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MODE OF ACTION OF ITURIN A, AN ANTIBIOTIC ISOLATED FROM *BACILLUS SUBTILIS*, ON *MICROCOCCUS LUTEUS*

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Iturin A has an antibacterial activity on *M. luteus* which is strongly reduced in presence of $MgCl_2$. Iturin A lyses *M. luteus* protoplast, this lysis is enhanced by EDTA and inhibited by $MgCl_2$. These results suggest an action of iturin A on cytoplasmic membrane with interactions of both lipophilic and polypeptidic moieties of the antibiotic, respectively with membrane lipids and membrane polar components. Polar interactions involve the participation of mineral ions as magnesium ions have a strong inhibition effect on the activity of iturin A. The effect of iturin A on the incorporation of radioactive thymidine, uracil, isoleucine and alanine seems unspecific and is probably a consequence of the primary action on cytoplasmic membrane.

INTRODUCTION

Iturin A is a peptidolipidic antibiotic which was found in various strains of *Bacillus subtilis* (1,2). Its structure is characterized by a C_{14} or C_{15} β-aminoacid included in a macrocyclic heptapeptide with D and L α-aminoacids (3,4). This antibiotic has a strong antifungal activity on a large variety of yeasts and fungi but its antibacterial activity is limited to a few bacteria specially *Micrococcus luteus* (5). We have studied the mode of action of iturin A on *M. luteus*.

MATERIAL AND METHODS

Antibiotics. Iturin A was prepared as described previously (2). Rifampicin was obtained from Sigma (U.S.A.), chloramphenicol from Mann Research Laboratories (U.S.A.) mytomycin from Schwaz-Mann (U.S.A.) and vancomycin from Lilly and Co. (U.K.).

Culture conditions. *M. luteus* NCTC 2665 was grown at 35°C on a brain-heart medium, 37 g/l (bioMérieux, France) and the growth was estimated by turbidimetry at 600 nm. In assays for the biosynthesis of peptidoglycan, an inhibitor of protein synthesis, chloramphenicol (100 µg/ml) was added to the medium.

Incorporation of radioactive precursors. |^{14}C| thymidine (53.8 mCi/mmol), |^{14}C| uracil (55 mCi/mmol), L-|^{14}C| isoleucine (240 mCi/mmol) and L-|^{14}C| alanine

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(50 mCi/mmol) were obtained from the Commissariat à l'Energie Atomique, Saclay, France. The radioactive compound was added to a log-phase culture of *M. luteus* (absorbance = 0.3), 1 µCi/ml for uracil and L-isoleucine, 1.1 µCi/ml for thymidine. When the absorbance reached 0.6, the antibiotic was added to the medium and after 10, 20, 30, 40, 50 min 1 ml aliquots of the culture were each treated with ice-cold 5 % trichloracetic acid. The resulting acid-insoluble fractions were collected on a Millipore filter (0.45 µ) and washed by 5 ml of 10 % trichloracetic acid, then 2 ml ethanol. The radioactivities of acid-insoluble fractions were determined by a liquid scintillation spectrometer (Packard Tricarb 2001) in a scintillation fluid containing, in 1 l of toluene, 5 g of 2,5 diphenyloxazole and 0.3 g of 1,4 bis (4-methyl 5-phenyloxazol-2 yl) benzene.

For the cell wall peptidoglycan synthesis the bacteria were grown in a brain-heart medium, collected by centrifugation and suspended in a new medium containing L-|^{14}C| alanine (2 µCi/ml) and chloramphenicol (100 µg/ml). After 15 min incubation, vancomycin or iturin A was added and the incorporation of radioactive alanine in the acid-insoluble fraction was measured as described above.

Preparation of *M. luteus* protoplasts. *M. luteus* was grown on 400 ml brain-heart medium for 15 h. The bacteria were collected by centrifugation, washed by 100 ml of 10 mM pH 8.0 Tris/HCl buffer and suspended in 50 ml of a solution 20 % sucrose in 20 mM pH 8.0 Tris/HCl buffer. Lysozyme was added to the final concentration 0.5 mg/ml. The suspension was stirred gently for 1 h at room temperature and the protoplast formation was controled by phase contrast microscopy. The protoplast suspension was diluted with the solution 20 % sucrose in 20 mM Tris/HCl buffer until the absorbance reached 0.35 and the antibiotic was added. In some assays 1 mM, 20 mM $MgCl_2$ or 20 mM EDTA were added to the protoplast suspension. The lysis of protoplasts was measured by plotting the absorbance at 600 nm with a Beckman spectrophotometer connected to a recorder.

RESULTS

1) Effect of Iturin A on the growth of *M. luteus*.

The inhibition of the growth of *M. luteus* by iturin A increased with the concentration of the antibiotic (Fig. 1). After 10 h of incubation the inhibition was 6 % with a concentration 10 µg/ml and 93 % with a concentration 100 µg/ml of iturin A.

2) Effect of Iturin A on the biosynthesis of macromolecules.

The *in vivo* biosynthesis of macromolecules in presence of various concentrations of iturin A was studied by measuring the incorporation of radioactive precursors into the trichloracetic acid precipitate (see Material and Methods). Control assays were simultaneously performed in absence of iturin A and in presence of a specific inhibitor of each biosynthesis (Fig. 2a, 2b, 2c).

In all experiments the effect of iturin A was weak for concentration 50 µg/ml but no incorporation of radioactive compounds was found when the concentration of iturin A reached 200 µg/ml. The incorporation of |^{14}C| thymidine was weak, even in absence of antibiotic, however, in presence of iturin A (200 µg/ml), no incorporation was found. Thus all the biosynthesis : protein,

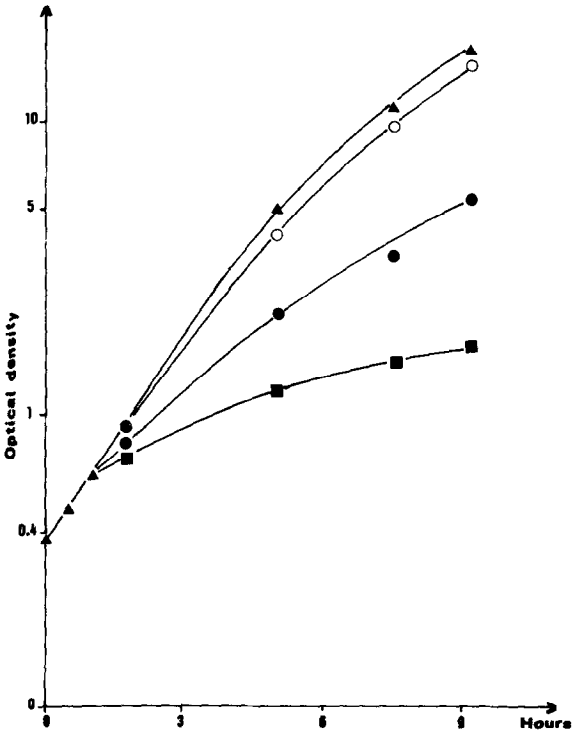

Figure 1. Effect on iturin A on growth of *M. luteus*. ▲—▲ control, ○—○ 10 µg/ml, ●—● 50 µg/ml, ■—■ 100 µg/ml.

RNA, DNA, peptidoglycan were similarly inhibited by iturin A and these results did not permit to state the primary site of activity of this antibiotic.

The antibacterial activity of iturin A which is limited to a few bacteria could be related with a specific composition of membrane or cell wall of these bacteria. The peptidoglycan of *M. luteus* has a structural peculiarity with head-to-tail peptide cross linkages (6) and its biosynthesis needs a specific transpeptidase (7). We have tested the possibility of an inhibition of peptidoglycan biosynthesis with an accumulation of radioactive precursors, using the experimental procedure of Chmara and Borowski (8). *M. luteus* was incubated in a medium containing chloramphenicol and L-[^{14}C] alanine (see Material and Methods) ; the bacterial suspension was heated 2 min, 100°C and analyzed by paper chromatography. Comparative experiments were performed with *M. luteus* without antibiotic and with vancomycin. There was no difference between the assays without antibiotic and with addition of iturin A while, in the assay with addition of vancomycin, a more intense radioactivity was observed in the precursors of peptidoglycan. These results ruled out the possibility of a specific activity of iturin A on the biosynthesis of peptidoglycan.

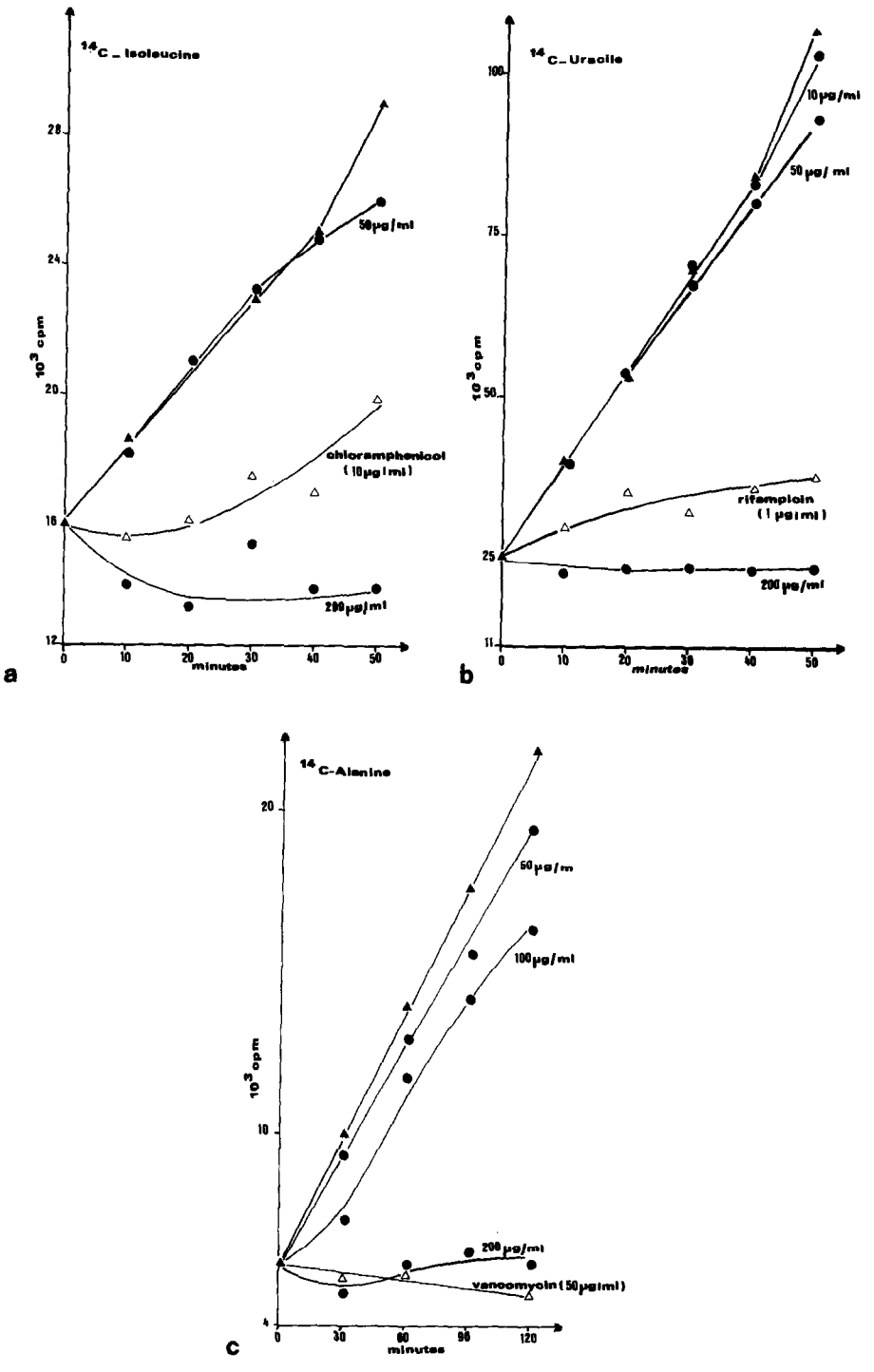

Figure 2. Incorporation of radioactive precursors into acid-insoluble fraction of *M. luteus* cells. ▲—▲ control, ●—● incubation in presence of various concentrations of iturin A, △—△ incubation in presence of standard antibiotic.

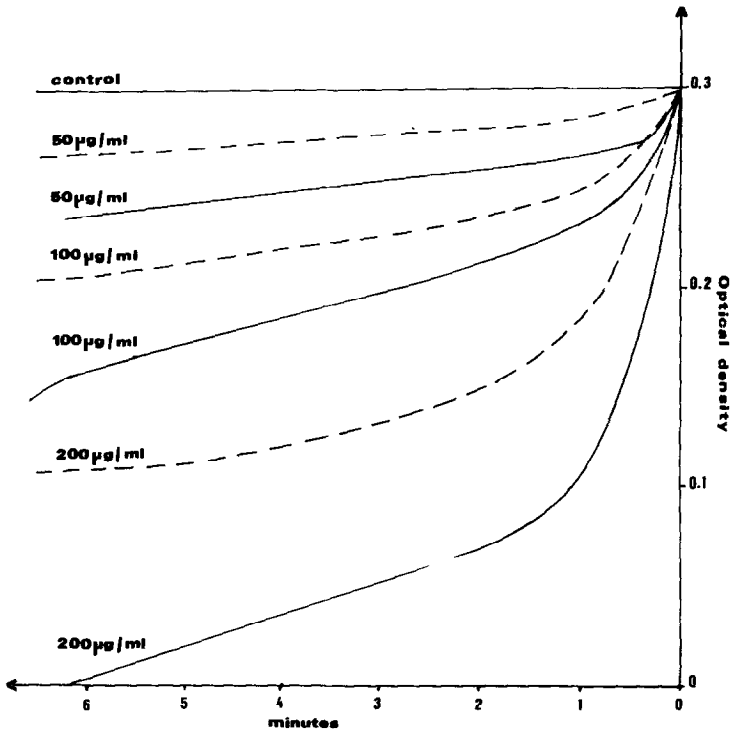

Figure 3. Effect on iturin A on a suspension of *M. luteus* protoplasts.
— — suspension in 20 % sucrose, 20 mM pH 8.0 Tris/HCl buffer
——— suspension in the same buffer + 20 mM EDTA.

3) Effect of Iturin A on *M. luteus* protoplasts.

The peptidolipidic structure of iturin A could agree with an activity on the cytoplasmic membrane involving binding of the lipid moiety of the antibiotic with membrane phospholipids. This possibility was tested on *M. luteus* protoplasts and the results are reported fig. 3. *M. luteus* protoplasts were sensitive to iturin A and the turbidity decreased when the concentration of antibiotic increased. In all experiments, lysis was rapid in the first 5 min and reached the maximum value in 10 min. The percentages of lysed protoplasts after 5 min are reported in table I.

Similar experiments were carried out by adding 1 mM, 20 mM $MgCl_2$ or 20 mM EDTA to the medium. The turbidity curves are shown fig. 4 and the percentages of protoplasts lysed after 5 min are reported in table I. The presence of 1 mM $MgCl_2$ had a strong protective effect and no lysis was observed with 20 mM $MgCl_2$, even for a high level of iturin A (200 µg/ml). On the other hand the lytic activity of the antibiotic was strongly enhanced by EDTA.

TABLE I

Lysis of M. *luteus* protoplasts by Iturin A.
Results are given in % of protoplasts lysed after 5 min.

Concentration of Iturin A mg/ml	Products added to the medium			
	0	1 mM $MgCl_2$	20 mM $MgCl_2$	20 mM EDTA
50	10	0	0	20
100	30	4	1	43
200	60	27	4	93

4) Effect of $MgCl_2$ on the activity of Iturin A against bacteria.

The protective effect of $MgCl_2$ against iturin A was studied with a culture of M. *luteus*. Various concentrations of iturin A (50 µg/ml, 100 µg/ml and 200 µg/ml) were added to the culture medium containing 20 mM or 60 mM $MgCl_2$ and the growth of bacteria was followed by turbidimetry at 600 nm. With all iturin A concentrations a strong inhibition of the antibiotic activity was found in presence of $MgCl_2$: 85 % for 20 mM $MgCl_2$ and 91 % for 60 mM $MgCl_2$.

DISCUSSION

The activity of several polypeptidic antibiotics is due to their binding to the cytoplasmic membrane with a disturbance of its function (9). This group includes polymyxins, tyrocidins and gramicidin S which are cyclic polypeptides. Another group of polypeptidic antibiotics has ionophoric properties : valinomycin and gramicidin A form a well-defined complex with potassium ions and thus the transport of these ions across the membrane is facilitated (9). Recently Rosenthal *et al* found that a peptidolipidic antibiotic, EM 49, split the outer membrane of *Escherichia coli* with a release of fragments in the medium (10).

The lysis of M. *luteus* protoplasts by iturin A agrees with an antibacterial action on the cytoplasmic membrane. The iturin A has a lipophilic moiety, a C_{11} or C_{12} aliphatic hydrocarbon chain and a hydrophilic moiety, a cyclic polypeptide. It can be postulated that the hydrocarbon chain penetrates in the hydrophobic zones of the cytoplasmic membrane and the polypeptide moiety is able to interact with polar groups of the membrane. The protective effect

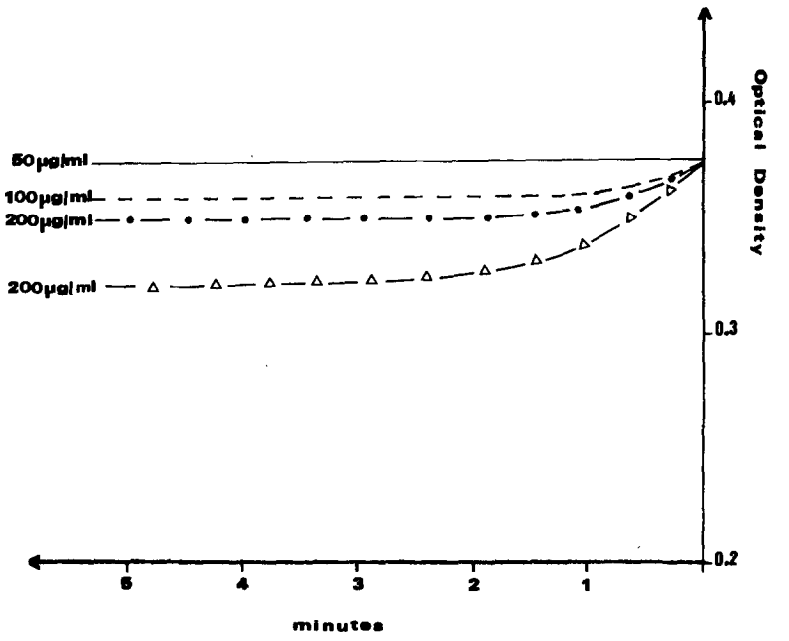

Figure 4. Effect of $MgCl_2$ on the lysis of *M. luteus* protoplasts by iturin A. Protoplasts were suspended in the sucrose-Tris/HCl buffer as described fig. 3. ——— addition of 50 µg/ml iturin A and 1 mM or 20 mM $MgCl_2$, — — — addition of 100 µg/ml iturin A and 1 mM or 20 mM $MgCl_2$, •—•—• addition of 200 µg/ml iturin A and 20 mM $MgCl_2$, △—△—△ addition of 200 µg/ml iturin A and 1 mM $MgCl_2$.

of magnesium ions on the bacteria and on the protoplasts and the enhancement of the lysis of protoplasts by EDTA agree with this hypothesis. It is not possible to know whether the cytoplasmic membrane is the only site of action of iturin A. With the protoplasts, the interaction membrane-iturin A might be sufficient to disturb the structure of the membrane and to give a lysis while on the bacteria this interaction couldinactivate metabolites or enzymes of the cytoplasmic membrane. Such a mechanism had been found for bacitracin which forms a complex with a membrane phospholipid, the polyisoprenyl pyrophosphate, an inter mediate in the peptidoglycan biosynthesis (11).

In conclusion, our results agree with an action of iturin A at the level of the cytoplasmic membrane ; moreover they suggest the possibility to use this antibiotic for studies of the function of bacterial membranes and of interactions lipids-peptides.

ACKNOWLEDGMENTS

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