Immunodiffusion Analysis of Isolates of
Xanthomonas cyamopsidis

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Immunodiffusion analysis of intact, sonically treated, and sonically treated and
heat-treated cell antigen preparations of isolates of races 0 and 1 of Xanthomonas
cyamopsidis of guar, Cyamopsis tetragonoloba, indicated that these races differ from
one another antigenically. The differentiating precipitin reactions are believed to
have been brought about by specific heat-stable surface antigens, possibly similar
to lipopolysaccharide somatic antigens of other slime-producing xanthomonads.
Because differences in host reaction to inoculation with the two isolates of race 1
were known to be minor, these isolates are considered to represent serotypes of
the race rather than distinct separate races. This conclusion is supported by the
antigenic behavior of isolates 14 and 19 to antiserum 19. The applicability of im-
munodiffusion analysis for the study of pathogenic variability in X. cyamopsidis is
demonstrated.

A new virulent race of Xanthomonas cyamopsidis Patel, Dhande, and Kulkarni, the cause of
bacterial blight of guar, Cyamopsis tetragonoloba (L.). Taub., was distinguishable by reaction of
differential guar cultivars inoculated with isolates which represented prevailing bacterial
populations in the Texas-Oklahoma area (5–8). Although host reaction is considered to be a
reliable criterion for characterization of new pathogenic races, it is not known if serological
diagnostic procedures can be applied in the characterization of races and serotypes of X. cyamopsidis. Serological procedures have been applied, however, to tomato and pepper isolates of X. vesicatoria (4), X. campestris, X. flavcum-
faciens, X. phaseoli, and X. phaseoli sojense (3). With the latter four species, a correlation was
found between host specificity and serological specificity.

This investigation was undertaken to test immunodiffusion analysis (IDA) on X. cyamopsidis
to determine whether isolates of races 0 and 1 of this pathogen can be distinguished, in absence of
their host, by their antigenic behavior, and to test IDA as a method of diagnosis of serotypes
which may escape recognition by direct host inoculation.

**MATERIALS AND METHODS**

Isolates. X. cyamopsidis isolates used in this investigation were isolate 1, representing the bacterial
population of race 0, and isolates 14 and 19, two highly virulent isolates representing race 1 (5). To prevent
these isolates from losing their virulence from repeated transfer to synthetic media, the isolates were grown on
nutrient agar only at 9- to 10-month intervals. Bacterial colonies were then suspended in sterile water in
screw-capped glass test tubes and stored at 5 C. Test inoculations of guar plants indicated no discernible
loss in virulence. Isolates 14 and 19 are on deposit at the American Type Culture Collection, Rockville,
Md., under accession numbers 25398 and 25399, respectively.

Antisera. New Zealand inbred male albino rabbits, each approximately 8 lb in weight, were immunized
by intravenous ear injection with live cell suspensions containing approximately 108 cell/ml (2) and diluted
1:1 in neutral sterile physiological saline (0.85% NaCl, w/v). The immunization schedule was as follows: 1 and 2 ml of cell suspension were administered at a 3-day interval during the first week, and 1 ml was
administered three times at a 7-day interval thereafter. The rabbits were bled 4 weeks after the last immuniza-
tion, and the blood serum was separated by centrifugation and stored with phenol at 5 C. The titer of the
antisera was determined by cross-agglutination against cell suspensions of isolates 1, 14, and 19 diluted in
neutral physiological saline in test tubes and incubated in a water bath at about 50 C for as long as 2.5 hr.

Immunodiffusion analysis. Aqueous suspensions of the three isolates of X. cyamopsidis were centrifuged
at 6,000 X g for 10 min, the supernatant water was discarded, and the cells were resuspended in neutral
saline. Cell suspensions used as test antigen preparations were submitted to one of the following pro-
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cedures: (i) cell suspensions were disrupted by three 30-sec cycles by means of a Fisher's Ultrasonicator (model BP-2) equipped with a 200-w, 60-cycle generator; (ii) cell suspensions were sonically treated and immediately heated in a water bath at 100 C for 5 min; or (iii) cell suspensions were left intact.

IDA of antigen-antiserum systems were made in petri plates containing 0.85% "Colab" Ionagar No. 2 to which was added sodium azide to prevent contamination. Tests were run with undiluted antisera and with antisera diluted to equivalent titer. Duplicate agar plates with three arrays of wells in either triangular or hexagonal pattern for cell suspensions and with a central well for a given antiserum were incubated at about 30 C. Plates were examined for precipitin arcs periodically up to 120 hr and then photographed. To determine whether cell antigens responsible for precipitation of antibodies in agar were identical to those responsible for cell agglutination in the titer test, absorption of the antiserum by intact unheated cell suspensions was performed in agar plates as for IDA.

RESULTS

Antiserum homologous for isolate 1 of race 0 agglutinated intact cells at dilutions as high as 1:5,120. Antisera homologous for isolates 14 and 19 of race 1 agglutinated intact cells at dilutions as high as 1:1,280 and 1:320, respectively, after 2.5 hr of incubation at about 50 C.

Antigens of intact cells of Xanthomonas isolates 1, 14, and 19 gave rise to single, well defined, and strong precipitin arcs only when their antigens interacted with antibodies of homologous antiserum, except in the homologous systems of isolates 14 and 19 and antiserum 19. Weak and poorly defined precipitates formed, however, in all other systems of intact cell preparations of the three isolates. IDA ran with antiserum diluted to equivalent titer did not change these results.

Single and strong precipitin arcs possibly identical to those which developed in homologous systems of intact cells also developed in homologous systems of sonically treated cells. Whereas a fairly well defined single precipitate developed in the homologous system of sonically treated cells of isolate 19 and antiserum 19, no antibody precipitation occurred in the system of isolate 14 and this antiserum, which suggests that these two isolates of race 1 differed antigenically. As in systems with intact cell preparations, weak and poorly defined precipitates also developed, but their patterns were not always consistent. Precipitates likewise developed in heterologous systems of sonically treated cells of isolates 14 and 19 and antiserum 1.

The antigenic behavior of sonically treated and heat-treated cell preparations indicated that each of the strong and well defined precipitin arcs that characterized the three isolates in homologous systems (Fig. 1) were heat-stable. The precipitates that developed in heterologous systems of isolates 14 and 19 to antiserum 1 as well as the weak and indefinite precipitates were, however,
heat-labile and apparently uncharacteristic of the isolates tested. The occurrence of two very faint parallel precipitates in the systems of sonically treated and heat-treated cell preparations of isolate 14 and its apparent homologous antiserum 19 was surprising.

In absorption tests of the antisera by intact, unheated cell preparations of the three Xanthomonas isolates, antibody precipitation occurred in all systems with one exception. Antiserum 1 was not precipitated by antigens of either isolate 14 or 19 as indicated by the strong precipitates that formed in homologous interactions with isolate 1.

**DISCUSSION**

Results of immunodiffusion analysis of relatively crude preparations of isolates of X. cyamopsidis representing race 0 and 1 demonstrated that these isolates differed from one another antigenically and provided additional support to our earlier characterization of these races made on the basis of blight reactions of differentiating cultivars of the host plant.

The strong precipitates that separated isolates of the two races in homologous systems were brought about, apparently, by specific, heat-stable, surface antigens similar to somatic antigens of other slime-producing xanthomonads. Absorption of the antiserum by intact, unheated cell preparations of these isolates further suggested that the differentiating surface antigens probably were identical to lipopolysaccharide somatic antigens that caused cell agglutination.

Because, as noted previously (5), differences in blight reaction of guar cultivars inoculated with isolates 14 and 19 of race 1 were of relative minor order, these isolates are considered to represent serotypes of this highly virulent race rather than distinct, separate races. This conclusion is supported by the antigenic behavior of cell preparations of isolates 14 and 19 to antiserum 19. The unique occurrence of the two parallel precipitin arcs in the system of sonically treated and heat-treated cell preparations may also be indicative of antigenic differences between the two isolates.

Under the conditions of almost exclusive cultivation of field-resistant guar in the Texas-Oklahoma area in recent years, it is reasonable to assume that the pathogen has been and is being subjected to change by selection exerted by the host or by mutation, or by both, with both acting as mechanisms in bacterial evolution (1). In light of these results and the applicability of IDA for the study of pathogenic variability of X. cyamopsidis, it is suggested that races and serotypes that exist or may arise in nature can be detected and characterized, even in the absence of host plants, by their antigenic behavior. Further studies of isolates that represent recognizable disease outbreaks, of the nature of antigens involved, and of relationships that may exist between specific surface antigens and virulence may contribute to an understanding of the epidemiology of this and other plant pathogenic bacteria.

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