Importance of Ribosome Purity in Ribosomal Serology

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ABSTRACT

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Antisera made from unwashed preparations of 50 S ribosomal subunits of *Agrobacterium* gave reproducible immuno-precipitation patterns when reacted with ammonium sulfate-washed ribosomes, but the patterns were not always reproducible when reacted with unwashed ribosomes. We suspected that this lack of reproducibility was due to a nonribosomal antigen associated with unwashed ribosomes. The association of this antigenic contaminant with the unwashed ribosomes was demonstrated when antiserum to heat-stable antigens of whole cells and antiserum to unwashed ribosomes reacted with both heat-stable antigens of whole cells and unwashed ribosomes to produce confluent precipitin bands. This contaminant was also associated with unwashed 50 S subunits. The contaminant was removed by ammonium sulfate fractionation and the subsequent sedimentation of the ribosomes in the presence of 0.6 M ammonium sulfate. The contaminant was not associated with either ammonium sulfate-washed ribosomes nor the proteins extracted from the highly purified 50 S subunits. Therefore, nonspecific binding of a somatic antigen to unwashed ribosomal particles appears to offer the most probable explanation for the additional antigenic response.

Species specific antisera to ribosomes have been used to demonstrate the potential utility of ribosomal serology to examine relationships between species (12). Schaad (8) was the first to apply ribosomal serology to phytopathogenic bacteria, and antisera to ribosomes extracted by his method were specific at the subspecies level. Surprisingly, these antisera cross-reacted with whole bacterial cells (9,10). When antisera to 50 S ribosomal subunits of *Agrobacterium* that were prepared following Schaad's method were tested against ribosomes of *Agrobacterium* and *Rhizobium*, 15 serological groups were tentatively identified (1). Subsequent ribosome samples from the same strains produced different precipitin reactions (2). In contrast, no serological differences could be detected when purified ribosome preparations were tested against these antisera. This lack of reproducibility in the reaction of ribosomes of *Agrobacterium* and *Rhizobium* and the cross-reaction between Schaad's ribosomal antisera and whole cells lead us to investigate if Schaad's ribosomal extraction method yielded ribosomes free of nonribosomal cellular contaminants. The serological comparisons of such ribosomes with rigorously purified ribosomes, proteins from highly purified 50 S ribosomal subunits, and somatic antigens indicated that the contaminant detected in Schaad's preparation was present in somatic antigen samples but not in pure ribosomes or proteins from highly purified 50 S subunits.

MATERIALS AND METHODS

Antigen preparation. Strain C58 of *Agrobacterium* was grown on 523 liquid medium (5) at 27 C on an orbital shaker. Cells in exponential growth-phase were harvested by centrifugation at 12,000 *g* for 15 min. The cell pellets were washed in 0.85% NaCl and recentrifuged. These cells were the source of five different antigenic preparations described below. Heat-stable antigens of whole cells (somatic antigens) were prepared from cells resuspended in normal saline and left 1 hr in a boiling water bath. Ribosomes were extracted in 0.5 M ammonium chloride following Schaad's method (8), which does not employ an ammonium sulfate fractionation procedure. In short, cell lysates were centrifuged at high-speed (180,000 *g*) for 3 hr; the ribosome pellets were resuspended and clarified by centrifugation (12,000 *g* for 15 min). Ribosomes were also extracted following a modification (2) of Kurland's procedure (6). Those extracted by Kurland's procedure were called washed ribosomes, whereas those prepared by Schaad's procedure were called unwashed ribosomes. The concentration of ribosomes was adjusted to 3 mg/ml, based on a specific extinction coefficient of 157 $(mg/ml)^{-1}$ cm^{-1} at 260 nm (11). The purity of the ribosomes was determined from the $A_{260/235nm}$ ratio as described by Schaad (8). Washed and unwashed 50 S ribosomal subunits were obtained from the washed and unwashed ribosome preparations by zonal sucrose density-gradient centrifugation (9). Proteins from washed 50 S ribosomal subunits were extracted in 66% glacial acetic acid (4). Protein concentrations were determined by the microassay procedure of Bradford (3) and adjusted to 3 mg/ml.

Antiserum production. New Zealand White rabbits were immunized with unwashed 50 S subunits, unwashed ribosomes, and somatic antigens from *Agrobacterium* strain C58. Preimmune sera were collected before immunization. Rabbits were given weekly injections and bled from the marginal ear vein 10 and 14 days after completion of the immunization schedule. Antiserum to unwashed 50 S subunits was prepared as described (2). Antiserum to unwashed ribosomes was obtained following two initial intravenous (IV) injections each containing 2 mg of unwashed ribosomes and three subsequent intramuscular (IM) injections, each containing 4 mg of unwashed ribosomes emulsified with incomplete Freund's adjuvant (IFA) (Difco, Detroit, MI). Antiserum to somatic antigens was developed from two IV injections, containing about 10^6 and 10^7 heat-treated bacterial cells, followed by two IM injections, each containing 10^8 heat-treated cells emulsified in IFA.

Immunodiffusion in gels. The serological analysis was performed using Ouchterlony double-immunodiffusion (7). The reactions of somatic antigens, unwashed ribosomes, washed ribosomes, and proteins of washed 50 S subunits were compared by running these four antigenic preparations in adjacent wells, so that all possible paired comparisons could be made, against antisera developed from somatic antigens, unwashed ribosomes, and unwashed 50 S subunits.

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RESULTS AND DISCUSSION

The presence of a contaminating molecule in the unwashed ribosome preparation was suspected when the $A_{260/235nm}$ ratio of unwashed ribosomes was compared with that of washed ribosomes. Unwashed ribosomes had a low ratio of about 1.6, whereas washed ribosomes had a reproducible ratio of 1.8.

The presence of a contaminating antigen on unwashed ribosomes was confirmed serologically by immunodiffusion tests. Confluent precipitin bands developed only between somatic antigens and unwashed ribosomes when reacted with antiserum to somatic antigens. Washed ribosomes and proteins isolated from washed 50 S ribosomal subunits did not react. The precipitin band common to unwashed ribosomes and somatic antigens formed near the antigen well and fused, indicating the presence of the same antigenic determinant in both preparations (Fig. 1A). Similarly, a precipitin band common to only somatic antigens and unwashed ribosomes was observed when these two antigenic preparations were tested with antiserum to unwashed ribosomes (Fig. 1B). In addition to this somatic band, antiserum to unwashed ribosomes reacted with washed ribosomes, proteins of washed 50 S subunits and unwashed ribosomes to produce the precipitin band nearer the antiserum well. This latter band was of ribosomal origin because it was shared only by the three ribosomal preparations and not the somatic antigens. The somatic precipitin band was not associated with washed ribosomes, suggesting that the somatic antigen, present in unwashed ribosomal preparations, was either a molecule that binds nonspecifically to ribosomes or a ribosomal protein that was released as a consequence of particle degradation in the unprocessed ribosomal samples. In the washed ribosomal preparations, degradation would be less likely and the antigenic site would essentially be masked. However, the somatic band was not associated with proteins extracted from washed 50 S ribosomal subunits, indicating that the somatic antigen was not an internal ribosomal protein but probably a somatic antigen that binds nonspecifically to unwashed ribosomes. This nonribosomal contaminant was released and precipitated by 20% ammonium sulfate or washed off the particles during sedimentation of ribosomes in the presence of ammonium sulfate.

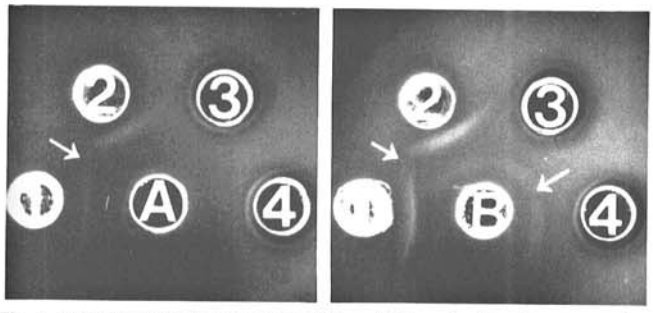

Fig. 1. Immunodiffusion patterns of four different antigenic preparations against two different antisera to *Agrobacterium* strain C58. The center well of **A** contains antiserum to somatic antigens, whereas the center well of **B** contains antiserum to unwashed ribosomes. Outer wells of A and B contain: somatic antigens (1), unwashed ribosomes (2), washed ribosomes (3), and proteins of washed 50 S ribosomal subunits (4). The precipitin bands are indicated by arrows.

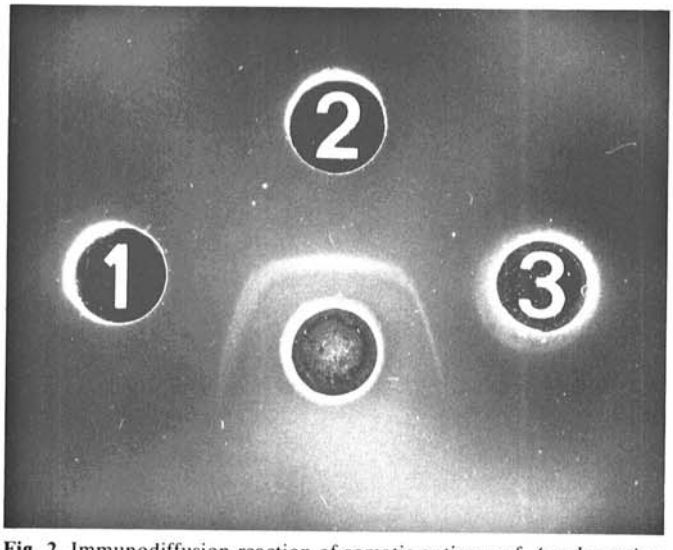

Fig. 2. Immunodiffusion reaction of somatic antigens of *Agrobacterium* strain C58 (center well) with antiserum to somatic antigens (1), antiserum to unwashed ribosomes (2), and antiserum to unwashed 50S ribosomal subunits (3).

Similarly, the presence of a nonribosomal contaminant in the unwashed 50 S subunit preparations that were used as immunogens in this study was deduced from the reaction between antiserum to unwashed 50 S subunits and somatic antigens. Ideally, the four different antigenic preparations should have been tested in the same gel against antiserum to unwashed 50 S subunits. However, this test was not workable because the weak reaction of somatic antigens was obscured by the strong reaction of ribosomal antigens with this antiserum (i.e., high ribosomal concentrations reacting with antibodies to ribosomes). To overcome this problem, unwashed 50 S subunit antiserum was compared with somatic antigen antiserum and unwashed ribosome antiserum. All three of these antisera reacted with somatic antigens (Fig. 2), illustrating that these antisera shared antibodies that recognized the same antigen. These results and results shown in Figure 1A clearly indicate that a nonribosomal contaminant was present in both the unwashed 50 S subunits used for immunization and the unwashed ribosomes.

In retrospect, the literature on ribosomal serology that reports cross-reactions between unwashed ribosomes and whole cell preparations (9,10) corroborates the presence of somatic contamination in ribosomes extracted by Schaad's method. Although serogrouping of ribosomes from *Xanthomonas* strains (9,10) may be valid, nonribosomal molecules may also have contributed to the differential precipitin patterns. The presence of a contaminant in both unwashed ribosomal extracts and unwashed 50 S subunits immunogens resulted in multiple and dissimilar precipitin patterns when antisera to unwashed 50 S subunits from *Agrobacterium* were reacted with unwashed ribosomes (2). This serological heterogeneity masked the true ribosomal relationships of these strains, because it most likely involved the reaction of antibodies to somatic antigens with nonribbsomal antigens that contaminated the unwashed ribosome preparations. The greatest hazard of previous procedures was the nonspecific binding of the antigenic contaminant. Because this contaminant could be removed by washing, it was not detected in every extraction of unwashed ribosomes and our results were not always reproducible. In contrast, when purified ribosomes were used as test-antigens the test was reproducible and a single homogenous serological group comprising both *Agrobacterium* and *Rhizobium* was revealed (2).

Our data indicate that Kurland's procedure (6) of ammonium sulfate fractionation is superior to the more commonly used method of Schaad (8) for ribosome purification because it minimizes contamination of these particles with antigens of somatic origin. This emphasizes the importance of using ribosome immunogens purified by Kurland's method to develop ribosomal antisera for use in comparative serology.

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