THE MODE OF ACTION OF NANAOMYCIN A IN GRAM-POSITIVE BACTERIA

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The mode of action of nanaomycin A on Gram-positive bacteria such as *Staphylococcus aureus*, *Bacillus cereus* and *Streptococcus faecalis* was investigated. Nanaomycin A inhibited the biosyntheses of protein, DNA, RNA and cell-wall peptidoglycan to a similar extent. It increased the exogenous respiration of *S. aureus* cells at the minimal inhibitory concentration. The cells preincubated with nanaomycin A showed stimulation of proton influx after addition of N,N′-dicyclo-hexylcarbodiimide, an inhibitor of Ca^{++}, Mg^{++}-ATPase. Nanaomycin A seems to interfere with the cytoplasmic membrane or to inhibit coupling of oxidative phosphorylation, followed by secondary inhibitory effect on protein, nucleic acids and cell-wall peptidoglycan biosyntheses.

Nanaomycin A is a new quinone antibiotic (Fig. 1) discovered by ŌMURA *et al.* in 1974[1~4]. It is produced by *Streptomyces rosa* var. *notoensis* and is mainly effective against Gram-positive bacteria, mycoplasmas and fungi.

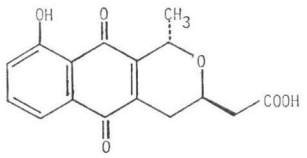
In this paper the mode of action of nanaomycin A against Gram-positive bacteria is presented.

Materials and Methods

Strains

Staphylococcus aureus FDA 209P, maintained at Kyowa Hakko Kogyo Co., Ltd., *Bacillus cereus* T, obtained from Dr. K. IZAKI, School of Agriculture, Tohoku University (Sendai), and *Streptococcus faecalis* ATCC 10541 were used.

Fig. 1. The structure of nanaomycin A.



Culture media

Nutrient broth (Difco, pH 7.0) was used for *S. aureus*. PM medium containing 0.5% peptone and 0.5% meat extract (pH 7.0) was used for *B. cereus* T. For *S. faecalis*, Tryptosoya broth (Nissan, pH 7.0) was used.

Growth of bacteria

Bacteria were grown in a Monod tube at 37°C with aeration. Drug was added after 3 hours of incubation and the optical density at 660 nm was measured.

Protein, DNA, RNA and cell-wall biosyntheses

Cultures of *S. aureus* or *B. cereus* T, grown overnight in PM medium at 37°C with aeration, were diluted 1/10 with fresh PM medium, then incubated at 37°C for 3 hours with aeration. To 4.0 ml of an exponentially growing culture, an isotope solution (0.5 ml) and an antibiotic solution (0.5 ml) were

Abbreviations used: TCA, trichloroacetic acid; Dpm, 2,6-diaminopimelic acid; CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; DCCD, *N,N′*-dicyclohexylcarbodiimide.

added, and the mixture (5.0 ml) was incubated in a Monod tube at 37°C. After 5, 10, 15 and 20 minutes, aliquots (0.5 ml) of the mixture were taken and added to 0.5 ml of 10% trichloroacetic acid (TCA) solution; the flocculated materials were collected on a Millipore filter (pore size, 0.22 μm). The radioactivity of the acid-insoluble fraction on the filter was counted in a scintillation counter using toluene scintillation fluid.

Protein, DNA and cell-wall peptidoglycan biosyntheses were measured from the radioactivities incorporated into the TCA-insoluble fraction, using as substrates [^{3}H]leucine (final concentration, 0.1 μCi/ml), [^{3}H]thymidine (10 μCi/ml) and [^{3}H]diaminopimelic acid (Dpm, 0.1 μCi/ml), respectively. RNA biosynthesis was evaluated from the radioactivity of the TCA-insoluble fraction incorporated from [^{3}H]uridine (0.1 μCi/ml) in the presence of 30 μg/ml cold uridine.

Endogenous respiration

Cells of *S. aureus* incubated in nutrient broth with aeration were harvested by centrifugation, washed with saline, and suspended in M/15 phosphate buffer (pH 7.0) to give a final OD_{660} of 0.80. After the suspension was incubated with aeration at 37°C for 2 hours, cells were harvested and resuspended in the same buffer (OD_{660}=0.61). An aliquot (0.5 ml) of the suspension was placed in the side arm of a Warburg's flask. The main compartment contained 0.2 ml of 2.7 mg/ml of glucose, 0.5 ml of the antibiotic solution and 1.1 ml of M/15 phosphate buffer (pH 7.0). The center well contained 0.2 ml of 20% potassium hydroxide with a piece of filter paper. The gas phase was air. Incubation was carried out at 37°C and oxygen uptake measured periodically.

Proton conduction

Proton conduction was monitored by the method described by FEINGOLD[5] and ROSENTHAL *et al.*[6] A culture of *S. aureus* or *S. faecalis* grown overnight at 37°C was diluted 1/10 with fresh medium and incubated with aeration for 3.5 hours at 37°C. The cells were harvested and washed twice with 0.1 M tris-(hydroxymethyl) aminomethane hydrochloride (pH 8.0). The cells (10^8 cells/ml) were centrifuged and washed twice with 50 mM potassium chloride, and finally suspended in the same solution. For the preincubation experiment, cells were incubated with nanaomycin A for 30 minutes in nutrient broth, washed with 50 mM potassium chloride, then resuspended in 50 mM potassium chloride. Before the experiment, the pH of fresh cell suspensions (2.0 ml) was brought to about 6 with 0.1 N hydrochloric acid and the drugs were added at various concentrations. The pH of the cell suspension was monitored periodically throughout the experiment.

Fig. 2. Effect of nanaomycin A on the growth of *Staphylococcus aureus* FDA 209P and *Bacillus cereus* T. Nanaomycin A was added at the time indicated by the arrows at the final concentrations of 0 (●), 0.78 (□), 1.56 (△), 3.12 (×) and 6.25 (○) μg/ml.

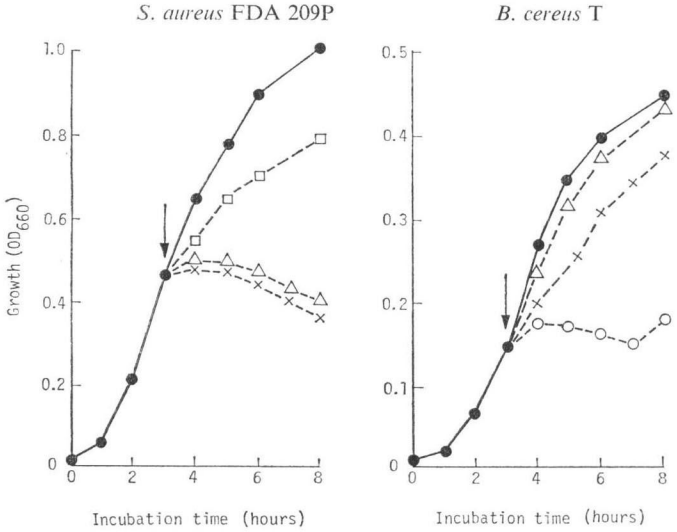


Fig. 3. Effect of nanaomycin A on nucleic acids and protein biosyntheses in *Staphylococcus aureus* FDA 209P.

Concentrations of nanaomycin A (μg/ml): 0 (●), 0.78 (□), 1.56 (△) and 3.12 (×).

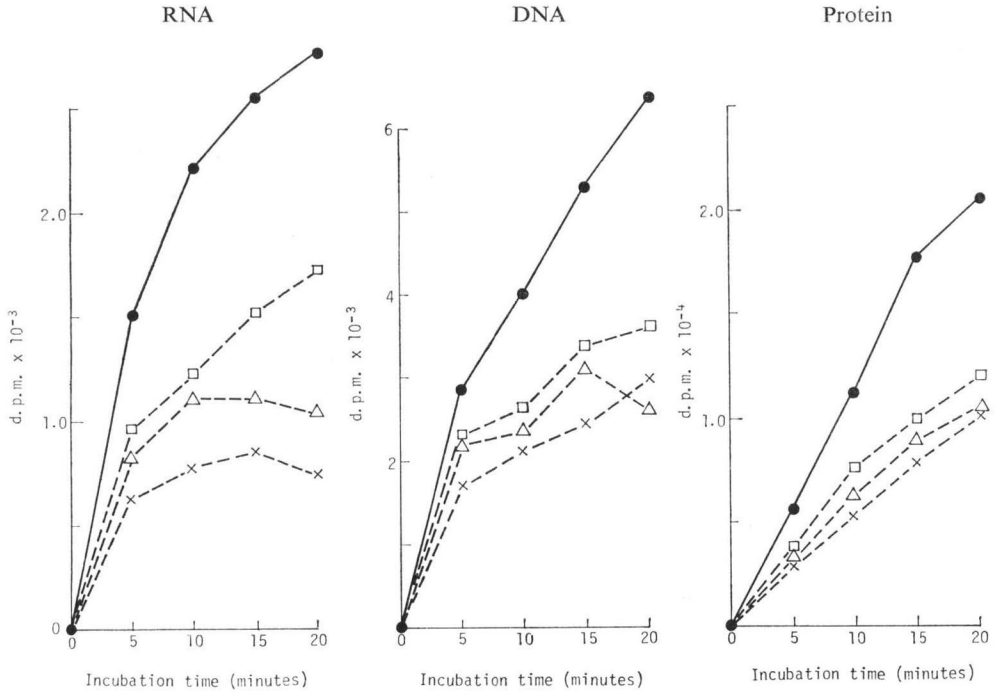


Fig. 4. Effect of nanaomycin A on the incorporation of [^{3}H] Dpm into cell-wall peptidoglycan of *Bacillus cereus* T.

Concentrations of nanaomycin A (μg/ml): 0 (●), 0.78 (□), 1.56 (△), 3.12 (×) and 6.25 (○).

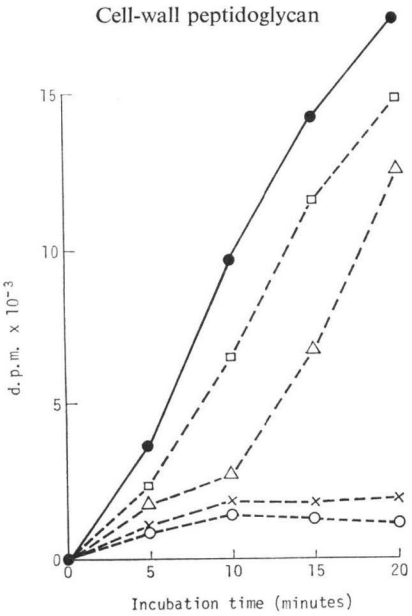
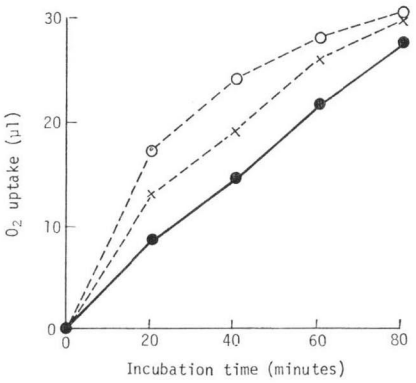


Fig. 5. Effect of nanaomycin A on the respiration of *Staphylococcus aureus* FDA 209P in the presence of glucose.

Concentrations of nanaomycin A (μg/ml): 0 (●), 3.12 (×) and 6.25 (○).



Chemicals and radiochemicals

Nanaomycin A was prepared by the method described previously[1,2]. Valinomycin was purchased from Boehringer Mannheim. These antibiotics were used after dissolution in ethanol.

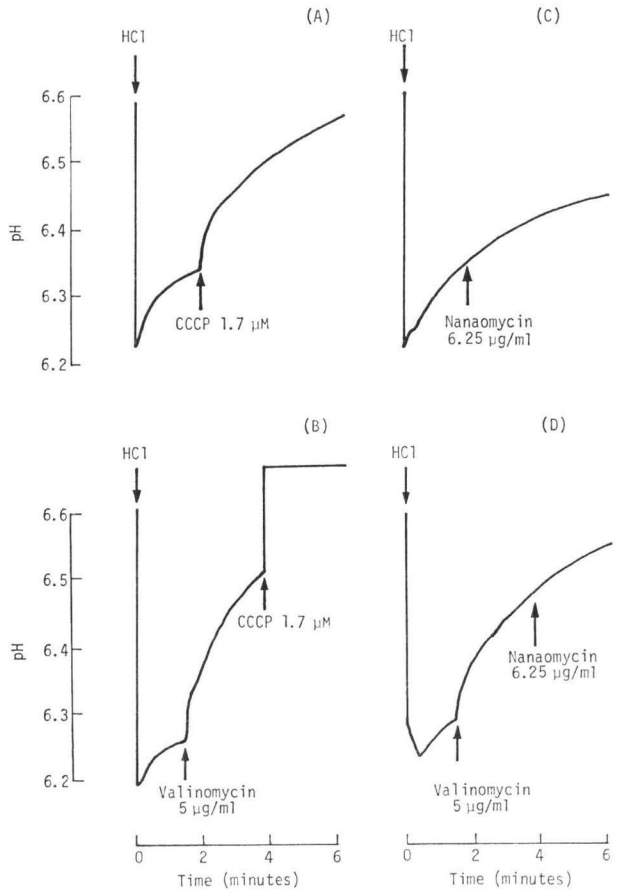
[6-^{3}H]Thymidine (5.0 Ci/mmole), [5-^{3}H]uridine (29.2 Ci/mmole) and 2,6-[^{3}H]Dpm (5.2 Ci/mmole) were purchased from the Radiochemical Center, Amersham; and L-[4,5-^{3}H]leucine (60 Ci/mmole) was purchased from New England Nuclear Co. Other chemicals were obtained commercially.

Results

The Effect of Nanaomycin A on Growth

Fig. 2 shows effect of nanaomycin A on bacterial growth when added to exponentially growing culture. The growth of *S. aureus* FDA 209P and *B. cereus* T was strongly inhibited by 1.56 and 6.25 μg/ml, respectively, of nanaomycin A. These concentrations correspond to the minimal inhibitory concentration of nanaomycin A (3.9 μg/ml) against *S. aureus* FDA 209P as obtained by the agar dilution method using nutrient agar[2].

Fig. 6. Effect of nanaomycin A on proton conduction to the cells of *Streptococcus faecalis* ATCC 10541.



Effects of Nanaomycin A on the Biosyntheses of Protein, Nucleic Acids and Cell-wall Peptidoglycan

As shown in Fig. 3, nanaomycin A (1.56 μg/ml) inhibited RNA and DNA biosyntheses in *S. aureus* at 5 minutes of incubation. It also inhibited protein biosynthesis at the same concentration of the drug, but the biosynthesis continued for 20 minutes. The incorporation of [^{3}H]Dpm into cell-wall of *B.*

cereus was severely inhibited by 3.12 μg/ml of nanaomycin A (Fig. 4).

Effect of Nanaomycin A on Respiration

Nanaomycin A stimulated the respiration (oxygen uptake) of *S. aureus* in the presence of glucose at a concentration of 3.12 μg/ml, and the stimulation became obvious immediately after the addition of nanaomycin A (Fig. 5).

Effect of Nanaomycin A on Proton Conduction

When a hydrochloric acid solution is added to a lightly buffered suspension of *S. faecalis*, the pH of the suspension falls abruptly and then rises slowly as H^+ passes into the cells[7]. As shown in Fig. 6, the tendency of pH to equilibrium was accelerated by carbamylcyanide-*m*-chlorophenylhydrazone (CCCP) and valinomycin, uncouplers of oxidative phosphorylation[7]; nanaomycin A had only slight effect, if any.

When the membrane potential is relaxed by an uncoupler, the cells tend to maintain the proton gradient through ATP hydrolysis which is catalysed by Ca^{++}, Mg^{++}-ATPase[8]. If the Ca^{++}, Mg^{++}-ATPase counteracts the effect of nanaomycin A on proton conduction, the inhibition of this enzyme should enhance the effect of nanaomycin A. As shown in Fig. 7, the addition of nanaomycin A to *N, N'*-dicyclohexylcarbodiimide (DCCD)-treated cells (*S. aureus*) showed an additional increase of 0.17 pH unit: DCCD is known to be a potent inhibitor of Ca^{++}, Mg^{++}-ATPase[9]. A similar effect was also observed when the order of addition of these two reagents was reversed: an additional increase of 0.26 pH unit was obtained. The value of pH gradient obtained was not significantly different from the control without drug

Fig. 7. Effects of nanaomycin A and valinomycin on proton conduction in *Staphylococcus aureus* FDA 209P.

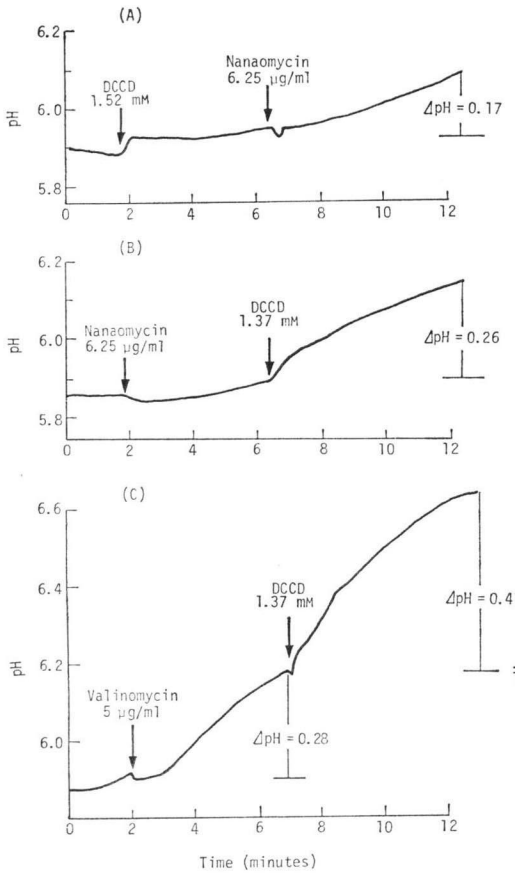
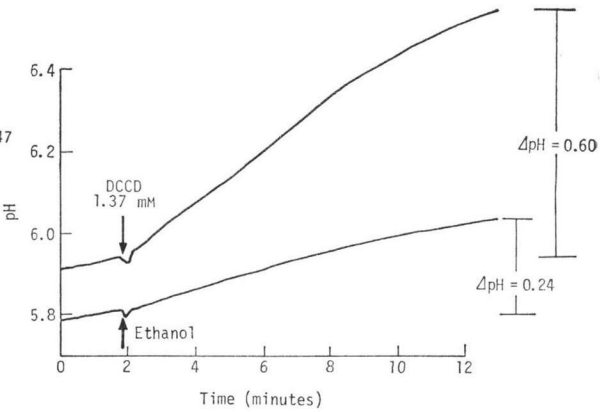


Fig. 8. Proton conduction by the cells pretreated with nanaomycin A and effect of DCCD on proton conduction.

The cells of *Staphylococcus aureus* were incubated in nutrient broth containing nanaomycin A (6.25 μg/ml) for 30 minutes at 37°C. With a suspension of cells in 50 mM potassium chloride, proton conduction was examined.



(data not shown). With valinomycin a larger effect was observed (Fig. 7 (C)).

On the other hand, upon the addition of DCCD to the cells of *S. aureus* preincubated with nanaomycin A for 30 minutes in nutrient broth, an additional increase of 0.60 pH unit was observed. Addition of ethanol (vehicle of DCCD) gave an increase of 0.24 pH unit (Fig. 8). These observations support the proposal that the increase in proton permeability stimulated by nanaomycin A is partly counteracted by Ca^{++}, Mg^{++}-ATPase.

Discussion

Nanaomycin A inhibited the growth of *B. cereus* T and *S. aureus* FDA 209P at concentrations of 1.56~6.25 μg/ml. Macromolecular biosyntheses (protein, DNA, RNA and cell wall peptidoglycan) were inhibited to similar degree in these organisms.

Cryomycin and valinomycin, uncouplers of oxidative phosphorylation, have been reported to stimulate the respiration and the proton conduction in *S. aureus* or *S. faecalis*[7,10]. Nanaomycin A stimulated the respiration of *S. aureus*, but did not increase proton uptake to any measurable extent at a concentration of 6.25 μg/ml. On the other hand, after the cells were pretreated with 6.25 μg/ml of nanaomycin A, the proton influx increased and was stimulated by DCCD, a potent inhibitor of Ca^{++}, Mg^{++}-ATPase[9], to the net charge of 0.60 pH unit (Fig. 8): the value was comparable to that obtained with valinomycin (Fig. 7 (C)). In potassium chloride solution, little synergetic effect between nanaomycin A and DCCD was observed, but the cells pretreated with nanaomycin A in nutrient broth showed the rapid increase of pH after the addition of DCCD.

From the above results, it is speculated that nanaomycin A interferes with the cytoplasmic membrane by altering and then disrupting its permeability in growing cells. The mechanism of action seems to differ from that of the so-called uncouplers of oxidative phosphorylation. Also, the disruption of membrane permeability may cause the inhibition of the incorporation of labeled precursors ([^{3}H]leucine, [^{3}H]thymidine, [^{3}H]uridine and [^{3}H]Dpm) into macromolecules.

Recently, OLENIK *et al.* reported that 2-hydroxy-3-(cyclohexylpropyl)-1,4-naphthoquinone binds preferentially to the membrane of *B. megaterium*[11]. Nanaomycin A is also a derivative of 1,4-naphthoquinone. The primary action of nanaomycin A must await further investigation.

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