STUDIES ON POLYMYXIN: ISOLATION AND IDENTIFICATION OF BACILLUS POLYMYXA AND DIFFERENTIATION OF POLYMYXIN FROM CERTAIN KNOWN ANTIBIOTICS

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Received for publication July 23, 1947

Polymyxin is an antibiotic substance occurring in the culture filtrates of *Bacillus polymyxa*. The isolated substance is unique in its specificity for gram-negative bacteria. A summary of the more important results obtained during the course of several years, including chemotherapeutic and toxicity data, has been reported (Stansly, Shepherd, and White, 1947). The present contribution is concerned with the isolation and identification of the antibiotic-producing organism and some early findings which both characterized and distinguished polymyxin from certain known antibiotics.

Isolation of Bacillus polymyxa. *Bacillus polymyxa* was isolated from soil in the course of a program designed to find new antibiotics for the chemotherapy of gram-negative bacterial infections. The test organism used in this search was *Salmonella schottmuelleri*. Our method for isolating antibiotic-producing organisms with a specific type of activity involves the preparation of pour plates of soil dilutions using a variety of media and cultural conditions. The plates are subsequently sprayed with a suspension of the test organism by means of an apparatus designed for the purpose (Stansly, 1947).

Identification of Bacillus polymyxa. The identification of *Bacillus polymyxa* was established by following the key to the identification of aerobic sporeforming bacteria by Smith, Gordon, and Clark (1946). In the preliminary work,[1] edition 5 of Bergey's *Manual of Determinative Bacteriology* (1939) and the galley proofs of edition 6 were found helpful.

An 18-hour broth culture consisted of gram-negative rods with few or no gram-positive cells. Older cultures showed vegetative cells and oval spores either free or central to terminal in adhering and swollen sporangia. Broth cultures at 30 C were turbid and had a ropy sediment. Indole was not formed. Nitrates were reduced to nitrites. Hydrogen sulfide was not produced. Acid and gas were formed from glucose, lactose, and sucrose. Acid but no gas was produced from rhamnose and a slight amount of acid but no gas from sorbitol. Starch was hydrolyzed. Acid and gas were produced from litmus milk, which was coagulated and reduced.

The existence of oval spores, central to terminal, and sporangia frequently adhering and swollen, plus the predominant gram-negative nature of the vegeta-

[1] The authors are indebted to Dr. Walter C. Tobie and Miss Marion H. Cook for the preliminary work which led to the conclusion that the antibiotic-producing organism had characteristics intermediate between those of *Bacillus polymyxa* and *Bacillus macerans*.

tive forms, placed the organism in group 2 in the classification of Smith, Gordon, and Clark. The fermentation of carbohydrates, such as glucose, lactose, and sucrose, with the formation of both acid and gas narrowed the possible identity of the organism to one of two species, namely, *Bacillus polymyxa* or *Bacillus macerans*. These two species may be distinguished in the following ways: (1) *B. polymyxa* produces acetylmethylcarbinol from the proper substrate, whereas *B. macerans* does not; (2) *B. polymyxa* does not produce an amylase which catalyzes the formation of crystalline dextrins from starch, whereas *B. macerans* produces this enzyme. Both of these criteria were used to identify the unknown organism.[2]

Production of acetylmethylcarbinol. Three known *B. polymyxa* strains (ATCC nos. 8523, 7047, and 7070), one *B. macerans* (ATCC no. 355), and the unidentified organism were inoculated in the recommended neopeptone medium and under the suggested conditions (Smith *et al.*, 1946). The test for acetylmethylcarbinol was made according to O'Meara (1931). *B. macerans* was negative for acetylmethylcarbinol on the third, fifth, seventh, and fourteenth day of incubation, whereas the isolated organism and the three polymyxa strains were positive at these times.

Formation of crystalline dextrins. The formation of crystalline dextrins from starch was detected by the iodine test of Tilden and Hudson (1942). The same strains of *B. polymyxa* and *B. macerans* were used as before, in a medium and under conditions recommended (Smith *et al.*, 1946), with the exception that Merck's soluble starch was used instead of Takamine or White Rose. *B. macerans* gave a positive test for crystalline dextrins (both hexagons and needles were observed) when tested after 2 weeks and again after 3 weeks of incubation. All three polymyxa strains and the antibiotic-producing organism were negative at these times.

The two foregoing critical tests supported each other in identifying the organism as a strain of *Bacillus polymyxa*, a species apparently first described in 1880 as *Clostridium polymyxa* (Smith *et al.*, 1946) and of current interest in the production of 2,3-butanediol by fermentation (Adams, 1946).

Antibacterial activity. When a colony of *Bacillus polymyxa* on an agar plate was sprayed with a suspension of *Salmonella schottmuelleri* or *Escherichia coli* and compared to a similar plate sprayed with *Staphylococcus aureus*, the difference in the inhibition zones of the gram-negative and the gram-positive organisms was striking, the former showing a wide zone (approximately 40 mm), the latter a relatively narrow zone (approximately 10 mm). It was this difference alone which stimulated further investigation since, at the time the investigation began, no antibiotic had been described which was more active against gram-negative bacteria than gram-positive bacteria.

At first some difficulty was experienced in demonstrating antibacterial activity in bacteria-free broth filtrates. This may have been due to the use of filters which removed the active principle. With the introduction of sintered

[2] The authors wish to thank Miss Nydia H. Ananenko for conducting these two tests in the identification of *Bacillus polymyxa*.

glass filters appreciable activity could be demonstrated. An early antibacterial spectrum obtained with filtered broth is given in table 1.[3]

As shown in table 1, crude fermentation liquor was highly active against the gram-negative bacteria but either inactive or relatively inactive against the gram-positive organisms, confirming and extending the previous findings with the *Bacillus polymyxa* colony. More striking than the results with crude fermentation liquor were those obtained with concentrates of polymyxin. These were relatively free of activity against gram-positive bacteria, even against those organisms, for example, *Diplococcus pneumoniae* SVI, which were some-

TABLE 1

*Antibacterial spectrum of polymyxin broth filtrates**

ORGANISM	MEDIUM†	HIGHEST INHIBITORY DILUTION‡
Escherichia coli	A, 1/16	1,024
Eberthella typhosa	A, 1/16	2,048
Shigella dysenteriae (Flexner)	A	512
Salmonella schottmuelleri	A, 1/16	128
Pseudomonas aeruginosa	A, 1/16	128
Klebsiella pneumoniae	A, 1/16	512
Streptococcus, group A, strain C203	A	8
Streptococcus, group B	A, 1/4	4
Streptococcus, group D	A, 1/2	0
Diplococcus pneumoniae, type I	A	32
Staphylococcus aureus	A, 1/16	0
Clostridium welchii	B	16
Erysipelothrix rhusiopathiae	B+	8

* The medium consisted of glucose, glycerol, tryptone, yeast extract, and inorganic salts, and was therefore far more complex than the routine production medium which was finally developed (Stansly *et al.*, 1947).

† A = Trypticase-soy-phosphate broth (Baltimore Biol. Lab.). A, 1/2, 1/4, and 1/16, designates the medium used at 1/2, 1/4, and 1/16 the concentration recommended by the manufacturer.

B = Brewer's thioglycolate broth.

B+ = Brewer's thioglycolate broth + bile and yeast extract.

‡ Inhibitory end point obtained by serial twofold broth dilution.

what affected by the crude liquor. Thus from table 1 it can be calculated that *E. coli* is 32 times more sensitive to the broth filtrate than is *D. pneumoniae*. With a partially purified preparation of polymyxin the ratio was found to be in excess of 2,048.[4]

A possible explanation for the difference in behavior of the liquor and concentrates was that the liquor contained at least two active substances, only one of which, the gram-negative principle, was present in the concentrates. In

[3] We wish to thank Mrs. Edith Jackson for conducting the antibacterial spectrum.

[4] We wish to thank Dr. H. J. White and Mrs. A. H. Clapp for the data on the purified preparations.

support of this explanation is the fact that, as described below, it has been possible to extract from the cells of *Bacillus polymyxa* a water-insoluble, ethanol-soluble substance which is highly active against *Staphylococcus aureus* and inactive against *E. coli*. It is suggested, therefore, that the low order of activity of metabolic liquors against gram-positive bacteria may be due to small amounts of this cellular substance escaping into the medium.

Ten grams of moist, unwashed *Bacillus polymyxa* cells and cellular debris, collected by centrifugation, were triturated with sand to a smooth paste. Fifty ml of 95 per cent ethanol were added and the suspension was shaken overnight at room temperature. To 40 ml of the alcoholic filtrate, 80 ml of water were added and the resulting precipitate was collected and dried. It was then dissolved in boiling 95 per cent ethanol and treated several times with charcoal to decolorize it. Water was added to the point of incipient turbidity and the solution cooled. The flocculent white precipitate was washed with ethanol and ether, and dried. A 200-mg per cent suspension was made in water and tested for activity against *E. coli* (MacLeod) and *S. aureus* (Barlow) by the agar streak method. The suspension inhibited *S. aureus* at 10 μg per ml and was inactive against *E. coli* at 1,000 μg per ml. The origin (Stokes *et al.*, 1942), solubility properties (insoluble in water, ether, chloroform, and acetone), and biological behavior are similar to those of tyrothricin, although its relationship to tyrothricin has not otherwise been determined.

Effect of blood on activity. Before therapeutic experiments were instituted it was felt desirable to determine the effect of blood on the antibacterial activity of polymyxin and to determine whether the active substance contained any hemolytic principle. On blood agar plates a colony of *Bacillus polymyxa* showed a very narrow but distinct zone of hemolysis. However, the antibacterial zone (*E. coli*) was much greater.

The experiment summarized in table 2 demonstrated that blood had no appreciable effect on the antibacterial activity of polymyxin, nor had polymyxin, in the concentrations used, any visible effect on blood. Also, the fact that the last tubes showing no growth apparently contained no viable cells suggested that polymyxin had bactericidal properties.

Differentiation of Polymyxin from Known Antibiotics

Polymyxin is active only against certain gram-negative bacteria (Stansly *et al.*, 1947). This fact alone would distinguish it from all known antibiotics. It may be worth while, however, to point out these and other differences insofar as the literature or actual comparisons in the laboratory permit.

Tyrothricin. The insolubility of tyrothricin and its components in water (Hotchkiss and Dubos, 1941; Dubos and Hotchkiss, 1942), its hemolytic activity (Dubos and Hotchkiss, 1942), its toxicity (Robinson and Molitor, 1942), and its greater activity for gram-positive compared to gram-negative organisms (Dubos and Hotchkiss, 1941) distinguished it from polymyxin.

Streptomycin and streptothricin. Both streptomycin and streptothricin have gram-positive activity, thus distinguishing them from polymyxin. Neverthe-

less, their similarity to polymyxin in certain other respects was notable. These were their basic nature (Waksman, Bugie, and Schatz, 1944), water solubility (Waksman and Schatz, 1945), high activity against certain gram-negative bacteria (Waksman *et al.*, 1944), similarity in concentration procedure (Waksman and Schatz, 1945; Stansly *et al.*, 1947), and high activity of streptomycin in the *Klebsiella pneumoniae* mouse infection (Heilman, 1945). In view of these similarities, it was felt desirable to compare polymyxin, streptomycin, and streptothricin experimentally to determine whether any close relationships existed among them.

The effect of the pH of the medium on the antibacterial activity of streptomycin and streptothricin is well known (Foster and Woodruff, 1943; Waksman

TABLE 2

*Effect of blood on the antibacterial activity of polymyxin and the effect of polymyxin on blood**

CONC. POLYMYXIN MG PER CENT	50 PER CENT BLOOD		20 PER CENT BLOOD		10 PER CENT BLOOD		NO BLOOD
	Growth	Hemolysis	Growth	Hemolysis	Growth	Hemolysis	Growth
32	−	−	−	−	−	−	−
1/8	−	−	−	−	−	−	−
1/16	−	−	−	−	−	−	−
1/32	−†	−	−	−	−	−	−
1/64	+		−†	−	−†	−	−†
1/128	+		+		+		−
1/256	+		+		+		+

* Serial twofold dilutions of a crude polymyxin concentrate were made in trypticase-soy-phosphate broth containing the indicated concentrations of defibrinated rabbit blood. Each tube contained a total of 2 ml and was inoculated with approximately 700 *E. coli* cells. Incubation was for 24 hours at 37 C, and the presence or absence of growth was determined by visual inspection. This was possible since the red blood cells had settled by this time.

† These tubes were plated out on agar (1 ml from the tube + 13 ml agar) and incubated for 48 hours at 37 C. No visible colonies appeared on any of the plates.

and Schatz, 1945) and seemed a plausible basis for comparison. Another, obviously, was an antibacterial spectrum with selected organisms. The results of tests using these criteria are shown in tables 3 and 4.

The anticipated increase in activity with increasing pH in the case of streptomycin and streptothricin (table 3) was confirmed, whereas polymyxin showed essentially no change in activity under the same circumstances. The data show that, under the conditions employed, streptomycin was 16 times more active at pH 8.5 than at pH 5.5 and streptothricin 78 times more active at pH 8.5 than at pH 5.5.

The data in table 4 indicate that the preparation of polymyxin used in this experiment was 16 times more active against *E. coli* than was streptomycin, but less than one sixteenth as active as streptomycin against *Bacillus mycoides*. Likewise, the preparation of streptothricin was twice as active as polymyxin against *E. coli* but over 80 times as active against *Bacillus subtilis*. These ob-

servations comprised presumptive evidence for the nonidentity of polymyxin with streptomycin or streptothricin. Cross-resistance experiments with polymyxin and streptomycin confirmed this presumption (White and Clapp, to be published). Additional biological and chemical properties which distinguish polymyxin from streptomycin and streptothricin have been found and will be reported elsewhere.

Subtilin. The relative insolubility of subtilin in water at neutrality (anonymous, 1946) and its inactivity against most gram-negative bacteria (Salle and Jann, 1945) distinguished subtilin from polymyxin. The susceptibility of

TABLE 3

Effect of pH of assay medium on the inhibition of E. coli

EXPERIMENT	ANTIBIOTIC†	CONC. IN MG PER CENT INHIBITING GROWTH OF E. COLI* AT INITIAL pH VALUES OF			
		5.5	6.5	7.5	8.5
1	Polymyxin	0.19	0.39	0.39	0.39
	Streptomycin	25.0	25.0	1.56	1.56
2	Polymyxin	0.19	0.09	0.09	0.09
	Streptothricin	1.56	0.39	0.09	0.02

* In T-S-P medium, agar streak method.

† Antibiotic solutions adjusted to pH 6.4 and titrated in media of indicated pH.

TABLE 4

Relative antibacterial activity of polymyxin, streptomycin, and streptothricin

EXPERIMENT	ANTIBIOTIC	MINIMUM EFFECTIVE CONC.* MG PER CENT		
		E. coli	*B. mycoides*	*B. subtilis*
1	Polymyxin	0.5	>32	
	Streptomycin	8.0	2	
2	Polymyxin	0.09		>2,000
	Streptothricin	0.04		25

* In T-S-P medium, agar streak method.

subtilin to decomposition by pepsin, trypsin, and pancreatin (anonymous, 1946) and the resistance of polymyxin to these enzymes (Stansly and Ananenko, to be published) confirmed the lack of identity.

Bacitracin (Johnson, Anker, and Meleney, 1945). Its activity against gram-positive bacteria and lack of activity against gram-negative bacteria were the only criteria available which served to distinguish bacitracin from polymyxin.

Eumycin (Johnson and Burdon, 1946). The solubility of eumycin in acetone and its inactivity against *Eberthella typhosa* and *E. coli* distinguished it from polymyxin.

Gramicidin S. Its insolubility in water (Belozersky and Passhina, 1944),

toxicity (Gause and Brazhnikova, 1944), hemolytic activity (Manevich and Pitskhelauri, 1944), and greater or equivalent activity against gram-positive organisms compared to gram-negative organisms (Gause and Brazhnikova, 1944) distinguished this substance from polymyxin.

Colistatin (Gause, 1946). Its higher activity against staphylococci than against *E. coli* and its inextractability from broth filtrates with normal butanol were characteristics distinguishing this recently described material from polymyxin.

Bacillin (Foster and Woodruff, 1945). Bacillin is equally effective against gram-positive and gram-negative bacteria. Blood neutralizes its activity *in vitro*. These facts distinguished bacillin from polymyxin.

Antibiotic from Bacillus licheniformis (Callow and Hart, 1946). Its greater activity against *S. aureus* than *E. coli*, activity against *Mycobacterium tuberculosis*, and apparent insolubility in ethanol distinguished this recently described material from polymyxin.

SUMMARY

The isolation and identification of *Bacillus polymyxa* as the organism producing the antibiotic polymyxin is described. Preliminary data on the biological activity of polymyxin which served both to distinguish and characterize the antibiotic are given. The points of distinction between polymyxin and some known antibiotics which bore a superficial resemblance to polymyxin are discussed.

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