INVESTIGATIONS UPON THE ANTIGENIC RELATIONSHIPS AMONG THE ROOT-NODULE BACTERIA OF THE SOYBEAN, COWPEA, AND LUPINE CROSS-INOCULATION GROUPS[1]

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Although several workers have investigated the serological characteristics of the organisms in the genus *Rhizobium*, few have given much consideration to the possible relationships between bacteria isolated from plants of the soybean, cowpea, and lupine groups. Moreover, none of these workers has had the benefit of recent discoveries in immunology, which have made new concepts and new techniques available for studies of the antigenic constitution of bacteria.

The frequency with which recent papers (Leonard, 1923; Sears and Carroll, 1927; Hansen and Tanner, 1931; Carroll, 1934; Walker and Brown, 1935; Raju, 1936; Reid, 1936; Conklin, 1936; Toxopeus, 1936; Bushnell and Sarles, 1937) have reported "inter-crossings" among the bacteria and plants of the soybean, cowpea, and lupine groups, and the consequent confusion attending upon the classification of both plants and bacteria in these groups, has made advisable some study of the antigenic constituents of these bacteria. Such a study must necessarily consider the roles played by flagellar and by capsular substances in determining the serological specificity of a bacterial strain. The agglutination test appeared to lend itself best to a study of this sort.

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METHODS

Antisera were prepared with strains of bacteria obtained from plants in each of the three groups. The strains were so selected that some formed nodules as readily on soybean as on cowpea and lupine plants, while others formed nodules on two, but not on all three, of the host plants. The ability of these strains to infect the different host plants was established by Reid (1936) in a number of consecutive greenhouse experiments made under the wide range of variation in length of day experienced in Madison, Wisconsin. The cross-inoculation relationships of the strains used as antigens are given in table 2.

Preparation of antigens

The organisms were grown on slants of yeast-water mannitol mineral-salts agar medium containing potato extract, which supplies an accessory growth factor (Sarles and Reid, 1935) favoring both the growth and production of capsular polysaccharides of the organisms from soybeans, cowpeas, and lupines. After 5 days at 28°C., the growth was washed from the agar and suspended in 0.5 per cent sodium chloride solution.

From each strain three antigens were prepared:

1. A suspension of the untreated cells was used as the "whole-cell antigen," referred to as the "whole-antigen."
2. A suspension of cells, heated for two hours in flowing steam to inactivate the "flagellar" proteins, provided the "heated antigen."
3. Cells believed to be capsule-less were obtained by treating the suspensions with warm 0.5 per cent saline solution at 65°C., (Hopkins, Peterson, and Fred, 1930) followed by centrifugation. Three successive washings with warm saline solution removed the gum, and the "flagellar" proteins were inactivated by heating the capsule-free cells in flowing steam for two hours. This capsule-less, flagella-less form of antigen is called, for want of a better term, the "washed-heated antigen," or "washed-antigen."

Preparation of immune sera

Each of the antigens, suspended in 0.5 per cent sodium chloride solution, was then injected intraperitoneally at intervals of 5

days into a separate rabbit. The dose increased from 1.0 ml. of the stock suspension at the first injection to 2.0 ml., then 3.0 ml., and finally to 3.5 ml. at the fourth and last injection. One week after the last injection each rabbit was bled from the ear; the blood was placed in the refrigerator for 24 hours; then the serum was removed and stored at 4°C. No preservative was used, except in the sera for Soybean strain 10, Cowpea strain 602, and Lupine strain 16,400 which were preserved with 0.5 per cent phenol.

Sera were prepared against antigenic modifications of 10 different strains of root-nodule bacteria: 4 from soybeans, 3 from cowpeas, and 3 from lupines. In some instances, in order to introduce more strains into the study, only 2 antigenic modifications of the strains were used; (in one other the rabbit being immunized died a few days before it was to have been bled, so that the serum it was to have provided was lost). In all, 25 different antisera were obtained, and are listed below:

WHOLE-CELL	HEATED	WASHED-HEATED
Soy 10	Soy 10	Soy 10
Soy 505	Soy 505	Soy 505
Soy 500	Soy 500	
Soy 507	Soy 507	
Cowpea 601	Cowpea 601	Cowpea 601
Cowpea 602	Cowpea 602	Cowpea 602
	Cowpea 603	
Lupine 804	Lupine 804	Lupine 804
Lupine 810	Lupine 810	
Lupine 16,400	Lupine 16,400	Lupine 16,400

Agglutination tests

These antisera were employed in simple cross-agglutination tests against the three modifications of "whole-cell," "heated," and "washed-heated" antigens prepared in the same manner as were the immunizing antigens from 54 strains of root-nodule bacteria from plants in the soybean, cowpea, and lupine cross-inoculation groups.

Whole-cell antigens prepared from rhizobia from alfalfa, clover, and pea plants were also tested against these antisera.

The test-antigens were suspended in 0.5 per cent saline solution containing 0.5 ml. of a 40 per cent solution of formaldehyde per 100 ml. of antigen. The dilutions of serum were also prepared with a similar salt-formalin solution in order to prevent growth of organisms in the lower dilutions during the period of incubation of the tests. The sera were used in dilutions of 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600, 1:2000, 1:3200, 1:4000, and 1:6400; sometimes other dilutions were interpolated in the lower ranges or added at the upper limits. The tubes were allowed to incubate 18 to 20 hours at 37°C. before the titers were recorded. Each test was performed at least twice.

RESULTS AND COMMENT

The data presented in the tables are those which provide the most information with the least amount of detail; the rest of the data obtained, while bearing out the general conclusions drawn from the cited figures, are not included because to present them would provide too cumbersome and too confusing a compilation.

The most striking feature of the results given in table 1 is the large number of "serological types" found among the strains studied. These results confirm those of Stevens (1923) who concluded that the agglutination test can not be used to identify all strains of a single species of the root-nodule bacteria. The antigenic constitution of different strains within a species is apparently dissimilar; hence an antiserum for one strain of, for example, *Rhizobium japonicum*, will not agglutinate all strains of that species. The many minus signs in this table, each marking an instance where there was no clumping of the test antigen by the serum employed, offer testimony to this limitation of the agglutination reaction.

The success of Lancefield (1933) and of Plummer (1935) in their serological classification of the streptococci by means of the precipitation reaction suggests that perhaps that method is better suited than is the agglutination reaction for distinguishing the species antigen as well as the more variable type-antigens

TABLE 1

Serological types: highest dilution at which clumping of whole-cell test-antigens was obtained with antisera prepared against whole-cells

WHOLE-CELL TEST ANTIGENS	ANTISERA PREPARED AGAINST WHOLE-CELL ANTIGENS OF:								
	Soy 505	Soy 10	Soy 500	Soy 507	Cowpea 601	Cowpea 602	Lupine 804	Lupine 810	Lupine 16,400
Rhizobium japonicum:									
Soy 8			1,600				400	400	
Soy 10	800	6,400	2,000	200	400	3,200	400	200	200
Soy 500	800		6,400						
Soy 505	6,400							800	
Soy 507				4,000			2,000		
Soy 508	400			400	100		4,000		
Soy 510	400	200	3,200				1,600		
Soy 516							400		
Rhizobium for cowpea:									
Cowpea 600					4,000				
Cowpea 601	2,000		100		4,000		100	2,000	2,000
Cowpea 602	800				100	100	400		
Cowpea 603	50		800						
Cowpea 605	6,400		100	100		200	100	400	200
Cowpea 606	6,400			200	4,000	400	4,000	200	
Cowpea 610							100		
Cowpea 617	800	800	800	400	400	4,000	3,200		
Rhizobium lupini:									
Lupine 801				100			800		
Lupine 804						100	1,600		
Lupine 807		200	400		100		100		100
Lupine 810	800		800				100	4,000	200
Lupine 812			100						
Lupine 16,400							200		12,800
Rhizobium meliloti									
Rhizobium trifolii									
Rhizobium leguminosarum									

In tables 1 and 2, blank spaces indicate negative results.

in the bacterial complex. This approach to the problem must yet be tried.

Just as obvious as are the serological types within a species,

TABLE 2

Relation between cross-inoculation and cross-agglutination

STRAIN	CROSS-INOCULATION; NODULES FORMED ON:			CROSS-AGGLUTINATION: HIGHEST DILUTION AT WHICH CLUMPING OF WHOLE-CELL ANTIGENS WAS OBTAINED WITH ANTISERA PREPARED WITH WHOLE CELLS:									
				Sera for Soybean Strains				Sera for Cowpea Strains			Sera for Lupine Strains		
	Soybeans	Cowpeas	Lupines	500	505	507	10	601	602	603*	804	810	16,400
Rhizobium japonicum:													
Soy 500	+	+	−	6,400	800					2,000			
Soy 505	+	+	−		6,400							800	
Soy 507	+	+	−			4,000					2,000		
Soy 10	+	+	−	2,000	800	200	6,400	400	3,200	100	400	4,000	200
Rhizobium for cowpea:													
Cowpea 601	+	+	−	100	2,000			400		3,200	100		2,000
Cowpea 602	−	+	+		800			100	12,800	100	400		
Cowpea 603	+	+	+	800	200					6,400			
Rhizobium lupini:													
Lupine 804	+	+	+		100				100		2,000		
Lupine 810	−	+	+	800	800						100	4,000	200
Lupine 16,400	−	−	+								200		12,800
Rhizobium meliloti	−	−	−										
Rhizobium trifolii	−	−	−										
Rhizobium leguminosarum:	−	−	−										

* Antiserum prepared with heated cells.

however, are the many instances in which antisera prepared against a strain of rhizobia from one cross-inoculation group caused agglutination of bacteria from the other two cross-inoculation groups as well. The serum of Lupine strain 804 is distinctive for its ability to agglutinate almost all strains of soybean bacteria that were tested, and the majority of the strains from plants in the cowpea group. The fact that the sera for Lupine strain 810, Cowpea strains 601 and 602 and Soybean strains 505 and 507 possess to some degree this same ability to agglutinate strains of other species makes it likely that this faculty is not a characteristic peculiar to Lupine strain 804, but that it is shared to a greater or lesser degree by many of the strains of root-nodule bacteria from these three cross-inoculation groups. It is noteworthy, however, that there is less cross-agglutination, just as there is less cross-inoculation (as shown in table 2) between strains from the soybean and lupine groups than there is between strains from either of these two groups with those from the cowpea group.

There seems to be no correlation between the ability of the strains to cross-inoculate and to cross-agglutinate. For instance, soybean strain 505, which forms nodules on both soybean and cowpea plants, but not on lupine plants, is not agglutinated by any of the antisera for cowpea bacteria, but is agglutinated to a fair titer by the serum for one strain of *Rhizobium lupini*. Soybean strain 500, which forms nodules on both soybean and cowpea plants, is agglutinated by sera from some strains of the soybean *Rhizobium*, but the serum from only one of the strains of the *Rhizobium* for cowpea will cause its agglutination. Another instance of this sort is that of Cowpea strain 603, which, although it forms nodules on plants in all three cross-inoculation groups, reacted with no other sera of the cowpea group, and only fairly well with the sera from two strains of soybean rhizobia.

On the other hand, strains which form nodules on plants in at least two of the three cross-inoculation groups, were agglutinated by sera for strains of bacteria derived from all three of the groups. Soybean strain 10, and Cowpea strains 601 and 602 are examples of this sort. Apparently the serological nature of the strains of

soybean, cowpea and lupine nodule bacteria tested is not related to their ability to cross-inoculate.

The results of the many agglutination tests in which various antigenic modifications of 54 strains of soybean, cowpea and lupine rhizobia were tested against the 25 different antisera are not presented. There are three reasons for omitting a detailed presentation of these data. In the first place, there is insufficient space for such a mass of tabulated information; secondly, whole-cell antigens used in agglutination tests with antisera prepared with whole cells gave in general the same reactions as did the various antigenic modifications; and finally, the titers secured with whole-cell antigens and whole-cell antisera were as a rule higher than those secured with the other antigenic modifications and their antisera.

However, these tests showed that strains belonging to the same serological type possessed similar antigenic constituents. This is illustrated in the following selected data, which show the highest titer in which agglutination occurred when the various antigenic modifications of certain strains were tested with antisera which would cause their agglutination.

ANTIGENS	ANTISERA EMPLOYED IN TESTS	WHOLE	HEATED	WASHED-HEATED
Cowpea 606 whole	Cowpea 601	6400	2000	800
Cowpea 606 heated	Cowpea 601	2000	800	200
Cowpea 606 washed-heated	Cowpea 601	800	400	200
Soy 505 whole	Soy 505	6400	400	400
Soy 505 heated	Soy 505	1600	400	400
Soy 505 washed-heated	Soy 505	400	800	200
Lupine 807 whole	Soy 505	1600	200	50
Lupine 807 heated	Soy 505	400	100	100
Lupine 807 washed-heated	Soy 505	100	100	100
Soy 508 whole	Lupine 804	6400	1600	400
Cowpea 605 whole	Soy 505	6400	1600	200
Soy 507 whole	Lupine 804	2000	400	100

This diminution of activity, both in agglutinability and in ability to stimulate antibody formation in the animal, strongly suggests the existence of three components in the antigenic complex of these root-nodule bacteria. In the cells that are relatively free from flagella and capsules is an antigen which stimulates the production of agglutinins. Its ability both to stimulate the production of these antibodies and to react with them is very limited, indicating that this antigenic fraction may be present in only very small amounts, or that the treatment the washed-heated antigens received may have denatured it to a considerable extent. The second antigenic substance is apparently associated with the flagella, since depriving the cells of their flagellar proteins by exposing them to heat lowers their ability to induce formation of agglutinins and to react with these antibodies to the degree that is possessed by untreated cells. Finally, there is a fraction, possessed by untreated cells, which may be associated with the polysaccharide capsular gum of the root-nodule bacteria.

It is disconcerting to note, however, that these antigenic constituents, even those of the washed-heated cells, are not found in all strains of a species; nor are they found in all strains that will cross-inoculate in a similar manner.

It is significant that there was no agglutination of the whole-cell test-antigens prepared from mixed strain cultures of alfalfa, clover, and pea rhizobia by the different antisera used in these tests. Apparently these rhizobia differ in their antigenic constitution from any of the soybean, cowpea or lupine strains that were used in these tests.

CONCLUSIONS

1. A great number of serological types exist among the root-nodule bacteria from plants of the soybean, cowpea, and lupine cross-inoculation groups.

2. The strains within a serological type possess antigenic constituents in common. These antigens are found in whole cells, heated cells and in washed-heated cells.

3. There seems to be no correlation between the ability of

strains of rhizobia from soybeans, cowpeas and lupines to cross-inoculate and to cross-agglutinate. One serological type may be made up of *Rhizobium* strains isolated from only one species of plant, but in other cases, of strains isolated from two or even three species of plants.

4. Antisera for the different antigenic modifications of soybean, cowpea and lupine bacteria failed to agglutinate whole-cell antigens of alfalfa, clover or pea bacteria.

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