Serogroups of *Agrobacterium tumefaciens* Biovar 3 Determined Using Somatic Antigens*

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Abstract

Serological specificity of *Agrobacterium tumefaciens* biovar 3 was tested by means of agglutination and Ouchterlony double diffusion tests, using rabbit antisera prepared by injecting heat-treated (100°C, 1 hr) cell suspensions of 4 Japanese strains of G-Ag-19, 26, 27 and 60, and strain NCPPB 2562, a representative strain for biovar 3 isolated in Greece. In the agglutination test, 39 strains of biovar 3 except strain G-Ag-19 were separated into 4 distinct groups (A-D) according to the titers against 5 antisera. The same group-specific reactions were observed in the Ouchterlony double diffusion test as those in the agglutination test. Each of the biovar 3 strains except strain G-Ag-19 formed several group-specific precipitin bands only in combination with the antiserum prepared against the member of the same group. These results suggest that the biovar 3 strains used are serologically heterogeneous and can be separated into 4 serogroups (A-D), which differ from one another in the reactivity of somatic antigens (heat-stable antigens).

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Key words: *Agrobacterium tumefaciens*, biovar 3, somatic antigen, serogroup, agglutination test, Ouchterlony double diffusion test.

INTRODUCTION

The incidence of grapevine crown gall disease has gradually increased in new plantations and nurseries in Japan¹²⁾. It is postulated that the frequent occurrence of the disease results from planting of symptomlessly infected vines produced by vegetative propagation and wounding of them by freezing temperatures⁵⁾. Thus, simple and convenient methods such as serodiagnosis for the detection of the causal agent, *Agrobacterium tumefaciens* biovar 3, are required to study the epidemiology and to index propagation materials.

Bishop *et al.*⁵⁾ produced a monoclonal antibody against biovar 3 and reported its application to diagnosis. However, Alarcon *et al.*¹⁾ and Miller and Vrugink¹⁰⁾ reported the presence of serological heterogeneity within each of the *Agrobacterium* biovars and suggested the existence of several "serotypes". Moreover, Bouzar *et al.*²⁻⁴⁾ presented the evidence that lipopolysaccharide obtained from strain B6 (*A. tumefaciens* biovar 1) induced the production of strain-specific antibodies. Therefore, further studies are required to assess the serological specificity of biovar 3, by using a larger number of strains derived from various sources.

In this paper, we examined the serological relationships among the biovar 3 strains, and classified them into serogroups according to the difference of somatic antigens.

MATERIALS AND METHODS

Bacteria. Serological relationships among the biovar 3 strains and specificity of antisera were

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examined by using 65 strains of *Agrobacterium*, including 12 overseas strains, listed in Table 1. In this paper, we follow the classical nomenclature of *Agrobacterium* presented in Bergey's Manual of Systematic Bacteriology⁸⁾ for the moment to avoid confusion, although another valid naming system was proposed¹¹⁾. Problems relating to the classification and nomenclature of *Agrobacterium* were discussed in the previous reports^{13,14)}. Three type strains of *Rhizobium* (Table 1), 12 species of phytopathogenic bacteria belonging to *Clavibacter*, *Erwinia*, *Pseudomonas* and *Xanthomonas*, and 2 strains of *Escherichia coli* were used for comparison.

Antisera. Strain NCPPB 2562⁸⁾, a representative strain for biovar 3 isolated in Greece, and four Japanese strains of G-Ag-19, 26, 27 and 60 identified as biovar 3¹²⁾ were used as antigens for the production of the antisera. Each strain was grown on a PPGA¹²⁾ slant for 48 hr at 28°C, suspended and washed in saline solution three times by centrifugation (7,000 rpm, 10 min). The final precipitate was resuspended into saline solution at a concentration of 10⁹ cfu/ml, and the suspension was heated at 100°C for 1 hr to destroy heat-labile antigens. Somatic antigens (heat-treated cells) thus prepared were injected intramuscularly twice [mixed with an equal volume (0.5 ml) of Freund's incomplete adjuvant] and intravenously 7 times (1 ml; twice, 1.5 ml; once, 2 ml; 4 times) to rabbits. Blood was collected on the 7th day after the final injection. Antiserum was heated at 56°C for 30 min to inactivate the complement and stored at -80°C.

Agglutination test. For testing the agglutination titer of antiserum, twofold dilutions of

Table 1. Strains of *Agrobacterium* and *Rhizobium* used in the present experiment

Taxon	Strain ^{a)}
Biovar 1	
<i>A. tumefaciens</i>	NCPPB 2437** (ATCC 23308) ^{b)} MAFF 03-01001, 03-01222, 03-01224, 03-01278
<i>A. rhizogenes</i>	MAFF 03-01724, 03-01725, 03-01726, 03-01727
<i>A. radiobacter</i>	IAM 12048** (ATCC 19358, NCPPB 3001) IAM 1527 (ATCC 6466) IAM 1526 (ATCC 4718)
Biovar 2	
<i>A. tumefaciens</i>	NCPPB 2303*, MAFF 03-01546
<i>A. rhizogenes</i>	IFO 13257** (ATCC 11325, NCPPB 2991)
<i>A. radiobacter</i>	Kerr 84* (NCPPB 2407)
Biovar 3	
<i>A. tumefaciens</i>	NCPPB 2562* YGAt 32-3, 33-1, 35-2 G-Ag-4, 9, 14, 19, 21, 23, 26, 27, 29, 31, 33, 35, 37, 39, 43, 45, 46, 48, 50, 52, 54, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67 K-Ag-1, 2
<i>A. rubi</i>	IFO 13261** (ATCC 13335, NCPPB 1854) IFO 13260 (ATCC 13334, NCPPB 1856)
Unclassified	
<i>A. tumefaciens</i>	NCPPB 1650, 1771 K-Ag-3, 4 Ch-Ag-4, 5, 7, 8
<i>Rhizobium meliloti</i>	IAM 12611** (ATCC 9930, IFO 14782)
<i>R. leguminosarum</i>	IAM 12609** (ATCC 10004, IFO 14778)
<i>R. loti</i>	IAM 13588** (ATCC 33669, IFO 14779)

a) For assignment of strains to taxa and their origin, see previous reports^{12,13)}.

*: representative strain for the corresponding biovar⁸⁾. **: type strain.

b) The numbers in parentheses are other strain designations.

Abbreviations for culture collections: ATCC, American Type Culture Collection; IAM, Institute of Applied Microbiology, University of Tokyo; IFO, Institute for Fermentation; MAFF, Ministry of Agriculture, Forestry and Fisheries, Japan; NCPPB, National Collection of Plant Pathogenic Bacteria.

antiserum were prepared from 1:20 to 1:40, 960 with 0.5 ml of saline for dilution in tubes. To each of the above tubes, an equal volume of antigen (living cell suspension at a concentration of 10^9 cfu/ml) was added. The tubes were incubated at 37°C for 2 hr and then kept at 4°C overnight, until agglutination was observed. The agglutination titer was expressed as the reciprocal of the highest dilution in which distinct agglutination occurred.

Ouchterlony double diffusion test. Sixteen ml of agar gel [Tris-HCl buffer (0.2 M, pH 7.4) 100 ml, polyethylene glycol 3 g, NaN_3 0.1 g, agar 1.5 g] was dispensed into a 9-cm petri dish. Wells, 4 mm in diameter and 4 mm apart, were cut in sets of six peripheral wells surrounding a center well. Twenty μl of undiluted antisera and antigens (heat-treated dense suspensions) were dispensed into the wells, and the plates were incubated at 28°C and observed for 1 week.

RESULTS

Specificity of antisera

Five antisera prepared against strains G-Ag-27, 26, 60, NCPPB 2562 and G-Ag-19 gave high agglutination titers of 2,562, 20,480, 10,240, 2,562 and 2,562, respectively, in homologous combinations (Table 2). In contrast, these antisera gave no visible agglutination against strains other than biovar 3, except that strain K84 (*A. radiobacter* biovar 2), *R. meliloti*, *R. loti*, *P. solanacearum* and *E. coli* showed extremely low titers ranging from 20 to 160 against some of these antisera (data not shown). Moreover, these antisera formed no precipitin bands in combination with strains other than biovar 3 (data not shown). Thus, all the antisera produced in the present experiment were found to be highly specific.

Agglutination test

To clarify the serological relationships among the biovar 3 strains, 40 strains of biovar 3 isolated from grapevine and kiwifruit were examined by cross-agglutination test. Thirty-nine strains except strain G-Ag-19 were divided into 4 distinct groups (A-D) according to the titers against 5 antisera (Table 2). Namely, 29 strains of group A, including strain G-Ag-27, showed high titers only in combination with the antiserum prepared against strain G-Ag-27, whereas they gave low titers against the others. Two kiwifruit isolates (K-Ag-1 and 2) also belonged to group A. Eight strains, which reacted strongly only with the antiserum prepared against strain G-Ag-26, were assigned to group B. The antisera prepared against strains G-Ag-60 and NCPPB 2562 were specific to only homologous strains, indicating that these two strains are serologically distinct and can be separated into groups C and D, respectively. Although strain G-Ag-19 exhibited strong reactions to all the 5 antisera, the antiserum prepared against

Table 2. Agglutination titers of antisera to heat-treated cells of biovar 3 against biovar 3 strains

Serogroup	Strain	Antiserum to heat-treated cells of				
		G-Ag-27	G-Ag-26	G-Ag-60	NCPPB 2562	G-Ag-19
A	G-Ag-4, 9, 14, 27, 29, 31, 33, 35, 37, 39, 43, 45, 50, 52, 54, 56, 57, 58, 59, 62, 63, 64, 65	640	160	40	20	40
	YGAt 32-3, 33-1, 35-2	5,120	640	640	160	80
	K-Ag-1, 2	(2,560) ^{a)}				
	NCPPB 1771					
		20	5,120	40	20	80
B	G-Ag-21, 23, 26, 46, 48, 61, 66, 67	80	20,480 (20,480) ^{a)}	320	160	160
C	G-Ag-60	40	160	10,240	160	80
D	NCPPB 2562	80	80	40	2,560	20
(not grouped)	G-Ag-19	5,120	10,240	5,120	2,560	2,560

a) Titer obtained in homologous combination.

Table 3. Ouchterlony double diffusion reaction of antisera to heat-treated cells of biovar 3 against biovar 3 strains

Serogroup ^{a)}	Number of strains ^{a)}	Common precipitin band ^{b)}	Antiserum to heat-treated cells of				
			G-Ag-27	G-Ag-26	G-Ag-60	NCPBP 2562	G-Ag-19
A	29	+ ^{c)}	>3 ^{d)}	0	0	0	0
B	8	+	0	>1	0	0	0
C	1	+	0	0	>2	0	0
D	1	+	0	0	0	>2	0
(G-Ag-19)	1	+	0	0	0	0	0

a) See Table 2.

b) See Fig. 1.

c) +: common bands which coalesced and fused completely with each other were produced against antisera to biovar 3.

d) Number of serogroup-specific bands formed against each antiserum.

Fig. 1. Ouchterlony double diffusion patterns of antisera to heat-treated cells of biovar 3 against representative strains of serogroups. Wells a, b, c, d and e contained antiserum to G-Ag-27 (serogroup A), G-Ag-26 (B), G-Ag-60 (C), NCPBP 2562 (D) and G-Ag-19 (ungrouped), respectively. Wells 1, 2, 3, 4, 5 and 6 contained heat-treated cells of G-Ag-27 (A), NCPBP 1771 (A), G-Ag-26 (B), G-Ag-60 (C), NCPBP 2562 (D) and G-Ag-19 (ungrouped), respectively. A single, common precipitin band which coalesced and fused completely with the others was produced (outermost; panel I, innermost; panel II). Serogroup A-specific bands were observed (near the center well; panel I, near the well a; panel II).

strain G-Ag-19 was found to be strain-specific, which indicated that strain G-Ag-19 is serologically unique.

Ouchterlony double diffusion test

The same group-specific reactions as those in the agglutination test were observed in the Ouchterlony double diffusion test (Table 3 and Fig. 1). Each of the 39 strains except strain G-Ag-19 formed several group-specific precipitin bands only in combination with the antiserum prepared against the member of the same group. On the other hand, a single, common precipitin band was produced in all the combinations, which coalesced and fused completely with the others (Fig. 1).

DISCUSSION

Alarcon *et al.*¹⁾ and Miller and Vrugink¹⁰⁾ suggested the existence of several "serotypes" within each of biovars 1, 2 and 3, because they observed serological heterogeneity in each of them. The variability in the reactions of the biovar 3 strains obtained in the agglutination and Ouchterlony double diffusion tests in the present experiment (Tables 2, 3 and Fig. 1) supports their results. Moreover, the data obtained in both methods were found to corroborate each other, and the biovar 3 strains tested could be separated into 4 distinct groups, which differed from one another in the reactivity of somatic antigens (heat-stable antigens). We concluded that these groups should be considered as "serogroups"

rather than “serovars”, because the antigens which were used to produce the antisera were limited to unrefined somatic ones.

The use of the serogroup-specific antisera prepared in the present experiment and the development of a suitable serological technique may enable the rapid differentiation among biovar 3 serogroups, which will be useful to study the epidemiological characteristics and to index propagation materials. However, further studies are needed to develop a practical serodiagnosis for this purpose by estimating the suitability and convenience of various serological techniques.

Keane *et al.*⁶⁾ and Lopez⁹⁾ produced specific antisera which enabled to distinguish biovars 1 and 2 serologically. As for biovar 3, Bishop *et al.*⁵⁾ and Ophel and Kerr¹¹⁾ reported the existence of serological specificity, by using a monoclonal antibody prepared against biovar 3. In the present experiment as well, the presence of a somatic antigen in common with all the biovar 3 strains was detected, because a single, common precipitin band which coalesced and fused completely with the others was produced in only the biovar 3 strains. This antigen may be associated with the serological specificity of biovar 3 observed by Bishop *et al.*⁵⁾ and Ophel and Kerr¹¹⁾.

Strain NCPPB 1771, treated as an aberrant strain hitherto^{7,8)}, showed the same serological reactions as serogroup A of biovar 3 (Table 2 and Fig. 1). We concluded that it should be assigned to biovar 3, because it was also similar to biovar 3 in terms of phenotypic characteristics and fatty acid methyl ester profiles in our experiments^{13,14)}. Strain G-Ag-19, which was found to be serologically unique (Tables 2 and 3), remains to be grouped. Detailed antigen analysis is required to clarify its position.

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和 文 摘 要

澤田宏之・今田 準・家城洋之： *Agrobacterium tumefaciens* biovar 3 の菌体抗原に基づく血清群分化

Agrobacterium tumefaciens biovar 3 の血清学的性質を調べるため、ブドウから分離した 5 菌株の biovar 3 (G-Ag-19, 26, 27, 60 および NCPPB 2562) を 100°C で 1 時間加熱処理し、得られた菌体抗原を免疫原として家兎を用いて抗血清を作製した。40 菌株(外国産の 12 菌株を含む)の biovar 3 を抗原として試験管内凝集反応を行ったところ、G-Ag-19 を除いた 39 菌株は、作製した 5 種の抗血清に対する凝集素価に従って A～D の四つのグループに分かれた。寒天ゲル内二重拡散法でも同様に四つのグループ間で反応に差が認められた。すなわち、全菌株に共通な 1 本の沈降帯のほかに、各グループごとに特異的な沈降帯が数本形成された。以上のことから、供試した 39 菌株の biovar 3 は、菌体抗原の相違に基づく四つの血清群 (serogroup) に類別できることが明らかとなった。