BACTERIAL VIRUSES SPECIFIC FOR THE YELLOW CHROMOGENIC BACTERIA OF WHEAT

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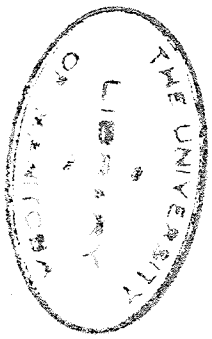

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Bacterial Viruses Specific for the Yellow Chromogenic Bacteria of Wheat.

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ABSTRACT.

Twenty bacterial viruses specific for the yellow chromogenic bacteria of wheat were isolated from wheat seed. In each case the virus lysed the homologous culture and certain other cultures, but was too specific for identifying these bacteria as a group. A composite virus was prepared by mixing equal portions of four viruses, selected because these viruses lysed the complete range of cultures from which individual viruses had been obtained. The composite virus lysed all cultures of the chromogen, but none of 18 cultures of Xanthomonas translucens, nor X. carotae, nor X. campestris, nor any of 22 other species. Likewise, the composite virus lysed 100% of 20 fresh isolates from wheat and 82% of 160 isolates from other cereal and forage seeds. On the basis of these results it is evident that the yellow chromogens constitute a distinct group of bacteria.

Evidence is presented which indicates that one virus did not exert an inhibitory effect on another when mixed, since the composite virus invariably lysed all cultures lysed by the single viruses. In fact, there was evidence of a phenomenon analogous to synergism in bacterial cultures, since the composite virus, in one case at least, lysed a culture not lysed by any of the single viruses used in the composite.

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INTRODUCTION

The occurrence of appreciable numbers of yellow chromogenic bacteria on wheat, up to 89% of the bacterial count (29), as well as on other cereal and forage seeds has been observed by many investigators during the last half century. James et al (14) studied the bacterial flora of wheat and found this type predominant on all samples. Since then a detailed study of these organisms has been carried out in this laboratory by a number of investigators.

Wilson (28) investigated 22 isolates and found that culturally, morphologically and physiologically they resembled the pathogen, Phytomonas translucens var. undulosa. (now Xanthomonas translucens f. sp. undulosa, Hagborg).

Stark (21) studied 38 isolates of these bacteria from wheat, oats, barley and flax. Slight physiological differences among these cultures were noticed, but these were not of sufficient importance to justify considering them to belong to different species.

Goldstein (11) carried out a comparative study on 13 cultures of yellow bacteria isolated from wheat and nine known cultures of the genus Xanthomonas. This investigator concluded these two groups to be so much alike as to warrant placing them in the same genus.

Steel (22) conducted a study on 31 isolates and seven Xanthomonas cultures. He confirmed the morphological, cultural and physiological findings of Wilson, Stark, and Goldstein. The main difference between these two groups was found to be in the utilization

of asparagine as the sole source of carbon and nitrogen. Tests for pathogenicity were made on 500 plants known to be susceptible to the different varieties of Xanthomonas translucens. None of the isolates produced disease, whereas under the same conditions the pathogens produced characteristic symptoms of disease. The pigments of the two groups were found to be similar with regard to the lipocyan reaction, extracting solvents, chromatographic adsorption, partition and color tests.

The identification of bacteria involves morphological, cultural and physiological procedures, and in case of pathogens host inoculations. These are time consuming. Specific sera have been used successfully for the quick identification of certain bacteria, but their development is complicated and often impracticable. In the past two decades the lytic reactions of specific bacterial viruses have proven helpful to a number of investigators in identifying bacteria.

Accordingly, this investigation was undertaken in the hope that a bacterial virus specific for the yellow chromogenic bacteria of wheat could be isolated and that it could be used for the rapid identification of these bacteria.

HISTORICAL

In 1915 Twort observed peculiar changes in colonies of organisms grown on solid media from vaccine lymph. After 24 hours these became transparent (7). D'Herelle, 1917, found that a filtrate from faeces of Shiga dysentery (now Shigella dysenteriae) cases, emulsified in broth and then incubated for 18 hours, inhibited the growth of a young culture of Shiga (7).

Ever since the lytic reactions of bacterial viruses were observed by these two scientists, their possible value as an aid in identifying bacteria has been considered. Among the first to investigate this possibility was Laird (17) who isolated a number of bacterial viruses for the purpose of identifying Rhizobium strains. Because of great variation in susceptibility of the strains to the viruses he did not consider this method promising. Evans (5), only a year later, found bacterial viruses useful in the identification of certain hemolytic streptococci. According to this investigator (5) Lancefield identified 47 of 56 strains of streptococci with "Clark phage".

Conn et al (2) used bacterial viruses as a criterion in classifying soil bacteria. Viruses developed from each of the six strains of Agrobacterium radiobacter lysed all the six strains, but were incapable of lysing any of the 33 Rhizobium cultures and practically none of the 14 organisms showing the morphology of Bacterium globiforme, nor seven miscellaneous soil types. Of the 14 bacterial viruses isolated

from cultures "showing the morphology typical of B. globiforme" six showed complete cross-lysis. Three of the six cultures showing complete lysis were subcultures of one strain. This, according to the authors, indicated that the bacterial virus method is a satisfactory means of identifying strains. Further, on the basis of cultures showing partial lysis, the authors justify Lochhead's observations that there were many types of bacteria in soil with "globuliform-like" morphology. The viruses isolated for Rhizobium strains of alfalfa, soy and lima beans were found strain specific and incapable of cross-lysis. Thus, viruses could be successfully used for their differentiation. But, as Laird (17) had found, these investigators were not successful in developing specific viruses for Rhizobium strains isolated from clover, peas and beans. The bacteria associated with the latter group were found to produce identical races of viruses capable of cross-lysis.

Fulton (10) isolated two bacterial viruses from Pseudomonas tabaci and the closely related P. angulatum. One virus lysed P. tabaci, P. angulatum and P. lachrymas, the other virus was found to lyse P. angulatum, P. tabaci, P. coronafaciens, P. phaseolicola and P. syringae. The viruses differed in thermal inactivation exposures and in morphology. Similar results were obtained by Sutton and Katznelson (20) with P_{26} and P_{11} viruses isolated from Pseudomonas pisi strains. The P_{26} virus lysed both P_{26} and P_{11} strains of P. pisi and 4 species of the same genus. The P_{11} virus was specific for the host culture only. But the viruses isolated for certain strains of P. coronafaciens lysed other strains

also and were found inactive against a number of other Pseudomonas species. Fifty nine bacterial viruses isolated from wheat from different parts of Canada, for avirulent strains of P. atrofaciens, were found to be specific to the virulent strains only. None of the 14 cultures belonging to 11 different species of Pseudomonas and the three virulent strains of P. atrofaciens were susceptible. The viruses isolated from three form species of Xanthomonas translucens reacted with host cultures only and, therefore, were regarded as too specific for species identification. Katznelson and Sutton (15) isolated specific viruses for Pseudomonas phaseolicola and Xanthomonas phaseoli. P. phaseolicola virus acted on all cultures of X. phaseoli and was completely inactive against X. phaseoli var. fuscus and var. sojensis. The latter two differ from X. phaseoli only in respect to host pathogenecity.

In later work these two investigators (16) reported the isolation of polyvirulent viruses for the identification of X. translucens. Twenty four of 28 cultures were lysed by the polyvirulent virus KPg 34. Thus, 91% of the cultures tested were assigned to X. translucens. Attempts to use form specific viruses to identify cultures of X. translucens f. spp. hordei, hordei-avenae and undulosa were unsuccessful. However, the use of specific viruses for the detection of homologous cultures of X. translucens f. sp. secalis gave encouraging results.

Thornberry et al (25) reported the use of bacterial viruses for the identification of Xanthomonas pruni. The virus was isolated in 1927, and was one of the first bacterial viruses recognized for plant

pathogenic bacteria. After 20 years storage, the 10^{-1} dilution of the virus lysed all cultures of X. pruni isolated from peaches, plums and apricots in various regions of Illinois, whereas cultures of X. campestris, X. lactucae-scariole, distinguishable from X. pruni by pathogenecity only, were not lysed.

Thornberry et al (24) found that the reactions of specific bacterial viruses for X. pruni were more reliable than cross-agglutination tests. Six isolates of X. pruni and 54 other species of the same genus were tested for susceptibility to the virus of X. pruni. Only six cultures of X. pruni were lysed. None of the other 54, including X. corylina, X. phaseoli var. sojensis and X. lespedezae, which according to cross-agglutination reactions should be placed into one serological group with X. pruni, were lysed.

Toshach (26) used bacterial viruses for typing Corynebacterium diphtheriae. This investigator was unable to obtain a definite pattern of susceptibility of C. diphtheriae strains as to type or source of these organisms, except in two instances where epidemiologically related strains showed uniform susceptibility to one of the viruses.

Fahey (6) undertook a detailed study in order to investigate the possibility of classifying C. diphtheriae strains by this method. From four original bacterial viruses a number of "host range mutants" were developed, and, by their reactions 68 strains of C. diphtheriae were grouped into nine distinct "phage types".

Fisk (8) reported that the lytic reactions of bacterial viruses were not altered by environment or time, and, therefore, this

method might be successfully used for typing Staphylococcus aureus (now Micrococcus pyogenes var. aureus) and for tracing the origin of infections. Ninety five cultures from related sources in different patients were typed with 27 viruses. Of special interest is the fact that a number of strains isolated from members of the same household were found susceptible to the same viruses, though, in one case, the bacterial strain differed in type of pigment. Fisk and Mordvin (9) confirmed the practical use of bacterial viruses for identifying strains of Staphylococcus aureus. Seventy eight cultures, isolated from different sources (throat, urine, furuncles, etc.) from 30 patients during a period of three years, were studied in detail. Thirty three mono- and two polyvalent bacterial viruses were employed. Cultures isolated from sources which presupposed their identity were lysed by the same bacterial viruses, while most of the cultures isolated from different sources reacted with homologous viruses. Only some of the cultures isolated from related sources were found to be of the same "phage type", though they differed in some other respects. However, several cultures isolated from similar sources were found to be different "phage types" and were considerably unlike in other respects. These investigators found this method of typing of S. aureus quite reliable and suggested its wider use.

Craigie and Yen (4) employed 18 bacterial viruses for typing B. typhosus (now Salmonella typhosa). The "Type II phages" exhibited high selective affinity for particular strains of B. typhosus. On the basis of susceptibility of cultures to particular bacterial viruses a

number of distinct types of B. typhosus were recognized.

Gunther (12) reported that the number of bacterial viruses originally used by Craigie and Yen (4) was reduced from 18 to eight when the modified method of isolation was employed. With eight viruses 65.7% of the 67 strains of typhoid bacilli were typed. The virus typing method also corroborated the epidemiological findings during the 1945 outbreak in Philadelphia, where 29 of 65 cases and the carrier were found to excrete Type F typhoid bacilli.

Wassermann and Seligmann (27) reported the isolation of four bacterial viruses for strains of Serratia marcescens. The viruses exhibited fair specificity, only three of 68 possible cross-lysings with 17 cultures occurred. One of the four viruses was found to be specific to its homologous culture only. The species specificity was checked on about 100 cultures of Enterobacteriaceae. Only few of them showed any degree of lysis when the first undiluted filtrate was used, but on dilution of the filtrate no lysis could be observed, whereas all S. marcescens strains were lysed.

Thomas (23) isolated viruses for nine species of plant pathogenic bacteria and found that each virus reacted best with the species from which it was isolated. A group of eight human pathogens was tested against a similar number of viruses for plant pathogens. All reactions were negative. Likewise when viruses for human pathogens were tested against bacteria isolated from plants, complete lysis was not observed in any case.

Hunter (13) used specific bacterial viruses in order to clarify the division of lactic streptococci into two species. A number of microbiologists had not recognized Streptococcus cremoris as a separate species and included it with S. lactis. This worker isolated a number of cultures and according to their characteristics divided them into two groups, S. lactis and S. cremoris, and then isolated bacterial viruses from representatives of each group. About 7000 tests for susceptibility were carried out. None of the cultures of S. cremoris was lysed by the bacterial viruses isolated from S. lactis. Three cultures of the S. lactis group reacted with viruses of both groups, and three others were susceptible to viruses of the S. cremoris group, but did not react with any other S. lactis virus. None of the viruses was found to attack cultures of S. fecalis and D (Lancefield) streptococci.

Further work on lactic streptococci classification was carried out by Nichols and Hoyele (18). In this investigation 375 strains of S. lactis and S. cremoris and their reactions with 78 bacterial viruses, involving some 60,000 tests, were studied. Strains of both species were grouped into 11 distinct "phage types". These investigators were of the opinion that, at the present time, this method provides the best available means of differentiating strains within these two species.

Reilly et al (19) reported that bacterial virus "M", obtained from Merck and Company, when tested against cultures of Streptomyces bikiniensis, S. violaceus and 12 strains of S. griseus, was found active against seven streptomycin producing strains of S. griseus only.

PROCEDURE

Thirty two cultures of typical yellow chromogenic bacteria were isolated from samples picked at random from 120 samples of six varieties of wheat collected in Western Canada. These cultures were used to isolate a bacterial virus specific for the yellow chromogenic bacteria of wheat. The method for isolation of bacterial viruses from cereal seeds employed by Katznelson and Sutton (16) was modified slightly. The method follows.

Twenty five g. ground wheat was placed in a sterile 250 ml. Erlenmyer flask containing 75 ml. nutrient broth* and inoculated with a 24 hr. nutrient broth culture of one of the isolates. This was incubated at 25°C. for about 24 hr., and the mixture was filtered twice, the first time through filter paper and the second through a sterile Seitz filter. One ml. of the bacteriologically sterile filtrate was added to 8 ml. nutrient broth, inoculated with one ml. of the culture, incubated as before, and filtered. Usually after two to four such passages, one drop of the filtrate inhibited one ml. of the culture. At that time each of one ml. of the filtrate, and of serial dilutions of it, was mixed with two ml. nutrient agar* heavily seeded with the culture and poured into a plate containing a sterile layer of solidified agar. After incubation at 25°C. for 24 hr. a plaque was fished from a plate showing discrete plaques into 8 ml. nutrient broth inoculated lightly with the culture. This enrichment process was continued through two to six passages, or

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* Difco nutrient broth plus 0.1% Merk's dextrose, and Difco nutrient agar were used throughout this investigation. Other sugars and yeast extract did not support growth of the yellow chromogenic bacteria as well as did dextrose.

until one drop of the filtrate inhibited one ml. of the culture.

By this method twenty viruses were obtained by using different cultures of the yellow chromogen. Three cultures were discarded because after a period of time they exhibited certain abnormal characters. The nine remaining cultures failed to yield viruses that completely lysed homologous cultures, or their titers could not be raised to reach the accepted range.

Katznelson and Sutton (15) suggested that the difficulty in isolating a species specific phage might be reduced to some extent by combining various strain-specific phages. In this investigation a composite virus was prepared by mixing equal portions of four virus filtrates. This particular combination was found necessary in order to cover the cross-lysing range of the 20 viruses.

The bacterial virus filtrates were stored in tightly closed glass bottles at 4°C. in a dark room. A number of duplicates was stored in frozen state in darkness and at room temperature exposed to light.

In testing for susceptibility two nutrient broth tubes were used for each culture of the yellow chromogenic bacteria. The same amount of inoculum was added to each tube from an 18-24 hr. culture. One tube received one drop of bacterial virus filtrate, the other was the control. The virus reactions were read after incubation for 18 to 24 hr. In testing for susceptibility in other than the yellow chromogenic bacteria three test tubes were employed for each culture. Two test tubes received equal amounts of inoculum from a young culture of the bacteria to be tested, the third was inoculated with equal amounts

from the virus-homologous culture of yellow chromogenic bacteria. One of the two and the third received one drop of the virus. Thus two controls were used. These were incubated at optimum temperature for growth as indicated in Bergey's Manual (1) and the results were read after the "virus-free" control showed satisfactory growth. Each test for susceptibility was carried out in duplicate.

Degrees of lysis were designated by ⁄⁄⁄ for complete lysis, ⁄⁄ for lysis with slight growth, ⁄ for partial lysis and - for no visible effect of the virus filtrate. Three plus and two plus lysis were grouped together and regarded as positive in the interpretation of results.

RESULTS

1. Virus Reactions.

Wheat seeds were found to harbour bacterial viruses capable of lysing the yellow chromogenic bacteria. From 29 cultures tested bacterial virus isolation was successful 20 times. Most of the virus isolates from single cultures were found to lyse certain other cultures of the yellow chromogenic bacteria but in no case all other cultures from which viruses were obtained.

Each of the 20 viruses was tested against the nine cultures from which virus isolation was not successful in this study. Eleven viruses lysed one to five cultures each, whereas nine viruses did not lyse any of these cultures. The results with the two groups of cultures are shown in Tables I and II.

TABLE I.

Cross-lysis by viruses from cultures from which viruses were obtained.

Bacterial Viruses

Cultures	III	IV	VI	X	XI	XII	XIII
3	+++	-	-	-	-	-	+++
4	-	+++	-	+++	-	-	-
6	-	+++	+++	+++	-	-	-
10	-	+++	-	+++	-	-	-
11	-	+	-	-	+++	-	-
12	-	+++	-	-	-	+++	+++
13	++	-	-	-	-	-	+++
14	-	+++	+++	+++	+++	-	-
16	-	+++	-	+++	-	-	-
17	-	++	-	+++	-	-	-
18	-	+++	-	+++	-	-	-
19	-	+++	-	-	-	-	-
20	-	+++	-	+++	-	-	-
21	-	+++	-	+++	-	-	-
22	-	+++	-	+++	-	-	-
24	-	+++	-	+++	-	-	-
28	-	+++	-	++	-	-	-
30	-	+++	-	+++	-	-	-
31	+++	-	-	-	-	-	+++
32	-	+++	-	+++	-	-	-