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The Thuggacins, Novel Antibacterial Macrolides from *Sorangium cellulosum* Acting against Selected Gram-positive Bacteria

Production, Antimicrobial Activity and Mechanism of Action

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Abstract In our screening program we found an activity against some Gram-positive bacteria, including mycobacteria in the culture supernatant of *Sorangium cellulosum* strain So ce895. The antibiotic responsible for this activity was isolated and named thuggacin. Initial studies towards the mechanism of action showed that thuggacin A inhibits a late step of the respiratory chain of some bacteria.

Keywords *Sorangium cellulosum*, antibiotics, macrolides, respiration, thuggacin

Introduction

In the last few years, we isolated many strains of the order myxobacteria from soil samples collected from all over the world. The screening program comprised tests for cytotoxic, antifungal and antibacterial activities accompanied by HPLC-diode array UV analysis (HPLC-UV-DAD). The results showed that myxobacteria are a rich source of new secondary metabolites [1]. *Sorangium cellulosum* strain So ce895 showed an activity especially against some mycobacteria with a HPLC and UV spectrum not yet known to us. The active compounds were isolated and the structures were elucidated [2] (Fig. 1).

This report gives some data on the production and the biological activities of thuggacins A, B and C. Thuggacin A acts on the respiration of some bacteria. Electron transport chains in microorganisms with their different enzyme complexes are described in [3]. NADH driven electron flow begins with the oxidation of NADH and the reduction of ubiquinone by NADH-oxidase. Then in several bacteria, *e.g. Micrococcus luteus* and *Mycobacterium phlei*, electrons are transferred to cytochrome c by an enzyme complex containing cytochromes bc_1. The last step is the reduction of oxygen by the cytochrome c oxidase (terminal oxidase), containing the cytochromes aa_3. Other bacteria like *Escherichia coli* oxidize the ubiquinone by an ubiquinol oxidase. In most cases bacteria have more than one terminal oxidase with different cytochromes.

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Materials and Methods

Fermentation

The producing strain was isolated from a soil sample in 1993.

The antibiotic was produced by fermentation. The medium consisted of soluble starch 0.6%, yeast extract 0.2%, KNO_3 0.04%, NH_4Cl 0.06%, $CaCl_2 \cdot 2H_2O$ 0.1%, $MgSO_4 \cdot 7H_2O$, 0.1%, glucose$\cdot H_2O$ 0.35%, Na-Fe-EDTA 8.0 mg/liter, and Amberlite adsorber resin XAD-16 1.0%.

The pH was adjusted with KOH (20%) to 7.4 before autoclaving and was kept at 7.0 with KOH (5.0%).

The fermentation conditions were: inoculum, 2.0% (v/v);

temperature, 30°C; stirring rate, 80/minute; aeration, 0.05 liters/liter culture/minute until the pO_2 decreased to 20%, and was kept at this value by variation of the stirring rate.

Isolation

The XAD was separated from the culture and the antibiotic was isolated as described elsewhere [2].

Biological Assays

MIC values were determined according to [4]. Bacteria, initial cell concentration of 10^5 cells/ml, were incubated overnight at 30°C in a medium containing peptone.

Macromolecular Synthesis

The synthesis of DNA, RNA and protein in *Micrococcus luteus* were measured as described in [4]. The precursors were [^{3}H-methyl]thymine (53.2 Ci/mmol), 2-[^{14}C]-uracil (60 Ci/mol) and U-[^{14}C]-protein hydrolysate (50 Ci/gramatom C).

Oxygen Consumption

An overnight culture of *Micrococcus luteus* was diluted to an optical density (623 nm) of 0.5 in growth medium. Measurement of oxygen was performed at 30°C in a RE K1-1 N device from Biolytik, Bochum, Germany.

Respiratory Chain Measurements

A crude fraction of cytoplasmic membranes of *Micrococcus luteus* was isolated as follows.

Cells from an overnight culture were harvested by centrifugation and washed twice with 50 mM phosphate buffer, pH 7.8. Then the cells were burst in a French pressure cell at 16,000 psi. The suspension was centrifuged at 15,000×g for 20 minutes and the supernatant at 160,000×g for 1.5 hours. The pellet was resuspended in phosphate buffer and frozen at −80°C. For measurements, it was thawed at room temperature. Cytoplasmic membranes of other microorganisms were isolated in the same way.

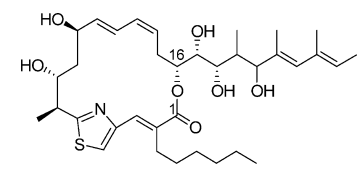
NADH oxidation and particular steps of the respiratory chain were measured as followed: NADH oxidase at 340 nm starting with 0.255 mM NADH, NADH dehydrogenase at 578 nm starting with 0.255 mM NADH and 0.066 mM 2,6-dichlorophenolindophenol and 2.0 mM KCN, NADH: cytochrome c reductase at 546 nm with 0.255 mM NADH and 0.05 mM horse heart cytochrome c and 2.0 mM KCN, cytochrome c oxidase at 550 nm with 100 mM reduced cytochrome c [5, 6]. The protein concentration of the cytoplasmic membrane in these tests was 0.028 mg/ml. The membranes were suspended in prewarmed (30°C) air-saturated phosphate buffer. Electron acceptor, if necessary, and inhibitor were added and incubated for 2 minutes. Then the reaction was started with NADH and the absorbence was measured at 30°C during 10 minutes.

Cytochrome difference spectra were recorded in an AMINCO DW 2000 UV/VIS spectrophotometer at 30°C according to [7]. Concentration of NADH: 1.4 mg/ml, thuggacin A: 20 μg/ml, KCN: 240 μg/ml, protein concentration of the membranes: 1.4 mg/ml. Preincubation of membranes was as above. Then NADH was added to the sample cuvette, whereas the reference cuvette contained membranes only. For measurement of the baseline there were membranes in both cuvettes without addition of NADH. When the reduction was at its maximum, the difference spectrum was recorded. In these tests the concentrations of the inhibitors thuggacin and KCN were higher than for measurement of enzyme reactions, because the concentration of the membranes was higher [7]. Beside this there was no experience with membranes from bacteria. So high concentrations were used.

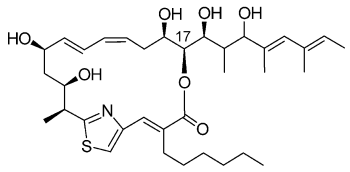
Results

Production of Thuggacin

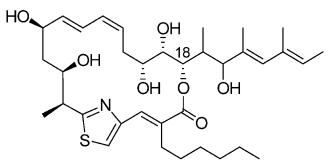
Fig. 2 shows that after a lag phase of 2 days the strain grew exponentially for 3 days, then growth slowed down. At day 7, the cells began to lump together. The production of thuggacin A did not begin unitil day 5 when exponential



Thuggacin A



Thuggacin B



Thuggacin C

Fig. 1 Structures of thuggacins A, B and C.

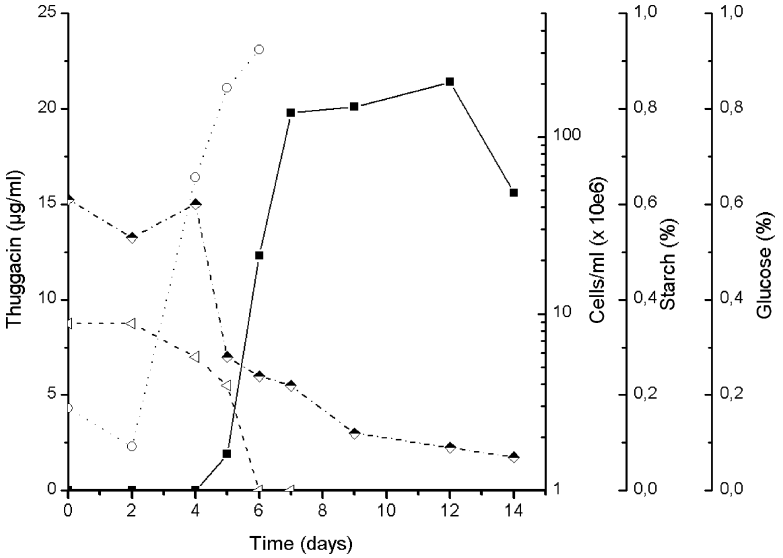


Fig. 2 Production of thuggacin A in a 10-liter fermentor.
—■— thuggacin A (µg/ml), ···○··· cells/ml, --◆-- starch (%), ---◁--- glucose (%).

growth slowed down. It continued for two days and reached a maximum value of the antibiotic of 21 µg/ml. After this the level remained constant for the next 5 days. The concentration of starch did not change during the first four days, but then the starch was quickly used up. Glucose, on the other hand, was used up by until day 6.

Biological Effect of Thuggacin

Thuggacin inhibited the growth of some Gram-positive bacteria, especially of *Micrococcus luteus* and of species of the genera *Corynebacterium* and *Mycobacterium*. But great differences of the MIC values were observed within these groups. The activities of thuggacins A and B were rather similar, but thuggacin C was much less active. They were not active against yeasts and fungi. The mouse fibroblast cell line L929 was moderately sensitive to thuggacin A (Table 1).

The mode of action of thuggacin A was studied using *Micrococcus luteus*. The syntheses of DNA, RNA and protein, measured as incorporation of radioactive precursors, were stopped immediately after the addition of the antibiotic (Fig. 3).

Results from the measurement of the oxygen consumption of *Micrococcus luteus* under the influence of thuggacin are presented in Fig. 4. It shows that respiration was inhibited to 100% with 2.5 ng/ml. The figure further gives the inhibition of NADH oxidation of cytoplasmic membranes of *M. luteus*. The maximum inhibition of NADH oxidation reached 90% at 10 ng/ml thuggacin A. As a control, with 60 µg/ml KCN, there also was a maximal inhibition of 90%. Fig. 4 also shows that the cytochrome oxidase, using reduced cytochrome c, was inhibited comparably to the inhibition of NADH oxidation. The system NADH-cytochrome c oxidoreductase was not influenced until at least 1000 ng/ml (data not shown). Fig. 5 shows difference spectra of the cytochromes in crude membranes of *M. luteus* reduced by NADH versus air oxidized membranes, with or without inhibitors. Thuggacin A gives a partial inhibition of the reduction of cytochrome

Table 1 MIC values of thuggacin A

Test organism	MIC (µg/ml)
Staphylococcus aureus GBF	>20
Bacillus subtilis GBF	5
Corynebacterium mediolanum GBF	5
Corynebacterium glutamicum DSM20300	0.006
Nocardia corallina GBF	0.006
Micrococcus luteus GBF	0.003
Mycobacterium phlei GBF	0.03
Mycobacterium chitae DSM43238	0.6
Mycobacterium smegmatis DSM43856	40
Mycobacterium diernhoferii DSM43218	20
Escherichia coli DSM5698	>20
Saccharomyces cerevisiae GBF	>80
	IC_{50} (µg/ml)
Mouse fibroblast cells L929	4

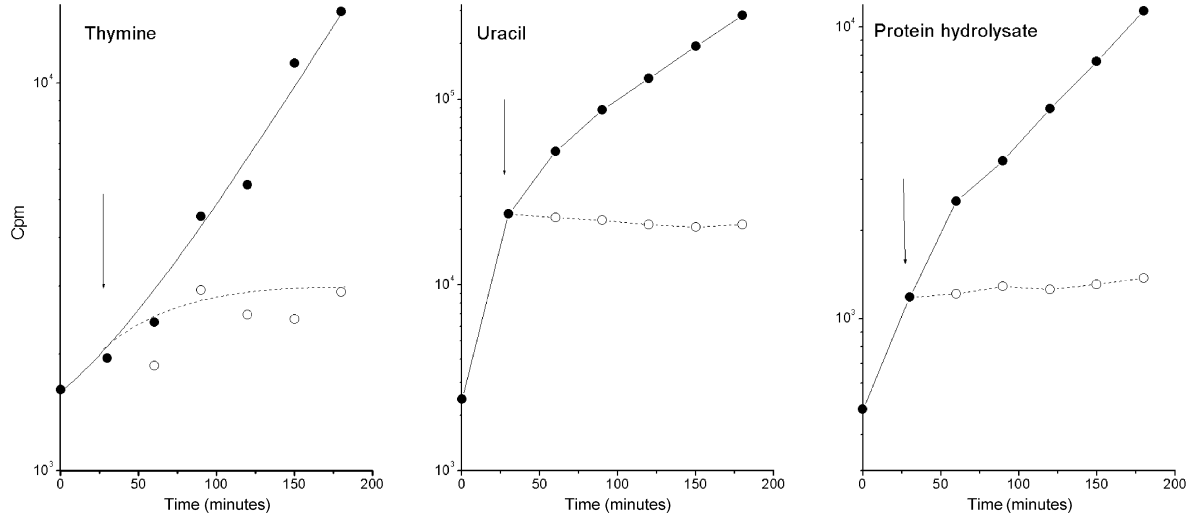


Fig. 3 Synthesis of some macromolecules in cells of *M. luteus*.

Incorporation of precursors of DNA, RNA and Protein was measured arrow shows time of addition of the antibiotic. ●; Control, ○; 20 ng thuggacin A/ml.

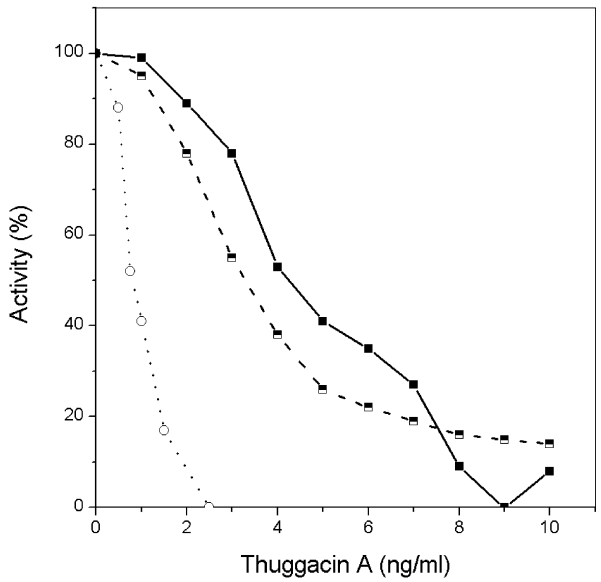


Fig. 4 Effects of thuggacin A on the oxygen consumption in *M. luteus*, and NADH oxidase and cytochrome c oxidase in cytoplasmic membranes of *M. luteus*.

100% activity of NADH oxidase was 0.0149 mM NADH minute^{-1} and of cytochrome c oxidase 0.117 μM cytochrome c minute^{-1}. ···○··· Oxygen demand of *M. luteus* cells, −−■−− NADH oxidation in CM from *M. luteus*, —■— cytochrome c oxidase in CM from *M. luteus*.

a (600.9 nm), cytochrome b (560.3 nm) and cytochrome c (551.7 nm). A similar, but not identical, result was obtained with KCN, as the reduction of cytochrome b was slightly less and that of cytochrome c slightly more inhibited than with thuggacin.

Cytoplasmic membranes (crude fractions) were also isolated from *Mycobacterium phlei*, *Corynebacterium glutamicum*, *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis*.

Only the respiratory chains of *M. phlei* (NADH oxidase, IC_{50}: 5.0 ng/ml) and *C. glutamicum* (NADH oxidase, IC_{50}: 15 ng/ml) showed similar sensitivities for thuggacin A as that of *M. luteus*.

Discussion

The thuggacins constitute a novel group of macrolide antibiotics (Fig. 1). They are produced by some strains of the myxobacterium *Sorangium cellulosum*. In strain So ce895 they were produced in the late log phase of growth and excreted into the medium up to values of about 20 μg/ml (Fig. 2).

Thuggacin proved to be active against some Gram-positive bacteria, all of them belonging to the Actinomycetales (Table 1).

The syntheses of three important macromolecules (DNA, RNA and protein) were inhibited immediately after the addition of the antibiotic (Fig. 3). Further measurements indicated that the energy supply of whole cells was interrupted by inhibition of the oxygen consumption (Fig. 4). Measurements of the activity of the respiratory chain in cytoplasmic membranes corroborated this result. Both, oxidation of NADH and of cytochrome c, were inhibited (Fig. 4), but not the reduction of added oxidized

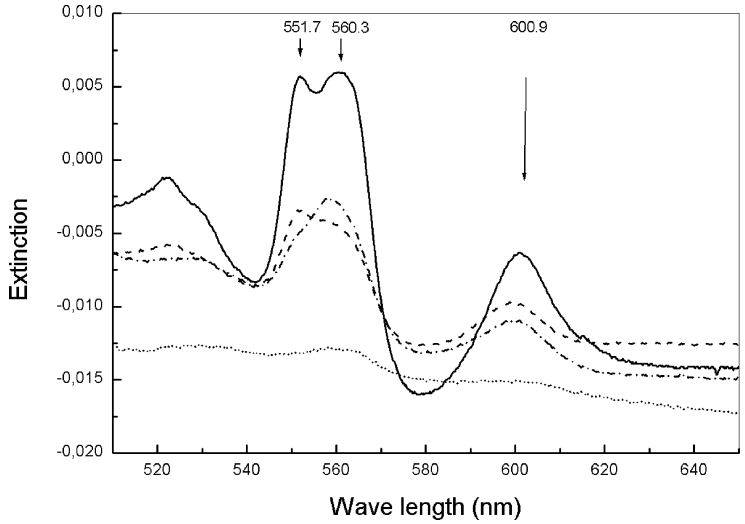


Fig. 5 Influence of thuggacin A on the reduction of cytochromes by NADH in membranes of *M. luteus* suspended in air-saturated buffer.

·····; Baseline without NADH and inhibitor, -·-·-; with NADH and KCN, – – –; with NADH and thuggacin, ——; with NADH alone.

cytochrome c by NADH. There remains unexplained difference between the total inhibition observed in whole cells and a non-inhibitable rest activity in membrane fractions. However, as described [8, 9], there are alternative electron pathways in *Micrococcus luteus* and other bacteria resulting in an incomplete inhibition by KCN. Fig. 5 gives the difference spectra with membranes of *M. luteus*. Both KCN and thuggacin show a similar inhibition of the reduction of cytochrome a.

On the other hand, inhibition at 551.7 (cytochrome c) and 560.3 nm (cytochrome b) differs slightly.

The data presented in Figs. 4 and 5 indicate that one of the late steps of the respiratory chain is the target of thuggacin. The small deviations between KCN and thuggacin in the inhibition of the cytochromes may be due to different binding sites. But the inhibition of the reduction of all the cytochromes is not yet completely understood.

Similar results as with *M. luteus* were obtained with membranes of *Mycobacterium phlei* and *Corynebacterium glutamicum*. The latter organism is of special interest because the molecular composition of the components of the respiratory chain is under investigation [9].

The tolerance of *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli* to thuggacin corresponds to the insensitivity of the NADH oxidation in cytoplasmic membranes of these organisms.

So thuggacins are an additional novel group of antibiotics from myxobacteria that acts on the electron transport chain [10, 11], but it seems to be selectively restricted to a few organisms. On the other hand, it may be of a certain interest because of its activity against *Mycobacterium tuberculosis* strain H37RV showing a MIC value of 8.0 μg/ml [Rüsch-Gerdes, S., Forschungszentrum Borstel, Germany, personal communication].

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