ANTIBIOTIC ACTIVITY OF MYXOBACTERIA IN RELATION TO THEIR BACTERIOLYTIC CAPACITY

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ABSTRACT

NORÉN, BÖRGE (University of Wisconsin, Madison) AND KENNETH B. RAPER. Antibiotic activity of myxobacteria in relation to their bacteriolytic capacity. J. Bacteriol. **84**:157–162. 1962—*Myxococcus virescens*, *M. fulvus*, *M. stipitatus*, *M. lacteus*, *Chondrococcus blasticus*, and *Chondromyces crocatus* were tested for antibacterial activity, and all were found to secrete products that inhibited the growth of gram-positive eubacteria. The amount of inhibition varied with the myxobacterium employed and with the eubacterium used as a test organism. The growth of gram-negative eubacteria was in no case visibly affected by the antibiotic products. The experiments performed failed to indicate any positive relationship between the antibacterial activity and the bacteriolytic capacity of the six myxobacteria investigated.

The ability of myxobacteria to produce antibiotic substances was first reported by Oxford (1947). Later investigations have revealed that myxobacteria other than *Myxococcus virescens*, the species investigated by Oxford, are capable of producing substances with antibacterial activity (Norén, 1953; Kato, 1955). It was assumed that the antibiotics produced by the myxobacteria play a positive role in the initial phase of the lysis of living eubacterial cells effected by many myxobacteria (Norén, 1953). However, to our knowledge, no investigation has been directed specifically to the question: does any direct connection exist between the antibiotic activtiy and the bacteriolytic capacity of the myxobacteria?

The present paper records (i) results relating to the antibacterial action of selected myxobacteria as tested against ten different eubacteria, and (ii) consideration of these results in relation to the bacteriolytic activity exerted by the same myxobacteria on the intact cells of the same eubacteria.

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MATERIALS AND METHODS

The myxobacteria employed included *Myxococcus fulvus* strain MF 71; *M. virescens* strain B 2; *M. stipitatus* strain MS 62-1; *M. lacteus* (Peterson, 1958) strain ML 56 B; *Chondrococcus blasticus* strain 53-2; and *Chondromyces crocatus* strain CC 59. *C. crocatus* was maintained as a mixed culture with *Aerobacter aerogenes*, whereas the remaining myxobacteria were isolated and propagated as pure cultures. All species were obtained from soils collected in the environs of Madison, Wis.

The eubacteria included: *A. aerogenes* strain 900; *Escherichia coli* strain 281; *Pseudomonas fluorescens* strain 112; *Serratia marcescens* strain 175; *Flavobacterium* sp. strain Weber L; *Micrococcus* sp. strain 101; *Sarcina lutea* strain 1018; *Bacillus subtilis* Sarles' strain; and *B. megaterium* strains M and KM. The two strains of *B. megaterium* were obtained by courtesy of C. Weibull, Central Bacteriological Laboratory of Stockholm City, Stockholm, Sweden, and the remaining cultures were secured from stocks maintained in the Department of Bacteriology, University of Wisconsin, Madison.

RESULTS

The production of antibiotic substances by the myxobacteria was first tested in the following way. The surface of a section of a petri plate containing agar with added salts (K_2HPO_4, 2.0 g; NaCl, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $CaCl_2$, 10 mg; $MnSO_4 \cdot 4H_2O$, 1.0 mg; agar, 15 g; distilled water, 1,000 ml; pH adjusted to 7.1) was covered with a very thick suspension of living cells of *A. aerogenes* previously grown for 24 hr in glucose-nutrient broth, harvested, and washed three times by centrifugation. The "eubacterial sec-

TABLE 1. *Inhibition of eubacteria*

Tested eubacteria	Zones (mm) of inhibition produced by myxobacteria*					
	M. virescens	*M. fulvus*	*M. stipitatus*	*M. luteus*	*C. blasticus*	*C. crocatus*
Bacillus megaterium KM	11	14	6	7	10	?
B. megaterium M	12	8.5	5	5	10	?
B. subtilis	12	17	4	4	10	1
Sarcina lutea	13	14	40	18	13	5
Micrococcus sp.	28	31	40	40	28	11
Flavobacterium sp.	0	0	0	0	0	0
Escherichia coli	0	0	0	0	0	0
Serratia marcescens	0	0	0	0	0	0
Pseudomonas fluorescens	0	0	0	0	0	0
Aerobacter aerogenes	0	0	0	0	0	0

* Incubation: 24 hr.

tion" was inoculated with several myxobacterial fruiting bodies. After some days, the number varying with the species, the developing myxobacterial swarm completely lysed the *Aerobacter* cells. Glucose-nutrient broth was then added to that part of the plate not covered by the myxobacterial swarm and, after the broth had been absorbed by the agar, the plates were inoculated with the selected eubacteria so that the latter formed streaks 40 mm long radiating from the swarm front. Eubacterial cultures grown for 24 hr in glucose-nutrient broth were used as inocula.

The influence of the myxobacteria on the growth of the added eubacteria was examined at 10, 24, and 48 hr after inoculation. In those cases where myxobacteria produced an inhibitory effect, no growth or reduced growth of the eubacteria occurred at some distance from the swarm front. The zones wherein eubacterial growth was inhibited were measured, and are summarized in Table 1. Controls for these experiments consisted of heavy streaks of living cells of *A. aerogenes*, to the edge of which perpendicular streaks of the other test eubacteria were inoculated as described above. In no case was the growth of the implanted eubacterial species inhibited by the previously placed *Aerobacter*.

The experiments thus performed revealed that the myxobacteria tested were able to produce an antibacterial agent(s) which, however, was active only on gram-positive eubacteria, the gram-negative species being unaffected by the agent(s). Even among the gram-positive organisms, there was appreciable variation in resistance to the inhibitory products. *Micrococcus* sp. and *S. lutea* appeared to be most susceptible.

It was of interest to determine whether the myxobacteria produced inhibitory substance(s) only in the presence of living eubacteria. A series of experiments was carried out in which the living cells in the "eubacterial section" were replaced by autoclaved cells of *A. aerogenes*. Antibacterial effects were obtained in this case also, but they were somewhat weaker than in the previous experiments. This possibly resulted from reduced myxobacterial growth, for, as a rule, myxobacteria grow less well on autoclaved eubacteria than on living cells (Singh, 1947; Oetker, 1953; Norén, 1960*a*). Unfortunately, the amount of growth could not be determined quantitatively in these comparisons.

A second series of experiments comprised tests of the inhibitory effect of a cell-free metabolic solution in which myxobacteria had been grown previously. These tests included all of the myxobacterial species mentioned above except *C. crocatus*, which, as previously noted, was not grown in pure culture. The myxobacteria were grown in 300-ml Erlenmeyer flasks with a thin layer of Pyrex glass wool covering the bottom of each. The flasks were filled to the upper surface of the glass wool with 50 ml of nutrient solution having the following composition: casein hydrolyzate, 1.0 g; asparagine, 1.0 g; K_2HPO_4, 2.0 g; NaCl, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $CaCl_2$, 10 mg; $MnSO_4 \cdot 4H_2O$, 1 mg; distilled water, 1,000 ml; (pH 7.2). Autoclaved cells of *A. aerogenes* were aseptically added to a concentration of 0.25 mg (dry wt) per 1 ml of nutrient liquid. The flasks were inoculated with mixtures of microcysts and vegetative cells of the myxobacteria and incubated at 30 C. Samples were removed from the flasks after 1, 2, 3, 4, 5, 6, and 8 days of incubation, and immediately filtered through Seitz filters.

Antibacterial activity was tested against *Micrococcus* sp. and measured by a serial dilution method. The cell-free metabolic solution was

TABLE 2. *Growth of Micrococcus sp. in a broth nutrient to which has been added the cell-free metabolic solutions of various myxobacteria**

Myxobacteria	Concn (%) of the cell-free metabolic solution added					
	30	20	10	5	2.5	0
Myxococcus virescens	0	0	0	2	4	5
M. fulvus	0	0	0	5	5	5
M. stipitatus	0	0	0	1	4	5
M. lacteus	0	2	5	5	5	5
Chondrococcus blasticus	0	0	0	1	3	5

* Relative amounts of growth are scored from 1 to 5.

added aseptically to nutrient broth to yield concentrations of 30, 20, 10, 5, and 2.5%. In each series, the final liquids contained identical concentrations of nutrient broth. The test solutions thus obtained were aseptically distributed into tubes (4 ml per tube), which were then inoculated with 0.05 ml of a 24-hr broth culture of *Micrococcus* sp. diluted to 1:6,400. Table 2 shows the comparative growth obtained after 9 hr at 30 C in continuously shaken cultures.

Whereas a concentration of the metabolic solution of 5 to 10% was necessary to produce a pronounced effect, it is obvious that the cell-free metabolic solutions, in which the myxobacteria had been permitted to attain maximal growth, contained substance(s) which inhibited the growth of *Micrococcus* sp. Sterile nutrient solution, of the type used for myxobacterial growth, when added to the nutrient broth inoculated with *Micrococcus* had either no effect or a stimulatory effect on the eubacterial growth.

Two pertinent observations, not recorded in the table, deserve mention. (i) The incubation period for myxobacteria was an important factor in obtaining an antibacterial effect from the metabolic solutions. The activity of the inhibitory agent appeared to reach its maximum after a few days and then decreased rapidly. The time of incubation necessary to reach peak activity varied with the myxobacterial species involved, and also with the amount and quality of the inoculum and the composition of the nutrient solution. In one experiment performed with *M. virescens*, an incubation of 3 days was sufficient to produce a maximal inhibitory effect by the metabolic solution, whereas in 2 additional days the peak was already passed. In another experiment, where the inoculum was more dilute, the metabolic solution of the 3-day culture was definitely less inhibitory than those of 5- and 6-day cultures. (ii) The way in which the eubacterial test cultures were incubated also influenced the results of the experiments. If the cultures were shaken continuously, a pronounced inhibition of eubacterial growth could be seen for about 10 hr (values in Table 2 refer to readings after 9 hr), but the effect then gradually weakened and was apparently overcome completely in 24 hr. On the other hand, in stationary cultures the inhibitory effects were marked even after an incubation period of 72 to 96 hr. Aeration of the test culture thus appeared to be an important factor in overcoming the effect of the inhibitory products formed by the myxobacteria tested. Under these conditions, aeration would be expected to permit a more vigorous growth of the eubacteria, or, alternatively, it might hasten the inactivation of the growth-inhibiting compound(s).

Since, as a rule, myxobacteria lyse more easily and consequently feed and grow better on gram-negative than on gram-positive bacteria (Singh, 1947; Oetker, 1953), it was puzzling that only gram-positive bacteria were susceptible to the antibacterial substances of the myxobacteria. To determine whether this was also the case with the specific eubacteria and myxobacteria involved in this investigation, some experiments were conducted to test the bacteriolytic activities of the myxobacteria. These experiments were carried out according to the method described by Singh (1947). The eubacteria for these trials were grown in a tryptone-glucose broth at 30 C for 24 hr, harvested by centrifugation, washed once in distilled water, and then resuspended in limited amounts of distilled water sufficient to give the same concentration of cells in all the eubacterial suspensions tested. From the final suspensions, 0.01 ml was applied on the surface of salt agar plates (composition given earlier) to form eubacterial streaks 40 mm long. The lytic activities of *M. virescens*, *M. fulvus*, *C. blasticus*, and *C. crocatus* were tested on all the eubacteria mentioned above. Fruiting bodies were used as inocula, one being placed at one end of each

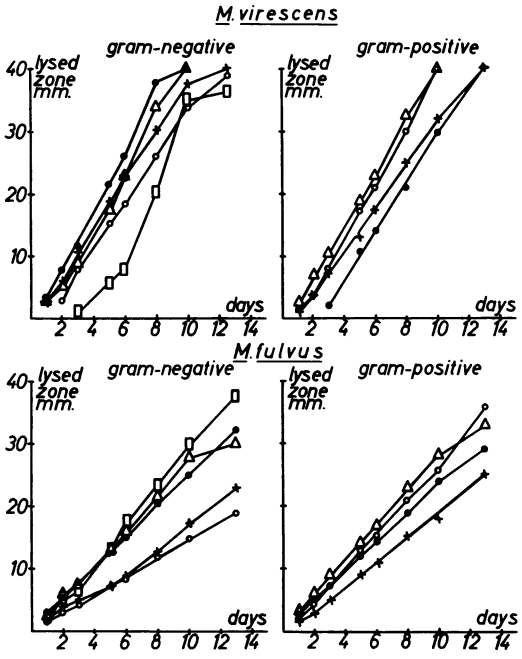

FIG. 1. *Lysis of gram-negative and of gram-positive eubacteria by Myxococcus virescens and Myxococcus fulvus. Gram-negative organisms:* (●) *Aerobacter aerogenes;* (+) *Escherichia coli;* (○) *Pseudomonas fluorescens;* (□) *Serratia marcescens;* (△) *Flavobacterium sp. Gram-positive organisms:* (●) *Micrococcus sp.;* (+) *Sarcina lutea;* (○) *Bacillus subtilis;* (△) *B. megaterium strain M = KM.*

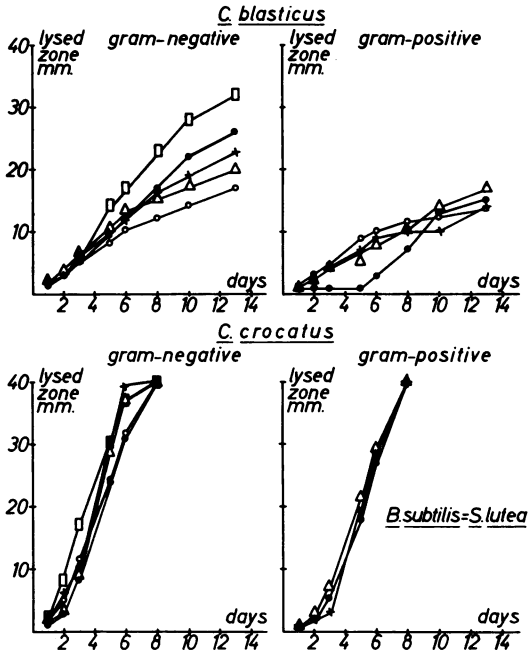

FIG. 2. *Lysis of gram-negative and gram-positive eubacteria by Chondrococcus blasticus* and *Chondromyces crocatus. Gram-negative organisms:* (●) *Aerobacter aerogenes;* (+) *Escherichia coli;* (○) *Pseudomonas fluorescens;* (□) *Serratia marcescens;* (△) *Flavobacterium sp. Gram-positive organisms:* (●) *Micrococcus sp.;* (+) *Sarcina lutea;* (○) *Bacillus subtilis;* (△) *B. megaterium strain M = strain KM.*

streak. The zones of the eubacterial streaks cleared by the growing myxobacterial swarms were measured over a period of approximately 2 weeks. The results are summarized in Fig. 1 and 2, where the values represent averages for four streaks.

From these graphs it is clear that the gram-positive species (antibiotic-sensitive eubacteria) were not subject to more rapid lysis than were the gram-negative, nonsensitive eubacteria. *Micrococcus* sp. and *S. lutea*, the species most susceptible to the antibiotic agents, were, in certain cases, lysed at the slowest rate. Furthermore, *B. megaterium*, the gram-positive bacterium apparently least sensitive to the antibiotic agents, was, as a rule, the one most rapidly lysed.

Another striking feature of the experiment was the different rates at which the six myxobacteria lysed the same eubacterium. For example, *M. virescens* lysed *S. marcescens* at a very slow rate during the first 6 days, although this eubacterium was the one most rapidly lysed by *C. blasticus*. *M. virescens* lysed *A. aerogenes* most rapidly of the gram-negative eubacteria and then, in decreasing order, *Flavobacterium* sp., *E. coli*, *P. fluorescens*, and *S. marcescens*. The order varied with the other myxobacteria.

DISCUSSION

These experiments clearly show that *M. virescens*, *M. fulvus*, *M. stipitatus*, *M. lacteus*, *C. blasticus*, and *C. crocatus* secrete products that inhibit the growth of gram-positive eubacteria. Not unexpectedly, the amount of inhibition varies with the myxobacterium and with the eubacterium used as a test organism. Of the

eubacteria tested, *Micrococcus* sp. and *S. lutea* were consistently more susceptible to the inhibitory agents produced. In general, the results obtained in this investigation are in accord with those obtained with *M. virescens* by Oxford (1947) and with *C. coralloides* by Norén (1953). Kato (1955), on the other hand, reported a strain of *M. fulvus* to produce an antibiotic that inhibited equally well the growth of gram-positive and gram-negative eubacteria. Both Oxford (1947) and Kato (1955) found the antibiotics in their myxobacterial cultures to be very unstable products, and, in our experience, the antibiotic of *M. virescens*, at least, appears to be an unstable compound which reaches a maximum at a certain period in the myxobacterial cycle and then vanishes more-or-less rapidly.

Inter-myxobacterial responses are interesting also. When cultivated on a salts agar supplemented with casein hydrolyzate (0.2%) and asparagine (0.2%), *C. blasticus*, which grew only poorly on this medium, secreted a product which inhibited the growth of *M. fulvus* but not that of *M. virescens*. Obviously the myxobacteria, like the eubacteria, are variously susceptible to the inhibitory products secreted by other members of the group. On the other hand, the antibiotic produced by *M. virescens* had no inhibitory effect on *M. fulvus*, i.e., the antibacterial product of *M. virescens* had an activity evidently not identical with that of *C. blasticus*.

The results obtained do not indicate any positive relationship between the antibacterial potential and the bacteriolytic capacity of the myxobacteria. The *Micrococcus* sp. was strongly susceptible to the inhibitory products of all the myxobacteria, but it was always lysed at a relatively slow rate. On the other hand, *A. aerogenes* was insensitive to the antibiotics, and yet it was lysed rapidly by all the myxobacteria tested. Thus, it is clear that, if a eubacterium is susceptible to an antibacterial substance produced by a myxobacterium, this fact alone does not mean that it is highly susceptible to the bacteriolytic agents of the same myxobacterium or vice versa.

From the observations made in the bacteriolytic experiments, two general conclusions may be drawn. (i) Certain qualitative differences exist between the bacteriolytic agents that are produced by the different myxobacteria. (ii) The eubacteria differ in their ability to withstand the bacteriolytic agents of myxobacteria, their resistance being governed by the qualities of the eubacterial cell walls and as well as the cytoplasms that they protect (Norén, 1955). From other investigations (Norén, 1960*a*, *b*) the cell walls would appear to constitute the more important factor limiting bacteriolysis.

Finally, from an ecological point of view, the recorded observations seem to be of interest. They may suggest that the myxobacterial swarms living in the soil are able to favorably influence their territories in two ways. The swarms with their antibiotic agents may hinder certain bacteria that are more difficult to lyse from entering their "Lebensraum"; other eubacteria, insensitive to these inhibitory substances, can grow contiguous with the swarm, be readily lysed, and so provide nutrition for the myxobacteria.

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