# ACTION OF MYCOSUBTILIN AND OF BACILLOMYCIN L ON *MICROCOCCUS LUTEUS* CELLS AND PROTOPLASTS

## Influence of the polarity of the antibiotics upon their action on the bacterial cytoplasmic membrane+

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### 1. Introduction

Mycosubtilin and bacillomycin L are antifungal agents which were isolated from strains of *Bacillus subtilis* [1,2], their structures have been recently determined [3,4]. These antibiotics are cyclic peptidolipids from iturin group [5] characterized by a liposoluble β-amino acid [6] linked to a peptidic moiety containing D and L α-amino acids. Mycosubtilin as iturin A, has no amino acid residue with an ionic side chain while bacillomycin L has 2 aspartyl residues [3,4,7].

A previous work has shown that the action of iturin A on *Micrococcus luteus* could involve an interaction between the antibiotic and the cytoplasmic membrane [8]. We have studied the activity of mycosubtilin and of bacillomycin L on *M. luteus* cells and protoplasts to find a possible correlation between the mode of action and the structural peculiarities of iturin A, mycosubtilin and bacillomycin L.

### 2. Materials and methods

2.1. *Antibiotics and other products*

Mycosubtilin was a gift of Dr H. B. Woodruff, Merck and Co., Rahway, NJ, and bacillomycin L was a gift of Dr G. H. Warren, Wyeth Inst. App. Biochem., PA. Chloramphenicol was obtained from Mann Res. Labs (USA), mytomycin from Schwaz-Mann (USA), rifampicin from Sigma Chemical Co. (USA) and vancomycin from Lilly and Co. (England). Radioactive compounds were obtained from the Commissariat à l'Energie Atomique, Saclay, France.

2.2. *Culture conditions and incorporation of radioactive precursors*

*M. luteus* NCTC 2665 was grown at 35°C on a brain–heart medium, (Bio-Mérieux, France) 37 g/l; the growth was measured by turbidimetry at 600 nm. The incorporation of [$^{14}$C]thymidine, [$^{14}$C]uracil, L-[$^{14}$C]isoleucine and L-[$^{14}$C]alanine was determined by measurement of the radioactivity in the trichloracetic precipitate from bacterial cells as in [8].

2.3. *Preparation of* M. luteus *protoplasts*

The protoplasts of *M. luteus* were prepared from lysozyme-treated cells in a Tris/HCl–sucrose buffer as in [8]. The lysis of protoplasts was followed by plotting the $A_{600}$ with a Beckman spectrophotometer connected to a recorder.

### 3. Results

3.1. *Action of antibiotics on the growth of* M. luteus

Mycosubtilin and bacillomycin L inhibited the growth of *M. luteus* (fig.1a,b). The inhibition increased with the concentration of the antibiotic:

+ This paper is dedicated to Professor E. Lederer, on occasion of his 70th birthday

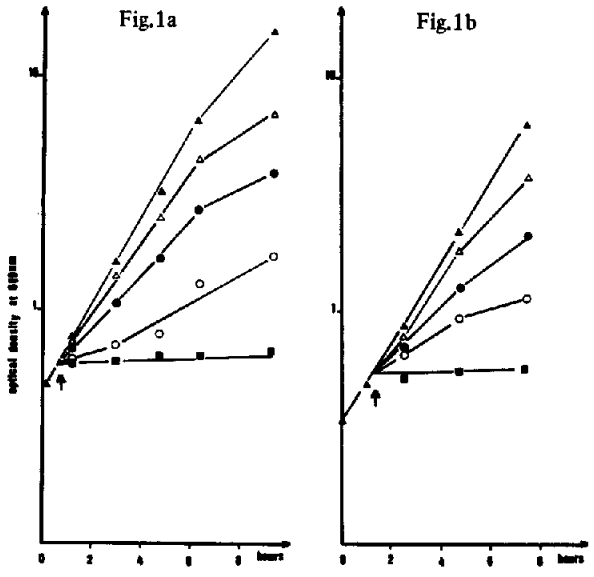


Fig.1. Effect of antibiotics on growth of *M. luteus*. a, mycosubtilin. b, bacillomycin L. (▲—▲) Control; (△—△) 20 μg/ml; (●—●) 40 μg/ml; (○—○) 100 μg/ml; (■—■) 200 μg/ml.

90% inhibition for 100 μg/ml and total inhibition for 200 μg/ml antibiotic. The effect of mycosubtilin and of bacillomycin L was quite similar to that of iturin A [8].

### 3.2. *Action of antibiotics on the incorporation of radioactive precursors of macromolecules*

The incorporation of radioactive precursors during the biosynthesis of macromolecules was determined by measuring the radioactivity into the trichloracetic acid precipitate (see section 2 and [8]). Control assays were performed in absence of antibiotic and in presence of an inhibitor, specific for each biosynthesis. The results are shown in fig.2 for mycosubtilin and fig.3 for bacillomycin L.

The biosyntheses of proteins, RNA and peptidoglycan were nearly totally inhibited in presence of 200 μg/ml each antibiotic. However the incorporation of isoleucine in proteins gave rather dispersed values with a high concentration of antibiotics (200 μg/ml). No explanation may be proposed for this latter

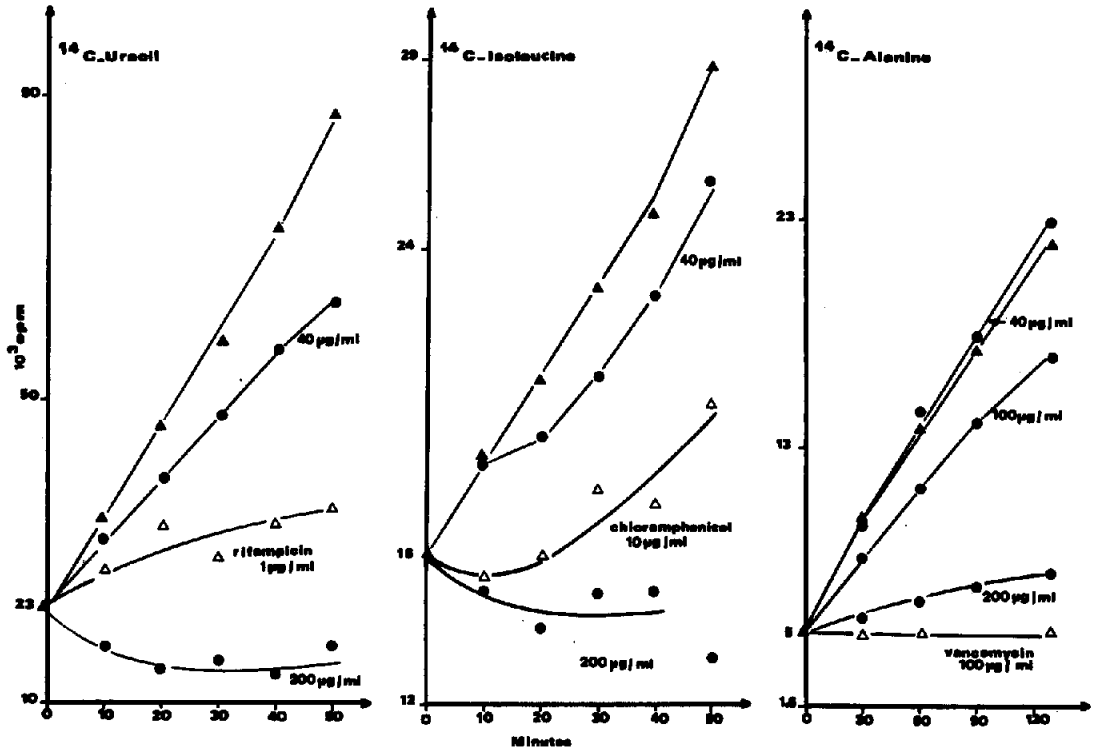


Fig.2. Incorporation of radioactive precursors into acid-insoluble fraction of *M. luteus* cells. (▲—▲) Control; (●—●) incubation in presence of various concentrations of mycosubtilin; (△—△) incubation in presence of standard antibiotic.

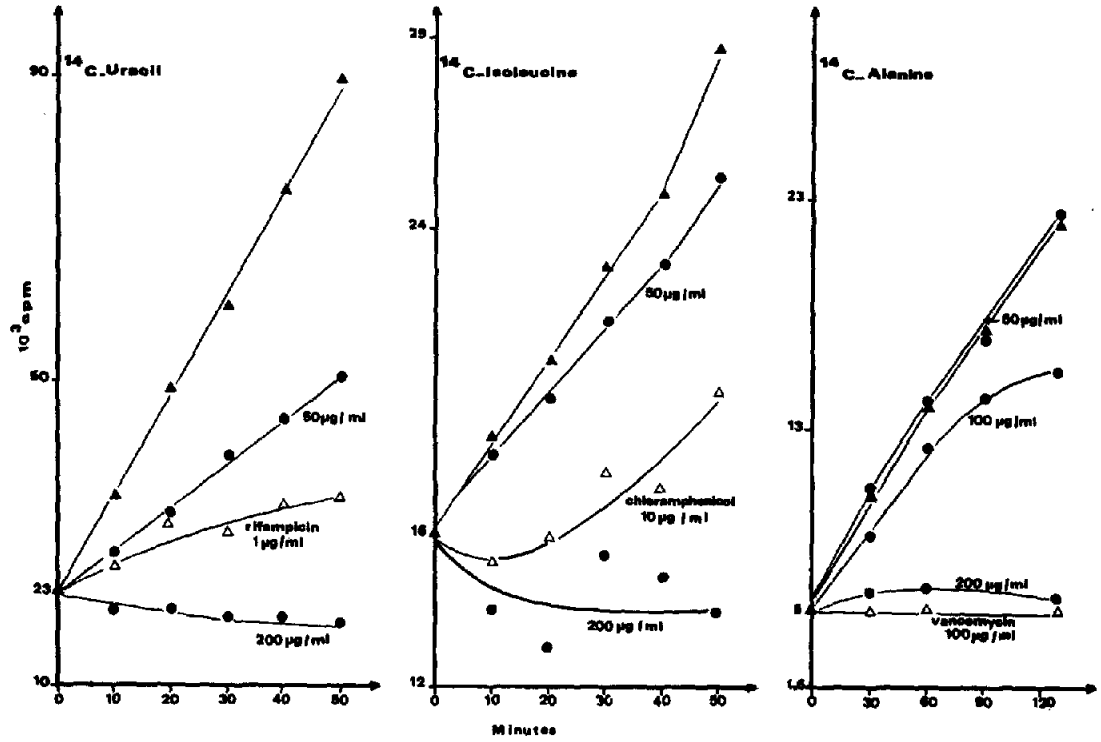


Fig.3. Incorporation of radioactive precursors into acid-insoluble fraction of *M. luteus* cells. (▲–▲) Control; (●–●) incubation in presence of various concentrations of bacillomycin L; (△–△) incubation in presence of standard antibiotic.

observation at the present time. The incorporation of thymidine was low, even in absence of antibiotic, but there is no incorporation in presence of 200 µg/ml of each antibiotic.

These results are quite similar to those of iturin A [8] and they do not permit to state the specific site of action of mycosubtilin, bacillomycin L and iturin A.

### 3.3. *Action of antibiotics on the protoplasts of* M. luteus

Previous investigations had shown a lytic action of iturin A upon the protoplasts of *M. luteus* [8]. A possible effect of mycosubtilin and of bacillomycin L upon these protoplasts was tested, the results are reported in fig.4. As a comparison the effect of iturin A in the same conditions [8] is also reported in fig.4.

Striking differences were observed between the lysis obtained with iturin A, mycosubtilin and bacillomycin L. Bacillomycin L had no action on the protoplasts of *M. luteus* at concentrations 50 µg/ml, 100 µg/ml, 200 µg/ml (curve a). After 5 min iturin gave respectively 10%, 30%, 60% lysis of protoplasts for 50 µg/ml, 100 µg/ml, 200 µg/ml of antibiotic (curves b–d). The lysis observed with mycosubtilin was strongly higher: 80% and 92% lysis for 12 µg/ml and 25 µg/ml antibiotic (curves e,f).

## 4. Discussion

Mycosubtilin and bacillomycin L have a strong antifungal activity against a large variety of yeasts and fungi and a restricted antibacterial activity against some species of *Micrococcus* [1,2]; their antibiotic spectrum is similar to that of iturin A. Mycosubtilin, bacillomcyin L and iturin A give the same inhibition of the growth of *M. luteus* and, with all these antibiotics, the incorporation of precursors of macromolecules is stopped or greatly reduced when a con-

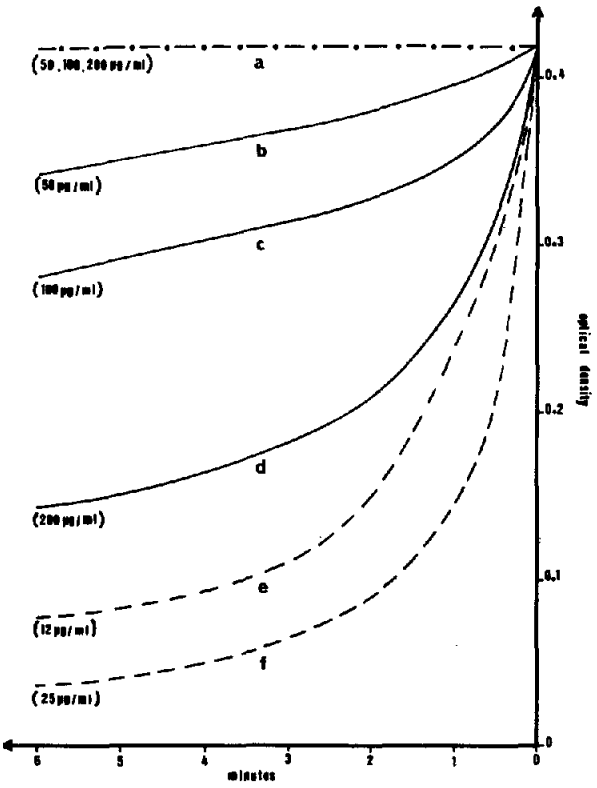


Fig.4. Effect of antibiotics on a suspension of *M. luteus* protoplasts: (–·–·) bacillomycin L; (—) iturin A; (– – –) mycosubtilin.

centration 200 µg/ml antibiotic is added to the culture medium. However, as all the macromolecule biosyntheses are sensitive, the primary site of action of these antibiotics cannot be defined. A good lytic activity of iturin A on the protoplasts of *M. luteus* has been shown [8] and these results had suggested an action of iturin A at the level of the bacterial cytoplasmic membrane.

The comparison of the effect of iturin A, mycosubtilin and bacillomycin L upon the protoplasts of *M. luteus* is very interesting. Mycosubtilin is the most active agent, the lysis is lower with iturin A and there is no lysis with bacillomycin L. These results are rather unexpected as similar activities upon whole bacteria were observed with the three antibiotics. The differences of lytic action upon protoplasts could be correlated to some structural differences between these antibiotics. The formula of iturin A, mycosubtilin and bacillomycin are reported below.

The 3 antibiotics have the same type of structure which is characterized by a hydrophobic hydrocarbon chain with 14–17 carbon atoms and a cyclopeptidic moiety. The polarity of this latter moiety is variable with the antibiotic: in bacillomycin L, 2 carboxylic groups are present in the side chain of 2 aspartic acid residues while iturin A and mycosubtilin have asparagine residues and no carboxylic group. The presence of ionisable groups could be correlated to

* βN $C_{14}$,$C_{15}$ → L-Asn → D-Tyr → D-Asn → L-Gln → L-Pro → D-Asn → L-Ser

Iturin A

* βN $C_{16}$,$C_{17}$ → L-Asn → L-Gln → L-Pro → D-Tyr → D-Asn → D-Asn → D-Ser → L-Asn

Mycosubtilin

* βN $C_{14}$,$C_{15}$ → L-Asp → D-Tyr → D-Asp → L-Ser → L-Gln → D-Ser → L-Thr

Bacillomycin L

* βN $C_{14}$,$C_{15}$ and βN $C_{16}$,$C_{17}$ are respectively *iso*$C_{14}$, *anteiso*$C_{15}$, *iso*$C_{16}$, *anteiso*$C_{17}$, β-aminoacids.

the inactivity of bacillomycin L upon *M. luteus* protoplasts. The structural differences between iturin A and mycosubtilin are less obvious: both these antibiotics have 2 polar residues, a tyrosyl and a seryl residue. However their positions in the peptide sequences are different: in iturin A the seryl unit is contiguous to the β-amino acid and the tyrosyl unit is separated from the β-amino acid by an asparaginyl unit only; in mycosubtilin the seryl and the tyrosyl units are much more distant from the β-amino acid. Thus the polarity of the β-amino acid environment is very different in the 3 antibiotics. These observations suggest a possible interaction of the hydrophobic moiety of the antibiotic with the cytoplasmic membrane, this interaction could be enhanced by an unpolar environment and partially or totally inhibited by the presence of polar or ionic groups near the lipophilic hydrocarbon chain of the antibiotic.

On account of the differences in their structures and in their effects on protoplasts, these antibiotics could provide a good material for the study of interactions between peptidolipids and cytoplasmic membranes.

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