Mutacin II is a bactericidal lantibiotic from *Streptococcus mutans*. It inhibits the growth of other streptococci as well as many other gram-positive microorganisms by a hitherto unknown mechanism. Mutacin II possesses bactericidal activity against susceptible cells. It transiently depolarizes the transmembrane electrical potential (ΔΨ) and the transmembrane pH gradient (ΔpH) and partially inhibits amino acid transport. However, it rapidly depletes the intracellular ATP pool in glucose-energized cells and prevents the generation of ATP. It is concluded that mutacin II does not belong to the group of pore-forming lantibiotics (type A) or to the type B lantibiotics, which inhibit phospholipases or interfere with peptidoglycan biosynthesis. Mutacin II acts by inhibiting essential enzyme functions at the level of metabolic energy generation, an activity that has not yet been classified for lantibiotics.

Mutans streptococci as well as many other microorganisms which inhabit the oral cavity produce antimicrobial substances of a proteinaceous nature (13). These are referred to as bacteriocins (27) or bacteriocin-like inhibitory substances (32). The term "mutacin" has been proposed for antimicrobial peptides originating from *Streptococcus mutans* (8). These antimicrobial peptides can play significant roles in primary colonization and give a competitive advantage to producer strains occupying critical ecological niches in dental plaque. Therefore, bacteriocins have several potential biotechnological applications and have been explored for their ability to provide protection against bacteria causing dental diseases (10, 11).

Recently, mutacin II was isolated from *S. mutans* T8 and partially characterized (21). It is a small (3,245 Da) peptide, and on the basis of chemical modification techniques and ion spray mass spectroscopy, it contains one β-methylanthionine and two lanthionines in addition to a didehydro amino acid (22). These data suggest that mutacin II belongs to the lantibiotic group of bacteriocins (12). Mutacin II is stable and biologically active over a wide range of pH values and temperatures (21).

In this study the mode of action of mutacin II has been analyzed. The results suggest that mutacin II acts in a bactericidal manner and that it interferes with the capacity of cells to generate metabolic energy. Its biological activity appears to be directed at the inhibition of enzyme functions.

**MATERIALS AND METHODS**

**Bacterial strains and media.** *S. mutans* NTCC 10449, *Streptococcus sanguis* NY101 (21), and *Lactococcus lactis* subsp. lactis IL1403 (4) were used as mutacin II-susceptible strains. Cells were grown in brain heart infusion broth or agar (Difco Laboratories, Detroit, Mich.) at 30°C (for *L. lactis*) or at 37°C (for the *Streptococcus spp. and Escherichia coli*) without aeration. Purified nisin was purchased from NBS Biologicals (North Mymms, Hatfield, Herts, United Kingdom) or obtained from Aplin & Barrett and stored at −20°C in acetic acid solution (pH 3.0) at 10 mg/ml.

**Purification of mutacin II and determination of its biological activity.** Mutacin II was purified as described by Novák et al. (21). Bacteriocin activity in prepared samples was determined by the agar spot tests (9). In short, 10 μl of a solution containing bacteriocin was spotted on an agar plate in serial twofold dilutions in demineralized water containing 0.5% (vol/vol) Tween 20. After the liquid had been completely absorbed by the agar, a top agar layer seeded with 0.1% of an overnight culture of susceptible *S. sanguis* NY101 was poured over the plates. The plates were incubated for 16 h at 37°C and examined for zones of growth inhibition. The highest dilution which produced a distinct zone of inhibition after this incubation period was defined as 1 arbitrary unit (AU). One arbitrary unit corresponded to about 0.035 μg of purified mutacin II and 0.025 μg of purified nisin.

**Effect of mutacin II on growth of susceptible cells.** Overnight cultures were diluted to approximately 6 × 10⁶ cells/ml and tested for their susceptibility to different concentrations of mutacin II. Briefly, aliquots of 0.1 ml samples were taken at 30-min intervals to determine the viable count by plating onto brain heart infusion agar. In order to detect lytic activity, optical density (OD) was determined either at 595 nm (Bio-kinetics reader EL 312c; Bio-Tek Instruments) or at 630 nm (DIAS microplate reader; Dynatech Laboratories, Guernsey, United Kingdom), depending on the instrument used in the experiment.

**Measurement of the transmembrane electrical potential.** The transmembrane potential (ΔΨ) in cells was determined with an electrode specific for the lipophilic cation tetraphenylphosphonium (31) as described previously (3).

**Measurement of intracellular pH.** The intracellular pH was determined by the use of the fluorescent pH probe 2,7-bis(2-carboxyethyl)-5,6-carboxyfluorescein (BCECF: Molecular Probes, Inc., Eugene, Ore.), which was trapped in *L. lactis* IL1403 cells by an acid shock (17). Cells suspended in 50 mM potassium phosphate (pH 6.0) were energized with 0.5% (wt/vol) glucose at 30°C, and the BCECF fluorescence was monitored with a Perkin-Elmer LS50B spectrofluorimeter with computer-controlled data acquisition and storage using excitation and emission wavelengths of 502 and 525 nm, respectively. The excitation and emission slit widths were 5.0 and 15.0 nm, respectively. Data were corrected for ATP-driven eflux of the fluorescent probe (16), and the fluorescent signal was calibrated by measuring the fluorescence at different pH values in the presence of 1 μM nigericin to equilibrate protons across the membrane (17). ΔpH was calculated from the difference between intracellular pH and medium pH.

**Transport assays.** The uptake of the amino acids [1-¹⁴C]-2-aminoisobutyric acid (AIB) (8.5 μM; 59 mCi/mmol) and [1-¹⁴C]-l-glutamate (1.75 μM; 285 mCi/mmol) was analyzed as described previously (3).

**Determination of intracellular ATP concentration.** Cells were harvested at late exponential growth phase (OD at 600 nm [OD₆₀₀] of 1.2), washed once with 100 mM potassium phosphate (pH 6.5), and concentrated to an OD₆₀₀ of about 3.0 (23). The cell suspension was energized with 0.5% (wt/vol) glucose, and aliquots of the suspension were taken at specific time points. Samples were mixed with half a volume of 14% (wt/vol) perchloric acid–9 mM Na₂EDTA. The supernatant was neutralized with half a volume of 1 M KOH–1 M KHCO₃. The
neutralized extract was diluted 40-fold into 40 mM Tris-SO₄, and just before the measurement 2 mM EDTA (pH 7.75) and 10 mM MgSO₄ were added. ATP was measured by the firefly luciferase assay as described previously (23).

RESULTS

Mutacin II is a bactericidal lantibiotic. To investigate the mode of action of the lantibiotic mutacin II against the susceptible cells, an overnight culture of _S. sanguis_ Ny101 was diluted in fresh brain heart infusion broth with and without 10 AU of mutacin II per ml and the viable count and optical density changes were determined as a function of time. After 1 h of incubation in the presence of mutacin II, 96% of the cells lost their ability to form colonies (Fig. 1A). After 3 h of treatment only 0.003% of the cells survived, but further incubation (i.e., 18 h) resulted in increases in both OD and colony forming ability, suggesting the appearance of resistant cells. Cell lysis, as detected by a change in OD of the cells, was not observed during the time of experiment. Stationary-phase (18 h) _Ny101_ cells were at least twofold more susceptible to mutacin II than exponentially growing cells (6 h) (data not shown). These data suggest that mutacin II acts in a bactericidal manner.

After prolonged (18 h) growth in the presence of the bacteriocin, a subpopulation of _Ny101_ cells was able to grow. Rapid growth of these cells (Ny101R) was observed when they were incubated in fresh growth medium, both in the absence (Fig. 1B, solid boxes) and in the presence (open boxes) of 30 AU of mutacin II per ml. This apparent resistance was lost when the cells were grown for 24 h in the absence of the bacteriocin (data not shown).

The surfactant cocolactobionamide enhances the bactericidal activity of mutacin II. Some lantibiotics act at the level of the cytoplasmic membrane of susceptible cells (7), and their activity may be enhanced in the presence of surfactants that are thought to increase access to the phospholipids (5). Growth of susceptible _S. mutans_ NTCC 10449 cells was monitored following treatment with a partially inhibiting concentration of mutacin II, the surfactant cocolactobionamide (1), and a combination of both. The data shown in Fig. 1C demonstrate that the surfactant has a synergistic antibacterial effect when added in combination with mutacin II, whereas little inhibition of growth is observed with surfactant alone.

Mutacin II transiently and partially depolarizes the transmembrane electrical potential and pH gradient. To study whether mutacin II was capable of permeabilizing the cytoplasmic membrane of susceptible cells, its effect on the transmembrane potential (ΔΨ) was determined by monitoring the distribution of the lipophilic cation tetrathylphosphonium in energized cells. The ΔΨ of susceptible _S. sanguis_ Ny101 cells that were either treated (Fig. 2B) or not treated (data not shown) with nigericin was transiently depolarized by 30 AU of mutacin II per ml. However, the dissipation was not complete, and it was only temporary. Under the same set of conditions,

![Graph](image-url)
30 AU of nisin per ml completely collapsed the Δψ (data not shown) (7). The Δψ recovered shortly after the cells were treated with mutacin II. In contrast, the potassium ionophore valinomycin elicited a complete dissipation of the Δψ (Fig. 2A). When susceptible cells were preincubated with mutacin II before the energy source glucose was added, no significant effect of repeated additions of mutacin II on the Δψ was observed (Fig. 2C). In no instance was a complete depolarization of the Δψ by mutacin II observed. Similar results were obtained with susceptible L. lactis IL1403 cells, whereas the Δψ of resistant S. sanguis Ny101 cells (Ny101R) was not affected by mutacin II (data not shown). These results suggest that mutacin II only temporarily affects the ion permeability of the cytoplasmic membrane of susceptible cells and that it does not form stable pores.

To study the effect of mutacin II on the transmembrane pH gradient (ΔpH) of susceptible cells, L. lactis IL1403 cells were loaded with the fluorescent pH indicator BCECF to monitor the intracellular pH. When cells were energized with glucose, a rapid increase in the intracellular pH was observed (Fig. 3). Dissipation of the Δψ by valinomycin (1 μM) resulted in a further increase in the intracellular pH. Addition of nigericin (1 μM), a K+∕H+ ionophore (Fig. 3, trace 1), or the lantibiotic nisin (30 AU/ml) (trace 4) rapidly reversed the intracellular pH to a level equal to that of the medium pH. Four repeated additions of 50 AU of mutacin II per ml each prior to one single addition of 200 AU of mutacin II per ml (data not shown) caused a small but reproducible dissipation of the pH gradient. These results indicate that mutacin II has only a minor effect on the proton permeability of the cytoplasmic membrane.

**Mutacin II inhibits the uptake of amino acids.** The influence of mutacin II on the proton motive force-dependent uptake of AIB (6, 15), a nonmetabolizable analog of alanine (33), by energized susceptible (Ny101) (Fig. 4A) and partially resistant (Ny101R) cells (Fig. 4B) was studied. AIB is rapidly accumulated by both Ny101 and Ny101R cells. Addition of valinomycin and nigericin (1 μM each) (Fig. 4A, solid boxes; added at the arrow) caused a slow but complete release of the accumulated AIB. On the other hand, addition of mutacin II (100 AU/ml; solid circles) resulted in only a partial release of accumulated AIB to the level observed when the cells were preincubated with mutacin prior to the addition of AIB (open circles). Mutacin II hardly affected the uptake of AIB by Ny101R cells (Fig. 4B). The partial inhibition of AIB uptake in susceptible cells is consistent with the observation that mutacin II elicits a partial collapse of the proton motive force.

Glutamate uptake by lactic acid bacteria is a phosphate-bond-linked (ATP-driven), unidirectional process that is not dependent on the proton motive force (24, 25). Cells of Ny101 efficiently retained the accumulated glutamate upon the addition of the ionophores valinomycin and nigericin (1 μM each) (Fig. 5, diamonds; added at arrow A), but complete release was effected by the pore forming lantibiotic nisin (solid boxes). In contrast, mutacin II (100 AU/ml) (solid circles) again caused only a partial release of the accumulated glutamate to the level that was observed when cells were preincubated with mutacin II prior to the addition of glutamate (open circles). Prior addition of valinomycin and nigericin had little effect on the mutacin II-induced release of glutamate, indicating that the proton motive force is not required for functioning of mutacin II. Mutacin II did not elicit glutamate release from Ny101R cells (data not shown). These results demonstrate that mutacin II transiently permeabilizes the membrane and that this permeabilization is accompanied by a short burst in which solutes like glutamate are released.

**Mutacin II depletes the intracellular ATP pool in glucose-energized cells.** To investigate whether mutacin II affects the
level of intracellular ATP, susceptible *L. lactis* IL1403 cells were energized with glucose and the impact of 100 AU of mutacin II per ml on the ATP level was measured. Addition of mutacin II to the cells resulted in an early complete depletion of the intracellular ATP pool (Fig. 6). In cells preincubated with mutacin II, virtually no ATP was generated upon the addition of glucose. These data demonstrate that mutacin II depletes the entire intracellular ATP pool and thereby dramatically affects the energetic status of susceptible cells.

**DISCUSSION**

Mutacin II is a bacteriocin produced by some strains of oral streptococci (32). In this study the effect of mutacin II on susceptible cells was analyzed. Mutacin II is bactericidal, but the mechanism of its activity is not yet clear. A transient and only partial dissipation of the Δψ and ΔpH occurred upon the addition of mutacin II. This effect was accompanied by a partial release of AIB and glutamate. Further additions of mutacin II did not result in a greater effect. In vivo labelling experiments using intracellular DNA with the specific membrane-impermeable fluorescent label propidium iodide demonstrated that mutacin II does not cause the disruption of the cells or a subset thereof (2). Therefore, it appears that the initial interaction of mutacin with the cells is responsible for the transient and incomplete permeabilization of the membrane for ions and small solutes. The principal biological activity of mutacin II appears not to rely on pore formation, in contrast to the activity of type A lantibiotics such as nisin (7, 28–30). A dramatic effect of mutacin II on the cellular ATP levels was observed. Addition of mutacin II caused a complete depletion of the intracellular ATP pool. Therefore, it seems likely that the partial depolarization of the proton motive force is the indirect result of the permeation of these molecules across the cytoplasmic membrane to gain access to the cytosol. There, it may interact with the primary target, for instance, with enzymes involved in the generation of metabolic energy at the substrate level. The mechanism by which mutacin II depletes the intracellular ATP pool is so far obscure. Mutacin may either inhibit the enzymes involved in ATP generation or activate ATPases to induce futile cycling and a depletion of available ATP. In this respect, barely any effect of mutacin II on the rate of glycolysis (as measured by the rate of acid production) of lactococcal cells (2) or on the uptake of the nonmetabolizable carbohydrate analog methyl-β-D-thiogalactopyranoside (19) was observed. On the other hand, mutacin II also does not appear to belong to the group of type B lantibiotics, which contains, e.g., cinnamycin, duramycin, mersacidin, and actagaridine (28), globular polypeptides that act as enzyme inhibitors. The primary target of these type B lantibiotics is peptidoglycan synthesis or inhibition of phospholipases. The primary target of mutacin II seems not to be related to either of these phenomena; rather, mutacin II seems to be directed at the inhibition of metabolic energy generation.

When cells are grown in the presence of mutacin II, a spontaneous development of cells that appear to be resistant to the lantibiotic occurs. Intriguingly, this phenomenon is not a stable attribute, since it requires that the cells remain exposed to the lantibiotic. The mechanism of this apparent resistance is not known, but the spontaneous occurrence suggests that there is...
heterotypem among the bacteria as far as their susceptibility to mutacin II is concerned. This may limit the use of mutacin II in dental applications. A phenotypically similar resistance has recently been described for pediciuin AcH (20). In the natural environment, however, the bacterium-killing effect of mutacin II may occur in combination with extracellular acid production. 

*S. mutans* and other lactic acid bacteria convert fermentable carbohydrates primarily to lactic acid (for a review see reference 34), and this helps these organisms to maintain their niche. For instance, lactic acid produced by food-grade microorganisms acts as a preservative (26), while lactic acid production by oral streptococci and lactobacilli has been implicated in the dissolution of tooth enamel and thus in the pathogenesis of dental caries (14). Therefore, combinational effects of the bacteriocins and extracellular acid production may suppress growth of competing microbial flora in the respective ecosystems.

In summary, mutacin II is bactericidal and inhibits cell metabolic processes in an unknown, voltage-independent manner, thereby causing cell death without visible lysis.

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