Asian J. Exp. Sci., Vol. 23, No. 1, 2009; 135-139

Determination of the Degree of Hydrophobicity - A Technique to Assess Bacterial Colonization on Leaf Surface and Root Region of Lotus Plant

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Abstract : Leaf surfaces and root regions are composed of diverse microorganisms including Gram positive and Gram negative bacteria as well as yeasts and fungi. Here we report the relative distribution of the bacterial populations on the leaf surface and root regions of lotus plant. A marked degree of heterogeneous bacterial populations were found on leaf surface and root region. The degree of hydrophobicity was studied and a high variability was observed which could be attributed to differences in arrays of surface molecules of bacterial cells and composition of biofilms.

Key words : Biofilm, leaf surface bacteria, hydrophobicity etc.

Introduction

Large bacterial populations colonize aerial plant leaves. Leaf associated bacterial communities can include members that cause disease, induce frost injury, alter plant growth through phytohormone production, protect plants from disease or frost injury, or fix atmospheric nitrogen. Plants foster larger populations of bacteria on non-waxy plants than plants with large amounts of cuticular waxes. The chemical composition and quantity of the waxes, as well as the morphology and density of the epicuticular waxes, are distinct for each plant species and often differ with plant growth stage and environment.

Cell surface properties are recognized as the key factors that influence bacterial adhesion to surfaces. Among the critical surface properties are surface hydrophobicity, extracellular polymers, and surface electrostatic charge (Ahn and Lee, 2003; Gannon *et al.,* 1991). Hydrophobic interactions define the strong attraction between hydrophobic molecules and surfaces in water. In biological systems hydrophobic interactions are the strongest long-range non-covalent interactions and are considered a determining factor in microbial adhesion to surfaces (Sanin *et al*., 2003). The effects of specific cuticular properties on colonization have been examined for several phytopathogenic fungi (Marcell and Beattle, 2002). A technique to quantify the population size and composition of biofilms component in communities of bacteria in the phyllosphere was carried out (Morris *et al*., 1998). The present studies are designed to determine the influence of leaf surface and root region as a habitat for colonization of bacterial species. Secondly, to evaluate the degree of hydrophobicity of bacterial isolates colonizing leaf surface and root region of lotus plants.

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Materials and Methods

The roots and broad-leaved Lotus *(Nelumbo nucifera*) were collected from the lake of Kandhar near Nanded in Maharashtra. Leaves were transported to the laboratory in clean plastic bags and stored at 4°C until analyzed (within 12 hours). Before use the soil adhering to the roots was removed in a stream of distilled water and then roots were rinsed multiple times in sterile buffer.

To obtain the naturally occurring bacterial populations, two procedures were followed as outlined. A direct impression of the upper surface of the leaf was made on an agar medium in a Petri dish. Fresh leaves were taken and cuticle peeled off and then cut into small squares and gently washed in 100ml of buffer. The flasks containing the buffer and leaf material pieces were gently rotated manually for 3 minutes. To eliminate small debris and the majority of isolated microbial cells, the washing solution was filtered across the polypropylene filter of 0.4ìm in diameter. The bacteria collected on the filter were gently suspended in 5 ml of buffer. 1 to 2 ml aliquots of serial dilutions of this suspension were plated on sterile agar plates and incubated at 25^0C temperature for further analysis The bacterial isolates were identified on the basis of various morphological and biochemical tests.

Three procedures were used to test hydrophobicity. The protocols for Microbial Adhesion to Hydrocarbon (MATH Assay), Bacterial Adhesion to Nitrocellulose Filter Assay (NCF Assay) and Agglutination in Salt Solution Assay (SAT Assay) were previously described in the literature and the degree of hydrophobicity was determined. The percentage of Microbial Adhesion to Hydrophobicity (MATH) was calculated by using the formula % of adhesion = 100 x O.D (Initial bacterial suspension) – O.D (aqueous phase) /O.D of initial bacterial suspension. The percentage of bacterial adhesion to Nitrocellulose Filter Assay (NCF) was calculated as: Percentage (%) of adhesion = 100 x (O.D. at 600 nm of initial bacterial suspension) - O.D. (600 nm) of filtrate / O.D. at 600 nm of initial bacterial suspension. In SAT assay, hydrophobicity was expressed as the lowest molarity in a mixture, which produced visual clumping. Molarities were converted to percentages by considering zero hydrophobicity as the aggregation at zero moles of ammonium sulfate per liter and 100% hydrophobicity as the aggregation at 4 moles of ammonium sulfate per liter.

Results and Discussion

The identification of different bacterial populations on the lotus leaf surface and root region was carried out on the basis of *Bergey's Manual of Systematic Bacteriology* (Holt *et al*., 1994). It is observed from the results that out of 7 isolates from leaf surface, some isolates are Gram-negative bacteria and others are Gram-positive bacteria. Among the seven isolates, five were found to be Gram-positive cocci and two were found to be Gram-negative rods. Among the different isolates *Streptococcus epidermidis* was found to be relatively dominant over other bacterial population. The other isolates were confined to only a small portion of the leaf surface. The results suggest that various isolates for attachment to the leaf surface varied considerably.

The results also depict that the bacteria from leaf isolates were identified as *Streptococcus epidermidis, Streptococcus saprophyticus, Streptococcus carnosus, Pseudomonas aeruginosa, Pseudomonas fluorescens, Micrococcus variens,* and *Micrococcus roseus.* The bacterial isolates from root portion were found to be *Bacillus subtilis, Bacillus licheniformis,* and *Bacillus mycoides.* Table-1 and Table-2 show the test of hydrophobicity for different methods used in the study.

It is observed from the results that the degree of hydrophobicity varied among the isolates at the leaf and root regions. According to the criteria proposed for hydrophobicity very few isolates were highly hydrophobic with the Microbial adhesion to hydrocarbon (MATH) assay using PBS or PUM buffer. However, 30% of the isolates showed strong hydrophobicity with agglutinations in salt solution (SAT) and 30% with the NCF assay.

Table 1 : Cell surface hydrophobicity of various isolates using different methods

S. No	Source	Bacterial strain	MATH		NCF	SAT
			PUM	PBS		
1	Leaf	*S.epidermidis*	23.9	11.7	36.5	1.5
2		*S.saprophyticus*	22.1	24.9	46.7	1
3		*S.carnosus*	41	34.1	55.3	1
4		*P.aeruginosa*	18	28.8	76.5	2
5		*P.fluorescens*	15.2	36.4	61.7	1.5
6		*M.variens*	26	15.6	42.9	1.5
7		*M.roseus*	9.7	41.5	78.7	1
8	Root	*B.subtilis*	25.6	46.9	59	2
9		*B.licheniformis*	16	30.4	83.5	1.5
10		*B.mycoides*	21.5	28.9	47.2	1.5

PUM = Phosphate Urea Magnesium Sulfate Buffer.
PBS = Phosphate Buffered Saline.

Table 2 : Degree of hydrophobicity of various isolates using different assay methods

Test	Values	Degree of Hydrophobicity	Isolates in Percentage (%)
SAT	0-1M	Strong	30
	1-2M	Moderate	50
	2-4M	Weak	20
	?4M	Negative	-
NCF	?75%	Strong	30
	50-75%	Moderate	20
	?50%	Negative	50
MATH (PBS)	?50%	Strong	-
	20-50%	Moderate	60
	?20%	Negative	40
MATH (PUM)	?50%	Strong	-
	20-50%	Moderate	80
	?20%	Negative	20

MATH = Microbial Adhesion to Hydrocarbon, NCF = Nitrocellulose Filters,
SAT = Agglutination in Salt Test.

Most of the isolates were considered to have moderate or weak hydrophobicity with SAT (50%) and MATH-PBS (60%), whereas 20% and 80% of the isolates were included in the moderately hydrophobicity group using the NCF and MATH-PUM assays.

It is apparent from this study that all ten isolates have shown variations in the degree of hydrophobicity. These differences could be attributed to the different criteria using SAT, NCF and MATH assays. The isolates studied showed wide differences in their hydrophobicity and observed that no consistent pattern of hydrophobicity exists for different isolates. These results confirm the high variability of the hydrophobicity reported by other authors. This variability may be the result of differences in arrays of surface molecules of bacterial cells, mainly fimbriae and proteins of the outer membrane.

Methods for measuring bacterial hydrophobicity differ somewhat in the precise properties they measure; and different types of interactions are considered when different methods are used to estimate hydrophobicity. Thus, the relative hydrophobicity estimated by hydrophobic interaction chromatography (HIC), microbial adhesion to hydrocarbons (MATH) and contact angle measurements for different bacterial species was dependent on the specific method tested (Dickson and Koohamaraie, 1989). They Characterized bacterial cells by measuring hydrophobicity (measured by water contact angle) and electrophoretic mobility (Van Loosdrecht *et al*., 1987; Mack *et al*., 1996). It was concluded that cell surface hydrophobicity was the major determining factor in attachment to negatively charged polystyrene. In the present study, an estimate of hydrophobicity with the NCF assay and MATH assays using PBS or PUM was significantly different. Strong hydrophobicity was observed with *Streptococcus epidermidis* using MATH assays. The complexity of cell surface mosaic resulting from hydrophobic and hydrophilic appendages and other macro molecular components might give rise to different sensitivities with different assay methods. Nevertheless, the correlation observed between the results obtained with SAT and the NCF and MATH assays in conjugation with the fact that the SAT assay is easily performed, highlights the convenience of this test for estimating the cell-hydrophobicity of various isolates. MATH assay using PBS and PUM buffer differ significantly from each other. Lower values of hydrophobicity detected in assays carried out with PUM buffer could be attributable to the different ionic strength of this buffer.

Among all the isolates studied, *Streptococcus epidermidis* and related coagulase-negative Staphylococci are now well established as major nosocomial pathogens associated with infections of indwelling medical devices. In *S.epidermidis*, a polymer of N-acetyl glucosamine, initially defined biologically as the polysaccharide intercellular adhesion (PIA) and chemically as Poly-N- acetyl-glucosamine (PNAG) has been identified as the molecule responsible for biofilm formation (Heilmann *et al*., 1996; McKenney *et al*., 1998). Some investigations into the ability of the *S.epidermidis* strains to form biofilm on inert surfaces and correlations with production of surface factors have been published (Hume *et al*., 2004). In order to make meaningful comparisons among different studies, it is important to determine which methodological differences can account for apparently disparate results. It can be inferred from the study that biofilm composition on leaf surface of lotus plant could play an important role in the colonization of bacterial population. We hypothesize that the biofilm on leaf surfaces should have the widest distribution of bacterial populations. Further studies can provide an insight on the protective mechanism of these isolates under varied conditions.

Acknowledgement

This is a part of the dissertation work carried out by **LR** in fulfillment of the degree in Biotechnology of this university and she is grateful to the School of Life Sciences for providing the facilities to carry out this small piece of work.

References

Ahn I. S. and Lee C. H. (2003): Kinetic studies of attachment and detachment of microbial cells from soil. *Environ. Technol.,* **24,** 411-418.

Dickson J.S and Koohamaraie M. (1989): Cell surface charge characteristics and their relationship to bacterial attachment to meat surfaces. *Applied and Environmental Microbiology.,* **55 (4)**, 832-836.

Gannon J. T., Manilal V. B. and Alexander M. (1991): Relationship between cell surface properties and transport of bacteria through soil. *Appl. Environ. Microbio.,* **57,** 190-193.

Heilmann C., Gerke C. and Predreau-Remington F. (1996): Characterization of Tn 917 insertion mutants of *Staphylococcus epidermidis* affected in biofilm formation. *Infection and Immunity.,* **64**, 277-282.

Holt J.G., Kreig N.R., Sneath P.H.A., Staley J.T. and Williams S.T. (1994): *Bergey's Manual of Systematic Bacteriology,* Vol. I and Vol. 2., 9th Edn, Williams & Wilkins, Baltimore, U.S. A..

Hume E., Baveja J., Murif B., Thissen H. (2004): The control of *Staphylococcus epidermidis* biofilm formation and in-vivo infection rates by covalently bound furanones. *Biomaterials.,* **25**, 5023-5030.

Mack D., Haeder M., Siemssen N. and Laufs R. (1996): Association of biofilm production of coagulase negative Staphylococci with expression of a specific polysaccharide intercellular adhesion. *J.Infect. Diseases.*, **174**, 881-884.

Marcell L.M. and Beattle G.A. (2002): Effect of leaf surface waxes on leaf colonization by *Pantoea agglomerans* and *Clavibacter michiganensis*. *Mol. Plant-Microbe Interactions.,* **15 (12)**, 1236-1244.

McKenney D., Hubner J., Muller E. and Wang E. (1998): The locus of *Staphylococcus epidermidis* encodes production of the capsular polysaccharide / adhesion. *Infection and Immunity*, 66**,** 4711-4720.

Morris C.E., Monier J.M. and Jacques M.A. (1998): A technique to quantify the population size and composition of the biofilm component in communities of bacteria in the phyllosphere. *Applied and Environmental Microbiology.,* **64(12)**, 4789-4795.

Sanin S. L., Sanin F. D. and Bryers J. D. (2003): Effect of starvation on the adhesive properties of xenobiotic degrading bacteria. *Process Biochem.,* **38,** 909-914.

Van Loosdrecht M.C.M., Lyklema J., Norde W., Schraa G. and Zehnder A.J.B. (1987): The role of bacterial cell wall hydrophobicity in adhesion. *Appied. Environ. Microbiol.,* **53**, 1893-1897.