Plant Pathology Journal

ISSN 1812-5387

science alert

ANSInet
an open access publisher
http://ansinet.com

Plant Pathology Journal 8 (1): 1-8, 2009
ISSN 1812-5387

Variation of Lipopolysaccharide among the Three Major *Agrobacterium* Species and the Effect of Environmental Stress on the Lipopolysaccharide Profile

[1]H.H. Arafat, [1]K. Tanaka, [2]H. Sawada and [1]K. Suzuki
[1]Department of Biological Science, Graduate School of Science,
Hiroshima University, Higashi Hiroshima 739-8526, Japan
[2]National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-8602, Japan

Abstract: Lipopplysaccharide (LPS) is a variable component among the bacterial species as wall as strains of a single species and this characteristic is helpful for discrimination between strains. However, we have only limited information about LPS variation and influence by environment in *Agrobacterium* strains. In this study, we analyzed variation of lipopolysaccharide (LPS) among 34 *Agrobacterium* strains; 9 strains of *A. tumefaciens*, 15 strains of *A. rhizogenes*, 9 strains of *A. vitis* and one *A. rubi* strain. Most of the *A. tumefaciens* strains and every *A. rhizogenes* strains had high and low molecular weight LPS molecules (LPS I and LPS II, respectively). On the contrary, every *A. vitis* strains and two exceptional *A. tumefaciens* strains lacked LPS I but had a single LPS II band. The LPS profiles were stable phenotype in the *Agrobacterium* strains. Abiotic stresses such as high salinity, high and low pH and high and low temperature were given to representative strains in each species. Only a little alternation in the LPS profiles was observed under the stress conditions except the high temperature to LPS I. Cultivation at 35°C or higher resulted in a significant size reduction of LPS I in *A. tumefaciens* C58 strain down to the size similar to that of LPS II which attenuated the tumor formation. On the contrary, cultivation at the high temperature induced the exceptional *A. tumefaciens* strain MAFF 03-01001 to synthesize LPS I, which was absent at lower temperature in the strain. This phenomenon has never been observed so far at least in the family *Rhizobiaceae*.

Key words: Agrobacteria, alteration by stress, discrimination under species level, LPS, pathogenicity

INTRODUCTION

Lipopolysaccharide (LPS) is a major and abundant structural component of the outer membrane in virtually all Gram-negative bacteria (Brade *et al*., 1999). LPS is a tripartite macromolecule, which consists of a hydrophobic domain known as lipid A (or endotoxin), the non repeating core oligosaccharide and the distal repeating O-antigen polysaccharide (Raetz and Whitfield, 2002). LPS displays a large variation in chemical composition and structure among different species as well as among strains of a single species (De Castro *et al*., 2008; Reuhs *et al*., 1998). Major structural heterogeneity of LPS molecules is found in the O-antigen polysaccharide.

Several studies of bacteria pathogenic to animals and plants have shown that LPS is important virulence factor (Lerouge and Vanderleyden, 2002). LPS also plays crucial roles in the interaction between nitrogen-fixing symbionts and leguminous plants (Campbell *et al*., 2002; Noel *et al*., 2000; Turska-Szewezuk *et al*., 2008). Several reports indicated competitive inhibitory effect of isolated LPS on tumorigenesis and attachment to plant cells by *Agrobacterium*. These results suggest contribution of *Agrobacterium* LPS to infection and colonization. Whatley *et al*. (1976) reported strains whose LPS activity in competitive attachment is less or absent. However, we have only limited information about LPS variation in *Agrobacterium*.

Analysis of LPS profiles provides merits as a technique for strain identification/discrimination (De Maagd *et al*., 1988; Santamaria *et al*., 1997) and for the diversity in a species (De Weger *et al*., 1987; Lindström *et al*., 1990). Nevertheless, it is prerequisite to verify the reliability of LPS profiles, since alternation of LPS profiles depending on environments were reported for many bacteria. Heat stress (35 and 40°C) changed the patterns of LPS mobility of some strains of tree rhizobia (Zahran *et al*., 1994). Also, some alternations in the LPS have been observed at 43°C for a *Rhizobium* sp. (*Cajanus*) (Nandal *et al*., 2005) and for *R. leguminosarum* under low oxygen concentration and low pH (Tao *et al*., 1992). To present knowledge no study has systematically shown the effect of environmental factors on the LPS of *Agrobacterium* species.

Corresponding Author: Katsunori Suzuki, Department of Biological Science, Graduate School of Science,
Hiroshima University, Higashi Hiroshima 739-8526, Japan
Tel: +0081-82-424-7455 Fax: +0081-82-424-0734

Most of pathogenic *Agrobacterium* strains have been classified into three species; *A. tumefaciens* (*R. radiobacter*, biovar 1), *A. rhizogenes* (*R. rhizogenes*, biovar 2) and *A. vitis* (*R. vitis*, biovar 3). The pathogenic strains cause either the crown gall or hairy root diseases, resulted in agricultural loss (Tzfira and Citovskg, 2008). It is difficult to cure the infected plants. Methods to control the diseases are limited. Thereby, it is important to develop rapid and sensitive technique to detect the causative agents and classify strains not only at species level but also below the species level in order to trace the origin of contamination. PCR methods have been developed to detect the tumor-inducing (Ti) and the root-inducing (Ri) plasmids (Sawada *et al*., 1995; Suzaki *et al*., 2004) and the PCR amplification of 16S rRNA gene followed by nucleotide sequence allows classification at species level (Sawada *et al*., 1993; Bautista-Zapanta *et al*., 2007). In order to investigate the origin and the route of contamination, classification below species level is desirable.

In the present study, we examined LPS profiles of various strains belonging to the major three pathogenic *Agrobacterium* species. We also analyzed the effect of environmental stress conditions on the agrobacterial LPS.

MATERIALS AND METHODS

Bacterial strains and culture condition: The investigated strains are listed in Table 1. *Agrobacterium* strains were cultured at 28°C in three different media. Yeast-Extract Mannitol (YEM) medium lacking $CaCO_3$, contains (L^{-1}) 10 g mannitol, 0.5 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 0.1 g NaCl and 0.4 g yeast extract (Difco). IFO medium contains (L^{-1}) 10 g polypeptone (Daigo), 2 g yeast extract (Difco) and 0.1 g $MgSO_4.7H_2O$ (pH 7.0). Luria Broth (LB) medium contains (L^{-1}) 5 g NaCl, 5 g yeast extract (Difco) and 10 g bacto-tryptone (Difco). The culture conditions were altered when indicated.

Preparation and electrophoretic separation of LPS: LPS was extracted by two different methods. The first method is according to Santamaria *et al*. (1999). This method was applied to compare LPS among many strains. The second method is the phenol-water method of Westphal and Jann (1965), with some modifications as mentioned below, in order to prepare detergent-free purified LPS samples. In the latter method, the water phase from the phenol-water mixture was dialyzed against water and then treated with ribnuclease A (10 μg mL^{-1} as a final concentration) in the

Table 1: *Agrobacterium* strains used in this study

	Strain	Origin	References
A. tumefaciens	C58	Cherry, USA	Bouzar and Moore (1987)
(Biovar 1; *R. radiobacter*)	IAM 1526	Soil, USA	Sawada and Ieki (1992)
	IAM 12048	Unknown, Netherlands	Sawada and Ieki (1992)
	MAFF 03-01278	Chrysanthemum, Shizuoka, Japan	Sawada and Ieki (1992)
	MAFF 03-01724	Melon, Chiba, Japan	Sawada and Ieki (1992)
	MAFF 03-01001	Cherry, Saitama, Japan	Sawada and Ieki (1992)
	MAFF 03-01224	Rose, Osaka, Japan	Sawada and Ieki (1992)
	MR4	Melon, Shizuoka, Japan	Sawada and Ieki (1992)
	NCPPB 2437^T	Unknown, Iowa, USA	Sawada and Ieki (1992)
A. rhizogenes	A4	Unknown, California, USA	Bouzar and Moore (1987)
(Biovar 2; *R. rhizogenes*)	Ch-Ag-2	Cherry, Yamagata, Japan	Sawada and Ieki (1992)
	Ch-Ag-6	Cherry, Okayama, Japan	Sawada and Ieki (1992)
	Ch-Ag-10	Cherry, Okayama, Japan	Sawada and Ieki (1992)
	Ch-Ag-14	Cherry, Okayama, Japan	Sawada and Ieki (1992)
	K84	Soil, Australia	Bouzar and Moore (1987)
	P-Ag-3	Pear, Mie, Japan	Sawada and Ieki (1992)
	P-Ag-5	Pear, Mie, Japan	Sawada and Ieki (1992)
	P-Ag-6	Pear, Nagasaki, Japan	Sawada and Ieki (1992)
	Pch-Ag-2	Peach, Okayama, Japan	Sawada and Ieki (1992)
	Pch-Ag-4	Peach, Okayama, Japan	Sawada and Ieki (1992)
	Pch-Ag-6	Peach, Okayama, Japan	Sawada and Ieki (1992)
	Pl-Ag-1	Plum, Okayama, Japan	Sawada and Ieki (1992)
	Ro-Ag-13	Rose, Yamagata, Japan	Sawada and Ieki (1992)
	IFO 13257^T	Apple, USA	Sawada and Ieki (1992
A. vitis	K-Ag-1	Kiwifruit, Hiroshima, Japan	Sawada and Ieki (1992)
(Biovar 3; *R. vitis*)	G-Ag-19	Grape, Nagano, Japan	Sawada and Ieki (1992)
	G-Ag-27	Grape, Nagano, Japan	Sawada and Ieki (1992)
	G-Ag-37	Grape, Nagano, Japan	Sawada and Ieki (1992)
	G-Ag-60	Grape, Aomori, Japan	Sawada and Ieki (1992)
	S4	Grapevine	Szegedi (1985)
	NCPPB 1771	Grape, Iran	Sawada and Ieki (1992)
	NCPPB 2562	Grape, Greece	Sawada and Ieki (1992)
	NCPPB 3554^T	Grapevine, Australia	Ophel and Kerr (1990)
A. rubi (*R. rubi*)	IFO 132614^T	*Rubus* sp., USA	Sawada and Ieki (1992)

presence of 10 mM Tris HCl (pH 7.5) for 2 h at 37°C, followed by lyophilization. The lyophilized material was dissolved in water and then precipitated by ultracentrifugation at 75000 rpm for 4 h. The precipitated LPS samples were re-dissolved in water. Electrophoresis was carried out by the discontinuous method of Laemmli (1970) in 0.5 mm thick 12% polyacrylamide slab gels containing SDS (SDS-PAGE). The gels were stained by the periodic acid-silver stain method as described by Tsai and Frasch (1982).

Affinity of LPS to polymyxin B: The LPS from the water phase of the phenol-water method was tested by affinity chromatography on columns of the polymyxin B agarose (Detoxi-Gel, Pierce, Rockford, IL). We applied 10 μg of LPS dissolved in 200 μL water to 0.5 mL bed volume of the polymyxin B agarose. The resulting elute was applied again to the same column. Then, the column was washed with 1.5 mL water. Finally, 1.5 mL of 1% sodium deoxycholate was added to the column. Each column fractions were checked by SDS-PAGE and silver staining.

Pathogenicity test: Bacterial cells were cultured overnight at either 25 or 35°C. The cells cultured at 35°C were further cultured at 25°C for 9 h. The cells were suspended and diluted with fresh medium and adjusted to OD_{660} = 1.0. Approximately 20 μL of the cell suspension was inoculated by piercing into stems of young plants using syringes and needles. The inoculated plantlets were kept at 27°C overnight, and then cultivated in a greenhouse. Tumor formation was scored at 35 days after the inoculation. The experiment was repeated twice with three replicates each time.

RESULTS

Characterization of LPS pro files among *Agrobacterium* strains: LPS was extracted from 34 strains cultivated in IFO and YEM media and then separated by SDS-PAGE in order to know how large variations or similarities in the LPS profiles among the strains in each of the three major pathogenic *Agrobacterium* species and *A. rubi* and among the species (Fig. 1). LPS profile of each strain

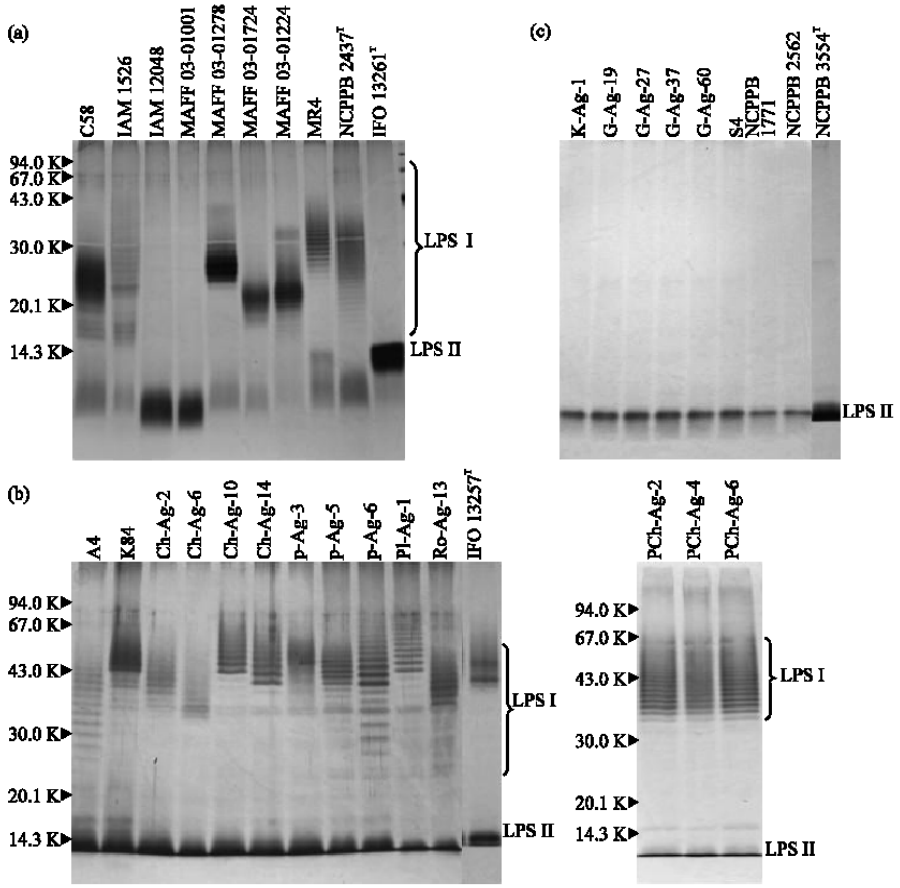

Fig. 1: Electrophoretic analysis of LPS molecules produced by *Agrobacterium* strains by SDS-PAGE. *Agrobacterium* strains were cultured on YEM medium till early stationary phase. LPS was extracted and separated by polyacrylamide gel and then stained by silver stain method as described in materials and methods (a) *A. tumefaciens* strains and *A. rubi*, (b) *A. rhizogenes* strains and (c) *A. vitis* strains

was identical between IFO and YEM cultures. We classified LPS into two categories: LPS I, a heterogeneous ladder of many bands ranging in electrophoretic mobility from 20 kDa to about 66 kDa protein size markers; LPS II, low molecular weight bands which migrated faster than the 14.3 kDa protein size marker. LPS I was largely variable among the examined strains. However, LPS II was generally a single band with the same mobility among the three species.

The greatest variation of LPS II profile in size and number was observed in *A. tumefaciens* strains among the three species (Fig. 1a). LPS I in strains C58, MAFF 03-01278, MAFF 03-01724 and MAFF 03-01224 looked as a stake of ladder from 20 to 30 kDa with some variation depending on the strains. LPS I in IAM 1526, MR4 and NCPPB 2437^T was distributing in larger size range from 25 to 43 kDa. We found two exceptional *A. tumefaciens* strains, IAM 12048 and MAFF 03-01001 contained no LPS I (Fig. 1a).

We observed similar but less variable ladder profiles of LPS I in 15 *A. rhizogenes* strains (Fig. 1b). Size of most LPS I molecules approximately ranged from 25 to 50 kDa with some variation depending on the strains. Contrary to most of *A. tumefaciens* and *A. rhizogenes* strains, 9 *A. vitis* strains contained no LPS I at all, similar to the two exceptional *A. tumefaciens* strains (Fig. 1c). LPS II was highly conserved in size and quantity among the *A. rhizogenes* strains and the *A. vitis* strains (Fig. 1b, c). In contrast, LPS II was variable in quantity among *A. tumefaciens* strains (Fig. 1a). An *A. tumefaciens* strain MR4 possessed an additional LPS II which is slightly larger than the standard LPS II common among strains. *A. rubi* IFO 13261^T strain had LPS II only. The LPS II in the *A. rubi* strain was slightly larger than the common LPS II and was similar with the additional LPS II of MR4 strain.

Effect of salt, pH and temperature on LPS profile: In order to understand how much LPS profile is influenced by environmental factors, we cultivated agrobacterial strains with the following modifications: high salinity, low and high pH and low and high temperatures. We used two strains from each species for this experiment namely, *A. tumefaciens* C58 and MAFF 03-01278 strains, *A. rhizogenes* A4 and Ch-Ag-2 strains and *A. vitis* K-Ag-1 and G-Ag-27 strains (Fig. 2a-c, respectively).

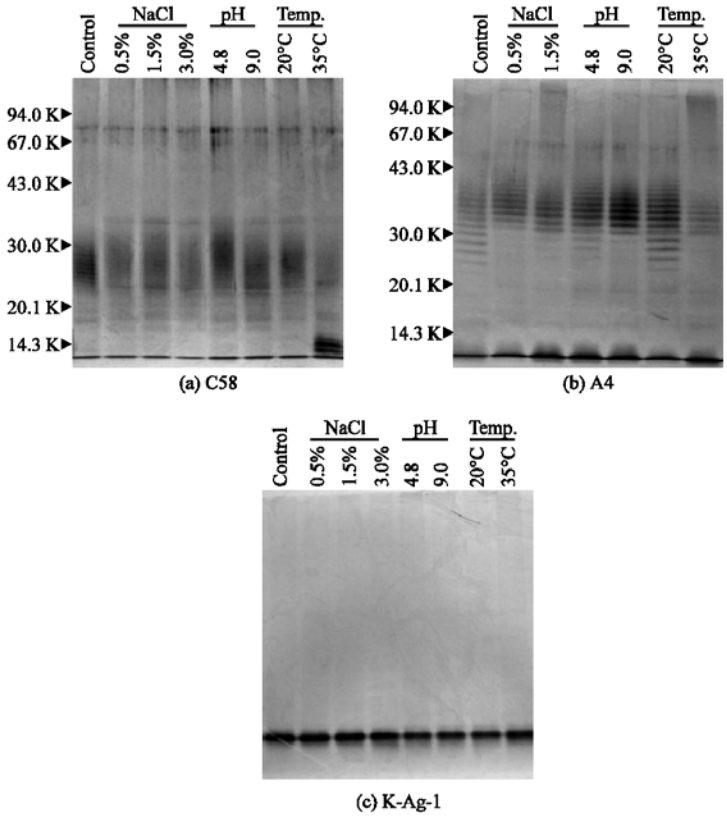

Fig. 2: Effect of environmental stress on LPS profile. *Agrobacterium* strains: (a) C58, (b) A4 and (c) K-Ag-1 were cultivated on IFO medium at 28°C. The culture condition was modified by adding NaCl, changing the pH and/or temperature as indicated

LPS was not altered by the acidic (pH 4.8) and alkaline (pH 9.0) shifts in C58 strain (Fig. 2a). The alkaline shift did not change LPS in MAFF 03-01278 (data not shown). The acidic medium did not permit MAFF03-01278 to grow. High salinity, namely addition of NaCl to medium (3%) had no effect on the LPS profiles in the two *A. tumefaciens* strains. The LPS profile was not altered at low temperature, whereas high temperatures (35 and 37°C) gave distinct effect. Most of the ladder of LPS I disappeared especially in C58 (Fig. 2, 3a) and vice versa, low molecular weight LPS (LPS II-like molecule) slightly larger than the common LPS II accumulated in the strain under the high temperature condition. High molecular weight LPS I molecules decreased at 35°C also in MAFF03-01278 (data not shown).

Only several of shorter LPS I molecules (27 and 28 kDa) disappeared in *A. rhizogenes* strain A4 by alkaline pH, high salinity as well as high temperature (Fig. 2b). Only the high temperature condition induced change in LPS I in *A. rhizogenes* strain Ch-Ag-2 (data not shown). The two *A. rhizogenes* strains did not grow at high NaCl concentration, which phenomenon is consistent with the earlier report (Kersters and De Ley, 1984). However, incubation in the high salt media did not alter the LPS II in the strains at all.

The acidic, alkaline and high salinity as well as low and high temperatures had no effect on the LPS profile in two *A. vitis* strains K-Ag-1 (Fig. 2c) and G-Ag-27 (data not shown). Similar to the *A. vitis* strains, the exceptional *A. tumefaciens* strain MAFF 03-01001 contained no LPS I as shown before in (Fig. 1c). However, the high temperature caused obvious alternations and modifications in the LPS of the strain (Fig. 3b). LPS I like molecules were formed abundantly at 36°C. No such molecules were detected in LPS formed at normal temperature, even when we applied larger quantity of the sample (first column in Fig. 3b). This phenomenon has never been seen at least in the family *Rhizobiaceae* as far as we know.

Affinity of the LPS altered by high temperature to polymyxin B: We examined by affinity chromatography the LPS II-like molecule induced by high temperature in C58 strain and the LPS I-like molecules induced by high temperature in MAFF 03-01001. The column retained the LPS II-like molecule and the LPS I-like molecule. Addition of 1% sodium-deoxycholate released the LPS II-like molecule and the LPS I-like molecules from the columns. Thereby, the LPS II-like molecule and the LPS I-like molecules that were induced at the high temperature in C58 and MAFF 03-01001, respectively, are confirmed as LPS molecules (Fig. 4a, b).

Effect of pre-culture at high temperature on pathogenicity and viability: We examined how much pathogenicity is influenced by pre-cultivation at high temperature, which largely altered LPS profile of the C58 strain. The C58 strain cultured at 35°C was cultured

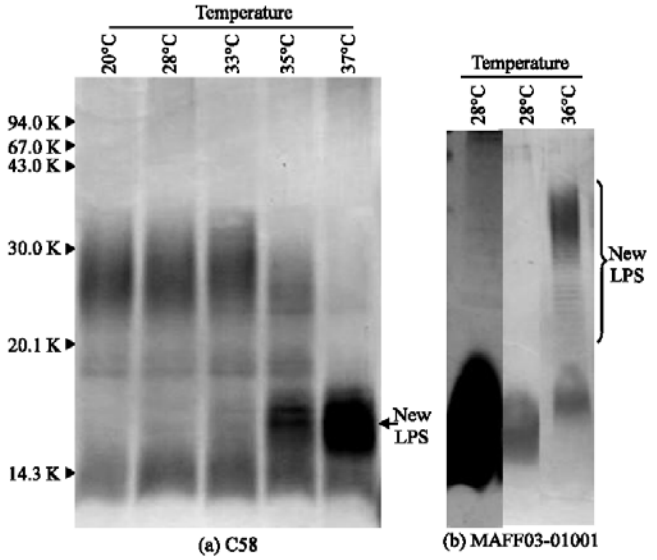

Fig. 3: Effect of different cultivation temperatures on the LPS profile. *Agrobacterium strains*. (a) C58 and (b) MAFF 03-01001 were cultivated on LB medium. In first column (b), twenty-fold larger quantity of LPS sample was applied than in the second column

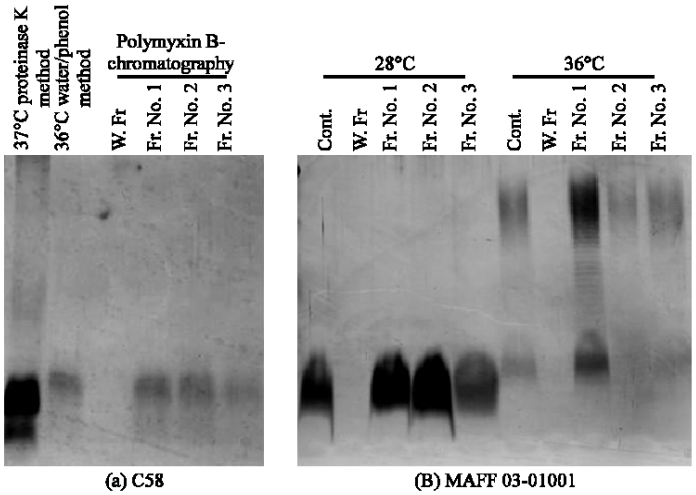

Fig. 4: Affinity chromatography by polymyxin B agarose column. LPS samples were prepared by the phenol/water method as described in materials and methods from *A. tumefaciens* strain C58 grown at 36°C (a) and from MAFF 03-01001 grown at 28°C (b, left) and at 36°C (b, right). Samples were applied to polymixin B column. Fr, eluate by water; F. No. 1-3; fraction number 1-3 of eluate by sodium-deoxycholate

Table 2: Effect of pre-culture at high temperature on pathogenicity of C58 strain

Agrobacterial cells applied (pre-culture)	Tumor size (diameter, mm)		
	Tobacco	Tomato	Kalanchoe
25°C	10.6±2.4	11.7±0.76	11.5±1.00
35°C*	5.5±2.8	5.9±3.15	6.0±6.26

*The cells were cultured for 9 h at 25°C before infection experiment, Data are shown as Mean±SD

further at 25°C for 9 h. As shown in Table 2, tumor formation was found to be constricted by the pre-culture at 35°C.

We analyzed the bacterial cells and we found that the cultivation at 35°C decreased the colony forming unit (cfu) by 6 folds. The cfu of a unit OD (at 660 nm) cell suspension was 5×10^9, 8×10^8 and 1×10^9 for cells cultivated at 25, 35 and 35°C plus 25°C for 9 h, respectively. Incubation after the inoculation was carried out at 27°C overnight.

DISCUSSION

In the present study, we analyzed LPS of the 33 strains belonging to the three major *Agrobacterium* species and one *A. rubi* type strain by SDS-PAGE. It is easy to analyze LPS profile because LPS is abundant in the bacterial cells and detectable with high sensitivity by the silver staining method. Reliability of analysis results depend on stability of LPS profile in relation with environmental difference.

All of the 9 *A. vitis* strains contained one band of LPS II but no LPS I. Seven among the nine *A. tumefaciens* strains and all the 15 *A. rhizogenes* strains contained LPS I and LPS II molecules. The difference between the three species was consistent to the pioneering study by two groups. Sakane and Yokota (1991) extracted LPS from six *A. tumefaciens* (biovar 1) and 7 *A. rhizogenes* (biovar 2), one *A. vitis* (biovar 3) and two *A. rubi* strains. They simply described that *A. tumefaciens* and *A. rhizogenes* strains are S-chemotype (LPS I and II type) and *A. vitis* and *A. rubi* strains are R-chemotype (LPS II type). Weibgen *et al.* (1993) analyzed LPS in two strains in each of *A. tumefaciens* and *A. vitis* and in 3 strains of *A. rhizogenes*. They showed that both of *A. tumefaciens* and *A. rhizogenes* strains have LPS I and LPS II, while *A. vitis* and *A. rubi* strains have LPS II only. In this study, we indicated that LPS profile of *A. rhizogenes* strains (Fig. 1b) were less variable than those of *A. tumefaciens* strains (Fig. 1a). Most of the LPS variation in *A. tumefaciens* and *A. rhizogenes* was observed in LPS I region. The large variation in *A. tumefaciens* and *A. rhizogenes* strains is significantly useful in intra-species identification/discrimination.

In this study, we found two exceptional strains IAM 12048 and MAFF 03-01001 among the highly variable *A. tumefaciens* strains. They did not contain LPS I at all like the *A. vitis* strains. This result suggests absence of some glycosyl transferase system for O-antigen synthesis in the two strains. Previously, we found that two *A. vitis* strains contained 16S-23S internal transcribed spacer (ITS) sequence same with that of *A. tumefaciens* MAFF 03-01001 strain, which suggests horizontal gene transfer between the two species and subsequent recombination (Bautista-Zapanta *et al.*, 2007).

The similarity in LPS profile supports the relationship between the *A. tumefaciens* and *A. vitis* strains. Also MR4 strain contained two types of LPS II, the common and an additional LPS II. The latter one was slightly larger in size than the common one and as large as LPS II of *A. rubi* IFO13261^T strain. The possession of the latter type LPS II by the two strains is reasonable when we consider the close relationship between *A. rubi* and *A. tumefaciens* according to the 16S rRNA gene sequence (Sawada *et al*., 1993; Willems and Collins, 1993).

In order to apply the LPS variation to discriminate strains, it is necessary to understand the influence by the environment. This report is the first one that deals with the effect of different environmental factors on the LPS profile in *Agrobacterium* species. LPS II was resistant to the stress conditions, whereas LPS I was relatively susceptible to some stress conditions depending on the strains. High temperature broadly altered LPS I profile. LPS profile of the *A. tumefaciens* strain C58 was unsusceptible to the stress conditions except the high temperature. Cultivation at 35°C or higher resulted in obvious decrease of high molecular weight LPS I and accumulation of new LPS II molecules, which suggests inhibition of addition of O-polysaccharide chain units in the LPS structure by high temperature. In addition, the formation of the LPS I molecules in MAFF 03-01001 at the high temperature observed in this study is an interesting phenomenon. This is the first observation of this type of LPS alteration. It is likely that some O-antigen biosynthetic genes in the strain are repressed at low temperature while the genes are de-repressed at the high temperature resulting in the formation of LPS I molecules. In conclusion, cultivation at temperature between 20°C and 28°C provides cells suitable for LPS profile analysis for comparison between strains. Cultivation at high temperature causes various types of alteration in LPS I.

In this study we indicated that the cultivation at high temperature resulted in decreased pathogenicity. We found 6-fold lower viability at high temperature than at normal temperature. Hamilton and Fall (1971) reported the decrease of pathogenic ability in C58 strain under the cultivation at 36°C for 48 h or more due to the loss of Ti-plasmid. It is clear that cultivation at high temperature induces serious and broad effects on *Agrobacterium* cell.

ACKNOWLEDGMENTS

HHA is a recipient of the Monbukagakusyo scholarship. This study was supported in part by the Japanese Ministry of Education, Science, Sports and Culture, Grant-in-Aid for Scientific Research (No. 20570221).

REFERENCES

Bautista-Zapanta, J., H.H. Arafat, K. Tanaka, H. Sawada and K. Suzuki, 2007. Variation of 16S-23S internally transcribed spacer sequence and intervening sequence in rDNA among the three major *Agrobacterium* species. Microbiol. Res., DOI 10.1016/j.micres.2007.08.003.

Bouzar, H. and L.W. Moore, 1987. Complementary methodologies to identify specific *Agrobacterium* strains. Applied Environ. Microbiol., 53: 2660-2665.

Brade, H., S.M. Opal, S.N. Vogel and D.C. Morrison, 1999. Endotoxin in Health and Disease. 15th Edn., Marcel Dekker, Inc., New York, ISBN: 0-8247-1944-1.

Campbell, G.R.O., B.L. Rheus and G.C. Walker, 2002. Chronic intracellular infection of alfalfa nodules by *Sinorhizobium meliloti* requires correct lipopolysaccharide core. Proc. Natl. Acad. Sci., USA., 99: 3938-3943.

De Castro, C., A. Molinaro, R. Lanzetta, A. Silipo and M. Parrilli, 2008. Lipopolysaccharide structures from *Agrobacterium* and *Rhizobiaceae* species. Carbohydr. Res., 343: 1924-1933.

De Maagd, R.A., C.V. Rossum and B.J.J. Lugtenberg, 1988. Recognition of individual strains of fast-growing rhizobia by using profiles of membrane proteins and lipopolysaccharides. J. Bacteriol., 170: 3782-3785.

De Weger, L.A., B. Jann, K. Jann and B. Lugtenberg, 1987. Lipopolysaccharides of *Pseudomonas* spp. that stimulate plant growth: Composition and use for strain identification. J. Bacteriol., 169: 1441-1446.

Hamilton, R.H. and M.Z. Fall, 1971. The loss of tumor-initiating ability in *Agrobacterium tumefaciens* by incubation at high temperature. Cell. Mol. Life Sci., 27: 229-230.

Kersters, K. and J. De Ley, 1984. Genus III *Agrobacterium* Conn 1942. In: Bergey's Manual of Systematic Bacteriology, Krieg, N.R. and J.G. Holt (Eds.). Williams and Wilkins, Baltimore, ISBN: 0-683-04108-8, pp: 244-254.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of head of bacteriophage T_4. Nature, 227: 680-685.

Lerouge, I. and J. Vanderleyden, 2002. O-antigen structural variation: Mechanisms and possible roles in animal/plant-microbe interactions. FEMS Microbiol. Rev., 26: 17-47.

Lindström, K., P. Lipsanen and S. Kaijalainen, 1990. Stability of markers used for identification of two *Rhizobium galegae* inoculant strains after five years in the field. Applied Environ. Microbiol., 56: 444-450.

Nandal, K., A.R. Sehrawat, A.S. Yadav, R.K. Vashishata and K.S. Boora, 2005. High temperature-induced changes in exopolysaccharides, lipopolysaccharides and protein profile of heat-resistant mutants of *Rhizobium* sp. (*Cajanus*). Microbiol. Res., 160: 367-373.

Noel, K.D., L.S. Forsberg and R.W. Carlson, 2000. Varying the abundance of O-antigen in *Rhizobium etli* and its effect on symbiosis with *Phaseolus vulgaris*. J. Bacteriol., 182: 5317-5324.

Ophel, K. and A. Kerr, 1990. *Agrobacterium vitis* sp. nov. for strains of *Agrobacterium* biovar 3 from grapevines. Int. J. Syst. Bacteriol., 40: 236-241.

Raetz, C.R.H. and C. Whitfield, 2002. Lipopolysaccharide endotoxins. Annu. Rev. Biochem., 71: 635-700.

Reuhs, B.L., D.P. Geller, J.S. Kim, J.E. Fox and V.S.K. Kolli *et al*., 1998. *Sinorhizobium fredii* and *Sinorhizobium meliloti* produce structurally conserved lipopolysaccharides and strain-specific K antigens. Applied Environ. Microbiol., 64: 4930-4938.

Sakane, T. and A. Yokota, 1991. Rapid method for LPS analysis with DOC-PAGE and PAS stain. IFO Res. Comm., 15: 42-51.

Santamaria, M., J. Corzo, M. Leon-Barrios and A.M. Gutierrez-Navarro, 1997. Characterization and differentiation of indigenous rhizobia isolated from Canarian shrub legumes of agricultural and ecological interest. Plant Soil., 190: 143-152.

Santamaria, M., F. Agius, J. Monza, A.M. Gutierrez-Navarro and J. Corzo, 1999. Comparative performance of enterobacterial repetitive intragenic consensus-polymerase chain reaction and lipopolysaccharide electrophoresis for the identification of *Bradyrhizobium* sp. (*Lotus*) strains. FEMS Microbiol. Ecol., 28: 163-168.

Sawada, H. and H. Ieki, 1992. Phenotypic characteristic of the genus *Agrobacterium*. Ann. Phytopathol. Soc. Jap., 58: 37-45.

Sawada, H., H. Ieki, H. Oyaizu and S. Matsumoto, 1993. Proposal for rejection of *Agrobacterium tumefaciens* and revised descriptions for the genus *Agrobacterium* and for *Agrobacterium radiobacter* and *Agrobacterium rhizogenes*. Int. J. Syst. Bacteriol., 43: 694-702.

Sawada, H., H. Ieki and I. Matsuda, 1995. PCR detection of Ti and Ri plasmids from phytopathogenic *Agrobacterium* strains. Applied Environ. Microbiol., 61: 828-831.

Suzaki, K., K. Yoshida and H. Sawada, 2004. Detection of tumorigenic *Agrobacterium* strains from infected apple saplings by colony PCR with improved PCR primers. J. Gen. Plant Pathol., 70: 342-347.

Szegedi, E., 1985. Host range and specific L (+)- tartrate utilization of biotype 3 *Agrobacterium tumefaciens*. Acta Phytopathol. Acad. Sci. Hungary, 20: 17-22.

Tao, H., N.J. Brewin and K.D. Noel, 1992. *Rhizobium leguminosarum* CFN42 lipopolysaccharide antigenic changes induced by environmental conditions. J. Bacteriol., 174: 2222-2229.

Tsai, C.M. and C.E. Frasch, 1982. A sensitive silver staining for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem., 119: 115-119.

Turska-Szewczuk, A., B. Lotocka, J. Kutkowska and J. Król *et al*., 2007. The incomplete substitution of lipopolysaccharide with o-chain prevents the establishment of effective symbiosis between *Mesorhizobium loti* NZP2213.1 and *Lotus corniculatus*. Microbiol. Res., DOI 10.1016/j.micres. 2006.11.011.

Tzfira, T. and V. Citovsky, 2008. *Agrobacterium:* From Biology to Biotechnology. 1st Edn., Springer Sci. + Business Media, LLC, New York, ISBN: 978-0-387-72289-4.

Weibgen, U., R. Russa, A. Yokota and H. Mayer, 1993. Taxonomic significance of the lipopolysaccharide composition of the three biovars of *Agrobacterium tumefaciens*. Syst. Applied Microbiol., 16: 177-182.

Westphal, O. and K. Jann, 1965. Bacterial lipopolysaccharides: Extraction with phenol-water and further applications of the procedure. Meth. Carbohydr. Chem., 5: 83-91.

Whatley, M.H., J.S. Bodwin, B.B. Lippincott and J.A. Lippincott, 1976. Role for *Agrobacterium* cell envelope lipopolysaccharide in infection site attachment. Infect. Immun., 13: 1080-1083.

Willems, A. and M.D. Collins, 1993. Phylogenetic analysis of rhizobia and agrobacteria based on 16S rRNA gene sequences. Int. J. Syst. Bacteriol., 43: 305-313.

Zahran, H.H., L.A. Räsänen, M. Karsisto and K. Lindström, 1994. Alteration of lipopolysaccharide and protein profiles in SDS-PAGE of rhizobia by osmotic and heat stress. World J. Microbiol. Biotechnol., 10: 100-105.