{Area}_{\text{experimental}}}{\text{Area}_{\text{control}}}\right) \times 100.
\]

Mutants showing a high sensitivity (60-100% colony size reduction) to nisin-CA were identified. High sensitivity mutants on at least 2 of the 4 replica experiments were then selected and categorized into functional clusters according to COG (Clusters of Orthologous Groups, http://www.ncbi.nlm.nih.gov/COG/; http://www.compsysbio.org/bacteriome/).

Flow cytometry analysis of genomic DNA content

Wild type *E. coli* strain JCM1649 and selected DNA-Keio mutants (Table 1) were each grown at 30 °C, 150 rpm, to mid-log phase and diluted to a cell density of $10^7$ cells/mL. The
cultures were combined with sub-inhibitory nisin-CA concentrations, HCl-CA (carrier solvent control), 0.2 mM novobiocin (positive control), or no-novobiocin (LB) control. Nisin concentrations used were selected according to the corresponding MIC\textsubscript{90} for each strain: \textit{ΔdnaQ} and \textit{K-12} were exposed to 0.25 and 0.50 mg/mL of nisin; \textit{ΔdnaG} and \textit{ΔtatD} were exposed to 0.75 and 1.0 mg/mL of nisin. After incubation for 2 hr at 37 °C, 1 mL aliquots were withdrawn (in duplicate) from each culture-treatment, centrifuged to pellet the cells and washed with ice-cold PBS (pH 7.4). Cells were fixed and permeabilized by resuspension in 1 mL of 70% ice-cold ethanol and stored overnight at 4 °C. Cells were then centrifuged, resuspended in ice-cold PBS, re-pelleted by centrifugation and resuspended in 200 µl of fresh propidium iodide (PI) solution (0.1 % v/v Triton X-100 in PBS, containing 0.2 mg/mL of RNAse and 20 µg/mL of PI). Cells were incubated with PI solution for 15 min in the dark at 37 °C. PI is a fluorescent dye that enters permeabilized cells where it intercalates into DNA and fluoresces in proportion to PI-DNA complex formation (Riccardi and Nicoletti 2006). Stained cells were subsequently analyzed by flow cytometry.

Flow cytometry was performed using a BD Accuri C6 (BD Biosciences, East Rutherford, NJ, USA) using an excitation wavelength of 488 nm, emission detector of 585±40 nm (FL2), and forward (0°±13°) and side scatter (90°±13°) detectors. Samples stained with PI were analyzed at medium speed (36 µl/min), and 10,000 events (cells) were measured per sample. Forward scatter (FSC) and side scatter (SSC) were simultaneously measured. Bacterial populations were gated in FSC-SSC-Dot Plots and stained cell counts (DNA content) were observed in a single-parameter histogram (PI relative fluorescence vs cell count).

**Checkerboard assays to assess drug interactions**
Checkerboard assays were done in 96-well microtitre plates according to the methods of Orhan et al. (2005) with modifications as follows. Each well contained a final volume of 200 μL: 100 μL of LB medium with $1 \times 10^4$ CFU of E. coli JCM 1649, 50 μL of nisin+CA solution (2× serial dilutions along the abscissa) and 50 μL of novobiocin or ampicillin solution (2× serial dilution along the ordinate). Plates were incubated at 30 °C for 20 hours and MIC was recorded as the lowest concentration at which no growth occurred based on OD$_{600}$ readings and verified by naked eye. The fractional inhibitory concentration index (ΣFIC) was calculated as: $\Sigma FIC = FIC_A + FIC_B$. $FIC_A$ is the MIC of drug A combined with drug B divided by the MIC of drug A alone. Similarly, $FIC_B$ is the MIC of drug B combined with drug A divided by the MIC of drug B alone.

Synergistic interaction is indicated when $\Sigma FIC \leq 0.5$, no significant interaction (indifferent) when $0.5 < \Sigma FIC < 2$, and $\Sigma FIC \geq 2$ indicates an antagonistic interaction between the two drugs.

RESULTS

Nisin and nisin-citric acid sensitivity

Boziaris and Adams (1999) showed that inhibition of Gram-negative bacteria by nisin is enhanced by addition of metal chelators such as EDTA or citric acid (CA). Similarly, we found that growth inhibition of E. coli by 1 mg/mL nisin is increased more than 10-fold with addition of 10 mM CA (pH 5) compared to nisin or citric acid alone (Figure 1). Combining nisin with chelators may thus expand the antimicrobial utility of nisin in food and other applications (Stevens et al. 1991; Fang and Tsai 2003). The observed synergistic activity is presumably due to disruption by citric acid of the E. coli outer membrane (OM). Lipopolysaccharides (LPS) in the OM are negatively charged and divalent cations such as Mg$^{2+}$ or Ca$^{2+}$ are necessary to neutralize this charge and allow polysaccharide units to cross-link and maintain an effective barrier. LPS
integrity may be compromised when these cations are chelated (Adams et al. 2014). Using citric acid as a chelator enabled us to use a high-throughput screen with the Keio knockout set to investigate the mode of action of nisin in *E. coli* as a Gram-negative model bacterium.

**Identification of Keio mutants highly sensitive to nisin-CA**

The set of 3900 *E. coli* knockout mutants was grown on LB agar medium with 20 mM citric acid (control, CA) and LB agar medium containing a semi-inhibitory concentration of nisin (1.56 mg/mL) with 20 mM citric acid (experimental, nisin-CA). The phenotypic screening was replicated four times and knockout strains showing 60-100% colony size reduction on an experimental plate compared to control in at least two trials were selected as mutants that were considered ‘highly sensitive’ to nisin. Averaged percent inhibition values of the selected mutant candidates from the four trials are presented in Supplemental Table 1. Under our experimental conditions, 1.6% of the Keio mutants (64/3900) were highly sensitive to nisin-CA.

Genes disrupted in the sensitive mutants could be classified by cellular process (COG) into eight functional categories (Figure 2). The major functional category encompassed mutants lacking non-essential genes involved in cell wall/membrane/envelope biogenesis, representing 22% of the most sensitive mutants (*P* = 0.01). The second largest category comprised mutants with disrupted genes of unknown function. Our strain sensitivity analysis may provide some insights into the function of these uncharacterized genes since they may belong to other COG groups identified here. Further studies are required to fully characterize these unknown function genes. The third largest category (14% of sensitive mutants, *P* = 0.1) encompassed mutants lacking genes related to cell cycle and DNA replication, recombination and repair.

Additional top categories include translation and post-translational modification (9%), carbohydrate transport and metabolism (9%) and ion transport and intracellular trafficking.
The effect of nisin on the top two functional categories, ‘cell wall/membrane/envelope biogenesis’ and ‘DNA replication, recombination, and repair’ were further investigated using secondary assays.

Examples of nisin-sensitive knockout mutants in the cell wall/membrane/envelope biogenesis category include *rffH* (antigen O biosynthesis), *rffA* (antigen ECA biosynthesis), *rfaP* and *rffP* (lipopolysaccharide biosynthesis), and *amiA* and *amiC* (cell wall organization). These genes encode proteins that contribute to the maintenance and stabilization of the OM and cell wall organization. Additional categories may also reflect this mode of action by nisin. For instance, *envC* and *damX* from the ‘Cell cycle, DNA replication, recombination and repair’ category both have septal ring-related functions. Likewise, *yidQ* and *ybaY* from the ‘unknown function’ category both encode predicted outer membrane proteins. Two other categories, carbohydrate transport and ion transport, may also be related to perturbation of cell membrane structure and function by nisin. Finding this major category of nisin-sensitive strains with disruptions in genes involved in cell wall/outer membrane function is consistent with the observations, based on studies with Gram-positive bacteria, that nisin induces pore formation in cell membranes and interferes with cell wall synthesis. We confirmed that our formulations of nisin disrupt artificial cell membranes using a liposome assay (Figure 1S in supplemental material).

**Nisin reduces *E. coli* DNA content and acts synergistically with novobiocin**

Inhibition of ‘DNA replication, recombination, or repair’ by nisin has not been previously reported and thus warranted further investigation. Among the strains in this category, mutants lacking genes such as *dnaQ* (DNA polymerase III epsilon subunit; 81.4% colony size reduction), *dnaG* (DNA primase; 64.7% colony size reduction) and *tatD* (quality control of Tat-exported FeS
proteins and Mg-dependent cytoplasmic DNase; 77.4% colony size reduction) were highly sensitive to nisin-CA in all four phenotypic screening experiments.

The effect of nisin on *E. coli* genomic DNA content was investigated by flow cytometry to determine whether nisin-CA impedes DNA replication. Intracellular DNA can be quantified in terms of fluorescence intensity when it is labeled with a fluorochrome such as propidium iodide (PI). PI molecules stoichiometrically intercalate into DNA to yield a fluorescent signal that can be utilized to estimate DNA content (Suzuki et al. 1997). JCM1649 wild type cells and nisin-sensitive knockout mutants, ΔdnaQ, ΔdnaG and ΔtatD were exposed to sub-inhibitory concentrations of nisin or novobiocin (positive control), along with the respective carrier controls that contained neither nisin nor novobiocin. The cells were then fixed and permeabilized with ethanol, stained with PI, and 10,000 cells from each treatment were analyzed by flow cytometry (Figure 3 and supplemental Table 2). In the absence of drug treatment, flow cytometry profiles were similar in all four *E. coli* strains examined. Upon nisin exposure, the four strains showed reductions of 57% (ΔdnaG), 26% (ΔdnaQ), 17% (ΔtatD), and 33% [JCM1649 (wt)] in the proportion of cells with PI-DNA complex (FL2-A = 10^3-10^4) compared to the corresponding carrier control (HCl-CA; shaded column in supplemental Table 2).

Similarly, for all four strains there was a marked reduction of PI-DNA signal in novobiocin-treated cells compared to the corresponding negative control containing no novobiocin [44% reduction for ΔdnaG, 45% for ΔdnaQ, 10% for ΔtatD, and 24% for JCM1649 (wt); supplemental Table 2].

Overall, the flow cytometry data support the inference from our high-throughput phenotypic screening using the Keio knockout set that nisin may interfere with cell cycle and DNA replication. Novobiocin is a coumarin produced by *Streptomyces* species, which is known
to inhibit DNA gyrase. DNA gyrase requires energy in the form of ATP to introduce negative supercoils and relieve the torsional strain occurring during DNA replication (Hardy and Cozzarelli, 2003; Fàbrega et al. 2009; Collin et al. 2011). DNA gyrase is composed of two subunits, gyrase A (GyrA), which contains an active site for DNA cleavage and the subunit B (GyrB) that contains an ATPase active site. Novobiocin obstructs DNA gyrase by competitively inhibiting the nucleotide binding site (GyrB). We observed a synergistic interaction with the combination of nisin and novobiocin in checkerboard assays (ΣFIC= 0.34, n=6), but no significant interaction with nisin and ampicillin (ΣFIC= 1.11, n=6) (supplemental Table 3). This may indicate that nisin and novobiocin block different pathways involved in DNA synthesis as opposed to ampicillin, which interferes with peptidoglycan synthesis in the cell wall (Tipper, 1985). While the specific target by which nisin putatively effects DNA synthesis is unknown, it appears distinct from that of ciprofloxacin, a quinolone antibiotic that inhibits activity of subunit A of gyrase (GyrA) and thus DNA replication (Collin et al. 2011), since seven E. coli strains having distinct GyrA or MarR mutations that confer resistance to ciprofloxacin did not show resistance to nisin compared to wild type (supplemental Table 4).

**DISCUSSION**

Finding antimicrobial substances with different modes of action is an important approach to dealing with the phenomenon of antimicrobial drug resistance. We investigated the mode of action of the bacteriocin nisin against the Gram-negative bacterium E. coli. From our high-throughput phenotypic screen we infer that, as with Gram positive bacteria, nisin perturbs cell membrane functions in Gram negative bacteria. Liposome-based assays suggest that nisin can interact directly with membrane lipids increasing membrane permeability. Our functional
analysis of sensitive *E. coli* mutants also indicated that nisin has a second mode of action in perturbing DNA replication. Consistent with this hypothesis, flow cytometry revealed that nisin exposure caused a decrease in the DNA content of *E. coli* cells similar to the DNA replication inhibitor, novobiocin. Also consistent with the hypothesis, nisin and novobiocin act synergistically to inhibit growth of *E. coli*. Of interest, there is evidence that the peptide microcin B17 (3.1 KDa), a bacteriocin produced by enterobacteria, perturbs gyrase activity in DNA replication (Yang et al. 2014). This toxin slows down DNA super-coiling and relaxation by gyrase. Microcin B17 undergoes post-translational modifications in which oxazole-thiazole fused rings are formed through cysteine residues. The nisin peptide (3.5 KDa) similarly undertakes post-translational modifications in which lanthionine rings are formed. That both microcin B17 and nisin possess similar ring structures is interesting in this context. The putative effect on DNA replication is also consistent with reports that nisin reduces cell proliferation, arrests cell cycle and increases DNA fragmentation in squamous cell carcinoma (Joo et al. 2012). Additional mechanistic studies are required to determine specific molecular target(s) that nisin may affect during DNA replication.

**ACKNOWLEDGEMENTS**

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64.4.470


doi.org/10.1016/0163-7258(85)90062-2


Figure Captions

Figure 1. *E. coli* sensitivity to nisin and citric acid based on drop-out assays. The different treatments are specified at the bottom of the figure; CA is citric acid adjusted to pH 5.5. Serial cell dilutions used for the analysis are indicated at left. Lane B shows that CA alone does not inhibit *E. coli* growth. Lane C shows that nisin, in the absence of CA, has no appreciable antimicrobial effect on *E. coli*. Lanes D, E and F show inhibitory effect of nisin in the presence of CA.

Figure 2. Functional distribution of sensitive mutants to nisin. Clusters of Orthologous Groups (COGs) of genes disrupted in the most sensitive mutant strains. Major known function COGs include cell wall/membrane/envelope biogenesis (22% of sensitive mutants) and cell cycle/DNA replication, recombination and repair (14%).

Figure 3. Similar to novobiocin, exposure to nisin results in reduction of DNA content in the *E. coli* deletion mutants ΔdnaG, ΔdnaQ and ΔtatD, and the wild type strain JCM1649. Flow cytometry showing DNA content of 10,000 *E. coli* cells of the corresponding strain exposed for 2 h to A) carrier-CA control (no nisin), B) nisin-CA, C) carrier control (no novobiocin), and D) novobiocin. Histograms show count of cells vs DNA content [pulse-area (FL2-A)]. FL2-A readings of $10^3 – 10^4$ (between dashed lines) are considered to be the fluorescent signal of DNA-PI complex, regions below $10^3$ are considered non-fluorescent.
Table 1. *E. coli* strains used in this study

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<th>Sensitivity test</th>
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<td>Nisin, Nisin-CA, novobiocin, ampicillin</td>
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<td>Wild type control for Cip-resistant strains</td>
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<td>deletion of DNA primase</td>
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<tr>
<td>ΔtatD</td>
<td>deletion of <em>tatD</em>, Mg-dependent cytoplasmic DNase</td>
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<td><strong>Ciprofloxacin (Cip)-resistant mutants</strong></td>
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Figure 1.

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Legend:
- A
- B
- C
- D
- F
- G

Concentrations:
- $10^{-5}$
- $10^{-6}$
- $10^{-7}$
- $10^{-8}$
Figure 2.
Figure 3.

A) nisin

B) + nisin

C) novobiocin

D) + novobiocin