SEARCH FOR BACTERIOPHAGES OF PASTEURELLA TULARENSIS AND BRUCELLA SPECIES

by

RALPH W. MITCHELL

A. B., Kansas University, 1952
D.V.M., Kansas State University, 1960

A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Bacteriology

KANSAS STATE UNIVERSITY
OF AGRICULTURE AND APPLIED SCIENCE

1961

TABLE OF CONTENTS

INTRODUCTION

The typing of Staphylococcus and Salmonella cultures by phage methods has been extremely valuable in differentiating strains of each genus and in tracing specific epidemics caused by them. Despite the enormous quantity of phage research, there are many species of bacteria for which phages have never been found; among these are Pasteurella tularensis and, until recently, Brucella species. These organisms, pathogenic for both man and other animals, present difficult medical problems throughout the world. A phage typing method, so useful with Staphylococcus and Salmonella cultures, could be particularly valuable in the identification of Pasteurella tularensis and Brucella strains.

At the time of initiation of research reported in this thesis, no phages for these organisms were available. Therefore, numerous cultures of P. tularensis and Brucella were examined for phage or phage-like substances with the ultimate aim of using these for rapid identification of strains. Before the research was completed, brucellaphages became available and results of experiments with these phages are included in this thesis.

The inclusion of these organisms within a single study was based upon the fact that the two genera have many characteristics in common and may have close taxonomic origins (Breed, et al. 1957). The most convincing evidence for such a relationship is the possession of at least one common surface antigen (Francis and Evans, 1926). Other closely related species of bacteria with common antigenic structures have been found to be susceptible to the same phage (Craigie and Yen,

1938a), therefore, this might be the case with P. tularensis and Brucella.

Early isolations of bacteriophage were from materials other than the bacterial culture itself; however, in recent years it has become a generally accepted technique to examine isolated cultures for the presence of phage. Many cultures contain lysogenic cells which can be induced to release phage under suitable conditions. Virulent phages may be carried with susceptible or resistant bacterial cultures and can be recovered by proper techniques. Therefore, attempts to isolate temperate or virulent bacteriophages were restricted to examination of filtrates from cultures of Brucella and P. tularensis.

REVIEW OF LITERATURE

Brucellaphages

Prior to 1950, there were few articles describing attempts to isolate bacteriophages for P. tularensis and Brucella. The earliest recorded search for bacteriophages active against Brucella was by Sanderson in 1925. He examined fetal membranes and meconium of aborted bovine fetuses for Bacillus abortus (Br. abortus) organisms and for phage active against this organism. He was successful in the isolation of the bacterial organisms from the tissues which he examined but he was unable to detect the presence of phage.

Gwatkin (1931) after conducting similar research, published the results of his examinations of normal and Brucella infected cows for the presence of Br. abortus phages. He found no evidence of phages for Brucella in specimens of feces, milk, and fetal material from both groups of animals.

In 1949 Smith expanded the field of materials which had been examined for brucella phages. He examined sewage as well as material from Brucella infected cows and isolated Brucella cultures, but was unable to obtain a phage for Brucella. He then experimented with the adaption of other known phages to Brucella but these experiments were also without success.

Several years prior to Smith's report an article by Sergienko, et al. (1940) appeared in a Russian publication stating they had isolated a phage for Brucella. However, their methods were not published and since they did not elaborate on their findings, it was not possible for other scientists to duplicate their work. This may have been the first successful isolation of brucellaphage.

In 1950 additional evidence for the existence of a brucellaphage was elucidated by Pickett and Nelson (1950) during their investigation of Brucella cultures isolated from human cases of brucellosis. They detected a lytic agent or phage in mucoid variant cultures of Brucella which had been freshly isolated from blood samples of human cases. The phage was reported to be feebly lytic but capable of forming plaques and causing conversion of smooth Br. abortus broth cultures to mucoid variant organisms and lysogenic variant organisms (Nelson and Pickett, 1951). Their phage preparations, however, failed to lyse cells when tested by other workers (Eisenstark, unpublished) and the question of brucellaphages was still not settled.

Carrere, et al. (1957) stated they had detected a brucellaphage in chick embryo cultures of Br. abortus recovered from a blood sample from a case of human brucellosis. Their phage, while it was capable of causing lysis of broth cultures of Br. abortus, showed no evidence

of plaque formation and could not be propagated on susceptible cultures. This lytic substance did not possess all of the characteristics needed to be classified as a phage.

In 1957 the Russian worker, Drozhevkina, published a report which stated that she had successfully recovered brucellaphages from freshly isolated strains of Brucella. She also cited Sergienko, et al. as being the original discoverers of brucellaphage. She indicated she had isolated brucellaphage from laboratory strains of Brucella, from blood samples of human cases of brucellosis and from the aborted fetuses of bovine cases of brucellosis. The phages were reported to be virulent and capable of lysing many strains of Brucella.

Mamatsashvili (1957) was also able to isolate brucellaphage from several sources including laboratory strains and fresh isolates of Brucella, dried and liquid Brucella vaccines, milk, urine, and fecal samples of cows vaccinated with attenuated strains of Br. abortus. His work suggests that the brucellaphages exist wherever the Brucella organism is found.

Shortly after brucellaphage became available to the Russian scientists more articles were published on the nature of the brucellaphage. Drozhevkina and Kharitonova (1958) found that certain cultures of Brucella were lysogenic and could be induced to produce virulent phage by repeated irradiation of the cultures with a quartz ultraviolet lamp. The irradiation resulted in changes in colony characteristics follwed by the breaking up on the colony with release of the phage. This method of isolation does not conform to the present accepted theories on lysogenic phage. Electron micrographs (Parnas et al., 1958) showed that the phage particle was a non-flagellated

circular elementary body 60 to 80 mu in diameter. This would indicate that the brucellaphage particle was approximately the size of Escherichia coli T2 phage. The absence of a tail is a major morphological difference between brucellaphage and many other phages; most phages have at least a rudimentary tail (Adams, 1959).

In summary, it may be stated that there is no clear-cut case of the isolation of brucellaphage in countries outside of Russia or her satellites. Judging from published articles by Russian scientists, their basic methods for the isolation of phages were not significantly different from the methods used in this country. It is puzzling, however, that all successful isolations of brucellaphage have been confined to that section of the world since the Brucella organism itself is ubiquitous. For this reason it may be suspected that Russian biologists have developed certain phage techniques which are not known outside of that country.

Pasteurella tularensis Phages

Reports of attempts to isolate a phage active against P. tularensis are not generally known and the published articles appear only in obscure scientific literature. An article by Vol'ferts in 1935 (Koliaditshaia et al., 1959) described a phage found in an old yolk culture of Pasteurella astewell tularensis (P. tularensis). The phage formed plaques in a film of P. astewell tularensis culture grown on cystine glucose chocolate agar but caused almost no clearing of a bacterial suspension in broth.

The only other report of an attempt to isolate a phage for P. tularensis was by Koliaditshaia et al., (1959) who observed plaques

in solid medium cultures of the vaccine strain, Gaiskii N. 15, of P. tularensis. They observed morphological changes in isolated colonies of this strain which they associated with the presence of a phage. The changes noted were the development of hemispherical alveoli on the surface of the colonies. Broth suspensions of these colonies, when inoculated onto blood agar plates, developed into cultures containing areas of lysis. The phage, when added to broth cultures of P. tularensis, did not produce broth lysis, but upon subculture of the exposed broth cultures to solid medium the phage present did produce areas of lysis in the culture which developed. This does not prove conclusively that the lytic substance was a phage; therefore, at this time there is no definite evidence that a phage for P. tularensis does exist.

MATERIALS AND METHODS

Cultures

The P. tularensis and Brucella cultures used in this research are listed in Table 1. Each cultures was given a Kansas State University code number and reference will be made to this number throughout this thesis. Following single colony isolation, Gram reactions and cellular morphology were determined for each of the P. tularensis strains after growth for forty-eight hours on glucose cysteine blood agar (GCBA) slants. The organisms were Gram negative rods or coccobacilli exhibiting various degrees of pleomorphism. Colonies on GCBA were small, gray, and raised with entire edges. Prolonged incubation usually produced a greenish discoloration of the medium surrounding

Table 1. Stock cultures of P. tularensis and Brucella species.

Pasteurella tularensis

Original Designation:	Kansas State Designation	Original Designation:	Kansas State Designation
38	HPt 1	Jap-A	HPt 44
38_1	HPt 2	Jap-B	HPt 45
38_2	HPt 3	Jap-C	HPt 46
38_3	HPt 4	Jap-H	HPt 47
38_4	HPt 5	SM-0	HPt 48
Li	HPt 6	SM-1	HPt 49
Li_1	HPt 7	SM-2	HPt 50
Li_2	HPt 8	SM-L	HPt 51
Ri	HPt 9	SM-L rabbit pass	HPt 52
Ri_{-1}	HPt 10	$SM-L_m$	HPt 53
Ri_{-2}	HPt 11	$SM-S_4$ Mouse pass	HPt 54
Ri_{-3}	HPt 12	$SM-S_4$ g.p. pass	HPt 55
Ri-1-L	HPt 13	$SM-S_4$ rabbit pass	HPt 56
$Ri-L_m$	HPt 14	$SM-S_4$ rabbit pass 10^{-1}	HPt 57
RL_4	HPt 15	$SM-S_4$ rabbit pass 10^{-1}	
Cruise	HPt 16	--immunized BLUE 10^{-5}	HPt 58
B.B.	HPt 17	$SM-S_4$-stabilized	HPt 59
425	HPt 18	SM-A	HPt 60
2532g	HPt 19	DEPUE	HPt 61
2533-2	HPt 20	$DEPUE_1$	HPt 62
25030	HPt 21	$DEPUE_2$	HPt 63
25009	HPt 22	HD	HPt 64
AL	HPt 24	HD_1	HPt 65
INCE	HPt 25	HD_2	HPt 66
AM-2-PS	HPt 26	SCHAD	HPt 67
$I_{16}SM$	HPt 27	$SCHAD_1$	HPt 68
$I_{16-2}SM$	HPt 28	$SCHAD_2$	HPt 69
$V_{16}-SM$	HPt 29	COLL	HPt 70
HLP	HPt 30	$COLL_1$	HPt 71
USSR	HPt 31	$COLL_2$	HPt 72
USSR g.p. pass	HPt 32	RUSS	HPt 73
NIIEG USSR BLUE	HPt 33	$RUSS_1$	HPt 74
NIIEG USSR GREY	HPt 34	$RUSS_2$	HPt 75
BLUE USSR mouse pass	HPt 35	CHURCH	HPt 76
BLUE USSR g.p. pass	HPt 36	$CHURCH_1$	HPt 77
BLUE mouse pass	HPt 37	$CHURCH_2$	HPt 78
BLUE rabbit pass	HPt 38	BISH	HPt 79
BLUE g.p. pass	HPt 39	$BISH_1$	HPt 80
BLUE g.p. pass 10^{-1}	HPt 40	$BISH_2$	HPt 81
Jap	HPt 41	CARR	HPt 82
Jap-1	HPt 42	$CARR_1$	HPt 83
Jap-2	HPt 43	$CARR_2$	HPt 84

Table 1. Cont.

Original Designation:	Kansas State Designation	Original Designation:	Kansas State Designation
41804 A	HPt 86	Aeromed	HPt 101
Canada Muskrat B-1	HPt 88	1240 F	HPt 102
Green	HPt 90	Vanenby	HPt 103
Nevada 14	HPt 94	Caraco Lake	HPt 104
MAX	HPt 96	Wig T83	HPt 105
O'hara	HPt 97	280	HPt 106
ATCC 6223	HPt 98	Townsend	HPt 107
Dugway	HPt 99	Nevada 46	HPt 108
Bitter Root	HPt 100	Schu IV	HPt 109

Brucella species

2583	Br.abortus	HBr 1	19	Br. abortus	HBr 42
544	Br.abortus	HBr 2	Carrere	Br. abortus	HBr 41
6232	Br.abortus	HBr 3	Unknown	Br.suis	HBr 50
6613	Br.abortus	HBr 4	Unknown	Br.melitensis	HBr 51
6611	Br.abortus	HBr 5	Unknown	Br. abortus	HBr 52
6610	Br.abortus	HBr 6	Unknown	Br.melitensis	HBr 53
4321	Br.abortus	HBr 7	Unknown	Br. abortus	HBr 54
11192	Br.abortus	HBr 8	Unknown	Br. suis	HBr 55
4315	Br.abortus	HBr 9	198	Br. abortus	HBr 56
4320	Br.abortus	HBr 10	Unknown	Br. suis	HBr 57
9014	Br.abortus	HBr 11	8200	Br.abortus	HBr A
4316	Br.abortus	HBr 12	3143	Br. suis	HBr B
4319	Br.abortus	HBr 13	3605	Br. abortus	HBr C
9153	Br.abortus	HBr 14	4493	Br.melitensis	HBr D
4314	Br.suis	HBr 15	3142	Br. suis	HBr E
9843	Br.suis	HBr 16	7472	Br. abortus	HBr F
4313	Br.suis	HBr 17	6373	Br. abortus	HBr G
7978	Br.suis	HBr 18	5259	Br. abortus	HBr H
9788	Br.suis	HBr 19	8226	Br. abortus	HBr I
4312	Br.suis	HBr 20	1478	Br. abortus	HBr J
6597	Br.suis	HBr 21	7470	Br.melitensis	HBr K
10001	Br.melitensis	HBr 22	4847	Br. abortus	HBr L
4306	Br.melitensis	HBr 23	624	Br. abortus	HBr M
9288	Br.melitensis	HBr 24	3511	Br.melitensis	HBr N
4309	Br.melitensis	HBr 25	8038	Br. abortus	HBr O
739	Br.melitensis	HBr 26	4490	Br. suis	HBr P
6616	Br.melitensis	HBr 27	7471	Br. abortus	HBr Q
4310	Br.melitensis	HBr 28	7226	Br.melitensis	HBr R
802	Br.melitensis	HBr 29	5061	Br. suis	HBr S
M1140b	Br.melitensis	HBr 35	8334	Br.melitensis	HBr T
4248	Br.melitensis	HBr 36	4487	Br. abortus	HBr U

the colony. All strains were tested with known positive tularensis antiserum (rabbit origin, titer 1-2560) and found to be agglutinated by a 1-50 dilution of the antiserum.

The Brucella cultures were examined for typical Gram reaction and cellular morphology after 24 hours growth on tryptose agar slants. Colonies on tryptose agar medium were small, semitransparent and raised with smooth circular margins and exhibited a light bluish color when viewed by transmitted light.

Bacteriophages

The brucellaphages were obtained through the courtesy of several investigators. The phage in each case was originally obtained from Russian workers. Phage 101 was donated by Dr. J. W. Foster of the University of Georgia, phage 3 by Dr. W. R. Stinebring of Rutgers University, and phage ONR was from Dr. C. Lamanna of the University of California at Berkeley. Phage stocks were prepared by the overlay agar technique (Swanstrom and Adams, 1951) using Br. abortus strain 8038 (HBR-O) as the propagating host. The titer of each phage preparation was determined by plating ten-fold dilutions of each filtrate on HBr-O. Titers of the stock phage were as follows:

Phage	Titer
101	1.9×10^{11}
3	4.9×10^{12}
ONR	1.0×10^{12}

After six months storage of the stock phages at 5° C and with repeated sampling it was found that each maintained a titer greater than 1×10^8 particles per ml.

Media

The basic media used for cultivation of P. tularensis and Brucella cultures are listed in Table 2. Snyder's medium (Snyder et al., 1946) and Won's (1958) spermidine medium were used as both liquid and solid media for growing P. tularensis. Tryptose medium was utilized in liquid and solid form for the cultivation of all Brucella cultures.

Table 2. Media used to cultivate P. tularensis and Brucella species.

Pasteurella tularensis

Glucose Cysteine Blood Agar:

Peptone	10.0 gms
NaCl	5.0 gms
Meat Extract	1.5 gms
Cysteine HCl	1.0 gms
Basamin-Busch yeast autolysate	1.0 gms
Agar	15.0 gms
Distilled water	1000.0 ml

This constitutes a basal medium; when used, glucose and blood were added, each to a final concentration of 5 per cent of the basal medium. Citrated or defibrinated blood of human or rabbit origin may be used as the source of blood.

Snyder's Yeast Medium (Modified):

Peptone	100.0 gms
Basamin-Busch yeast autolysate	100.0 gms
Glucose	250.0 gms
NaCl	100.0 gms
Distilled water	1000.0 ml

The concentrated medium was stored in the refrigerator until needed. To prepare the medium for use add the following ingredients to 30 ml of concentrated medium: (1) 300 ml of distilled water, (2) 300 mg of cysteine HCl, (3) 5 gms of agar (omit for broth). Medium was improved for isolation and culture purposed by the addition of 5 per cent blood.

Table 2. Cont.

Won's Spermidine Medium:

Spermidine phosphate	10.0 mgms
N-acetyl glucosamine	1.0 gms
D-Histidine HCl	5.0 gms
DL-Glutamic Acid	1.0 gms
NaCl	5.0 gms
Basamin-Busch yeast autolysate	5.0 gms
Peptone	10.0 gms
Orotic Acid	100.0 mgms
Glucose (50% sterile solution)	20.0 ml
Cysteine (20% sterile solution)	5.0 ml
Distilled water	1000.0 ml

Fifteen grams of agar were added to make a solid medium. To make overlay agar medium add only 7.5 gms of agar per liter.

Brucella

Tryptose Medium:

Tryptose	20.0 gms
Glucose	1.0 gms
NaCl	5.0 gms
Thiamine HCl	5.0 mgms
Distilled water	1000.0 ml

For solid medium add 15 gms of agar and for overlay agar medium add 7.5 gms of agar per liter.

General Testing Methods

All cultures of P. tularensis and Brucella were incubated at 37° C under normal atmospheric conditions without humidity control or increased CO_2 concentration. Liquid cultures were incubated at 37° C on a shaking machine.

Overlay agar technique. The overlay agar technique used in this research was based on the technique described by Adams (1959). In utilizing this technique Brucella cultures were grown on tryptose medium

and P. tularensis on spermidine medium. The adapted procedure was as follows:

1. Plates containing 20 to 25 ml of an appropriate medium with a 1.5 per cent agar base (base medium) were poured and allowed to harden and dry.

2. Broth cultures of the cultures to be tested were incubated until the cell concentration was approximately 10^8 cells per ml.

3. Tubes containing 2.0 ml of the appropriate medium with 0.75 per cent agar base (overlay agar) were melted and plated in a 45° C water bath.

4. To prepare the overlay plates 0.2 ml of the broth culture was added to a tube of overlay agar. The tube was shaken lightly and the contents poured on the surface of a plate containing the hardened base medium. The plates were left at room temperature until the overlay agar hardened.

Filtrates to be tested for the presence of phage were either added to the cell overlay agar mixture before plating or spotted on the surface of the plate after the cell-overlay agar mixture had been plated. The spotting procedure consisted of placing a drop of the filtrate (dispensed from a one ml syringe equipped with a 26 guage needle) onto the surface of the cell-overlay agar layer after the agar had hardened and dried.

Spreading technique. The spreading technique was employed for obtaining lawn cultures of P. tularensis on GCBA medium. At the time this method was utilized there was no medium available on which P. tularensis would grown readily by the overlay technique and have

sufficient transparency to observe areas of lysis. The spreading technique involved transferring P. tularensis cells from a 24 hour GCBA slant culture to the surface of a petri dish containing fresh GCBA medium and spreading the cells as a thin layer over the surface of the plate by means of a glass rod. The test filtrates were spotted on the fresh inoculum as previously described. After 24 hours incubation the cells had covered the surface of the plate with a gray film of confluent culture except in areas where the filtrates had been spotted.

Preparation of culture filtrates. Filtrates of P. tularensis and Brucella cultures were prepared by filtering broth cultures or cell suspensions through Selas 03 porosity filters. The filtrates were kept in screw cap vials and stored at -10° C until ready for use. Periodic tests for sterility of the filtrates were made by inoculating 0.1 ml of the filtrate into an appropriate medium and incubating the medium for 5 days.

RESULTS

Experiment I. Tests for lysogeny in Pasteurella tularensis

Cultures of P. tularensis were inoculated into tubes of Snyder's broth medium, incubated for 24 hours, filtered, and filtrates were frozen for storage. Each filtrate was identified by the letters PPt preceding the culture number from which the filtrate had been prepared.

The filtrates were examined for temperate phage or other inhibitory substances by spot testing each of the filtrates on Brucella and P. tularensis cultures. P. tularensis cultures were prepared by the

spreading technique on GCBA and by the overlay technique on spermidine agar. Brucella cultures were prepared by the overlay technique on tryptose agar. All plates of the test cultures were incubated for 18 hours before they were examined.

The results are summarized in Table 3. Visible clearing was interpreted as an inhibition or lysis of the test culture by a substance in the filtrate. Broth controls did not produce any observable changes in the test cultures.

P. tularensis cultures prepared by the spreading technique did not grow satisfactorily in those areas containing filtrates and broth controls. For this reason it was difficult to differentiate phage lysis from inhibition due to other causes. After the initial experiments the spreading technique was discarded in favor of the overlay agar technique utilizing spermidine medium.

Experiment II. Characterization of Inhibitory Substance

Titration of Filtrates. Ten-fold dilutions of filtrates PPt-29, 32, and 47 were made in tryptose broth and plated in overlay agar containing Brucella cultures HBr-4, 36, and 42. The dilutions were carried out to the 10^{-4} dilution on each filtrate. A one-tenth ml quantity of each dilution and the undiluted stock filtrate was plated on each of the test cultures. After 24 hours incubation plaques were observed on those plates containing PPt-47. The plaques were 2 to 3 mm in diameter and each was centered with a minute colony. The minute colonies were picked and tested for the presence of temperate phage but it was never possible to demonstrate that cells in colonies were lysogenic.

Table 3. Tests for inhibitory agents in filtrates from P. tularensis cultures.

Host*	Filtrates				
	PPt-25	PPt-29	PPt-32	PPt-47	PPt-62
Spread culture technique					
HPt-16	-	-	-	-	+
HPt-20	-	-	+	+	+
HPt-21	-	-	-	-	+
HPt-22	-	-	-	-	+
HPt-29	-	-	+	+	-
HPt-73	-	-	+	-	-
Overlay culture technique					
HPt-29	-	-	+	+	-
HPt-32	-	-	+	-	-
HPt-73	+	+	+	+	+
HBr-B	-	-	+	+	-
HBr-C	-	-	-	+	-
HBr-D	-	-	-	+	-
HBr-F	-	-	-	+	-
HBr-G	-	-	-	+	-
HBr-I	-	-	-	+	-
HBr-J	+	+	+	+	-
HBr-M	-	-	-	+	-
HBr-O	+	-	-	+	-
HBr-Q	+	+	+	+	-
HBr-T	-	-	-	+	-
HBr-W	-	+	-	+	-
HBr-X	-	-	-	+	-
HBr-4	-	+	+	+	-
HBr-7	-	-	-	-	+
HBr-35	-	+	+	+	-
HBr-36	-	-	-	+	-
HBr-42	-	+	+	+	-

* Spread cultures are listed separately because the techniques of the test are different even though the results were similar.

* Spermidine medium was used for the overlay culture of P. tularensis and tryptose medium for the overlay cultures of Brucella.

Positive signs indicate visible clearing of the test culture in the area covered by the filtrate; negative signs indicate no visible clearing.

The plaque-forming ability of this filtrate was further investigated by preparing ten-fold serial dilutions of the filtrate (10^{-1} to 10^{-4}) and plating 0.2 ml of each dilution in the presence of HBr-0.

Three plates were prepared from each dilution and one plate from the undiluted stock filtrate. The plates were incubated for 18 hours and examined for the presence of plaques. Approximately 175 plaques were formed on the overlay plate of the undiluted stock filtrate and 15 to 20 plaques on each of the plates of the 10^{-1} dilution. The plaques were small and cloudy but visible by transmitted light. Photographs of the plates containing plaques are shown in Plate I.

Recovery of Inhibitory Substance from Areas of Clearing. The areas of clearing in Brucella cultures produced by the positive filtrates utilizing the spot test method were removed from the plates and suspended in 2 ml of tryptose broth. The mixtures were treated with 1 drop of chloroform and stored at 5° C. After 24 hours the mixture had separated into a gelatinous sediment and a slightly cloudy supernatant fluid. The supernatant fluid of each mixture and the corresponding stock filtrate were spotted on overlay plates of Brucella. The plates were incubated for 18 hours, after which they were examined for areas of lysis or clearing.

The only areas of clearing were those produced by the stock filtrates; the supernatant fluids did not produce any apparent changes in the test cultures.

Attempts to Recover Inhibitory Substance from Plaques. Cultures HBr-0 and HPt-73 were plated in overlay with a 0.2 ml quantity of filtrate PPt-47. After 18 hours incubation each of the plates contained approximately 150 plaques per plate. The cells and plaques were removed from each plate by adding 5 ml of tryptose broth to each plate and mixing the cell-plaque layer into the broth. The mixture was poured from the plate and filtered. The plaque filtrates and the stock

Plate I

Photograph 1. Overlay plate of Brucella culture HBr-J with isolated plaques produced by filtrate PPt-47.

Photograph 2. Overlay plate of Brucella culture HBr-O with area of clearing in test culture produced by PPt-47.

Plate I

filtrate PPt-47 were spot tested on overlay plates of cultures of Brucella and P. tularensis.

After 24 hours incubation there were no changes produced by the plaque filtrates in any of the test cultures. Other attempts were made to recover the lytic substance but it was never possible to produce lysis in test cultures with material recovered from plaques or areas of clearing.

Heat Stability of the Inhibitory Substance. A 0.5 ml quantity of each of the filtrates PPt-29 and PPt-32 was heated in a water bath to a temperature of 80° C for 5 minutes. Following the heating process the filtrates were immediately cooled to room temperature and spotted on overlay cultures of P. tularensis and Brucella. The heated filtrates were then returned to the water bath and immersed in boiling water for 5 minutes. Following the boiling process the filtrates were again cooled and spotted on the overlay cultures previously mentioned. The corresponding stock filtrates were spotted on the same cultures as controls. The results are given in Table 4.

Action of Inhibitory Agent on Broth Cultures. Three test tubes, each containing 8.0 ml of tryptose broth, were inoculated with 1.0 ml of a tryptose broth culture of HBr-J containing approximately 10^9 cells per ml. Optical density determinations were made on each of the three tubes immediately after addition of the cells. The optical density determinations were made with a Bausch and Lomb spectrophotometer at 600 millimicron wave length. After determining the optical density the tubes were returned to the 37° C incubator for one hour and a second optical density determination was made on each tube. At this

Table 4. Heat stability of *F. tularensis* filtrates.

Test Cultures	PPt-29			PPt-32		
	Stock Filtrate	80° C Filtrate	Boiled Filtrate	Stock Filtrate	80° C Filtrate	Boiled Filtrate
HPt-20	NL	NL	NL	Lysis	Lysis	Sl. lysis
HPt-22	NL	NL	NL	NL	NL	NL
HPt-29	NL	NL	NL	Lysis	Lysis	Sl. lysis
HPt-32	NL	NL	NL	Sl. lysis	NL	NL
HPt-73	Lysis	Lysis	NL	Lysis	Sl. lysis	NL
HBr-J	Lysis	Lysis	Sl. lysis	Lysis	Lysis	NL
NBr-O	NL	NL	NL	NL	NL	NL
NBr-Q	Lysis	Sl. lysis	NL	Lysis	Lysis	Sl. lysis

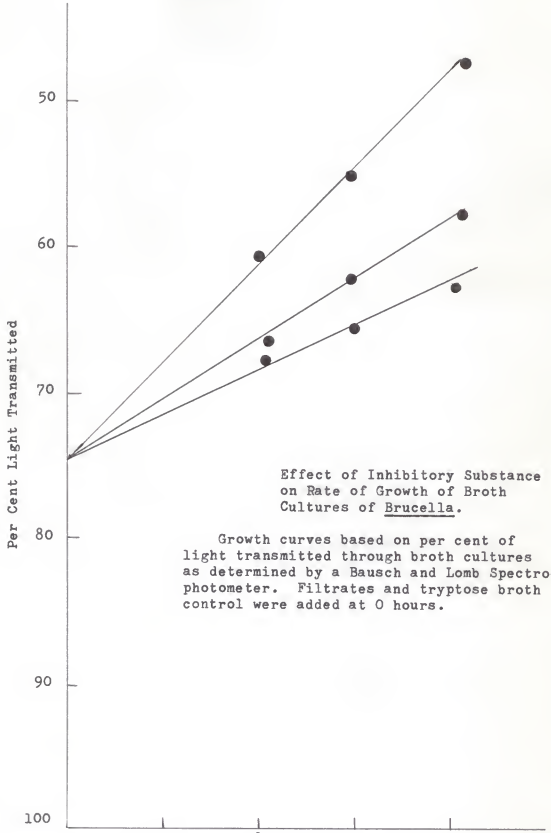
NL - no visible lysis in the area covered by the spot of filtrate.
Lysis - large area of lysis approximately the size of the spot of filtrate.
Sl. lysis - some clearing in the area covered by the spot of filtrate but not confluent lysis.

time 1.0 ml of stock filtrate PPt-47 was added to one tube (A), a second tube (B) received 1.0 ml of filtrate PPt-47 which had been immersed in boiling water for 5 minutes and the third tube (C) received 1.0 ml of tryptose broth. A third optical density reading was made immediately following the addition of the filtrates and broth and the cultures were returned to the incubator. During the ensuing incubation period optical density determinations of each of the cultures were made at 30 minutes, one hour, and two hours incubation. The accompanying graph, Figure 1, illustrates the results.

Action of the Inhibitory Agent on Rough Strains of Brucella. Rough strains of Brucella were developed by streaking cultures HBr-4 and HBr-42 on tryptose agar containing 5 per cent Br. abortus antiserum; titer, 1-1280 (Heney, 1933). The growth was flat and thin with a distinct bluish cast. Subcultures into tryptose broth containing 5 per cent brucella antiserum grew slowly, forming a granular sediment. Gram stains of the broth cultures showed the cells to be Gram negative rods with some pleomorphic large round forms. On this basis the cultures were considered to be typical rough strains of Brucella.

The rough cultures and the corresponding smooth cultures were plated on overlay plates of tryptose agar and spotted with filtrate PPt-47 and a sterile broth control. The plates were incubated for 24 hours and examined for areas of lysis.

Inhibition of both the smooth and rough cultures was produced by filtrate PPt-47 and no inhibition by the broth controls. The degrees of clearing was approximately the same on all plates.



Effect of Inhibitory Substance on Rate of Growth of Broth Cultures of Brucella.

Growth curves based on per cent of light transmitted through broth cultures as determined by a Bausch and Lomb Spectrophotometer. Filtrates and tryptose broth control were added at 0 hours.

Figure 1

Experiment III. Factors Effecting the Production and Release of the Inhibitory Substance

Culture Conditions for Production of Inhibitory Substance. Following unsuccessful attempts to propagate the inhibitory substance, other methods for the production of the inhibitory substance were examined. Three cultures of P. tularensis (HPt-29, 32, and 47) which had given positive results were selected for further study. The cultures were submitted to various conditions of growth in an effort to increase the quantity of inhibitory substance and to determine the optimum conditions for its production. The various conditions examined are listed below:

Sp-1. Cultures grown in spermidine broth for 24 hours and filtered.
Sp-2. Cultures grown in spermidine broth for 48 hours and filtered.
Sp-3. Cultures grown in spermidine broth for 72 hours and filtered.
Sp-4. Cultures grown in spermidine broth for 48 hours, frozen and thawed, then filtered.
Sp-5. Cultures grown in spermidine broth for 48 hours, stored at room temperature for 5 days, then filtered.
Sp-6. Cultures grown in spermidine broth without glucose for 24 hours and filtered.
Sp-7. Cultures grown in spermidine broth without glucose for 48 hours and filtered.
Sp-8. Cultures grown in spermidine broth without glucose for 72 hours and filtered.
Sp-9. Cultures grown on spermidine agar slants for 4 days, suspended in saline and filtered.
Sp-10. Cultures grown on spermidine agar slants without glucose for 4 days, suspended in saline and filtered.

The "Sp" filtrates were spot tested on overlay plates of Brucella and P. tularensis cultures. The results obtained following 24 hours incubation at 37° C are presented in Table 5.

Induction of P. tularensis. This experiment was an attempt to induce the formation of lytic substance by ultra-violet irradiation of cultures of P. tularensis. The P. tularensis cultures used were HPt-20, 22, 29, 32,

Table 5. Tests of "Sp" filtrates.

	Filtrates																													
	Sp-1			Sp-2			Sp-3			Sp-4			Sp-5			Sp-6			Sp-7			Sp-8			Sp-9			Sp-10		
FPt-29	32	47	29	32	47	29	32	47	29	32	47	29	32	47	29	32	47	29	32	47	29	32	47	29	32	47	29	32	47	
HBr-H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HBr-J	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	+	+	+	+	+	+
HBr-N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HBr-O	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	+	+	+	+	+	+
HBr-P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HBr-44	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HPt-29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HPt-32	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HPt-47	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HPt-73	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	+	+	+	+	+	+

+ indicate filtrates which produced lysis of the corresponding culture.

42, 47, 62, and 80. The cultures were maintained and routinely transferred in Snyder's broth medium; a condition under which it had not been possible for the cultures to produce detectable amounts of lytic substance. At the time of irradiation the cultures were in the log phase of growth with approximately 10^8 cells per ml. Each culture was irradiated for 20 seconds with a 15 watt ultra-violet lamp at a distance of 18 inches with a culture depth of 3 to 5 mm. After irradiation the cultures were incubated for 30 minutes and filtered. A filtrate of a non-irradiated duplicate control culture was made for each culture irradiated. The irradiated and control culture filtrates were spot tested on overlay plates of ten cultures of P. tularensis and ten cultures of Brucella.

Examination of the plates after 24 hours incubation did not reveal any areas of lysis. It was apparent that the irradiation time of the experiment did not induce the formation or release of detectable quantities of a lytic substance.

A portion of this experiment was repeated using cultures HPt-29, HPt-32, and HPt-47 as the cultures to be irradiated. The cultures were 18 hour spermidine broth cultures with approximately 10^8 cells per ml. Samples were taken at 10, 20, 40, 80, and 160 seconds following irradiation with a 15 watt ultra-violet lamp at a distance of 15 inches. The medium at the time of exposure was 3 to 5 mm deep and was constantly mixed by a magnetic stirrer. The irradiated and control cultures were not filtered but were spotted directly on overlay plates of test cultures HBr-J, HBr-0, and HPt-73.

The irradiated and control cultures were not able to produce visible lysis or inhibition of the overlay test cultures.

Experiment IV. Tests for Lysogeny in Brucella Cultures

The Brucella cultures were subcultured into tubes of tryptose broth and incubated for 24 hours. Filtrates of the broth cultures were spot tested on overlay plates of P. tularensis and Brucella cultures. After 24 hours incubation of the test cultures the plates were examined for areas of lysis.

There were no visible areas of lysis on the test cultures indicating that the Brucella cultures did not spontaneously produce a phage or inhibitory substance or that none of the hosts were sensitive.

Experiment V. Induction of Brucella

Radiation of Broth Cultures. Thirty-five cultures of Brucella were grown in tryptose broth to a concentration of approximately 10^7 cells per ml. Each culture was irradiated for 20 seconds with a 15 watt ultraviolet lamp at a distance of 15 inches and a culture depth of approximately 5 mm. The cultures were continuously mixed with a magnetic stirrer during the period of irradiation. After irradiation the cultures were incubated for 30 minutes and filtered. The filtrates were spot tested on overlay plates of P. tularensis and Brucella cultures. The test cultures were incubated for 24 hours and examined for areas of lysis.

There were no areas of lysis or inhibition of the test cultures indicating that the bacterial cultures were not induced by low amounts of irradiation to release phage or other lytic substances.

A second irradiation experiment was performed under the same conditions as those in the previous experiment except that the cells were irradiated for 5 minutes instead of 20 seconds. After irradiation the

cultures were divided into 2 aliquots; one aliquot was incubated for 20 minutes and filtered and the other was incubated for 4 hours and filtered. The filtrates were spotted on overlay plates of Brucella and P. tularensis cultures.

After 24 hours incubation of the test cultures there were no areas of visible lysis or inhibition caused by the filtrates, indicating that the irradiation did not induce cells to release phage or lytic substances.

Radiation of cultures on solid medium. Droshevkina and Karitonova (1958) irradiated lysogenic Brucella cultures on solid medium and found that the colonies which developed were dark, irregular, and radially striated with eroded areas.

In an attempt to verify these results, thirty-two cultures of Brucella were streaked on tryptose agar plates and incubated for 6 hours. The young cultures were irradiated with a 15 watt ultra-violet lamp at a distance of 12 inches for 5 minutes. The plates were incubated for 5 days to let the surviving colonies develop. One or two of the surviving colonies were picked and streaked on fresh tryptose agar plates, incubated for 6 hours, irradiated as before, and incubated again until colonies appeared. This procedure was repeated two more times before the plates were discarded. Each time subcultures were made, the surviving colonies were studied carefully for the appearance of lysogenic colonies as described by Droshevkina and Kharitonova (1958). During the experiment there was no apparent change in colony characteristics. Colonies which developed from irradiated cells did not differ visibly from colonies from non-irradiated cells.

Experiment VI. Host Range Experiments with Brucellaphages

The next phase of research was to examine the three known Brucella phages.and compare their characteristics with those of the inhibitory agents isolated from P. tularensis. A preliminary screening test was performed to determine the host range of each phage filtrate. This was done by the spot test method on overlay plates of Brucella and P. tularensis cultures. The concentrated stock filtrates of all three phages were found to produce spot lysis of most of the cultures of Br. abortus and Br. suis, although there was some apparent variation in the susceptibility among the different cultures of each species. Most of the cultures of Br. melitensis and all of the cultures of P. tularensis were resistant to the phages.

Experiment VII. Efficiency of Plating of Brucellaphages on Brucella

Since variation in susceptibility of the Brucella cultures to each of the phages was observed, the efficiency of plating (EOP) of each of the phages on each culture of Brucella was determined. This might result in a pattern whereby it would be possible to identify the Brucella cultures by the use of phage.

Hundred fold dilutions were made of each of the phages and a 0.01 ml quantity of each dilution and the stock phage was spotted on overlay plates of each of the Brucella cultures. The plates were incubated for 24 hours and examined for lysis by each of the filtrate dilutions. The end-point dilution was the highest dilution of phage which would give confluent lysis of the cells within the area covered by the spot of phage diluent.

Confluent lysis with the 10^{-6} dilution of phage was considered 100 per cent EOP when the stock phage filtrate titers were greater than 1×10^{8} phage particles per ml. The results are recorded in Table 6.

DISCUSSION

The initial objective of the research described in this thesis was the isolation of phages for P. tularensis and Brucella. With this objective in mind, all inhibitory substances isolated were examined for phage characteristics. A precursory examination of the results of experiments with the inhibitory substances found in filtrates of P. tularensis indicate that the substance is not a phage but does exhibit some phage characteristics. The inhibitory substance was found in filtrates of P. tularensis cultures and did produce plaques and areas of clearing when plated on sensitive cultures of Brucella and P. tularensis. However, it was never possible to recover a substance from the plaques and areas of clearing which would inhibit or lyse other cultures of P. tularensis or Brucella. It is therefore concluded that the inhibitory substance was a bacteriocin rather than a phage. Guelin (1943) reported a similar situation when he found that filtrates of broth cultures of Escherichia coli 36 contained a lytic substance which could produce plaques when plated on sensitive strains of E. coli. However, he could not recover a lytic substance from the plaques and he concluded that the lytic substance was a colicin rather than a phage.

The host specificity of the inhibitory substance and the sporadic nature of its release are characteristics which suggest that it is not an antibiotic or toxic metabolite. Toxic metabolites and antibiotics tend to

Table 6. Efficiency of Plating of three Brucellaphages when tested on 38 hosts.

Host	Phage 3	Phage 101	Phage ONR
Br. abortus			
HBr-A	0.01%	0.01%	1.00%
C	100.00	100.00	0.01
F	1.00	1.00	1.00
G	1.00	0.01	1.00
H	0.01	0.01	0.01
I	1.00	1.00	100.00
J	100.00	100.00	1.00
L	0.00	0.00	0.00
M	0.01	1.00	1.00
O	100.00	1.00	0.01
Q	100.00	100.00	1.00
X	100.00	100.00	0.0001
4	100.00	100.00	1.00
10	0.00	0.00	0.00
11	100.00	100.00	100.00
39	100.00	100.00	100.00
52	0.01	0.01	0.01
54	1.00	1.00	1.00
56	100.00	100.00	0.01
Br. suis			
HBr-B	1.00	0.01	0.01
E	0.0001	0.01	0.0001
P	1.00	0.01	0.0001
S	0.01	0.01	0.0001
17	1.00	0.01	1.00
18	0.01	0.01	0.01
20	0.00	0.00	0.00
50	0.01	0.01	0.01
55	0.01	0.01	0.01
57	1.00	100.00	1.00
Br. melitensis			
HBr-D	0.00	0.00	0.00
K	0.00	0.00	0.00
N	0.00	0.00	0.00
R	0.0001	0.00	0.0001
T	0.00	0.00	0.00
23	0.00	0.00	0.00
37	0.00	0.00	0.00
51	0.00	0.00	0.00
53	0.0001	0.0001	0.0001

accumulate in the medium as the culture matures, whereas the inhibitory substance was not necessarily associated with old cultures or cultures which were undergoing rapid metabolic activity. Host specificity would suggest that it required a specific chemical structure on the surface of susceptible cells before it could affect the cell. It must also be assumed that the specific chemical structure was a constituent of both P. tularensis and Brucella since the inhibitory substance could affect both P. tularensis and Brucella. Common antigens are known to exist between P. tularensis and Brucella and it is thought that some part of the common antigen might be the receptor site for the inhibitory substance.

Additional evidence that the receptor site was not the type specific antigen of Brucella is the fact that rough cells which were not agglutinated by specific antiserum were susceptible to the action of the inhibitory agent. A tentative conclusion can be made that the common antigen of P. tularensis and Brucella was the receptor site of the lytic substance.

The graph of Experiment II-5 gives some indication of the inhibitory action of this substance. The curve of control culture C was characteristic of Brucella during the log phase of growth. The growth curve of culture A showed a definite decrease in rate of growth following the addition of the unheated filtrate, but there was no decided drop in optical density readings to show that a significant number of cells had been lysed. This indicated that the inhibitory substance had an initial effect on some of the cells but there was not the prolonged effect which would have been found if the inhibitory substance were undergoing multiplication. It does suggest, however, that either it completely inhibited a portion of the cells or slowed the growth rate of the whole culture.

Heat stability is a characteristic of bacteriocins as evidenced by the fact that some colicins can withstand temperatures above 100° C for several minutes with only a partial loss in activity (Frederieq, 1957). The inhibitory substance of P. tularensis was found to lose none or only a small part of its inhibitory activity after being boiled for 5 minutes. This effect is illustrated in Experiment II-5 by the growth curve of culture B following the addition of 1 ml of boiled filtrate.

It was found in Experiment III that the inhibitory agent appeared only in filtrates of cultures grown on solid medium and in filtrates of young broth cultures. The presence or absence of glucose in the medium was not a factor in the production of the inhibitory substance; however, glucose was needed in the medium for any appreciable growth of the culture. The results suggest that the inhibitory substance was destroyed or adsorbed to susceptible organisms upon prolonged incubation of the broth cultures.

The conclusions to be drawn at this time are that the inhibitory substance found in filtrates of P. tularensis cultures had some of the characteristics of bacteriocins but it was never possible to produce sufficient quantity of the substance for a critical analysis.

The unsuccessful attempts to isolate a brucellaphage from Brucella cultures does not conclusively prove that a phage was not present but only that none could be demonstrated by the methods employed. The brucella-phages used in this research which were originally isolated in Russia behaved as typical phages. The three phages are similar and do not seem to have any distinguishing individual characteristics as far as plaque morphology and host range are concerned. There were variations in the efficiency of

plating of the three phages on the various cultures of Brucella but, in general, they were able to lyse most cultures of Br. abortus and Br. suis and a few cultures of Br. melitensis, thus there may be some relationship between the type of specific antigen of Brucella and the receptor site of the phage. The type specific antigen of Brucella is composed of two antigenic components, A and M, which vary quantitatively among the three species. Br. abortus and Br. suis have approximately the same quantity of A and M components in their type specific antigens and it is difficult to differentiate the two species on the basis of serological tests (Wilson and Miles, 1932). There is usually sufficient differences in the quantities of A and M components of the type specific antigen of Br. melitensis to differentiate this species from the other two by means of serological tests.

It is apparent from the research that the inhibitory substance found in filtrates of P. tularensis cultures did not resemble the bacteriophages of Brucella. The brucellaphages appear to be true phages and the inhibitory substance has been tentatively classified as a bacteriocin.

SUMMARY

A substance, capable of producing plaques in overlay cultures of Brucella and P. tularensis was detected in filtrates of P. tularensis cultures. The substance, found only in low concentrations in the filtrates, was not consistantly produced even under similar cultural conditions. The substance was relatively heat stable, withstanding temperatures of 80° C for 5 minutes but partially destroyed by boiling for 5 minutes. Although the inhibitory substance was capable of forming plaques in sensitive

cultures, it could not be demonstrated in material recovered from plaques or areas of clearing.

It was concluded that the substance was either not reproduced, or was so modified by reproduction that it was non-detectable by the test methods used. It has been tentatively classified as a bacteriocin.

Three known brucellaphages were found to have similar host ranges, all three being able to lyse most cultures of Br. abortus and Br. suis and few cultures of Br. melitensis. Some differences were found among the three phages based upon their efficiency of plating on each of the cultures of Brucella. None of the phages were able to lyse cultures of P. tularensis.

ACKNOWLEDGMENTS

The author wishes to acknowledge the excellent advice and invaluable aid offered many times throughout the research by Dr. A. Eisenstark. The author also feels he is deeply indebted to Mr. Frank Newman for technical assistance and encouragement during the past few years. The experimental research was supported by funds from the Biological Laboratory of the Army Chemical Corps.

BIBLIOGRAPHY

Adams, M. H.
Bacteriophages. Interscience Publishers. New York, N. Y. 1959.

Breed, R. S., E. G. D. Murray,, and N. R. Smith
Bergey's Manual of Determinative Bacteriology. 7th Ed. Williams and Wilkins Co. Baltimore, Md. 1957.

Carrere, L., J. Roux and J. Mandin
Obtention de souches lysogenes de Corynebacterium parvum et de Brucella melitensis. Comptes Rendus Des Seances de la Societe de Biologie et de ses Filales. 150:599-600. 1957.

Craigie, J. and C. H. Yen
The Demonstration of Types of Bacillus typhosa by means of Preparations of Type II Phage. Can. Pub. Health J. 29:448-463. 1938.

Drozhevkina, M. S.
Brucella Bacteriophage and the Prospects of its Utilization. J. Microbiol. Epidemiol. and Immunobiol. 28(9):3-7. 1957.

Drozhevkina, M. S. and T. I. Kharitonova.
Lysogeny in Brucella. Prob. in Virology. 2:93-97. 1958.

Eisenstark, A.
Personal Communication. 1958.

Francis, E. and A. C. Evans
Agglutination, Cross-agglutination, and Agglutination Adsorption in Tularemia. Pub. Health Rep. 41(26):1273-1295. 1926.

Fredericq, P.
Colicins. Ann. Rev. Microb. 11:7-22. 1957.

Guelin, A.
Lyse Bacterienne Provoquee par une souche de Bacterium coli. Ann. Inst. Past. 69:382-394. 1943.

Gwatkin, R.
Search for a Brucella Bacteriophage. J. Inf. Dis. 48:404-407. 1931.

Henry, B. S.
Dissociation in the Genus Brucella. J. Inf. Dis. 52:374-402. 1933.

Koliaditskaia, L. S., K. V. Kuchina and A. A. Shmurygina
Tularemia Phage. J. Microbiol., Epidemiol., and Immunobiol. 30:13-16. 1959.

Manatsashvili, E. G.
Some Properties of Brucella Bacteriophage. J. Microbiol., Epidemiol., and Immunobiol. 28(9):8-11. 1957.

Nelson, E. L. and M. J. Pickett
The Recovery of L Forms of Brucella and Their Relation to Brucella Phage. J. Inf. Dis. 89:226-231. 1951.

Parnas, J., A. Feltynowski and W. Bulekowski
Anti-Brucella brucei Phage. Bulletin de L'Academie Polonaise Des Sciences. 150(6):5. 1958.

Pickett, M. J. and E. L. Nelson
Brucella Bacteriophage. J. Hyg. 48:500-503. 1950.

Sanderson, E. S.
Bacteriophage Tests on the Meconium of Aborted Fetuses. J. Exp. Med. 42:561-564. 1925.

Sergienko, F. E., V. M. Shul'tts, and A. L. Matovich
Mikrobiol. Zh. 7:175-179. Cited in Drozhevkina, M. S., Brucella Bacteriophage and the Prospects of Its Utilization. J. Microbiol., Epidemiol. and Immunobiol. 28(9):3-7. (1957) 1940.

Smith, H. W.
A Search for Bacteriophages Active Upon Bacteria of the Brucella Genus. J. Hyg. 47:414-415. 1949.

Snyder, P. L., R. A. Penfield, F. B. Engley and J. C. Creasy.
Cultivation of Bacterium tularensis in Peptone Media. Proc. Soc. Exptl. Biol. and Med. 63:26-30. 1946.

Swanstrom, J. and M. H. Adams
Agar Layer Method of Production of High Titer Phage Stocks. Proc. Soc. Exptl. Biol. and Med. 78:372-375. 1951.

Vol'ferts
Vest. Mikrobiol., Epidemiol. Parazit. 2. Cited from Koliaditshaia, L. S. et al. Tularemia Phage. J. Microbiol., Epidemiol., and Immunobiol. 30:13-16. (1959) 1935.

Wilson, G. S. and A. A. Miles
The Serological Differentiation of Smooth Strains of the Brucella Group. Brit. J. Exptl. Path. 13:1 1932.

Won, W. D.
New Medium for the Cultivation of Pasteurella tularensis. J. Bact. 75:287-289. 1958.

SEARCH FOR BACTERIOPHAGES OF PASTEURELLA TULARENSIS AND BRUCELLA SPECIES

by

RALPH W. MITCHELL

A. B., Kansas University, 1952
D.V.M., Kansas State University, 1960

AN ABSTRACT OF A THESIS

Submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Bacteriology

KANSAS STATE UNIVERSITY
OF AGRICULTURE AND APPLIED SCIENCE

1961

Filtrates from broth cultures of the Brucella gave no indication of the presence of a phage or phage-like substance. Also, attempts at the induction of lysogeny with ultra-violet irradiation were unsuccessful.

Since no bacteriophages could be detected by the usual techniques and their production could not be induced by ultra-violet light, it was concluded that (1) the cultures were not lysogenic, (2) lysogeny is not a common occurrence in Brucella and P. tularensis, or (3) the methods employed were not adequate for detection of phage production by these organisms.

Another phase of the research dealt with the study of three brucellaphages of Russian origin. Due to the inability to produce these phages from cultures of Brucella or to find them in nature in the U. S. it was assumed that the phages might possess unusual properties. This assumption was discarded when it was found that these phages could be treated the same as any other phage. A study of the host range of the brucellaphages indicated that all three possessed a wide spectrum of host range. Cultures of Brucella abortus and Brucella suis are highly sensitive to the action of the phage, whereas, Brucella melitensis has a low level of sensitivity.

This study has shown that (1) the lysogenic state is not prevalent in cultures of Brucella and P. tularensis, (2) an inhibitory substance is produced by some strains of P. tularensis which has selective activity for P. tularensis and Brucella cultures, and (3) brucellaphages of Russian origin possess the same properties as other phages and may be useful in differentiation of the Brucella species.

This study was initiated to determine the extent of lysogeny and the conditions necessary for the establishment of lysogeny in strains of Pasteurella tularensis and Brucella species. A review of the literature revealed that phages for P. tularensis and Brucella have been isolated and that the lysogenic state does exist in these organisms. Due to the fact that the only successful isolates of these phages occurred outside of the United States, two possibilities seem apparent: (1) the methods necessary for isolation and propagation are different from the procedures used by phage workers in this country, or (2) the viruses are indigenous to certain countries.

Numerous strains of P. tularensis and Brucella obtained from various sources were used in the study. In the initial phase of the research filtrates from broth cultures of each of the organisms were prepared. These filtrates were spot-tested on each strain in order to detect the lytic activity of any substance present in the filtrates.

Several strains of P. tularensis were capable of secreting an inhibitory agent which possessed selective activity on cultures of P. tularensis and Brucella. This agent produced plaques on sensitive cultures Brucella and P. tularensis, but could not be propagated by any method on these same strains. It was obvious, therefore, that the substance was not a phage. Attempts made to characterize the agent met with the immediate difficulty that the inhibitory substance was only sporadically produced by actively growing cultures of P. tularensis. This observation, in addition to other analyses, led to the conclusion that the active agent was a bacteriocin. The limited amount of material available made further characterization of the substance difficult.