THE MYXOVIRESCINS, A FAMILY OF ANTIBIOTICS FROM *MYXOCOCCUS VIRESCENS* (MYXOBACTERALES)*

K. GERTH, H. IRSCHIK, H. REICHENBACH and W. TROWITZSCH**

GBF, Gesellschaft für Biotechnologische Forschung,
Abt. Mikrobiologie and **Abt. Niedermolekulare
Naturstoffe, Mascheroder Weg 1, D-3300 Braunschweig, FRG

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The myxobacterium, *Myxococcus virescens* strain Mx v48 produced a family of at least 12 closely related antibiotics, the myxovirescins***. At a concentration of 1 to 5 μg/ml, the main component, myxovirescin A, was bactericidal for many Gram-negative bacteria, in particular enterobacteria, and at 20 to 50 μg/ml it also inhibited some pseudomonads and Gram-positive bacteria. The antibiotics seem to interfere with cell wall synthesis. The molecular formula of myxovirescin A was $C_{35}H_{61}NO_8$. It is a new antibiotic.

During a screening program for antibiotics from gliding bacteria, an activity against *Escherichia coli* was discovered in the culture supernatant of *Myxococcus virescens* strain Mx v48. This article describes the production and isolation of this activity and some of its biological and chemical properties.

Organism, Culture Conditions, and Strain Improvement

Myxococcus virescens strain Mx v48 (deposited at the German Collection of Microorganisms in Göttingen under number DSM 1888) was isolated in 1977 from rabbit dung collected on the island of Tenerife, Spain. All preliminary experiments were performed in modified peptone liquid medium (PLM) containing (per liter) 10 g of peptone from casein (tryptically digested, Merck, Darmstadt), 250 mg of $MgSO_4 \cdot 7H_2O$, 50 mg of $CaCl_2 \cdot 2H_2O$, and 1 mg of $CoCl_2 \cdot 6H_2O$; pH 7.2; autoclaved. Batch cultures of 100 ml were incubated in 250-ml Erlenmeyer flasks at 32°C on a rotary shaker at 160 cycles per minute. Under these conditions, Mx v48 grew as a cell suspension with a generation time of 5 to 6 hours. The antibiotic activity appeared in the culture supernatant. As the yield was very low (about 0.2 μg/ml), the antibiotic could be demonstrated by the paper disk method only after concentrating the broth 50: 1. No antibiotic could be extracted from the cells with acetone or methanol. The activity was excreted only during exponential growth and disappeared again before the culture entered the stationary phase. Growth of the producing organism was inhibited by its own antibiotic by 50% at a concentration of 0.25 μg/ml. We therefore isolated mutants that were resistant against the antibiotic. These, as all other mutants isolated in the course of this study, were obtained by treat ment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. One of the mutants, Mx v48-M3, was resistant against 30 to 40 μg/ml of the antibiotic. It still produced only 0.2 μg/ml, but production was now at the end of the growth phase, and the antibiotic remained stable during the following stationary phase. We then looked for mutants with higher yields. Mutagenized cultures of Mx v48-M3 were plated, and after 10 days the developing colonies were covered with a thin layer of agar containing cells of *Escherichia*

* Article No. 8 on antibiotics from gliding bacteria.
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coli. After 12 hours, inhibition zones could be observed in the lawn of the indicator bacterium, and producing colonies with zones larger than average were picked, purified, and tested in liquid culture. Mutant Mx v48-M7 produced 0.5 μg/ml, and mutant Mx v48-M8 (DSM 2177) obtained from Mx v48-M7 by the same procedure 2.0 μg/ml. Next we selected clones which were able to grow in the presence of 0.5% $(NH_4)_2SO_4$ by cultivating Mx v48-M8 in PLM to which increasing amounts of the salt were added. Surprisingly, some of these clones were superior producers of the antibiotic, as *e.g.* Mx v48-M10 with 8 μg/ml, and it was produced in presence or absence of $(NH_4)_2SO_4$. The production behavior and the generation time of the strain were the same as those of the initial mutant Mx v48-M3.

Fermentation

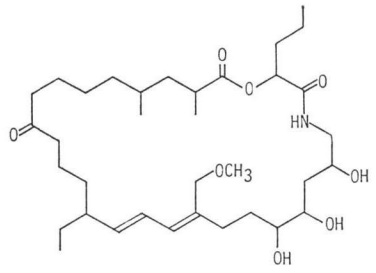
For mass production of the antibiotic, Mx v48 and its mutants were grown in fermentors (Giovanola Frères, Manthey, Switzerland) equipped with a circulating pump stirrer system ("intensor system") and containing 300 liters or 700 liters of the following medium: corn steep, powdered, 0.4%; soy flour 0.1%; peptone from casein (tryptically digested, Merck, Darmstadt) 0.1%; $MgSO_4 \cdot 7H_2O$ 0.025%; $CaCl_2 \cdot 2H_2O$ 0.005%; $CoCl_2 \cdot 6H_2O$ 0.0001%; silicone antifoam agent (Merck, Darmstadt) 0.015% (v/v). The fermentors were inoculated with log-phase cultures from 50-liter fermentors in the ratio 1: 10. The pH was adjusted to 7.4 with acetic acid and kept constant during the fermentation. The temperature was 32°C, the agitation 400 r.p.m., and the aeration rate 0.2 v/v/m. The bacteria grew exponentially during the first 10 hours. Then followed a linear growth phase of about 30 hours, during which the pO_2 remained nearly constant. After this, the pO_2 began to increase, and the antibiotic was produced. The fermentation was stopped 20 hours after the beginning of the production phase.

Isolation of the Antibiotic

The culture broth was separated from the cells and from insoluble constituents of the medium by centrifugation. Extraction of the broth with ethyl acetate yielded the crude antibiotic. The crude extract was concentrated under reduced pressure at 40°C. Distribution between heptane and methanol eliminated contaminating material, leaving the antibiotic in the methanol phase. The concentrated methanol phase was further purified by preparative HPLC (Waters Prep LC 500) on a reversed phase silica gel column (RP-8) with methanol - water, 9: 1 as the eluant. At this stage the active fraction consisted of at least 12 chemically different and biologically active compounds which could be separated by repeated HPLC on silica gel RP-18. One of these components was formed in large excess and was named myxovirescin A[1] (Fig. 1).

On silica gel thin-layer chromatograms (HP-TLC Kieselgel GO F 254, Merck, Darmstadt), the myxovirescins separated at best incompletely. The Rf values were: with ethylacetate 0.1; with dichloromethane - methanol (15: 1) 0.43; with hexane - isopropanol (4: 1) 0.17; and with hexane - acetone (1: 1) 0.14. Upon spraying the chromatogram with 1% hydroxylamine +5% $FeCl_3$ in 50% H_2SO_4, the myxovirescins appeared as a red-orange spot which turned blue upon standing.

Fig. 1. Chemical structure of myxovirescin A[1].



The physico-chemical data on the antibiotics are presented by TROWITZSCH *et al.*[1].

Biological Properties

All biological experiments were performed

Table 1. The antibiotic spectrum of myxovirescin A.

Test organism	Diameter of inhibition zone* (mm)	MIC in liquid culture** (μg/ml)
Escherichia coli	13	1
Klebsiella pneumoniae	12	
Proteus mirabilis	11	
Proteus morganii	10	
Serratia marcescens	12	
Salmonella typhimurium	10	
Aerobacter aerogenes	8	
Myxococcus virescens Mx v48	10	0.5
Azotobacter agilis	11	
Alcaligenes eutrophus	12	
Agrobacterium tumefaciens	0	
Rhizobium meliloti	0	
Vibrio extorquens	0	
Pseudomonas acidovorans	0	
Pseudomonas aeruginosa	0	30
Caryophanon latum	18	
Arthrobacter rubellus	0	
Brevibacterium ammoniagenes	0	
Nocardia flava	14	
Nocardia corallina	9	
Streptomyces heteropsis	8	
Streptococcus faecalis	0	
Staphylococcus aureus	0	30
Micrococcus luteus	0	
Bacillus subtilis	0	
Bacillus megaterium	0	30
Bacillus polymyxa	0	
Candida albicans	0	>100
Mucor hiemalis	0	
Penicillium digitatum	0	

* Tested by the agar diffusion technique, using paper disks of 6 mm diameter with 10 μg of myxovirescin A. The test agar contained: peptone from casein (tryptically digested, Merck, Darmstadt) 0.5%, meat extract (Oxoid) 0.1%, yeast extract (Difco) 0.1%, agar 1.5%, and was seeded with the respective test organism.

** Determined by the serial dilution assay in peptone-glucose liquid medium: peptone from casein (tryptically digested, Merck, Darmstadt) 1%, glucose 0.2%, $MgSO_4 \cdot 7H_2O$ 0.2%.

Fig. 2. Dose-response curves for different bacteria exposed to myxovirescin A.

Myxococcus virescens Mx v48 (wild strain) and Mx v48-M8 (myxovirescin-resistant mutant); *Staphylococcus aureus; Escherichia coli; Pseudomonas aeruginosa.* The tests were performed in peptone-glucose liquid medium.

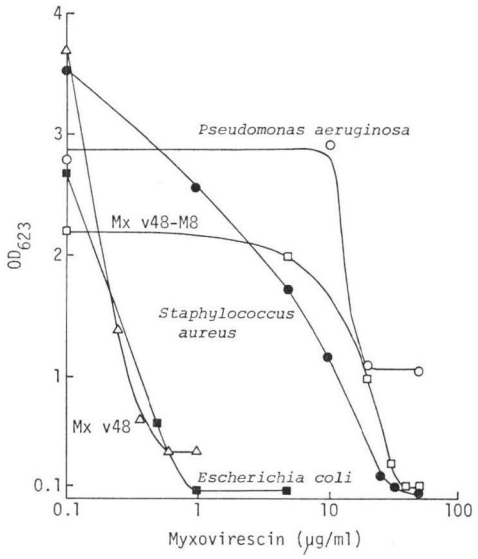
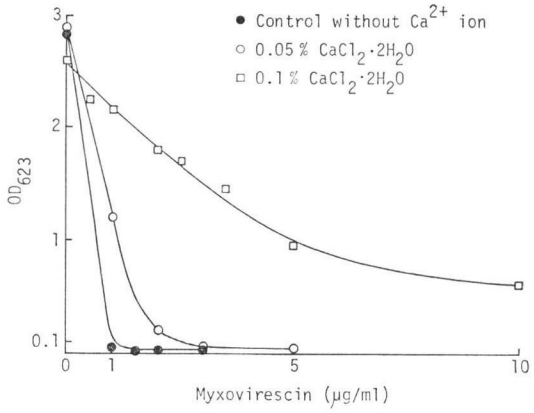


Fig. 3. Effect of Ca^{2+} ions on the inhibition of *Escherichia coli* by myxovirescin A.

The experiments were performed in peptone-glucose liquid medium.



with myxovirescin A only. The compound inhibited numerous Gram-negative bacteria, especially members of the Enterobacteriaceae, but at higher concentrations also some pseudomonads and Gram-positive bacteria (Table 1). When myxovirescin A (2 μg/ml) was added to a culture of *Escherichia coli*, 99.7% of the cells lost the ability to form colonies within 30 minutes. The effect of the antibiotic was thus relatively slow but irreversible.

Fig. 4. Synergistic action of EDTA on the inhibition of *Escherichia coli* by myxovirescin A.

The experiments were performed in peptone-glucose liquid medium.

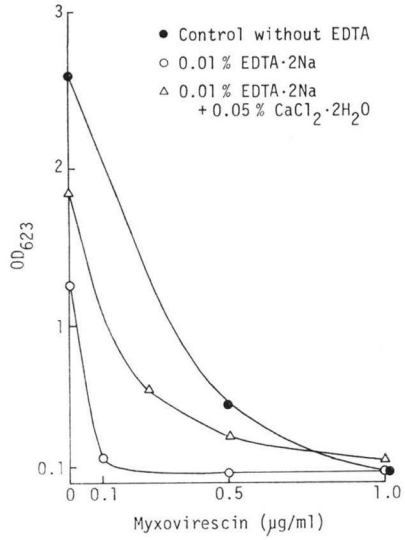
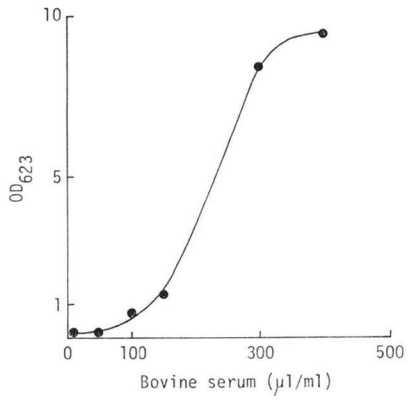


Fig. 5. Protective effect of bovine serum on *Escherichia coli* in presence of myxovirescin A.

Increasing amounts of bovine serum were made up to a total of 1 ml with peptone-glucose liquid medium; 2 μg of myxovirescin A and 0.1 ml of a suspension of *Escherichia coli* were added. The cultures were incubated over night at 32°C under shaking. The O.D. was determined at 623 nm and corrected by the O.D. of the uninoculated control.



The dose response curves of 4 Gram-negative bacteria tested differed in a characteristic way from that of *Staphylococcus aureus* (Fig. 2). While the former quickly reached the inhibition maximum when the antibiotic concentration was increased beyond the threshold concentration at which an effect could be observed, growth of *S. aureus* was gradually inhibited over a wide range of concentrations beginning at very low levels.

The minimal inhibitory concentration (MIC) for *E. coli*, but not for *S. aureus*, was strongly influenced by the presence of calcium ions (Fig. 3). Already 0.1% $CaCl_2 \cdot 2H_2O$ (about 7 mM) made *E. coli* highly resistant to myxovirescin A. Magnesium and barium ions had no such effect.

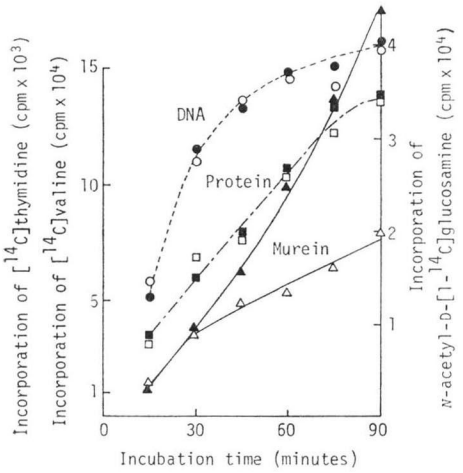
EDTA acted synergistically with myxovirescin A (Fig. 4). With *Pseudomonas aeruginosa*, the MIC was reduced by 0.01% EDTA (disodium salt) from 30 μg/ml to 7 μg/ml. The effect of EDTA could partially be reversed by the addition of calcium ions (Fig. 4).

E. coli could also be protected against myxovirescin A by bovine serum (Fig. 5). In bovine serum (Oxoid) with 0.5% peptone and 0.5% glucose, the MIC was 20 μg/ml instead of 1 μg/ml. A 10% addition to peptone liquid medium of the albumin fraction of bovine serum increased the MIC only from 1 to 3 μg/ml. However, when small quantities of lecithin (0.5 mg/ml) were added to peptone liquid medium, the inhibitory effect of myxovirescin A (2 μg/ml) on *E. coli* A1 was completely neutralized. Cholesterol, in contrast, did not interfere with the activity of the antibiotic.

To get an idea about the mechanism of action of myxovirescin A, its influence on DNA, protein and murein synthesis was studied with the usual tracer technique. *E. coli* A1 was grown in a medium containing: 0.2% peptone from casein, tryptically digested; 0.2% glucose; 0.05% sodium citrate; 0.1% $MgSO_4 \cdot 7H_2O$; 0.1% $(NH_4)_2SO_4$; 0.01% EDTA, disodium salt; trace elements; and 15 mM phosphate buffer pH 7.0. The cells were harvested by centrifugation and suspended in fresh medium to give an O.D. (623 nm) of 3. To these cultures were added 0.5 μCi/ml of either [2-^{14}C]thymidine, or L-[U-^{14}C]-valine, or *N*-acetyl-D-[1-^{14}C]glucosamine (all purchased from Amersham-Buchler, Braunschweig). The

Fig. 6. Effect of myxovirescin A on DNA, protein and murein synthesis by *Escherichia coli* A1.

For experimental details, see text. Incorporation of [^{14}C]thymidine: ●=control without the antibiotic; ○=with 2 μg/ml of myxovirescin A. Incorporation of [^{14}C]valine: ■=control; □=with 2 μg/ml of myxovirescin A. Incorporation of [^{14}C]-*N*-acetyl-glucosamine: ▲=control; △=with 2 μg/ml of myxovirescin A.



cultures with labeled *N*-acetyl-glucosamine and with labeled thymidine also contained 30 μg/ml of the respective unlabeled compound. For each tracer, two cultures were run in parallel; one with 2 μg/ml of myxovirescin A, the other without the inhibitor. The cultures were shaken at 37°C, and 0.5 ml samples were taken every 15 minutes. Each sample was mixed with 0.5 ml of ice cold 14% trichloroacetic acid and, after standing for 2 hours on an ice bath, filtered through a glass microfiber filter (GF/B, Whatman). The filters were washed with 10 ml of water, and their radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer. As can be seen (Fig. 6), myxovirescin A did not interfere with DNA and protein synthesis over a period of 90 minutes, but strongly inhibited incorporation of *N*-acetyl-glucosamine, supposedly into the cell wall, beginning after about 30 minutes.

Discussion

It appears that the myxovirescins are a family of new antibiotics, with myxovirescin A as the main representative[1] (Fig. 1). After the new antibiotics myxothiazol[2,3] and myxovalargin (D.P.A. MÜNCHEN, Patent 29,24,006, Dec. 18, 1980), and two compounds that were already known from other bacteria, *viz.* althiomycin[4] and pyrrolnitrin[5], this is the fifth antibiotic isolated by us from myxobacteria.

Myxovirescin A is a particularly efficient inhibitor of enterobacteria (MIC around 1 μg/ml; Table 1; Fig. 2), but also other Gram-negative bacteria, including pseudomonads, and even some Gram-positive organisms were inhibited at acceptable concentrations (MIC around 30 μg/ml). The producing organism itself was originally very sensitive against its own antibiotic (MIC 0.5 μg/ml). Eukaryotes seem to be completely resistant. The inhibitory spectrum would fit to the observation that myxovirescin A blocked rather specifically the incorporation of *N*-acetyl-glucosamine into cellular macromolecules, *i. e.* very probably interferes with murein synthesis (Fig. 6). This hypothesis is supported by other observations. Thus, the antibiotic acts rather slowly, but its effect is irreversible. Or, the dose response curves for Gram-negative bacteria and for Gram-positive *Staphylococcus aureus* differ substantially, with the Gram-negative ones showing a much sharper reaction. The synergistic effect of EDTA in the case of Gram-negative bacteria (Fig. 4) may have to do with changes at the cell surface. Either permeability for the antibiotic is increased, although the rather low MIC values for enterobacteria seem to indicate that permeability is not a serious problem; or surface layers are destabilized by complex formation of EDTA with divalent cations. Murein defects caused by the antibiotic would thus sooner become deleterious. The protective effect of calcium ions (Fig. 3) could be interpreted on the same line.

Addition of serum to the test medium sharply decreased the activity of myxovirescin A (Fig. 5). Serum contains appreciable amounts of phospholipids and cholesterol (up to 0.3% each), and it appears that the phospholipids of the serum are the interfering agents. For, addition of the albumin fraction of serum influenced the MIC but little, while small quantities of lecithin totally abolished the effect of the antibiotic.

The myxovirescins seem not to be uncommon among myxobacteria. After the antibiotics had been

isolated and characterized from *Myxococcus virescens* strain Mx v48, they were also found in *M. fulvus* Mx f49 (isolated in 1977 from rabbit dung collected near Alicante, Spain), in *M. virescens* Mx v10 (isolated in 1977 from rabbit dung collected on Tenerife, Spain), in *M. xanthus* Mx x35 (isolated in 1979 from sheep dung collected near Braunschweig, FRG), in *M. xanthus* Mx x36 and Mx x39 (both isolated in 1980 from goat dung collected near Madras, India, and kindly supplied to us by Dr. T. S. CHANDRA, Madras), and in *M. xanthus* Mx x46 (isolated in 1980 from soil collected near Rio de Janeiro, Brazil, and kindly supplied to us by Mr. G. WIDERA, GBF). There are also similarities between the myxovirescins and the antibiotic TA from *M. xanthus* TA[6,7], the chemical structure of which is not yet known, notably with respect to the mechanism of action[8] and the producing organism. According to published data, antibiotic TA differs, however, from the myxovirescins in its inhibitory spectrum (no inhibition of *Serratia marcescens*, inhibition of *Salmonella typhimurium* and *Proteus morganii* only at very high concentrations, good activity against several *Bacillus* strains), in its UV and IR spectrum, molecular weight and molar extinction coefficient. Contrary to the myxovirescin family, antibiotic TA is described as a single compound.

Acknowledgements

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