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Two new methods, one of broad specificity and the other of narrow specificity, were developed for the identification of agrobacteria. The former method was based on ribosomal antigens that were common to members of the closely related Agrobacterium and Rhizobium genera. Antisera to unwashed 50 S ribosomal subunits from five Agrobacterium strains were tested in gel immunodiffusion against ammonium sulfate-washed ribosomes from 34 Agrobacterium, one Rhizobium meliloti and three Rhizobium leguminosarum strains, and nine other genera outside the Rhizobiaceae. Only the agrobacteria and rhizobia reacted to form a single band of precipitation common to each of the strains tested. However, when the same antisera were reacted against unwashed ribosome preparations from the same strains, heterogeneous precipitation patterns with multiple bands were produced. The heterogeneous patterns were due to heat-stable cell surface antigens contaminating the unwashed ribosomes. We hypothesized that the

heterogeneous patterns represented antigenic differences among strains. Subsequently, antisera to unwashed ribosomes from six different Agrobacterium strains were tested against water-phenol extracts from 39 Agrobacterium strains; a precipitin band formed only with the homologous Agrobacterium strain. The strain-specific serological reaction was due to lipopolysaccharide antigens present as contaminants in the ribosomal preparation. The serological uniqueness of each strain was confirmed by EcoRI digestion profiles of total DNA. The profiles were different for each of the Agrobacterium strains. The utility of the broadly specific method was demonstrated when antisera to 50 S ribosomal subunits were used successfully to identify putative agrobacteria isolated from a natural habitat. In contrast, the strain specific method allowed the identification of Agrobacterium radiobacter K84 strains recovered six months after they were introduced into the environment.

SEROLOGICAL IDENTIFICATION OF COMMON AND STRAIN-SPECIFIC ANTIGENS IN AGROBACTERIUM

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SEROLOGICAL IDENTIFICATION OF COMMON AND STRAIN-SPECIFIC ANTIGENS IN *AGROBACTERIUM*

INTRODUCTION

Taxonomic tools available for the identification of members of the genus *Agrobacterium* are based on cultural, morphological, physiological, and pathogenicity tests. These tests are often laborious and time-consuming, and in some instances unstable (i.e., plasmid-borne pathogenicity genes) and unreliable for characterization of unknown *Agrobacterium* isolates. These problems have impeded in-depth studies of the ecology of *Agrobacterium*.

For the identification of human pathogens, rapid diagnostic methods often rely on the use of antisera. The successful utilization of antisera in clinical bacteriology led us to explore serology as a method to precisely and rapidly identify agrobacteria. Recently, ribosomes were used as antigens to develop antisera for the identification of phytopathogenic erwiniae and xanthomonads (55). Ribosomal antisera were used because ribosomes are simpler antigenically than whole cells and contain both highly conserved and moderately variable proteins (23); thus, ribosomes have the potential to induce the production of antibodies with a specificity broad enough to identify very closely related

bacteria. The first objective of this research was to investigate the specificity of antisera to 50 S ribosomal subunits of Agrobacterium and their potential to identify uncharacterized Agrobacterium isolates.

Antisera with such a broad specificity would be of limited use in ecological studies involving a particular Agrobacterium strain because of the ubiquity of the agrobacteria. To study the fate of a specific bacterial strain introduced into the environment, scientists have relied upon the use of antibiotic-resistant mutants (46) that can be recovered by plating on a medium amended with the corresponding antibiotic. However, such mutants may be affected in other genes that are important for the survival of the bacterium and its overall competitive abilities; for example, antibiotic-resistant mutants have been reported to be impaired genetically (11, 39). Because these mutants may no longer be representative of their parental strains in ecological studies, another objective of this research was to investigate alternative methods for identification that are based on natural markers present in the wild-type bacterium. These methods would be useful for monitoring a specific strain of Agrobacterium in nature.

CHAPTER I

SEROLOGICAL RELATIONSHIP BETWEEN 50 S RIBOSOMAL SUBUNITS FROM STRAINS OF AGROBACTERIUM AND RHIZOBIUM

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SUMMARY

Antisera to 50 S ribosomal subunits of five strains of Agrobacterium were developed and tested for specificity against ribosomes from various bacterial species in immunodiffusion gels. Two methods of ribosome extraction were compared. When unwashed ribosomes from 34 Agrobacterium and four Rhizobium strains were tested against the five antisera, heterogeneous precipitation patterns with multiple bands were produced. In contrast, when washed ribosomes from these strains were tested against the five antisera, a single precipitin band developed that was common to all 38 strains, showing that 50 S ribosomal subunits of Agrobacterium and Rhizobium are serologically identical. The five antisera did

not react with species outside the Rhizobiaceae and were therefore specific to the Agrobacterium-Rhizobium group. Any of these antisera could be used in diagnostic tests to determine if an unknown isolate belonged to the Agrobacterium-Rhizobium group.

INTRODUCTION

Comparative serology provides valuable information about relationships between prokaryotes (57) and has been helpful for rapid identification of various phytopathogenic bacteria (55). Unfortunately, in the case of Agrobacterium most of the serological studies are contradictory. In some reports serological specificity was observed at the species level (36, 40, 59), whereas in others it was at the subspecies level (3, 20, 44). Aside from an ambiguous taxonomy, this discrepancy probably arises from the use of antisera developed against a mosaic of different antigens (i.e., whole cells) and the presence of plasmids in Agrobacterium spp. that code for additional antigens (2, 22, 30, 60, 61).

Stable characters are essential for a practical classification that reflects true similarities among bacteria. Ribosomes appear to be an ideal choice to reveal serological relationships among the agrobacteria because these particles are 1) present in all cellular organisms, 2) simpler antigenically than whole cells, and 3) contain both highly conserved and moderately variable proteins (23). The potential utility of ribosomal serology was demonstrated initially by the development of specific ribosomal antisera (69),and subsequently this method was applied to some bacterial plant pathogens (52, 53, 62) but not Agrobacterium.

The objectives of the present research were to determine the serological specificity of ribosomes of Agrobacterium and investigate the serological relationships between the agrobacteria and the closely related rhizobia. We have found that antisera to 50 S subunits of each of five different Agrobacterium strains gave reactions of identity with purified ribosomes from Agrobacterium and Rhizobium in Ouchterlony double diffusion tests. Furthermore, ribosomes from Pseudomonas solanacearum, P. syringae, Xanthomonas campestris, Erwinia carotovora, Escherichia coli, Salmonella typhimurium, Bacillus subtilis and Clavibacter michiganense failed to react with these antisera.

MATERIALS AND METHODS

Organisms and cultivation: To provide genetic diversity, 34 strains of Agrobacterium representing different species and biovars were isolated from different hosts grown in widely diverse geographical regions (Table I.1) In addition, 13 strains of closely or distantly related bacterial species were included for comparative purposes (Table I.2). The bacteria were maintained on potato-dextrose-agar (Difco, Detroit, Michigan) supplemented with 5% (w/v) calcium carbonate and cultured in 2.8-L Fernbach flasks containing 1.5 L of 523 liquid medium (34) at 27°C on an orbital shaker. Because the yield of cells cultured in 523 was very low for Agrobacterium strains U11, K47, CG64 and 6/6, these strains had to be grown in YGP liquid medium (Yeast-extract, 0.4%; glucose, 2.0%; peptone, 0.4%; and ammonium sulfate, 0.5%). Rhizobium strains were grown in yeast-mannitol liquid medium (66). Cells in exponential-growth phase were harvested by low-speed centrifugation (10,000 x g for 15 min). The cell-pellets were washed in 0.85% sodium chloride, recentrifuged and stored at -20°C.

Production of antisera: Five female New Zealand White rabbits were immunized with 50 S ribosomal subunits of Agrobacterium strains B6, C58, M63/79, U11 and CG64. These 50 S subunits were prepared by sucrose-gradient centrifugation of ammonium chloride-washed and dissociated 70 S ribosomes as described (53). Before beginning the immunization, pre-immune sera were collected from marginal ear

veins of each animal. Immunization consisted of intramuscular injections of emulsions prepared from equal volumes of 50 S subunits and incomplete Freund's adjuvant (Difco). Injections of 1.5, 2.5, 3.5, and 4.5 mg of 50 S subunits were administered sequentially at 10-day intervals; concentrations were determined as described (52). The five different antisera were harvested by ear-bleeding 10 and 14 days after the last injection. Antisera from the two bleedings were not combined, even though their serological activity was the same. For comparative purposes an antiserum to 70 S ribosomes of E. coli, pooled from six rabbits and prepared by Antibodies Inc. (Davis, California), was provided by Dr. H.W. Schaup, Department of Biochemistry and Biophysics, Oregon State University.

Preparation of test-antigens: In contrast to the complex procedure required to prepare 50 S ribosomal subunits (used as immunogens for injects), a faster isolation method was needed for testing ribosomal antigens from multiple bacterial strains against the 50 S subunit antisera. Two methods were compared: the differential centrifugation method developed by Schaad to obtain unwashed ribosomes (52) and a modification of Kurland's procedure to prepare salt-washed ribosomes (38). In the latter procedure the cell lysate was centrifuged and ammonium sulfate was added to the supernatant to a concentration of 20% to precipitate nonribosomal proteins. The precipitate was removed by a low-speed centrifugation and the ammonium sulfate concentration of the

supernatant was raised to 40%. After another low-speed centrifugation, the ribosome pellet was resuspended in TSM buffer (10 mM Tris base, 3 mM succinic acid, 10 mM $MgCl_2$, 6 mM 2-mercaptoethanol, pH 8.0). The salt was removed by overnight dialysis against TSM, and the ribosome solution was then adjusted to 0.6 M ammonium sulfate. The ribosomes were pelleted by high-speed centrifugation (3 hr at 180,000 x g) and subsequently resuspended in TSM. After clarification of the suspension by another low-speed centrifugation, the concentration of ribosomes was derived from absorbance at 260 nm and adjusted to 3 mg/ml as previously described (52). The purity of the ribosomes was determined from the $A_{260/235nm}$ ratio as described by Schaad (52).

Gel immunodiffusion: Ouchterlony double-immunodiffusion (48) was used for the serological analysis. Gels were prepared with 8.5 g of NaCl, 7.5 g of SeaKem ME agarose (FMC Bioproducts, Rockland, Maine), 2.0 g of $MgCl_2.6H_20$, 0.2 g of NaN_3, 10 ml of 1% trypan blue solution and 990 ml of distilled water. The suspension was autoclaved and 15 ml aliquots were poured in 100-mm-diameter plastic petri dishes. Wells (3.5 mm diameter) were cut in the gel; the central well was filled with 10-25 µl of antiserum and the outer well was filled with 5-25 µl test-antigen. The gels were then incubated in a moist chamber at room temperature for three days before being read. To investigate the serological relatedness of the 50 S ribosomal subunits from the different bacterial strains tested, washed and unwashed ribosomes from each strain were reacted

with each of the five antisera. Terminology and interpretation of results of immunodiffusion tests have been described (14). Because spur formation in immunodiffusion tests is dependent on the antigen-antibody ratio, the agar diffusion method of Piazzi (49) was used to determine the optimal antigen and antiserum concentrations.

RESULTS

Washed ribosome preparations from 34 Agrobacterium and four Rhizobium strains reacted identically with all five antisera to 50 S ribosomal subunits of Agrobacterium strains B6, C58, M63/79, U11 and CG64 (Table I.3). No serological differences were detected between the 50 S ribosomal subunits of the Agrobacterium and Rhizobium strains. In all instances a single, sharply defined, reproducible precipitin band developed midway between the antigen and antiserum wells (Fig. I.1). Furthermore, precipitin band junctions among the antigens were confluent, illustrating the serological identity of the ribosomes from this group of bacteria.

In contrast, when suspensions of unwashed ribosomes were tested against the same antisera, the number and sharpness of the precipitin bands differed greatly among the strains and spurs developed (Fig. I.2). This increase in number of bands with different migration patterns suggested that multiple serological groups existed among the agrobacteria and rhizobia (Table I.3). However, the idea of multiple serogroups became questionable when different preparations of crude ribosomes, extracted at different times from the same strains, produced different reactions (Fig. I.2D).

The differences in the serological reaction between washed and unwashed ribosomes were also reflected in their $A_{260/235\ nm}$

ratios. Unwashed ribosomes had a variable ratio ranging from 1.0 to 1.7 whereas purified (washed) ribosomes had a reproducible ratio of about 1.8.

Antisera to 50 S subunits of Agrobacterium were specific to washed and unwashed ribosomes from the Agrobacterium-Rhizobium group; these five antisera did not react with species outside this group (Table I.3). Ribosomes from agrobacteria and rhizobia did not react with antiserum to ribosomes of E. coli. However, the E. coli antiserum did react with ribosomes extracted from Erwinia carotovora; both E. coli and E. carotovora belong to the Enterobacteriaceae.

DISCUSSION

The 50 S ribosomal subunits from Agrobacterium strains of diverse origin and taxonomic affiliation were serologically identical, indicating that these sub-cellular particles have conserved antigenic components. These data, obtained using washed ribosomes as test-antigens, are in agreement with the reported structural similarities among ribosomes of closely related species (69). Similarly, 50 S ribosomal subunits of Agrobacterium and Rhizobium strains were serologically identical in tests with antisera to 50 S ribosomal subunits of Agrobacterium, which corroborates the close relatedness between Agrobacterium and Rhizobium previously demonstrated by different methods of analysis (18, 24, 26, 27, 29, 31, 45, 59, 68, 70). The data from the above reports and the present study show that Agrobacterium and Rhizobium are closer to each other than is reflected by the current nomenclature presented in the most recent edition of Bergey's manual (33), a nomenclature based primarily on pathogenic-symbiotic differentiations.

In contrast to the single homogeneous precipitin band obtained with washed ribosomes, the unwashed ribosomal preparations resulted in inconsistent and heterogeneous precipitation patterns. The lower $A_{260/235\ nm}$ ratio in unwashed ribosomal extracts suggests the presence of nonribosomal proteins, and the wide range in the ratios among unwashed ribosomal extracts indicates varying amounts of

these impurities. The presence of contaminants in unwashed ribosomes is probably responsible for the inconsistency in the precipitin patterns of unwashed ribosomes. This serological diversity was eliminated by removal of nonribosomal proteins during the purification process in the presence of ammonium sulfate. The presence of nonspecifically bound contaminant on ribosomes extracted by Schaad's method (8) probably explains the unexpected reaction of ribosomal antisera with whole cells of Xanthomonas in immunofluorescence staining (53) and the production in gel immunodiffusion of a common specific band between ribosomes and fixed whole cells (62). This specific band was reported to be a membrane glycoprotein (63). It will be of interest to know if this glycoprotein is equivalent to our contaminant; however, isolation and purification of the contaminant in our preparation is still in the preliminary stage.

The importance of using purified ribosomal particles to prepare an antiserum is emphasized in our study. However, obtaining pure ribosomes for immunization is the major drawback of ribosomal serology. Once antisera to purified (washed) ribosomes are available, unwashed ribosomes which are extracted faster may be preferred as test-antigens. The value of ribosomal serology lies in the uniqueness of bacterial ribosomes which elicit specific antisera. The present data demonstrate that 50 S ribosomal subunits are serologically identical and conserved in the Agrobacterium Rhizobium group. Furthermore, antigenicity of the 50

S subunits was not altered by the presence or absence of plasmids as observed when whole cells were used as immunogens (22, 30, 61). In contrast, 50 S ribosomal subunit antisera provide a reliable tool for identification of strains of the Agrobacterium-Rhizobium group isolated from nature.

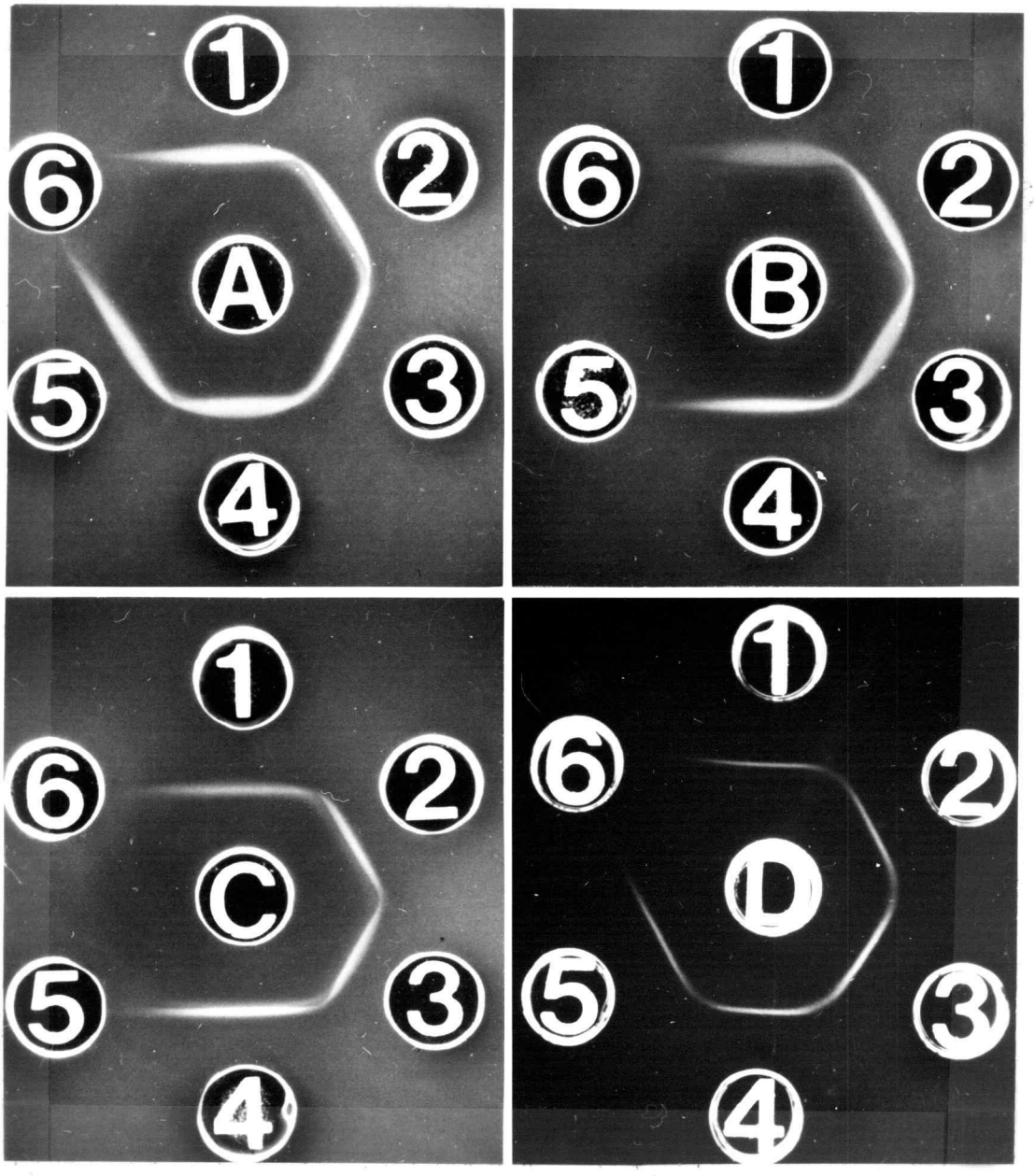

Fig. I.1 Immunodiffusion patterns of antisera to 50 S ribosomal subunits of Agrobacterium against washed ribosomes. Center wells of A, B, C and D contain antiserum, respectively, to M63/79, U11, CG64 and C58. Outer wells of A contain M63/79 (1), T20/73 (2), A4 (3), CG56 (4), Rhizobium meliloti YA15 (5) and Clavibacter michiganense (6). Outer wells of B contain U11 (1), K30 (2), CG56 (3), R. leguminosarum 127K12b (4), Pseudomonas syringae (5) and M9/79 (identity unknown) (6). Outer wells of C contain CG64 (1), Ag63 (2), GA002 (3), AB2/73 (4), Bacillus subtilis (5) and Erwinia carotovora (6). Outer wells of D contain C58 (1), B6 (2), K84 (3), N2/79 (4), A4 (5) and P. solanacearum (6). This precipitin band is represented by the third subcolumn of each antiserum in Table I.3.

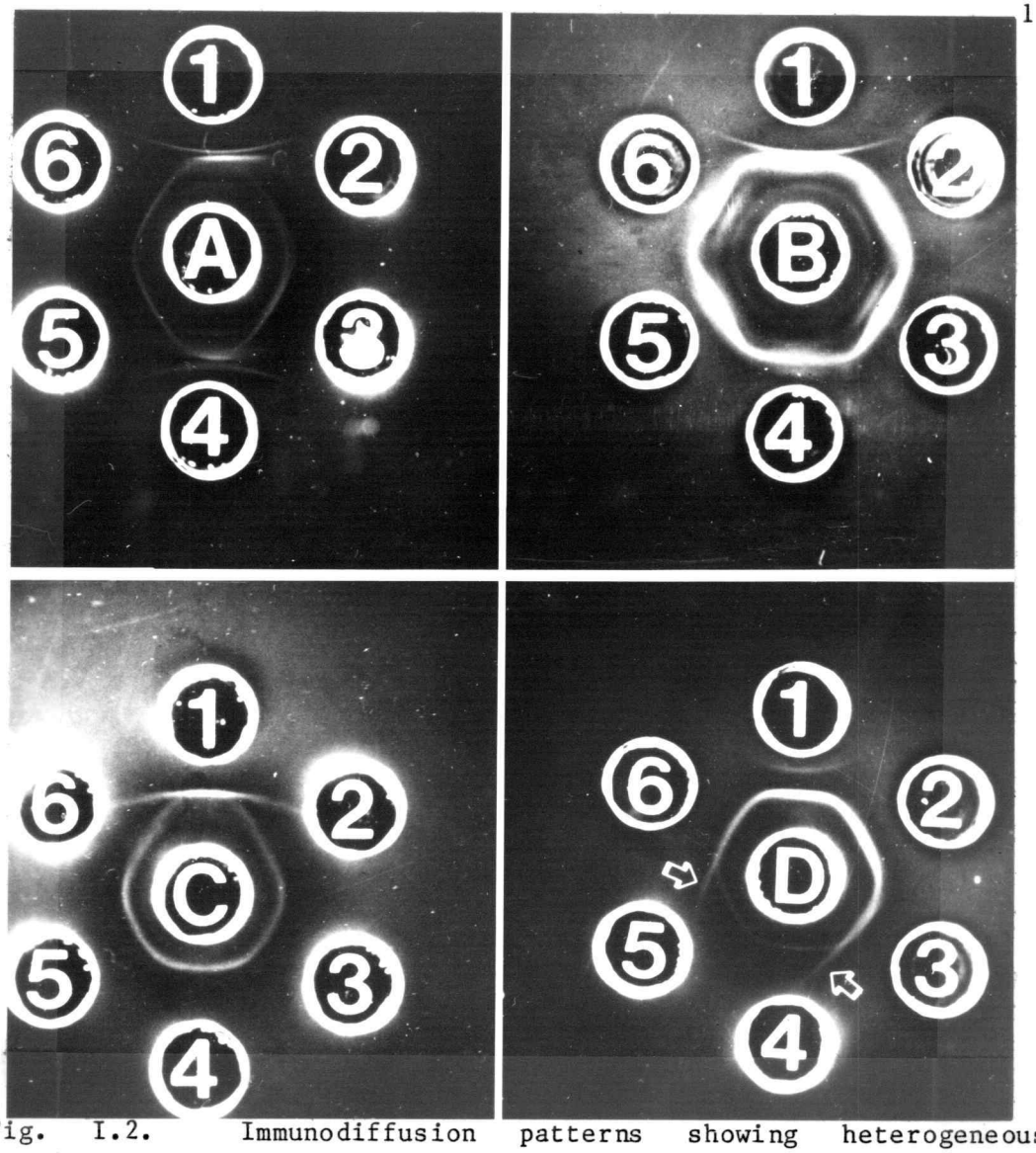

Fig. I.2. Immunodiffusion patterns showing heterogeneous precipitation bands when antisera to 50 S ribosomal subunits of Agrobacterium were tested against unwashed ribosomes. Center wells A and B contain antiserum to B6, and center wells C and D contain antiserum to M63/79. Outer wells of A contain B6 (1, 4), AB2/73 (2), G2/79 (3), K84 (5) and U11 (6). Outer wells of B contain B6 (1), CG48 (2), M63/79 (3), A4 (4), and C58 (5, 6). Outer wells of C contain M63/79 (1), AB2/73 (2), U11 (3), K84 (4), CG48 (5) and G2/79 (6). Outer wells of D contain M63/79 (1) and unwashed ribosomes extracted from A4 at one time (2, 3, 6) and another time (4, 5). Spur formation (arrows) indicates loss of antigens during the second extraction (4, 5), illustrating a lack of reproducibility between different preparations of unwashed ribosomes. The precipitin band that is continuous between the various Agrobacterium and Rhizobium strains shown in this figure is identical to the single band shown in Fig. I.1 above (data shown in ref. 8). Differences in band intensity are due to varying ribosome concentrations in the unwashed ribosomal preparations.

Table I.1 Source and biovar designation of *Agrobacterium* strains used as antigens

Strain	Species name[a]	Biovar affiliation[b]	Origin	Location	Source
T20/73	*radiobacter*	1	Rose	Oregon	1[c]
K84	*radiobacter*	2	Soil	Australia	7
K30	*tumefaciens*	1	Peach	Australia	7
B6	*tumefaciens*	1	Apple	Iowa	2
C58	*tumefaciens*	1	Cherry	New York	5
G2/79	*tumefaciens*	1	Cottonwood	Oklahoma	1
M63/79	*tumefaciens*	1	Cottonwood	Oklahoma	1
G18/79	*tumefaciens*	1	Poplar	Oklahoma	1
GA001	*tumefaciens*	1	Pecan	Georgia	1
GA002	*tumefaciens*	1	Pecan	Georgia	1
GA012	*tumefaciens*	1	Pecan	Georgia	1
GA015	*tumefaciens*	1	Pecan	Georgia	1
GA105	*tumefaciens*	1	Pecan	Georgia	1
H27/79	*tumefaciens*	1	Rose	Colombia	1
S1/73	*tumefaciens*	1	Lippia	Arizona	1
AB2/73	*tumefaciens*	2	Lippia	Arizona	1
B234	*tumefaciens*	2	INA[d]	California	4
GA003	*tumefaciens*	2	Pecan	Georgia	1
M3/73	*tumefaciens*	2	Birch	Oregon	1
U11	*tumefaciens*	2	Willow	Oregon	1
6/6	*tumefaciens*	3	Grapevine	Hungary	11
Ag63	*tumefaciens*	3	Grapevine	Greece	9
CG48	*tumefaciens*	3	Grapevine	New York	3
CG54	*tumefaciens*	3	Grapevine	New York	3
CG56	*tumefaciens*	3	Grapevine	New York	3
CG64	*tumefaciens*	3	Grapevine	New York	3
K47	*rhizogenes*	2	INA	Australia	7
UCBPP604	*rhizogenes*	2	INA	California	10
A4	*rhizogenes*	2	INA	California	6
RR5	*rubi*	1	Raspberry	Oregon	1
N2/79	*rubi*	2	Raspberry	Oregon	1
TR2	*rubi*	2	Raspberry	Washington	8
NT1	plasmid deficient mutant of C58				
A4R1	plasmid deficient mutant of A4				

[a] Species names based on Bergey's manual (37).
[b] Biovar affiliation based on physiological and biochemical tests (47).
[c] 1 = Authors; 2 = R. Baker, Colorado State Univ.; 3 = T. Burr, New York St. Ag. Exp. Station; 4 = J. De Vay, Univ. California, Davis; 5 = R. Dickey, Cornell Univ.; 6 = R. Durbin, Univ. Wisconsin; 7 = A. Kerr, Waite Inst., Australia; 8 = E. Nester, Univ. Washington; 9 = C. Panagopoulos, Greece; 10 = M. Starr, Univ. California Davis; 11 = S. Sule, Hungary.
[d] Information not available.

Table I.2. Bacterial species, other than Agrobacterium, that were used as antigens

Species	Strain	Origin	Source
Rhizobium meliloti	YA15	INA[a] 2[b]	
Rhizobium leguminosarum	128A12	INA 9	
Rhizobium leguminosarum bv. trifolii	162S7a	INA 9	
Rhizobium leguminosarum bv. phaseoli	127K12b	INA 9	
Pseudomonas solanacearum	51	Potato	8
Pseudomonas syringae pv. syringae	B3	Peach	4
Xanthomonas campestris pv. campestris	B24	Broccoli	1
Erwinia carotovora subsp. carotovora	EC105	INA 7	
Escherichia coli	CDC01A	Swine	6
Salmonella typhimurium	E26	mutant	3
Bacillus subtilis	J42	INA 1	
Clavibacter michiganense pv. michiganense	1	INA 5	
Unknown[c]	M9/79	INA 1	

[a] Information not available.

[b] 1 = Authors; 2 = L. Barber, Oregon St. Univ.; 3 = N. Bigley, Univ. Chicago; 4 = H. English, Univ. California, Davis; 5 = E. Echandi, North Carolina St. Univ.; 6 = W. Ewing, Center for Disease Control, Atlanta; 7 = R. Goodman, Univ. Missouri; 8 = A. Kelman, Univ. Wisconsin; 9 = R. Smith, Nitragin Co., Milwaukee, WI.

[c] This strain was mislabelled in our culture collection as A. tumefaciens.

Table I.3. Immunodiffusion reactions of antiserum to 50S ribosomal subunits from five Agrobacterium strains against unwashed and washed ribosomes

Antigens		Antiserum to 50 S subunits of *Agrobacterium*														
		B6[a]			C58			M63/79			U11			CG64		
Unwashed ribosomes:																
Agrobacterium Rhizobium:																
B6[b]	(1)[c]	I	I	I	I	I	I	-	I	I	III	III	I	I	I	I
GA105	(1)	III	I	I	III	I	I	-	I	I	III	III	I	III	I	I
S1/73	(2)	III	I	I	III	I	I	-	III	I	III	I	I	-	I	I
M63/79	(1)	-	I	I	I	I	I	I	I	I	III	III	I	I	I	I
C58	(11)	-	I	I	I	I	I	-	I	I	III	III	I	I	I	I
A4	(8)	-	I	I	I	I	I	-	I	I	III	I	I	III	I	I
CG48	(1)	-	I	I		nt[d]		-	-	I	-	-	I	-	I	I
K30	(2)	III	-	I	III	I	I	-	-	I	II	I	I	-	III	I
TR2	(1)	II	III	I	III	I	I	-	I	I	II	I	I	-	I	I
CG54	(1)	-	III	I	III	I	I	-	I	I	III	I	I	-	I	I
U11	(1)	-	-	I	-	I	I	-	-	I	I	I	I	-	III	I
K84	(5)	-	-	I	-	I	I	-	-	I	III	I	I	-	III	I
AB2/73	(1)	-	-	I	III	I	I	-	-	I	III	I	I	-	III	I
G2/79	(1)	-	-	I	III	I	I	-	-	I	II	III	I	-	III	I
128A12	(1)	-	-	I	-	I	I	-	-	I	-	-	I	III	I	I
Others[e]		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Washed ribosomes:																
Agrobacterium-*Rhizobium*		-	-	I	-	-	I	-	-	I	-	-	I	-	-	I
Others		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Key to symbols: I = Reaction of complete fusion, II = noninteraction (precipitin lines cross), III = partial fusion (spur), - = no band of precipitation.

Table I.3 (continued)

[a] At the optimum antigen-antibody ratio, up to four precipitin bands could be observed with unwashed ribosomes (Fig. I.2), whereas a single band developed with washed ribosomes of the Agrobacterium-Rhizobium group (Fig. I.1). Three subcolumns are used to illustrate the reaction and position of the three bands nearest the antigen well: left (band nearest the antigen well), middle (intermediate band) and right (band farthest from the antigen well).

[b] Representative strain for a particular serological group (4). Those Agrobacterium and Rhizobium strains which had the same precipitin patterns were grouped together. Based on the pattern combinations shown in this table, 15 serogroups were identified.

[c] The number in parenthesis is the total number of Agrobacterium and Rhizobium strains belonging to that particular serological group (footnote b). Strain YA15 is included in the C58 group, whereas strains 127K12b and 162S7a were grouped with A4.

[d] Not tested.

[e] Pseudomonas solanacearum, P. syringae, Xanthomonas campestris, Erwinia carotovora, Escherichia coli, Salmonella typhimurium, Bacillus subtilis, Clavibacter michiganense and M9/79 (identity unknown).

CHAPTER II

NONSPECIFIC BINDING OF CELL SURFACE ANTIGENS TO RIBOSOMES OF AGROBACTERIUM

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SUMMARY

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 or 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.

INTRODUCTION

Species specific antisera to ribosomes have been used to demonstrate the potential utility of ribosomal serology to examine relationships between species (69). Schaad (52) 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 (53, 62). 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 (4). Subsequent ribosome samples from the same strains produced different precipitin reactions (7). 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 led 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 (34) at 27°C on an orbital shaker. Cells in exponential growth-phase were harvested by centrifugation at 10,000 x 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 one hour in a boiling water bath. Ribosomes were extracted in 0.5 M ammonium chloride following Schaad's method (52) which does not employ an ammonium sulfate fractionation procedure. In short, cell lysates were centrifuged at high-speed (180,000 x g) for 3 hr; the ribosome pellets were resuspended and clarified by centrifugation (10,000 x g for 15 min). Ribosomes were also extracted following a modification (7) of Kurland's procedure (38). 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 (64). The purity of the ribosomes was determined from the $A_{260/235nm}$ ratio as described by Schaad (52). Washed and unwashed 50 S ribosomal subunits were obtained from the washed and unwashed ribosome preparations by zonal sucrose density-gradient centrifugation (53). Proteins from washed 50 S ribosomal subunits

were extracted in 66% glacial acetic acid (28). Protein concentrations were determined by the microassay procedure of Bradford (10) 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. Pre-immune 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 (7). 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, Michigan). Antiserum to somatic antigens was developed from two IV injections, containing respectively 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 (48). 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.

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. II.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. II.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 which 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.

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. II.2), illustrating that these antisera shared antibodies that recognized the same antigen. These results and results shown in Fig. II.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 (53, 62) corroborates the presence of somatic contamination in ribosomes extracted by Schaad's method. Although, serogrouping of ribosomes from Xanthomonas strains (53, 62) 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 (7). This serological heterogeneity masked the true ribosomal relationships of these strains, because it most likely involved the reaction of antibodies to somatic antigens with nonribosomal 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 homogeneous serological group comprising both Agrobacterium and Rhizobium was revealed (7).

Our data indicate that Kurland's procedure (38) of ammonium sulfate fractionation is superior to the more commonly used method of Schaad (52) 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.

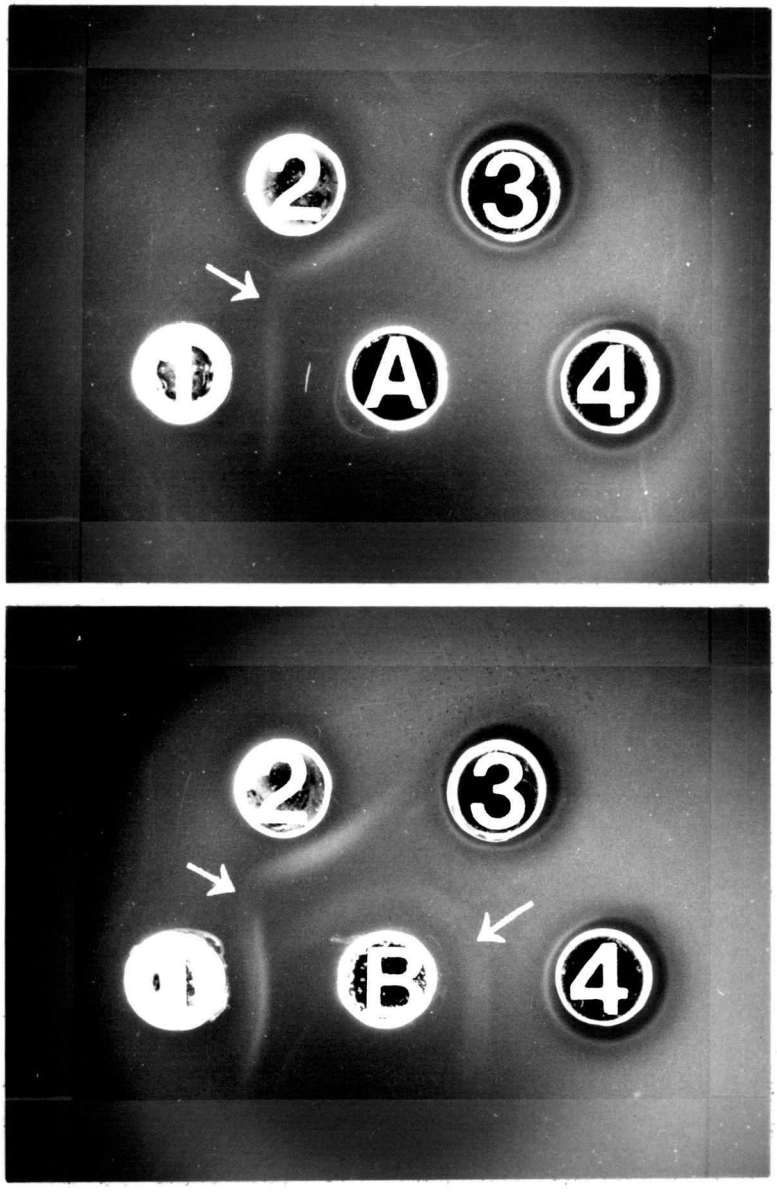

Fig. II.1 Immunodiffusion patterns of four different antigenic preparations against two different antisera to Agrobacterium strain C58. The center well of A contains antiserum to somatic antigens, whereas the center well of B contains antiserum to unwashed ribosomes. Outer wells of A and B contain: somatic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

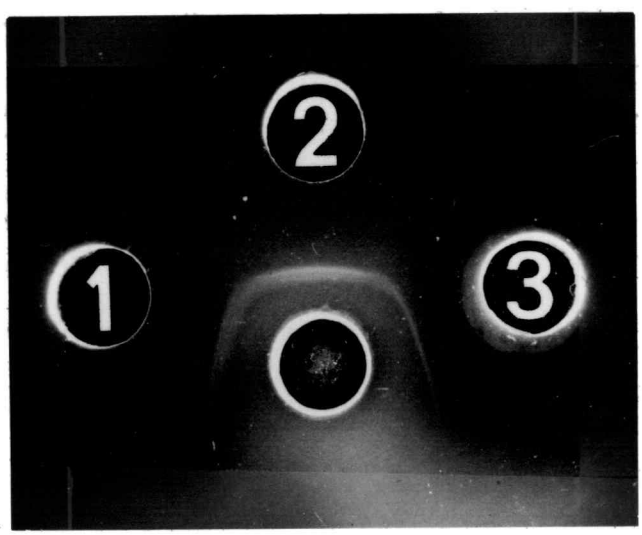

Fig. II.2 Immunodiffusion reaction of somatic antigens of Agrobacterium strain C58 (center well) with antiserum to somatic antigens (1), antiserum to unwashed ribosomes (2) and antiserum to unwashed 50 S ribosomal subunits (3).

CHAPTER III

COMPLEMENTARY METHODOLOGIES TO IDENTIFY SPECIFIC *AGROBACTERIUM* STRAINS

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SUMMARY

Serological techniques and restriction enzyme cleavage patterns of total DNA were used to differentiate strains of *Agrobacterium* spp. Forty five wild-type and plasmid-cured *Agrobacterium* strains were tested by immunodiffusion and immunofluorescence against polyclonal antisera to a crude ribosome preparation from *Agrobacterium* strains K84, U11, B6, A323, NT1, and C58. In immunodiffusion gels, these antisera reacted only with water-phenol extracts of the homologous strain, producing a single, strain-specific precipitin line. In contrast, when the same antisera were used in immunofluorescence staining, cross-reactions occurred with a limited number of heterologous *Agrobacterium* strains. However, the cross-reacting heterologous cells fluoresced

generally less brightly than the homologous cells. When the EcoR1-digested DNA profiles from the same Agrobacterium strains were compared, 34 distinct cleavage patterns were observed. The DNA profiles were the same for all strains sharing a common chromosomal background and correlated with the strain-specific serological reaction. The presence or absence of plasmid DNA did not alter the strain-specific serological reaction or the DNA cleavage patterns. Both the serological reaction and the restriction enzyme digestion of total DNA were complementary to each other. These methods were used successfully to identify A. radiobacter K84 strains which were recovered six months after being inoculated to young trees in the field.

INTRODUCTION

Ecological studies of Agrobacterium spp. have been hampered by time-consuming methods of identification. In search for rapid and accurate methods of identification for the agrobacteria, we developed antisera against 50 S ribosomal subunits (7) which were useful in identifying Agrobacterium strains isolated from a natural habitat (5). However, these antisera have a broad specificity for the Agrobacterium-Rhizobium group and are not useful for monitoring individual strains in the environment. This broad serological specificity was obtained with purified ribosomal antigens (7). In contrast, when crude ribosomal antigens (i.e. ribosomal preparations carrying other cellular antigens) were used, the serological reaction was specific to small groups of Agrobacterium strains (unpublished data). These observations suggested that antisera developed against crude (i.e., unwashed) ribosomes would be potentially more specific than our antisera to 50 S ribosomal subunits. In the present study, the reaction of antisera to crude ribosomes was strain specific in gel immunodiffusion tests against water-phenol extracts (WPE) of whole cells. However, a few strains cross-reacted when whole cells were stained by immunofluorescence. Therefore, another method was sought to complement the serology, and we investigated the specificity of restriction endonuclease digests of total cellular DNA. This method was reported specific below the species level for the genera Rhizobium (43) and Bradyrhizobium (58), two other members of the

family Rhizobiaceae, and proved strain specific for the Agrobacterium strains examined in this paper.

MATERIALS AND METHODS

Organisms and cultivation: Thirty nine Agrobacterium strains (Table III.1) and six derivative strains lacking indigenous plasmids (Table III.2) were used in this study. This collection of strains included different biovars and species representing diverse environments and geographical regions. In addition, 12 strains of both closely and distantly related bacterial species were included to test the specificity of the antisera (see reference 7). Unless specified otherwise, the bacteria were grown for 24 hr in MGY broth (mannitol, 0.5%; L-glutamic acid, 0.2%; yeast-extract, 0.1%; K_2HPO_4, 0.05%; NaCl, 0.02%; $MgSO_4.7H_2O$, 0.02%; pH 7.2) at 27°C on an orbital shaker. Cells were harvested in exponential growth phase by centrifugation (10,000 x g for 15 min).

Serological analyses: Six female New Zealand White rabbits were used for antiserum production. Crude ribosomal preparations of Agrobacterium strains K84, U11, B6, C58, NT1, and A323 were used as immunogens. The ribosomes were pelleted from cell lysates by centrifugation at 180,000 x g for 3 hr; the ribosome pellets were suspended and clarified by centrifugation at 10,000 x g for 15 min. Antiserum production was performed as described previously (8). The serological analyses employed both immunodiffusion and indirect immunofluorescence.

The procedure for immunodiffusion was performed as described previously (7), except that the central well was filled with 20 µl of antiserum and the outer well with 20 µl of test antigen. In contrast to crude ribosomes, which were used as immunogens, test antigens consisted of water-phenol extracts (WPE) from whole bacterial cells (16). Phenol extracts somatic antigens which were suspected to bind nonspecifically to crude (i.e., unwashed) ribosomes (8) and to be responsible for the unexpected reaction of ribosomal antisera with cell surface antigens (53, 62). WPE were prepared as follows: the bacteria were grown overnight on slants of nutrient agar (Difco Laboratories, Detroit, Michigan) and suspended in 0.5 ml of distilled water; 20 µl of 90% liquid phenol was added to the suspension and mixed thoroughly for 30-45 sec. WPE from each strain were reacted with each of the six antisera. Terminology and interpretation of results of immunodiffusion tests have been described previously (14).

Indirect immunofluorescence staining was performed on bacterial smears made on multiwell slides (Cel-line Associates, Inc., Minotola, New Jersey). Smears were made with 10 µl of a cell suspension (about 10^7 CFU/ml) which was prepared by suspending bacterial growth from a 24-hr old nutrient agar slant culture in 0.85% NaCl. The smears were allowed to air dry at room temperature before 10 µl of an antiserum was deposited on each smear. Neither heat nor chemical fixation of the bacterial smear was found

necessary. The slide was then incubated in a humidity chamber for 30 min, immersed for 10 min in 0.1 M phosphate-buffered saline at pH 8.0, rinsed in distilled water, and allowed to air dry. The smears were then stained with 10 µl of fluorescein isothiocyanate conjugated goat anti-rabbit immunoglobulin G (Gibco Laboratories, Grand Island, New York). All manipulations after deposition of the fluorescent anti-rabbit antibodies were performed in the dark: the slide was incubated in a humidity chamber for 30 min, immersed for 10 min in phosphate-buffered saline, rinsed in distilled water and air dried. The slide was then mounted in carbonate-buffered glycerin as described (54). Block titration tests (25) were performed to determine the optimum dilution of rabbit and anti-rabbit sera to be used. These dilutions ranged from 1:2 to 1:8 for the different crude ribosomes antisera, and from 1:20 to 1:50 for the anti-rabbit immunoglobulin G. To confirm that a negative reaction of immunofluorescence did not result from the absence of bacteria from the field of view, the bacterial smears were also examined by phase-contrast microscopy.

Two negative controls were run for each group of slides tested. One control, to detect nonspecific background fluorescence, consisted of a primary incubation with the smear coated with pre-immune rabbit serum, followed by a second incubation with fluorescein isothiocyanate-labelled anti-rabbit immunoglobulin G. The second control, for the detection of any

inherent Agrobacterium fluorescence, consisted of a primary incubation only.

A Zeiss Universal photomicroscope, equipped with attachments for epifluorescence microscopy (which included a high-pressure mercury lamp [HBO-50W]), was used for examination of the prepared slides. A BP-450-2490 exciter filter, a 510 reflector, and an LP-520 barrier filter (transmission range above 520 nm) were used under UV epi-illumination at a magnification of x400.

Restriction endonuclease analysis: Cultures used for isolation of DNA were grown to the stationary phase with shaking in 3 ml of MGY broth. The cells were pelleted at 4°C, suspended in 0.3 ml of TEG buffer (10 mM Tris-hydrochloride, 50 mM EDTA, 1% glucose, pH 8.0) and lysed by adding 30 µl of 10% sodium dodecyl sulfate (Bio-Rad Laboratories, Richmond, California). The lysate was mixed by repeated gentle inversion of the tube, and incubated at 65-70°C for 10 min. Proteins were extracted twice from this mixture with an equal volume of phenol-chloroform-isoamyl alcohol (24:24:1, vol/vol) saturated with TE buffer (10 mM Tris-hydrochloride, 1 mM EDTA, pH 8.0) (42). The DNA was ethanol-precipitated, washed (first in 70% ethanol and then in 95% ethanol), pelleted by centrifugation, and thoroughly dried under vacuum at room temperature for at least 30 min. The dried DNA was resuspended in 35 ul of TE buffer, and digested at 37°C for 1-3 hr by adding 60 units of the restriction endonuclease EcoR1 (United

States Biochemical Corp., Cleveland, Ohio) and 4 ul of 10 X EcoR1 digestion buffer (1 M NaCl, 10 mM dithiothreitol, 0.5 M Tris-hydrochloride, 0.1 M $MgCl_2$) (42). RNase A (20 µg; Sigma Chemical Co., St. Louis, Missouri) was added 10 min before the end of this incubation period. Digestion was stopped by adding 4 µl of 0.15 M EDTA. Gel loading buffer Type II (bromophenol blue, 0.25%; xylene cyanol, 0.25%; Ficoll type 400, 15%) (42) was added to the DNA suspension before electrophoresis. DNA samples of 5 µl each were loaded on a 0.7% agarose (Ultra-pure DNA grade agarose, Bio-Rad) gel and electrophoresed for 3 hr at 85 mA (30 v). The gel was stained for 30 min in ethidium bromide (0.75 µg/ml) and photographed over a UV transilluminator (Chromato-vue, Ultra-violet Products Inc., San Gabriel, California) with a Polaroid model MP-4 land camera equipped with a yellow wratten No. 9 UV filter (Eastman Kodak Co., Rochester, New York).

RESULTS

Strain-specific reaction in gel immunodiffusion: A single, sharply defined, reproducible precipitin band was formed in all instances when antiserum to crude ribosomes was reacted with a WPE of the homologous Agrobacterium strain (Table III.3, Fig. III.1). When the same antiserum was reacted with WPE from heterologous strains, no precipitin band could be detected. An exception to this strain specificity was observed when the K84 antiserum cross-reacted with CG-48 to produce a diffuse precipitin band and with I27/83 to form a reaction of partial fusion (partial identity) (Table III.3., Fig. III.1A & B). The wild-type strain C58 and plasmid mutants thereof (NT1, A136, and A323) reacted identically against antisera to C58, NT1 and A323, producing precipitin lines which completely fused (Table III.3); similarly, precipitin lines produced by the K84-antiserum against WPE of the wild-type K84 and its mutants K434 and K84Agr-, fused completely (Table III.3, Fig. III.1B). These reactions of complete fusion (identity) were anticipated because the mutants of C58 and K84 share the same chromosomal background with their parental strains (Table III.2). When mixtures of two antisera were tested against individual WPE of the homologous strains, a reaction of noninteraction (nonidentity, precipitin lines cross) developed (Fig. III.1C). This test was repeated with all combinations, and none of the strain-specific antisera recognized the strain-specific antigen(s) present in each heterologous strain (data not shown).

Cross-reactions in immunofluorescence: As expected from the immunodiffusion data, the antisera reacted with mutant strains sharing the same chromosomal background as the homologous strains. However, in contrast to the strain-specific reaction recorded in gel immunodiffusion, all six crude ribosomal antisera cross-reacted to some extent in indirect immunofluorescence with whole cells from some of the other Agrobacterium strains. The number of cross-reacting strains (not including derivative strains sharing the same chromosomal background) ranged from as few as two for B6 antiserum, to as many as nine different strains with K84 antiserum (Table III.4). Fluorescence intensity of cross-reacting heterologous strains was generally weaker than that of the homologous strain.

Different DNA digestion profiles of Agrobacterium strains: Agarose gel electrophoregrams of DNA fragments obtained from complete digestion of total cellular DNA by EcoR1 are presented in Fig. III.2. DNA fragments from strains belonging to a variety of different species and biovars showed clear and reproducible banding patterns that were distinguishable from one another. The clearest differences were among the largest EcoR1-fragments. However, there also appeared to be conservation of a large DNA fragment (located near the position of the 21.2-kilobase-pair-lambda fragment) among most (but not all) of the biovar 2 strains (Fig. III.2B). Digestion profiles of strains with common chromosomal background

were identical, both qualitatively and quantitatively: K84Agr-, K434, and K84 shared the same digestion patterns; A323, A136, NT1, and C58 had a common profile; and, A4 and A4R1 had identical banding patterns (Fig. III.2D). EcoR1-digestion of total DNA from 39 wild-type strains revealed 34 distinct cleavage patterns. Of those strains sharing the same cleavage pattern, the A. tumefaciens biovar 1 strains GA001 and GA002 were isolated from the same gall. Similarly, A. tumefaciens strains GA012 and GA015 were both biovar 2, inhabited the same tumor, and had the same DNA banding pattern. These two strains also had the unique phenotypic characteristic of producing a diffusible pigment in culture which turned the medium dark blue. Three other groups of strains produced similar DNA profiles but we do not have information about their precise origin. These were: A. tumefaciens biovar 1 strains G2/79 and G18/79, isolated from galls collected from different plant species in the same nursery in Oklahoma; A. tumefaciens biovar 3 strains 3/2 and 15/9, isolated from Hungarian grapevine by S. Sule; and A. rhizogenes biovar 2 strains K47 and UCBPP-604, obtained respectively from A. Kerr (Waite Institute, Glen Osmond, South Australia) and M. Starr (Univ. California, Davis).

The reproducibility of the cleavage patterns was demonstrated by the identical profiles obtained when we tested different DNA preparations from the same strain and using different EcoR1

batches. Reproducibility of our results indicates that digestion of total Agrobacterium DNA and of lambda bacteriophage DNA reached completion.

DISCUSSION

In gel immunodiffusion, a strain-specific precipitin line was obtained when crude ribosomes antisera were tested against WPE of the homologous Agrobacterium strain. The strain-specific reaction by the different antisera was due to antigenically different molecules in each case because 1) the different antisera did not recognize the strain-specific antigen of heterologous strains, and 2) a reaction of noninteraction (nonidentity) developed when these antisera were combined together and tested against individual WPE of the homologous strains (Fig III.1). The specific reaction was repeated for cured strains having the same chromosomal background. The presence or absence of tumorigenic, rhizogenic or agrocinogenic plasmids did not affect the serological reaction, indicating that the strain-specific antigen(s) was coded for by a chromosomal gene(s). Although the present immunodiffusion analysis demonstrates the antigenic diversity among a large group of heterogenous agrobacteria, characterization of the strain-specific antigen(s) from different strains would probably reveal relatedness in both structure and function. It seems unlikely that there would be functionally unique molecules in every Agrobacterium strain. Strain-specific immunogens, present in Salmonella ribosomal preparations, were identified as cell surface lipopolysaccharides (21). These observations and the fact that our antisera reacted with cell surface antigens and WPE (water-phenol is used to extract lipopolysaccharides; [67]) suggest that lipopolysaccharides

contaminate our crude ribosomal preparations. Efforts are under way to isolate and characterize the molecule(s) responsible for eliciting the strain-specific antibodies.

The strain-specificity which was observed when crude ribosome antisera were reacted with WPE in gel immunodiffusion did not hold when the same antisera were tested by indirect immunofluorescence against whole cells of different Agrobacterium strains. Cross-reactions of antisera with heterologous strains suggest that some cell surface antigens are common or similar among certain Agrobacterium strains, hence the greater cross-reactivity observed in immunofluorescence. These cross-reacting antigens either were not extracted by the selective water-phenol extraction procedure or were too large to diffuse through the agarose gel matrix. However, differences in the intensity of the fluorescence staining were observed. The lower intensity of fluorescence observed with some of the cross-reacting heterologous strains may reflect either weaker antigen-antibody affinities caused by similar but not identical antigens, or lower concentrations of the same antigen(s) at the cell surface of the cross-reacting heterologous strains. Cross-reactivity in immunofluorescence tests followed neither taxonomic affiliations (i.e. species and biovar) nor host of origin.

Profiles of EcoR1-fragments obtained from digestion of total cellular DNA were reproducible and specific to strains sharing a

common chromosomal background and correlated perfectly with the strain-specific serological reaction. The presence of extrachromosomal plasmid DNA did not alter these cleavage patterns; similar results were reported with the low copy number plasmids present in Campylobacter jejuni (9). We concluded that any variation between DNA profiles was due to genomic differences between strains and that these differences could be used as fingerprints for the confirmatory identification of individual Agrobacterium strains. The fact that strains isolated from the same gall had identical physiological characters and DNA fingerprints (GA001 and GA002; GA012 and GA015) suggests strongly that these were isolates of the same strain. Three additional groups of strains shared identical physiological characters and digestion profiles, but we lacked sufficient historical information to confirm whether these strains actually shared the same chromosome or whether indeed they were different but closely related strains. In the case of K47 and UCBPP-604, we found that both strains came from the collection of M. Starr with the respective code numbers ICPB TR-105 and ICPB TR-108. Therefore, it seems unlikely that these two very closely related strains were isolated from two different continents, as we previously supposed.

Both the serological method and the EcoR1-digestion of total DNA were useful tools to identify individual Agrobacterium strains. The serological tests were very easy and simple to perform once antisera were available. In contrast, EcoR1-digestion

of total DNA can be time consuming when large numbers of isolates are to be analyzed. However, EcoR1 digests can be performed readily on small numbers of strains as a confirmatory step to identification. When monitoring a strain released into the environment, we propose that the simple and rapid serological technique be used first to identify the released strain. EcoR1-digestion would verify which one of these serologically identified isolates was the reference strain. We have effectively used this approach to confirm the identity of A. radiobacter K84 strains which were recovered from roots and tumors of seedling trees (inoculated with K84 before transplanting) which had been grown in the field for six months (unpublished data). Of 97 Agrobacterium isolates recovered from these field-grown trees, 19 were nonpathogenic, produced agrocin 84 in-vitro, and developed the typical strain specific reaction against the K84-antiserum. One pathogenic isolate (I27/83) cross-reacted with the K84-antiserum (Fig. III.1B) but did not produce agrocin 84. Comparison of the I27/83 DNA profile with that of K84 confirmed that I27/83 was not a K84 strain which would have received a tumorigenic plasmid (Fig. III.2B).

This application of these two methods for the accurate identification of K84 introduced into the environment, and the fact that strains sharing the same chromosomal background and originating from different laboratories had the same strain-specific antigen and identical DNA profiles, demonstrates the

stability of the antigens and the DNA profiles over time. Such stability is critical to the practical use of these complementary methodologies to assess the fate of a specific Agrobacterium strain which would be released into the environment. A major contribution of these methodologies is that they reveal natural cellular markers that are strain-specific and thus offer an alternative to strains with selected spontaneous antibiotic resistance which may be impaired genetically (39). A further improvement of this dual method would be to reduce the cross-reactivity of the antiserum by cross-adsorption to allow use of the more sensitive technique of immunofluorescence to detect bacteria in-situ.

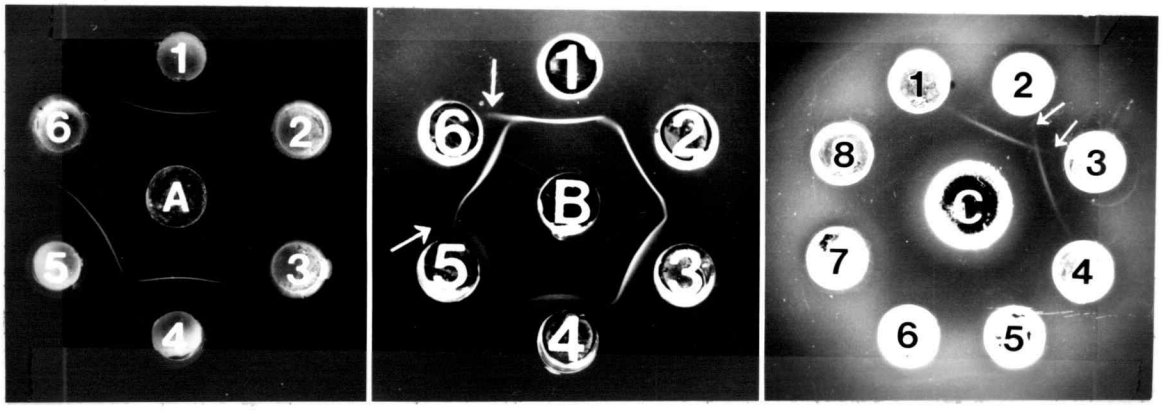

Fig. III.1. Immunodiffusion reactions between WPE of whole Agrobacterium cells and antisera to crude Agrobacterium ribosomes. The center wells of panels A and B contain K84 antiserum. Outer wells of panel A contain WPE of strains K84 (wells 1, 4, and 5), CG-48 (wells 2 and 3), and C58 (well 6). Outer wells of panel B contain WPE of strains K84 (wells 1, 4, and 5), K434 (well 2), K84Agr$^-$ (well 3), and I27/83 (well 6); a reaction of partial fusion (single spur) (arrows) developed between K84 and I27/83. The center well of panel C contains a mixture of antisera to strains B6 and U11 (1:1, vol/vol). Outer wells of panel C contain WPE of strains C58 (well 1), B6 (well 2), U11 (well 3), K84 (well 4), K434 (well 5), A323 (well 6), NT1 (well 7), and A136 (well 8); the strain-specific precipitin lines of B6 and U11 cross each other (reaction of noninteraction) producing spurs (arrows).

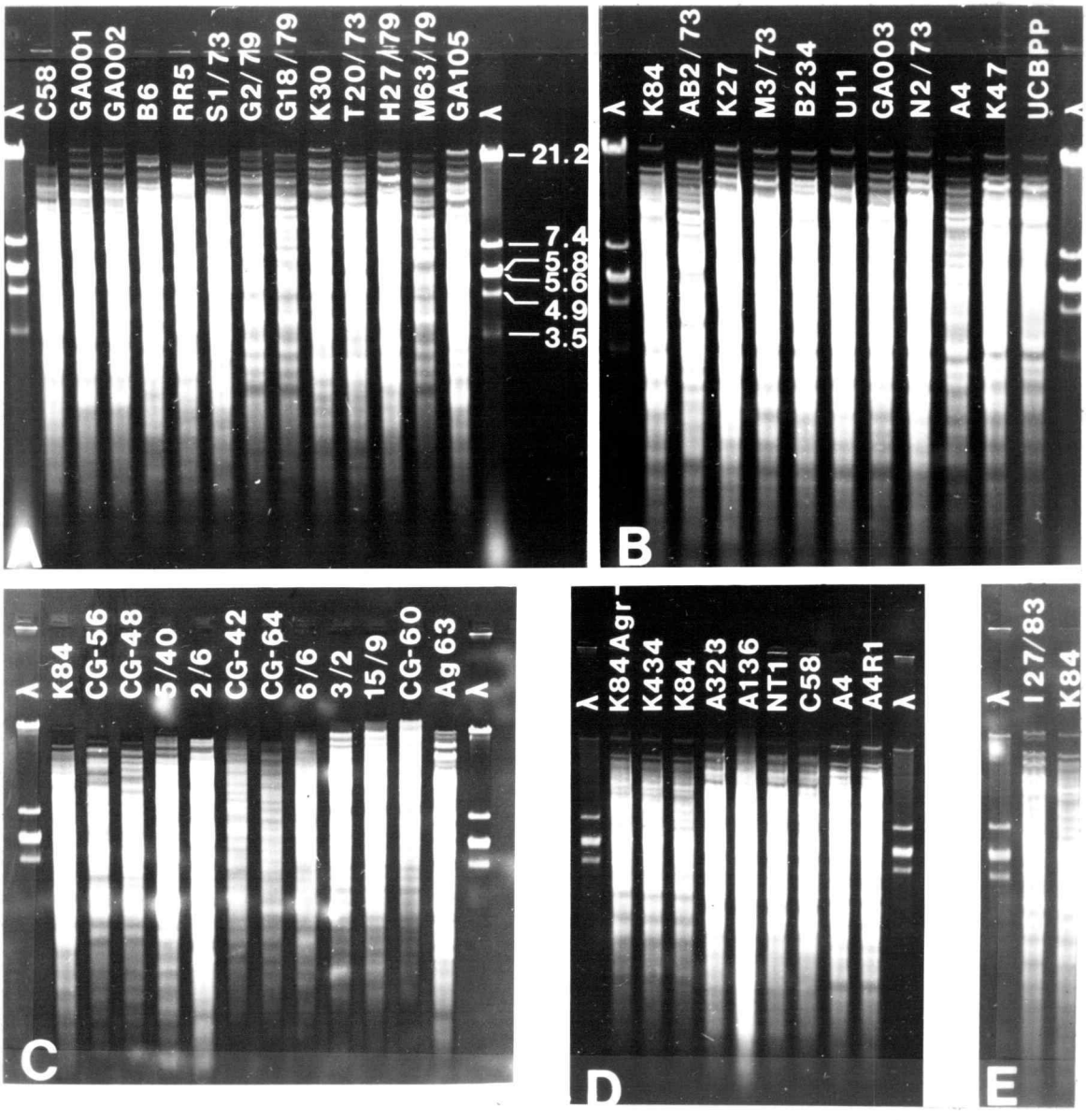

Fig. III.2. Agarose gel electrophoresis of total Agrobacterium DNA after digestion with EcoR1. (A, B, and C) DNA profiles of biovar 1, biovar 2, and biovar 3 strains, respectively, illustrating the uniqueness of banding patterns for individual strains. (D) Identical profiles between the wild type strains K84, C58, and A4 and their plasmid mutants. (E) Different DNA profiles of strains I27/83 and K84. EcoR1 digests of lambda DNA were run in each gel as size markers; fragment size (kilobase pairs) is shown to the right of panel A.

Table III.1. Sources of *Agrobacterium* strains

Strain	Biovar	Origin	Location	Source[a]
A. tumefaciens:				
B6	1	Apple	Iowa	R. Baker
C58	1	Cherry	New York	R. Dickey
G2/79	1	Cottonwood	Oklahoma	
G18/79	1	Poplar	Oklahoma	
GA001	1	Pecan	Georgia	
GA002	1	Pecan	Georgia	
GA012	1	Pecan	Georgia	
GA015	1	Pecan	Georgia	
GA105	1	Pecan	Georgia	
H27/79	1	Rose	Columbia	
K24	1	INA	Australia	A. Kerr
K30	1	Peach	Australia	A. Kerr
M63/79	1	Cottonwood	Oklahoma	
S1/73	1	Lippia	Arizona	
AB2/73	2	Lippia	Arizona	
B234	2	INA	California	J. De Vay
GA003	2	Pecan	Georgia	
I27/83	2	Cherry	Washington	
K27	2	Poplar	INA	A. Kerr
M3/73	2	Birch	Oregon	
U11	2	Willow	Oregon	
Ag63	3	Almond	Greece	
CG-42	3	Grapevine	New York	T. Burr
CG-48	3	Grapevine	New York	T. Burr
CG-56	3	Grapevine	New York	T. Burr
CG-60	3	Grapevine	New York	T. Burr
CG-64	3	Grapevine	New York	T. Burr
2/6	3	Grapevine	Hungary	S. Sule
3/2	3	Grapevine	Hungary	S. Sule
5/40	3	Grapevine	Hungary	S. Sule
6/6	3	Grapevine	Hungary	S. Sule
15/9	3	Grapevine	Hungary	S. Sule
A. rhizogenes:				
A4	2	INA	California	R. Durbin
UCBPP-604	2	INA	California	M. Starr
K47	2	INA	Australia	A. Kerr
A. rubi:				
RR5	1	Raspberry	Oregon	
N2/73	2	Raspberry	Oregon	

Table III.1 (continued)

A. radiobacter:			
T20/73	1	Rose	Oregon
K84	2	Soil	Australia

[a] Unless specified otherwise, strains were from our laboratory.

Table III.2. Plasmids of wild-types and derivative strains of *A. tumefaciens* C58, *A. rhizogenes* A4, and *A. radiobacter* K84

Strain	Chromosome	Plasmids	Source
C58	C58	pAtC58, pTiC58	R. Dickey
A136	C58	pAtC58	S. Farrand
NT1	C58	pAtC58	E. Nester
A323	C58	pAtC58, pAgK84	E. Nester
K84	K84	pAtK84, pAgK84	A. Kerr
K434	K84	pAtK84	S. Farrand
K84Agr$^-$	K84	pAtK84	This laboratory
A4	A4	pArA4, pRiA4	R. Durbin
A4R1	A4	pArA4	This laboratory

Table III.3. Reactions of crude ribosome antisera of *Agrobacterium* strains with water-phenol extracts in gel immunodiffusion

WPE antigens	Reaction with crude ribosome antisera of *Agrobacterium* strain[a]:					
	U11	K84	B6	C58[b]	NT1[b]	A323[b]
B6	-	-	I	-	-	-
C58[b]	-	-	-	I	I	I
NT1[b]	-	-	-	I	I	I
A323[b]	-	-	-	I	I	I
A136[b]	-	-	-	I	I	I
U11	I	-	-	-	-	-
K84[c]	-	I	-	-	-	-
K434[c]	-	I	-	-	-	-
K84Agr-[c]	-	I	-	-	-	-
I27/83	-	III	-	-	-	-
CG-48	-	III	-	-	-	-
34 other *Agrobacterium* strains[d]	-	-	-	-	-	-
12 other bacterial species	-	-	-	-	-	-

[a] Symbols of reaction types (14): I = complete fusion; III = partial fusion (spur); - = no precipitin lines.
[b] Have a C58 chromosomal background (Table III.2).
[c] Have a K84 chromosomal background (Table III.2).
[d] Listed in Table III.1.

Table III.4. Indirect-immunofluorescence staining of whole bacterial cells with antisera to crude ribosomes of *Agrobacterium* strains

Whole cell antigens	Staining with crude ribosome antisera of *Agrobacterium* strain[a]:					
	U11	K84	B6	C58[b]	NT1[b]	A323[b]
B6	–	–	3	2	2	1
C58[b]	1	–	2	3	3	3
NT1[b]	–	–	2	3	3	3
A323[b]	–	–	2	3	3	3
A136[b]	–	–	2	3	3	3
G2/79	–	–	–	–	1	–
G18/79	–	–	–	–	1	–
GA002	–	1	–	–	–	–
H27/79	–	2	–	1	–	1
K24	–	–	–	2	2	2
S1/73	–	1	–	–	–	–
B234	1	1	–	–	–	–
M3/73	1	1	–	–	–	–
U11	2	2	–	–	1	–
K84[c]	2	3	–	–	–	–
K434[c]	2	3	–	–	–	–
K84Agr-[c]	1	3	–	–	–	–
I27/83	–	3	–	–	–	–
CG-48	–	3	–	–	–	–
N2/79	–	1	–	–	–	–
2/6	–	–	2	–	1	–
24 other *Agrobacterium* strains[d]	–	–	–	–	–	–
12 other bacterial species	–	–	–	–	–	–

[a] Symbols: 3 = bright fluorescence; 2 = moderate fluorescence; 1 = pale fluorescence; – = no fluorescence.
[b] Have a C58 chromosomal background (Table III.2).
[c] Have a K84 chromosomal background (Table III.2).
[d] Listed in Table III.1.

CHAPTER IV

LIPOPOLYSACCHARIDES FROM AGROBACTERIUM TUMEFACIENS B6 INDUCE THE PRODUCTION OF STRAIN-SPECIFIC ANTIBODIES

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SUMMARY

Preparations of lipopolysaccharides from Agrobacterium tumefaciens strain B6 elicited rabbit antibodies which reacted with water-phenol extracts of whole cells from strain B6 to form a strain-specific precipitin band in gel immunodiffusion plates. This antiserum to B6 lipopolysaccharides did not react with water-phenol extracts from 38 other Agrobacterium strains nor twelve bacterial species from eight other genera. An additional precipitin band, although only slightly visible, developed against lipopolysaccharides from B6 and nine of the other 38 Agrobacterium strains tested. In this study, the lipopolysaccharides were identified as the contaminating strain-specific antigens associated

earlier with ribosomal preparations (H. Bouzar and L. W. Moore, Appl. Environ. Microbiol. 53:2660-2665, 1987). These lipopolysaccharide contaminants were removed from the ribosomal preparation by an initial salt cut of 20% ammonium sulfate and sedimentation of the ribosomes in 0.6 M ammonium sulfate.

INTRODUCTION

Studies of the ecology of Agrobacterium spp. are hampered by the lack of sensitive and specific methods for detection of the target organism among the many other microorganisms found in nature. In search for methods that would permit the monitoring of target Agrobacterium strains, we developed antisera to ribosomes from six strains of Agrobacterium. These antisera were strain-specific when they were reacted against water-phenol extracts (WPE) of whole cells (6). However, these strain-specific antisera cross-reacted with whole Agrobacterium cells, suggesting that the ribosome preparations used for immunization were also carrying contaminants from the surface of the bacterium cell (6, 8). These contaminating antigens were not detected in purified ribosome preparations which were obtained following salt fractionation and a subsequent sedimentation in 0.6 M ammonium sulfate (8).

The objectives of the present study were to identify the antigen(s) which elicited the strain-specific antibodies. We hypothesized that the strain-specific activity was due to lipopolysaccharides (LPS) because 1) the antiserum cross-reacted with cell surface antigens (6), 2) the cross-reacting antigens were heat-stable (8), and 3) readily extracted by water-phenol (6). In this paper, we present evidence that unwashed ribosome preparations of Agrobacterium tumefaciens strain B6 carry lipopolysaccharides as contaminants, and an antiserum to these contaminants was strain-specific.

MATERIALS AND METHODS

Antigen preparations. Strain B6 of Agrobacterium tumefaciens was grown overnight in nutrient broth (Difco Laboratories, Detroit, Michigan) at 27°C on an orbital shaker, washed several times in 0.02 M phosphate-buffered saline (PBS) to remove exopolysaccharides, and harvested by low-speed centrifugation (10,000 x g for 15 min). These cells were the source of the following antigenic preparations: washed ribosomes (WR), glutaraldehyde-fixed cells (GFC), and lipopolysaccharides (LPS).

Preparations of WR were obtained following a modification (7) of Kurland's procedure (38). In this procedure, the cell lysate was centrifuged and ammonium sulfate added to the supernatant to a concentration of 20%. The precipitate of this first salt cut was removed by low-speed centrifugation. The ammonium sulfate concentration of the supernatant was then raised to 40% to precipitate the ribosomes which were subsequently pelleted by a low-speed centrifugation. The supernatant of this second salt cut was removed and the ribosome-salt pellet was resuspended in TSM buffer (10 mM Tris-base, 3 mM succinic acid, 10 mM $MgCl_2$, 6 mM 2-mercaptoethanol, pH 8.0) and dialyzed overnight against TSM. The suspension was washed in 0.6 M ammonium sulfate by a high-speed centrifugation (180,000 x g for 3 hr). The supernatant from this ultra-centrifugation was removed and the WR pellet was resuspended

and clarified by a low-speed centrifugation. The ribosome concentration was adjusted to 3 mg/ml (7).

GFC were prepared by the method of Allan and Kelman (1) using cell concentrations adjusted to about 10^9 CFU/ml.

LPS were extracted by the hot phenol-water procedure of Westphal and Jann (67), a method commonly used to extract LPS from gram-negative bacteria (41). The bacterial cells (5 g) were suspended in 60 ml distilled water and heated to 67°C; an equal volume of 90% (vol/vol) aqueous phenol at 67°C was added to the suspension, and the mixture was stirred at that temperature for 15 min. After cooling to 4°C, the phases were separated by centrifugation (10,000 x g for 15 min). The aqueous phase was set aside and the phenol phase was heated to 67°C. Sixty ml of 67°C distilled water were added to the hot phenol phase and the above procedure was repeated. The two aqueous phases were combined and dialysed at room temperature against running deionized water for 24 hr. The insoluble fraction was sedimented at 80,000 x g for 8 hr. The sediment was resuspended in water and centrifuged at 105,000 x g for 3 hr. The pellet was resuspended in water and freeze dried. Before use, the LPS concentration was adjusted in PBS to 1 mg/ml.

Antisera production. Antisera were produced in 8-10 week-old New Zealand White rabbits against different antigenic preparations

obtained from Agrobacterium tumefaciens strain B6. The strain-specific unwashed ribosome (UWR) antiserum was obtained from a previous study (8). The GFC antiserum was developed following the method of Allan and Kelman (1). Because purified LPS of gram-negative bacteria have been recognized generally as poor immunogens (32) and as potent endotoxins (41), we used two different methods to produce anti-LPS sera. In the first method, one rabbit was immunized following a modification of Vaitukaitis' procedure (65). The immunization consisted of two series of multiple intradermal injections administered 5 days apart. A total of 500 µg LPS was administered each time. Before injection, the LPS suspension was emulsified with an equal volume of Freund's incomplete adjuvant (Difco). Blood was collected from the marginal ear vein one month after the second injection series and subsequently at weekly intervals for three weeks. In the second method, six intravenous (IV) injections, each containing 75 µg LPS, were administered weekly to a second rabbit. The animal was earbled ten days after the last injection and subsequently at weekly intervals for four weeks. A booster injection (500 µg of LPS) consisting of multiple intradermal injections was given two months after the last IV; the rabbit was earbled three weeks later.

Serological analyses for the identification of strain-specific antigens. All tests for serological relatedness were performed in gel immunodiffusion as described previously (6). However, to increase the resolution of the precipitin bands, larger wells (5 mm

in diameter) were cut in the gel and the antiserum-well was filled twice (at a 15 hr interval) to provide a final volume of 80 µl.

To test whether the three antisera described above were strain-specific, they were reacted against water-phenol extracts of whole cells (WPE) from 39 Agrobacterium strains (listed in ref. 6) and 12 other bacterial species (listed in ref. 7). Because the strain-specific reaction between UWR antisera and these WPE might be due to LPS, we compared the serological reaction of the LPS preparation to that of WPE. The LPS and WPE were reacted against both UWR antiserum and LPS antiserum.

To assure that neither proteins nor nucleic acids contributed to the serological reaction of the LPS preparation, samples of the LPS preparation were treated with appropriate enzymes for 2 hr at 37°C or autoclaved for 15 min at 121°C. Protein digestion was performed using 5 µg of proteinase K (EM Biochemicals, Darmstadt, Germany) per µg of protein (12); protein concentration was measured by the Bio-Rad Protein Micro Assay (Bio-Rad Laboratories, Richmond, California) with lysozyme as a standard. At the end of the reaction, proteinase K activity was stopped by the addition of 1 µl of 0.3 mM phenylmethylsulfonyl fluoride per 10 ug of proteinase K (12). Nucleic acids were digested by the addition of 5 µg of deoxyribonuclease I (pancreatic DNase, Sigma Chemicals Co., St. Louis, Missouri) and 5 µg of ribonuclease A (pancreatic RNase type I-A, Sigma) per ug of nucleic acid (51). The concentration of

nucleic acids was derived from absorbance at 260 nm (56). The effect of each treatment on the antigenic reaction of the LPS preparation was determined by immunodiffusion using the LPS and UWR antisera.

Lastly, to identify the step at which the strain-specific contaminant was removed during the preparation of WR, we compared the reaction of the different by-products of the purification procedure with that of WR in which the strain-specific contaminant was not detected (8). The different by-products analyzed included the precipitate from the first salt cut (FSC) which was dissolved in PBS, the supernatant from the second salt cut (SSC), and the high-speed centrifugation supernatant (HSS).

RESULTS AND DISCUSSION

Lipopolysaccharides (LPS) were identified as the antigenic substances responsible for the strain-specific activity that was reported when antiserum to unwashed ribosomes (UWR) was reacted against water-phenol extracts (WPE) (6; Fig. IV.2A). The reaction of the UWR antiserum with LPS from strain B6 revealed the presence of LPS antibodies in this antiserum which indicates that LPS were present as contaminants in the UWR preparations used for immunization. This reaction of UWR antiserum with LPS antigens was observed as a band of precipitation which fused completely with the precipitin band produced by the strain-specific reaction of the UWR antiserum with WPE of strain B6 (Fig. IV.1A). This serological reaction of identity illustrates the presence of identical antigenic determinants in both the strain-specific antigen and the LPS preparation. Washed ribosomes (WR) did not produce the strain-specific precipitin band (Fig. IV.1A), thus confirming our previous report that WR no longer carry the contaminant present in UWR (8). As expected, WR did react with ribosomal antibodies present in the UWR antiserum (Fig. IV.1A).

Confirmation of the presence of LPS in WPE and their absence in WR preparations was obtained with antiserum to LPS. LPS antibodies were elicited only after multiple intradermal injections of LPS and even then the antibody response was weak as visualized by the development of faint precipitin bands (Fig. IV.1B, IV.2B,

and IV.3A). The animal subjected to two series of intradermal injections gave a stronger reaction than the animal subjected to intravenous injections followed by one series of intradermal injections. Therefore, in our experiment we used the former LPS antiserum. The reaction pattern of LPS antiserum against WPE, WR and LPS was similar to that obtained with the UWR antiserum (Fig. IV.1). The LPS and WPE precipitin bands fused completely, but this common precipitin band could not be detected with the WR (Fig. IV.1B), confirming that the strain-specific antigen contaminating UWR was common to both the WPE and LPS preparations.

The identical bands obtained with the UWR and LPS antisera demonstrate that the strain-specific antigen present in WPE and UWR preparations is also present in LPS preparations. Although our LPS preparation was contaminated with 1.5% proteins and 2.5% nucleic acids, the strain-specificity of the LPS preparation was still present following autoclaving and enzymatic treatments. Because the serological reaction was unaffected by these treatments, we conclude that the strain-specific reaction was not due to protein or nucleic acid contaminants.

LPS from Agrobacterium tumefaciens strain B6 elicited strain-specific antibodies which did not react with WPE from 39 Agrobacterium strains and 12 other bacterial species. Only WPE from strain B6 reacted with the LPS antiserum to B6 to produce a strain-specific precipitin band (Fig. IV.2B). Similarly, the

strain-specificity of bacterial LPS has been reported in different species of the closely related genus Rhizobium (13), as well as other genera (19, 21, 50).

In contrast to the strain-specific reaction of LPS antiserum, a second but faint precipitin line, which could not be reproduced on photographic paper, also devloped when WPE of strain B6 and nine other Agrobacterium strains (A4, C58, K24, K30, GA012, GA003, 2/6, 6/6, and CG-60) were reacted with the LPS antiserum. This faint precipitin band was also observed with difficulty when WPE of these seven heterologous strains were tested against the strain-specific UWR antiserum. This additional antigen shared by the nine Agrobacterium strains may reflect relatively conserved LPS antigens that may be useful for serotyping the agrobacteria. O-specific antigens of LPS are the chemical basis for serological classification of other gram-negative bacteria (16, 35).

Strain-specific antibodies were also detected in the antiserum to GFC. This antiserum reacted only with WPE from strain B6 to produce a band that precipitated near the antiserum well (Fig. IV.2C). A second antigen was revealed by the GFC antiserum. The resulting precipitin band which precipitated next to the antigen well was common to 31 of the 39 Agrobacterium strains tested (Fig. IV.2C). This precipitin band, common to most Agrobacterium strains tested, was also observed with the Rhizobium meliloti strain tested but not the other Rhizobium spp. The presence of an antigen common

to Agrobacterium and R. meliloti is not surprising since these bacteria are very closely related (26, 31). This common antigen may prove useful for the broader serological grouping of Agrobacterium and Rhizobium meliloti.

The steps where LPS was removed during ribosome purification were identified by comparing the three different by-products of the ribosome purification procedure (precipitate of the first salt cut [FSC], the supernatant of the second salt cut [SSC], and the high-speed centrifugation supernatant [HSS]) with antigenic preparations of WR, WPE and LPS using both LPS and UWR antisera. The LPS antiserum reacted against the LPS, WPE, FSC and HSS antigens but not against SSC or WR (Fig. IV.3A). The individual precipitin bands formed against these various antigens were confluent at their junctions, illustrating the serological identity of the precipitated antigens present in the LPS, WPE, FSC and HSS preparations. In contrast, multiple precipitin bands were formed when the different by-products of the ribosome purification procedure and WR were reacted against the UWR antiserum. These complex precipitin bands, which were probably due to ribosomal antigens, were difficult to read, making interpretations difficult. However, the presence of the strain-specific (LPS) antigen band was still visible in the LPS, WPE, and HSS preparations (Fig. IV.3B). LPS antigens were not detected in the WR preparation using either the LPS or UWR antiserum. From the reaction of the LPS antiserum with the different by-products of the

ribosome purification procedure, we were able to determine that the first salt cut and the sedimentation of the ribosomes in 0.6 M ammonium sulfate were the two major steps where LPS were removed from ribosomes.

Contamination of UWR with LPS was not surprising for LPS were reported to be the antigenic determinants in "ribosomal" vaccines prepared from Salmonella (21). LPS were also reported to contaminate proteins of Esherichia coli (15), suggesting that LPS contaminations of bacterial antigens may be more common than we previously suspected. Such LPS contaminants elicit the production of LPS antibodies which may result in serological reactions that could be attributed falsely to other molecules. Such was the case when we first initiated a serological study of Agrobacterium ribosomes and mistakenly concluded that the heterogeneous reactions associated with our contaminated ribosomal preparations were of ribosomal origin (7).

One of the objectives of this study was to isolate and characterize the antigen responsible for the strain-specific reaction so that specific antisera could be prepared and used to detect individual strains of Agrobacterium introduced into the environment. We now know that LPS were responsible for the strain-specific activity. However, antisera to B6 LPS also cross-react with a few other Agrobacterium strains, thus diminishing their utility for the preparation of strain-specific polyclonal

antisera. In addition, purified Agrobacterium LPS were poor immunogens in the rabbit system, which makes it difficult to develop anti-LPS sera. It appears now that the best way to obtain strain-specific antibodies is to prepare monoclonal antibodies. Presently, we are in the process of immunizing mice with heat-killed bacteria to obtain hybridomas that secrete antibodies specific only to LPS from the homologous strain.

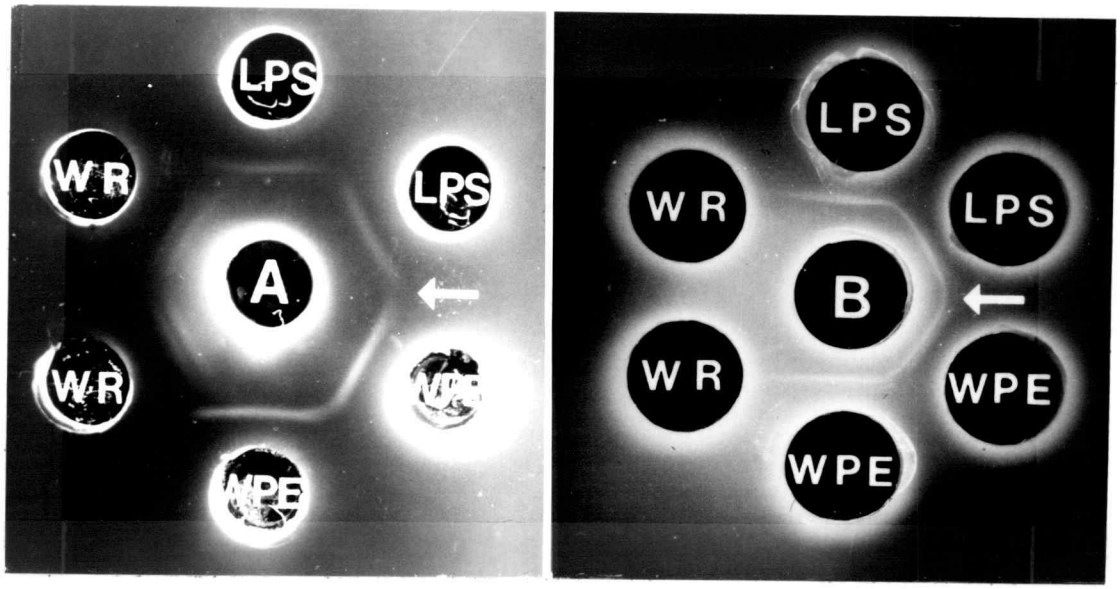

Fig. IV.1 Immunodiffusion reactions of three different antigenic preparations from Agrobacterium tumefaciens strain B6 against antisera to unwashed ribosomes (UWR) and to lipopolysaccharides (LPS). The center well of panel A contains UWR antiserum, whereas the center well of panel B contains LPS antiserum. Outer wells of A and B contain LPS, water-phenol extracts of whole cells (WPE), and washed ribosomes (WR). A precipitin band was produced by LPS and WPE against both antisera. At their junctions the bands coalesced in a reaction of complete fusion (arrow). No similar band was observed with WR.

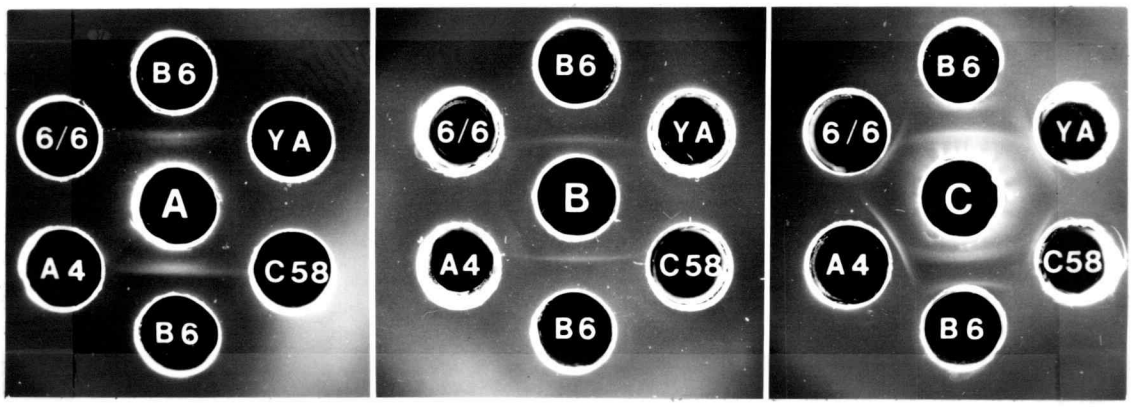

Fig. IV.2 Strain-specific immunodiffusion reactions of three different antisera to A. tumefaciens strain B6. The center wells of panels A, B, and C contain respectively: antiserum to unwashed ribosomes from strain B6, antiserum to B6 lipopolysaccharides, and antiserum to glutaraldahyde-fixed B6 cells (GFC). Outer wells of A, B, and C contain water-phenol extracts of Agrobacterium strains B6, C58, A4, and 6/6, and of Rhizobium meliloti strain YA-15. A strain-specific precipitin band was produced by all three antisera against B6 only. A band which precipitated next to the antigen wells was also produced by the GFC antiserum; this band was common to 31 of 39 Agrobacterium strains and the Rhizobium meliloti strain tested.

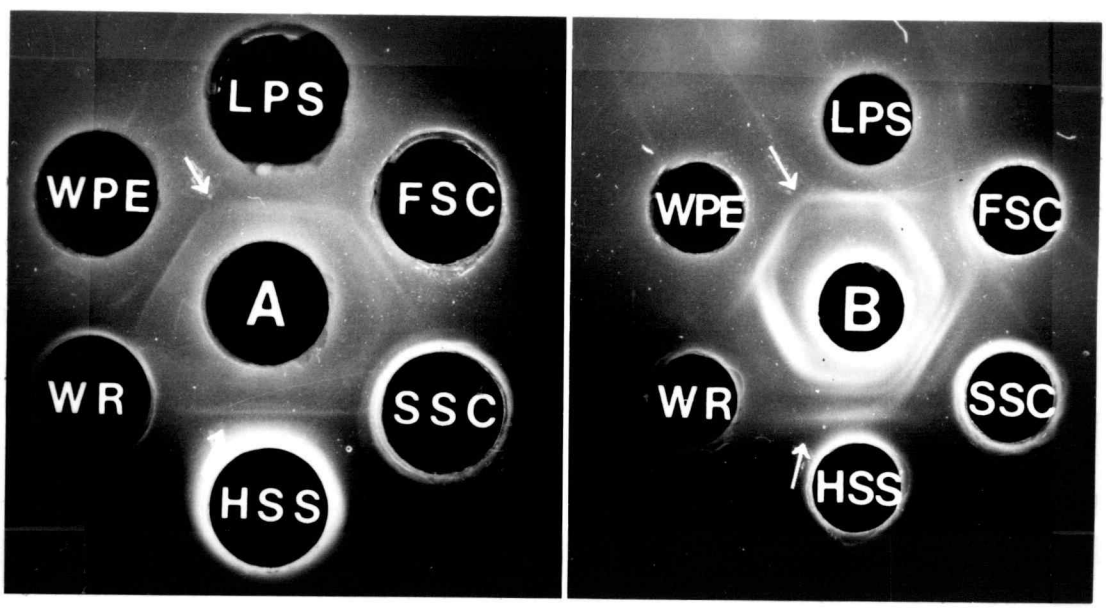

Fig. IV.3 Immunodiffusion reactions of antisera to lipopolysaccharides (LPS) and to unwashed ribosomes (UWR) against LPS, water-phenol extracts of whole cells (WPE), washed ribosomes (WR), and the different by-products of the ribosome purification procedure (i.e., the precipitate from the first salt cut [FSC], the supernatant from the second salt cut [SSC], and the high-speed centrifugation supernatant [HSS]). The center wells of panels A and B contain respectively LPS antiserum and UWR antiserum. Outer wells of A and B contain LPS, FSC, SSC, HSS, WR, and WPE. A precipitin band common to LPS, FSC, HSS, and WPE was produced against the LPS antiserum (arrows; panel A). The outermost precipitin band produced by the UWR antiserum was only observed with LPS, HSS, and WPE (arrows; panel B). In both tests, this band was not detected with WR.

CONCLUSIONS

Serological techniques developed during this thesis research can be used to identify agrobacteria at different levels of specificity, depending on the targeted antigen. The presence of ribosomal antigens that are common to both Agrobacterium and Rhizobium allows the development of antisera to 50 S ribosomal subunits which are broadly specific. In contrast, the diversity of cell surface lipopolysaccharide antigens allows the production of very specific antisera which can differentiate between individual strains of Agrobacterium.

The value of ribosomal serology lies in the uniqueness of ribosomal antigens which elicit antisera specific to very closely related bacteria. The large ribosomal subunits of members of the Agrobacterium-Rhizobium group are serologically identical. Antisera to 50 S ribosomal subunits provide a reliable tool for identification of strains of the Agrobacterium-Rhizobium group isolated from nature. These antisera were used to confirm the identity of naturally occuring nonpathogenic Agrobacterium strains which did not fit the reported physiological groupings of members of this genus (5). One drawback to using ribosomal serology is the limited number of strains that can be tested because of the complexity of the methods used to isolate and wash the ribosomes for immunization and for test-antigens.

Kurland's procedure (38) for isolation of ribosomes is superior to Schaad's methods (52) because it yielded ribosomes freed of nonribosomal contaminants. Kurland's ammonium sulfate washing is a ncecessary step to show the serological identity of ribosomes from Agrobacterium and Rhizobium. Use of unwashed ribosome preparations result in heterogeneous precipitation patterns with multiple bands due to the presence of cell surface contaminants that bind nonspecifically to the unwashed ribosomes.

On the other hand, these contaminants induced the production of strain-specific antibodies. The contaminants of interest are lipopolysaccharide antigens which are removed by an initial salt cut with 20% ammonium sulfate and subsequent sedimentation of ribosomes in 0.6 M ammonium sulfate.

The strain-specificity of the serological reaction can be substantiated by DNA restriction profile analysis. Total DNA from strains of Agrobacterium digested with the restriction enzyme EcoRI have characteristic DNA banding patterns. The restriction fragments length polymorphisms of individual Agrobacterium strains correlated with the strain-specific serological reaction. The complementarity of the DNA profiles and the serology was used to rapidly and accurately identify Agrobacterium strains released into the environment (6).

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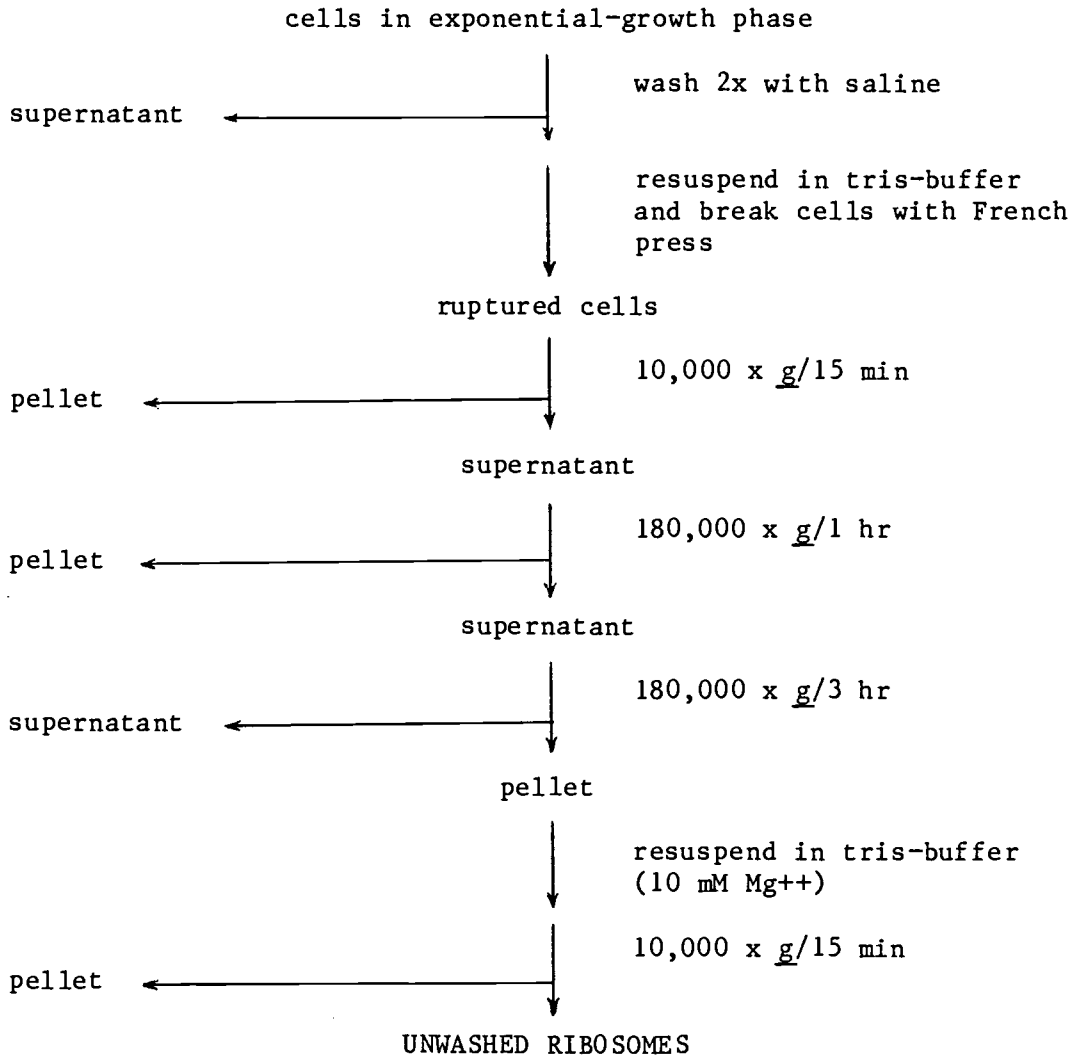
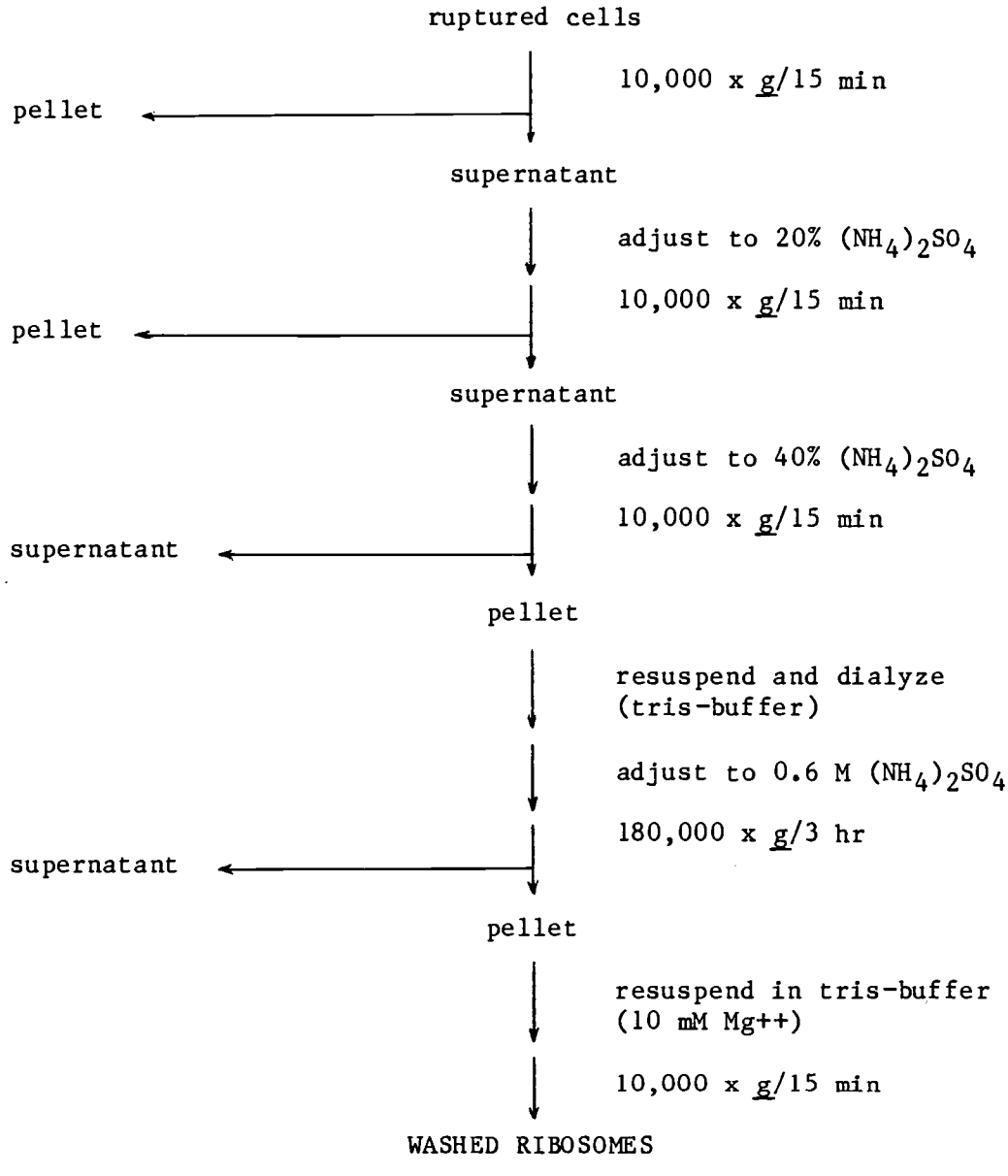
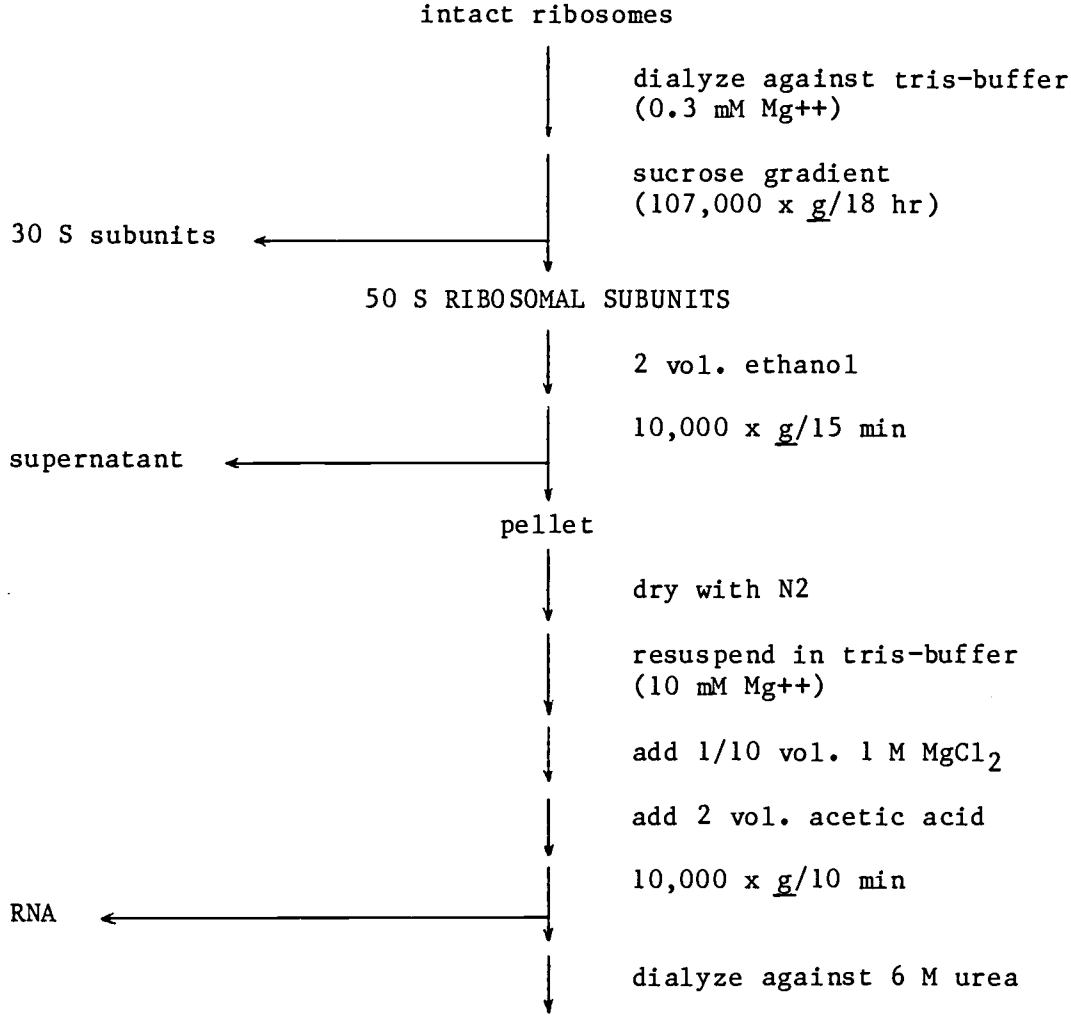
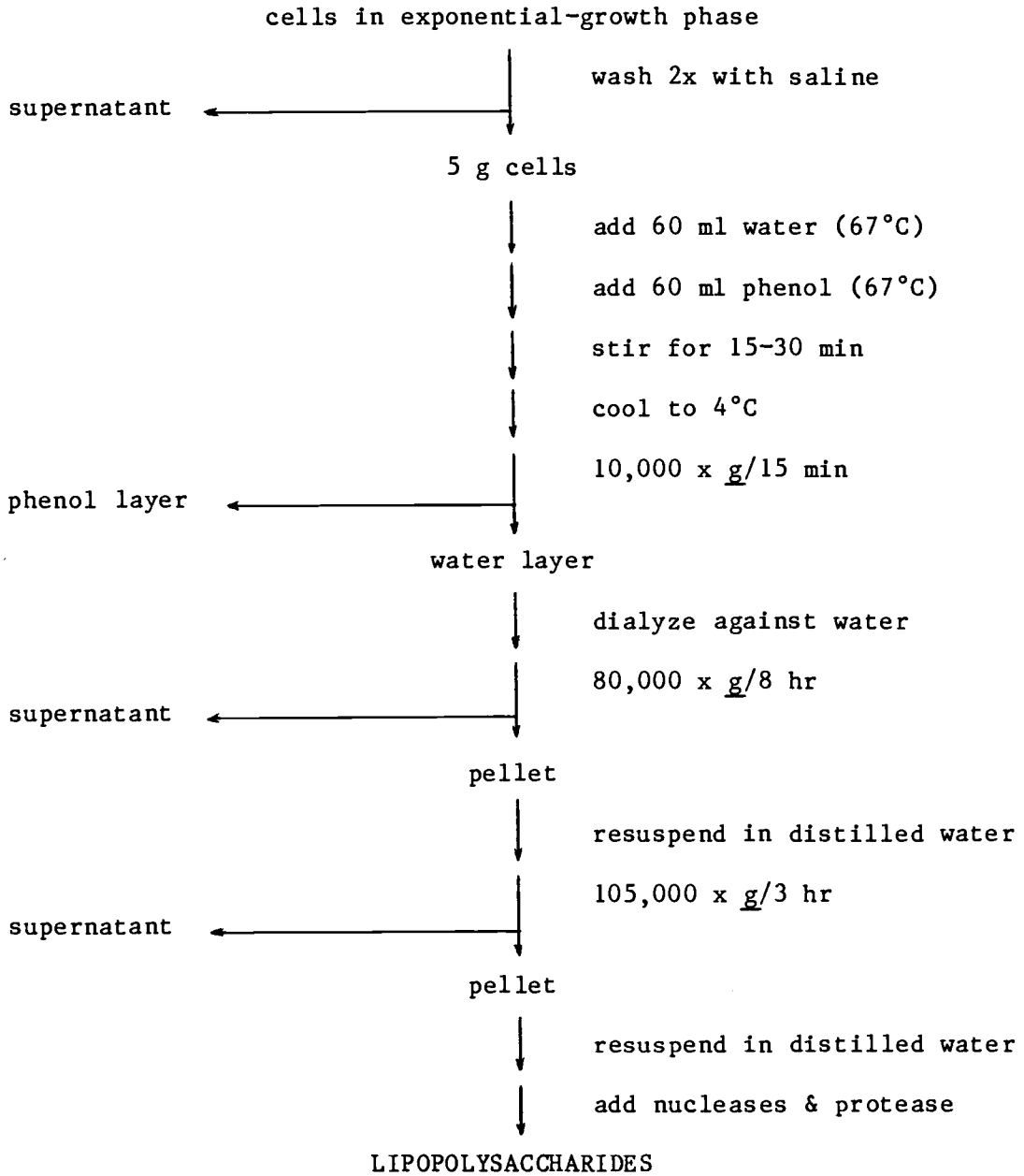
APPENDIX


cells in exponential-growth phase
wash 2x with saline
supernatant
resuspend in tris-buffer
and break cells with French
press
ruptured cells
10,000 x g/15 min
pellet
supernatant
180,000 x g/1 hr
pellet
supernatant
180,000 x g/3 hr
supernatant
pellet
resuspend in tris-buffer
(10 mM Mg++)
10,000 x g/15 min
pellet
UNWASHED RIBOSOMES


ruptured cells
10,000 x g/15 min
pellet
supernatant
adjust to 20% $(NH_4)_2SO_4$
10,000 x g/15 min
pellet
supernatant
adjust to 40% $(NH_4)_2SO_4$
10,000 x g/15 min
supernatant
pellet
resuspend and dialyze (tris-buffer)
adjust to 0.6 M $(NH_4)_2SO_4$
180,000 x g/3 hr
supernatant
pellet
resuspend in tris-buffer (10 mM Mg++)
10,000 x g/15 min
WASHED RIBOSOMES


intact ribosomes
dialyze against tris-buffer
(0.3 mM Mg++)
sucrose gradient
(107,000 x g/18 hr)
30 S subunits
50 S RIBOSOMAL SUBUNITS
2 vol. ethanol
10,000 x g/15 min
supernatant
pellet
dry with N2
resuspend in tris-buffer
(10 mM Mg++)
add 1/10 vol. 1 M MgCl2
add 2 vol. acetic acid
10,000 x g/10 min
RNA
dialyze against 6 M urea
PROTEINS FROM 50 S SUBUNITS


cells in exponential-growth phase
wash 2x with saline
supernatant
5 g cells
add 60 ml water (67°C)
add 60 ml phenol (67°C)
stir for 15-30 min
cool to 4°C
10,000 x g/15 min
phenol layer
water layer
dialyze against water
80,000 x g/8 hr
supernatant
pellet
resuspend in distilled water
105,000 x g/3 hr
supernatant
pellet
resuspend in distilled water
add nucleases & protease
LIPOPOLYSACCHARIDES