Techniques

Lipopolysaccharide from *Agrobacterium tumefaciens* B6 Induces the Production of Strain-Specific Antibodies

Hacene Bouzar, Larry W. Moore, and Henry W. Schaup

First and second authors, Department of Botany and Plant Pathology, Oregon State University, Corvallis 97331; third author, Department of Biochemistry and Biophysics, Oregon State University, Corvallis 97331. Present address of first author: Département de Phytopathologie, Institut National d'Enseignement Supérieur en Agronomie, Universite de Blida, B.P. 24 Douirete, Blida, Algeria.
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ABSTRACT

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A lipopolysaccharide (LPS) preparation from *Agrobacterium tumefaciens* strain B6 elicited rabbit antibodies that reacted with water-phenol extracts of whole cells to form a strain-specific precipitin band in gel immunodiffusion plates. This antiserum to B6 LPS did not react with water-phenol extracts from 38 other *Agrobacterium* strains or 12 bacterial species from eight other genera. An additional precipitin band, although only slightly visible, developed against LPS from B6 and nine of the other 38 *Agrobacterium* strains tested. In this study, LPS was identified as the contaminating strain-specific antigen associated with ribosomal preparations described in earlier studies. LPS was removed from the ribosomal preparation by initial precipitation with 20% ammonium sulfate and sedimentation of the ribosomes in 0.6 M ammonium sulfate.

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 a 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 of whole cells (2). However, these strain-specific antisera cross-reacted with whole *Agrobacterium* cells, suggesting that the ribosome preparations used for immunization also contained contaminants from the surface of the bacterium (2,4). These contaminating antigens were not detected in purified ribosome preparations obtained following salt fractionation and a subsequent sedimentation in 0.6 M ammonium sulfate (4).

The objective of the present study was to identify the antigen(s) that elicited the strain-specific antibodies. We hypothesized that the strain-specific activity was due to a lipopolysaccharide (LPS) because the antiserum cross-reacted with a cell surface antigen, the cross-reacting antigen was heat-stable and was readily extracted by water-phenol (2,4). In this paper, we present evidence that unwashed-ribosome preparations of *Agrobacterium tumefaciens* strain B6 are contaminated with LPS, and that an antiserum to this contaminant is strain-specific.

MATERIALS AND METHODS

Antigen preparations. Strain B6 of *Agrobacterium tumefaciens* was grown overnight in nutrient broth (Difco Laboratories, Detroit, MI) at 27 C on an orbital shaker, washed several times in

0.02 M phosphate-buffered saline (PBS, pH 7.2) to remove exopolysaccharides, and harvested by centrifugation at 10,000 *g* for 15 min. These cells were the source of the following antigenic preparations: washed-ribosomes, glutaraldehyde-fixed cells, and LPS.

Preparations of washed-ribosomes were obtained following a modification (3) of Kurland's procedure (15). In this procedure, the cell lysate was centrifuged (10,000 *g* for 15 min) and ammonium sulfate added to the supernatant solution to a concentration of 20%. The precipitate of this first salt cut was removed by centrifugation at 10,000 *g* for 15 min. The ammonium sulfate concentration of the supernatant was then raised to 40% to precipitate the ribosomes, which were subsequently pelleted by centrifugation as described above. 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 $MgCl_2$, and 6 mM 2-mercaptoethanol, pH 8) and dialyzed overnight against TSM. The suspension was washed in 0.6 M ammonium sulfate by centrifugation at 180,000 *g* for 3 hr. The supernatant was removed and the pellet of washed-ribosomes was resuspended in TSM buffer and clarified by centrifugation at 10,000 *g* for 15 min. The ribosome concentration, which was based on a specific extinction coefficient of 157 for a 1% solution at 260 nm, was adjusted to 3 mg/ml (3).

Glutaraldehyde-fixed cells were prepared by the method of Allan and Kelman (1) using cell concentrations adjusted to about 10^9 cfu/ml (100 Klett units).

LPS were extracted by the hot phenol-water procedure of Westphal and Jann (21), a method commonly used to extract LPS from Gram-negative bacteria (16). The bacterial cells (5 g) were suspended in 60 ml of distilled water and heated to 67 C; an equal volume of 90% (v/v) 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 *g* for 20 min). The aqueous phase was set aside and the phenol phase was heated to 67 C. Sixty milliliters of 67 C distilled water was added to the hot phenol phase and the above procedure was repeated. The two aqueous phases were combined and dialyzed at room temperature against running deionized water for 24 hr. The insoluble fraction was sedimented at 80,000 *g* for 8 hr. The sediment was resuspended in water and centrifuged at 105,000 *g* for 3 hr. The pellet was resuspended in PBS and lyophilized. Before use, the LPS concentration was adjusted to 1 mg/ml.

Production of antisera. Antisera were produced in 8–10 wk-old New Zealand White rabbits against different antigenic preparations obtained from *A. tumefaciens* strain B6. Before beginning the immunization, preimmune sera were collected from marginal ear veins of each animal. The strain-specific antiserum to unwashed ribosomes was obtained from a previous study (2). The antiserum to glutaraldehyde-fixed cells was developed following the method of Allan and Kelman (1). Because purified LPS of Gram-negative bacteria have been recognized generally as poor immunogens (13) and as potent endotoxins (16), we used two different methods to produce anti-LPS sera. In the first method, one rabbit was immunized following a modification of Vaitukaitis's procedure (20). The immunization consisted of two series of multiple intradermal injections administered 5 days apart. A total of 500 μg of 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 1 mo after the second injection series and subsequently at weekly intervals for 3 wk. In the second method, six intravenous injections, each containing 75 μg of LPS, were administered weekly to a second rabbit. The animal was ear-bled 10 days after the last injection and subsequently at weekly intervals for 4 wk. A booster injection (500 μg of LPS) consisting of multiple intradermal injections was given 2 mo after the last intravenous injection; the rabbit was ear-bled 3 wk later.

Serological analyses for the identification of strain-specific antigens. All tests for serological relatedness were performed by gel immunodiffusion as described previously (2). However, to improve 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) for 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 from 39 *Agrobacterium* strains (Table 1) and 12 other bacterial species (Table 2). Because the strain-specific reaction between unwashed-ribosome antisera and these water-phenol extracts might be due to LPS, we compared the serological reaction of the B6 LPS preparation to that of water-phenol extract of B6. The LPS and water-phenol extract were reacted against both unwashed-ribosome 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 with 5 μg of proteinase K (EM Biochemicals, Darmstadt, West Germany) per microgram of protein (5); protein concentration was measured by the Bio-Rad Protein Micro Assay (Bio-Rad Laboratories, Richmond, CA) with lysozyme as a standard. Proteinase K activity was stopped as described previously (5). Nucleic acids were digested by the addition of 5 μg of deoxyribonuclease I (pancreatic DNase, Sigma, St. Louis, MO) and 5 μg of ribonuclease A (pancreatic RNase type I-A, Sigma) per microgram of nucleic acid (18). The concentration of nucleic acids was derived from absorbance at 260 nm (19). The effect of each

TABLE 1. Sources of *Agrobacterium* strains used as antigens

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	Colombia	
K24	1	INA[b]	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	
A. radiobacter				
T20/73	1	Rose	Oregon	
K84	2	Soil	Australia	

[a] Unless specified otherwise, strains were from our laboratory.
[b] Information not available.

treatment on the antigenic reaction of the LPS preparation was determined by immunodiffusion against antiserum to unwashed-ribosomes.

Lastly, to identify the step at which the strain-specific contaminant was removed during the preparation of washed ribosomes, we compared the reaction of the different by-products of the purification procedure with that of washed ribosomes in which the strain-specific contaminant was not detected (4). The different by-products analyzed included the precipitate from the first salt cut that was dissolved in PBS, the supernatant from the second salt cut, and the high-speed centrifugation supernatant.

RESULTS AND DISCUSSION

LPS was identified as the antigenic substance responsible for the strain-specific activity that was reported when antiserum to unwashed ribosomes was reacted against water-phenol extracts (2; Fig. 1A). The reaction between antiserum to unwashed ribosomes and LPS from strain B6 revealed the presence of anti-LPS antibodies in this antiserum. This suggests that LPS was present as a contaminant in the preparation used for immunization. This reaction was observed as a band of precipitation that fused completely with that produced by the strain-specific reaction of the antiserum to unwashed ribosomes with water-phenol extract of strain B6 (Fig. 2A). The serological reaction of identity illustrates the presence of identical antigenic determinants in both the strain-specific antigen and the LPS preparation. Washed ribosomes did not produce the strain-specific precipitin band (Fig. 2A), thus confirming our previous report that washed ribosomes no longer carry the contaminant present in unwashed ribosomes (4). As expected, washed ribosomes did react with anti-ribosome antibodies present in the antiserum to unwashed ribosomes (Fig. 2A).

TABLE 2. Bacterial species, other than *Agrobacterium*, that were used as antigens

Species	Strain	Origin	Source
Rhizobium meliloti	YA-15	INA[a]	2[b]
R. leguminosarum	128A12	INA	9
R. l. bv. *trifolii*	162S7a	INA	9
R. l. bv. *phaseoli*	127K12b	INA	9
Pseudomonas solanacearum	51	Potato	8
Pseudomonas syringae pv. *syringae*	B3	Peach	4
Xanthomonas campestris pv. *campestris*	B24	Borccoli	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

[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.

Confirmation of the presence of LPS in the water-phenol extract and its absence from the washed ribosome preparation was obtained with antiserum to LPS. Anti-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 (Figs. 1B, 2B, and 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 antiserum. The reaction pattern of LPS antiserum against water-phenol extract, washed ribosomes, and LPS was similar to that obtained with the antiserum directed against unwashed-ribosomes (Fig. 2). The LPS and water-phenol extract precipitin bands fused completely, but this common precipitin band could not be detected with the washed ribosome preparation (Fig. 2B), confirming that the strain-specific antigen contaminating unwashed ribosomes was common to both the water-phenol extract and LPS preparations.

The identical bands obtained with the unwashed ribosome and LPS antisera demonstrate that the strain-specific antigen present in the water-phenol extract and the unwashed ribosome preparation is also present in the LPS preparation. Although our LPS preparation was contaminated with 1.5% proteins and 2.5% nucleic acids, the strain-specificity of the LPS preparation was retained following autoclaving and enzymatic treatments.

LPS from *A. tumefaciens* strain B6 elicited strain-specific antibodies that did not react with water-phenol extracts from 39 other *Agrobacterium* strains and 12 other bacterial species (Fig. 1B). Similarly, the strain-specificity of bacterial LPS has been reported in different species of the closely related genus *Rhizobium* (6), as well as other genera (9,10,17).

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 developed when water-phenol extracts 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 (Fig. 1B). This faint precipitin band was also observed with difficulty when water-phenol extracts of these nine heterologous strains were tested against the strain-specific antiserum to unwashed ribosomes. This additional antigen shared

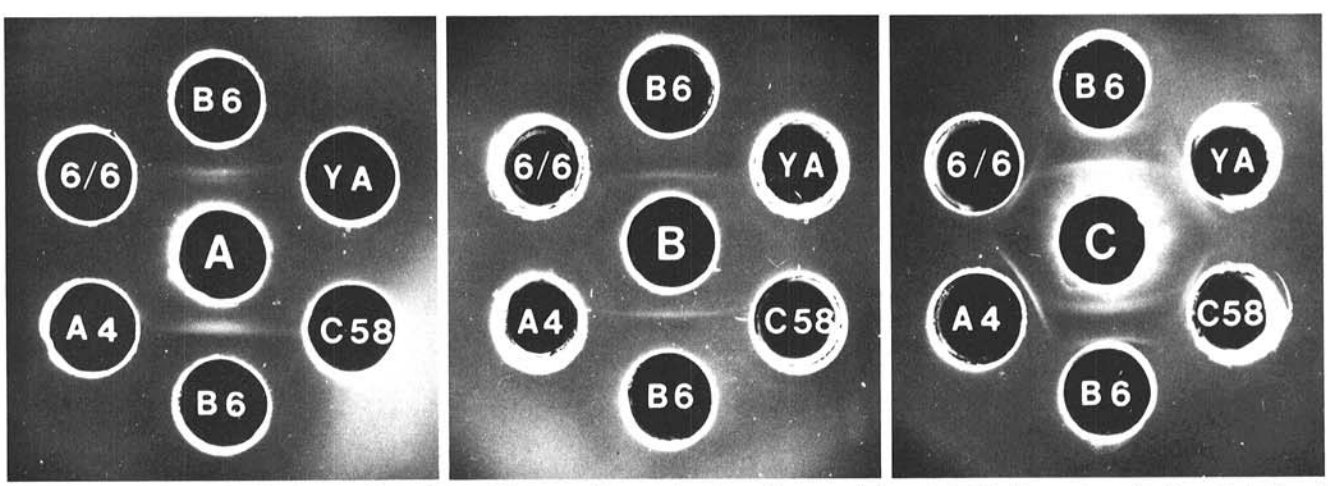

Fig. 1. Strain-specific immunodiffusion reactions of three different antisera to *Agrobacterium tumefaciens* strain B6. The center wells of panels A, B, and C contain, respectively: antiserum to unwashed-ribosomes from strain B6, antiserum to B6 lipopolysaccharide, and antiserum to glutaraldehyde-fixed B6 cells. 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 that precipitated next to the antigen wells was also produced by the antiserum to glutaraldehyde-fixed cells; this band was common to 31 of 39 *Agrobacterium* strains and the *Rhizobium meliloti* strain tested.

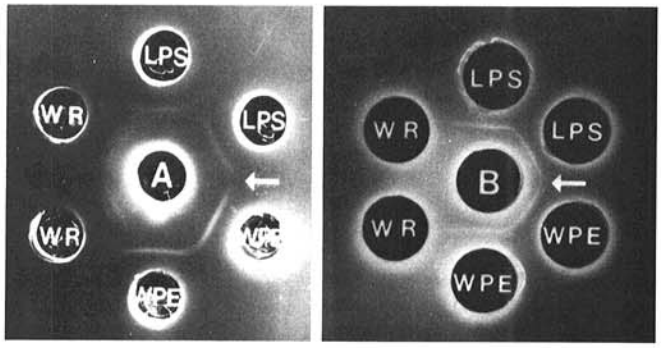

Fig. 2. Immunodiffusion reactions of three different antigenic preparations from *Agrobacterium tumefaciens* strain B6 against antisera to unwashed-ribosomes and to LPS. The center well of panel A contains antiserum directed against unwashed-ribosomes, whereas the center well of panel B contains LPS 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.

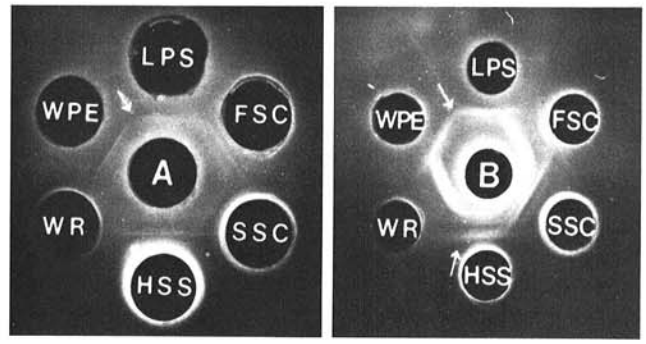

Fig. 3. Immunodiffusion reactions of antisera to LPS and to unwashed-ribosomes against LPS, water-phenol extract of whole cells (WPE), washed-ribosomes (WR), and the different by-products of the ribosome purification procedure (i.e., the precipitate from the first salt cut [FSC], the supernatant from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

by the nine *Agrobacterium* strains may reflect conserved LPS antigens that may be useful for serotyping the agrobacteria. 0-specific antigens of LPS are the chemical basis for the serological classification of other Gram-negative bacteria (8,14).

Strain-specific antibodies were also detected in the antiserum to glutaraldehyde-fixed cells. This antiserum reacted only with water-phenol extract from strain B6 to produce a band that precipitated near the antiserum well (Fig. 1C). A second antigen was revealed by the antiserum directed against glutaraldehyde-fixed cells. The resulting precipitin band, which precipitated next to the antigen well was common to 31 of the 39 *Agrobacterium* strains tested (Fig. 1C). 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 (11,12). This common antigen may prove useful for the rapid serological identification of *Agrobacterium* and *R. meliloti* isolates.

The steps where LPS was removed during ribosome purification were identified by comparing the three different by-products of the ribosome purification procedure (i.e., the precipitate of the first salt cut, the supernatant of the second salt cut, and the supernatant from the high-speed centrifugation) with antigenic preparations of washed-ribosomes, water-phenol extract, and LPS using both antisera to LPS and to unwashed-ribosomes. The LPS antiserum reacted against the LPS, the water-phenol extract, the first salt cut, and the high-speed supernatant but not against the second salt cut or washed-ribosomes (Fig. 3A). The individual precipitin bands that developed against these various preparations were confluent at their junctions, illustrating the serological identity of the precipitated antigens present in the LPS, water-phenol extract, first salt cut, and high-speed supernatant preparations. In contrast, multiple precipitin bands were formed when the different by-products of the ribosome purification procedure and washed-ribosomes were reacted against the antiserum to unwashed-ribosomes. 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 (i.e., LPS) antigen band was still visible in the LPS, water-phenol extract, and high-speed supernatant preparations (Fig. 3B). LPS antigen was not detected in the washed-ribosome preparation using either the LPS or unwashed-ribosome 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 was removed from ribosomes.

Contamination of unwashed-ribosomes with LPS was not surprising, because LPS was reported to be the antigenic determinant in ribosomal vaccines prepared from *Salmonella* (10). LPS was also reported to contaminate proteins of *Escherichia coli* (7), 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 (3).

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 was responsible for the strain-specific activity. However, antisera to B6 LPS produced against a few other *Agrobacterium* strains a faint precipitin band; this cross-reactivity diminishes the utility of LPS for the preparation of strain-specific polyclonal antisera. In addition, purified *Agrobacterium* LPS was poorly immunogenic 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.

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