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Screening and Characterization of Phylloplane Bacteria Producing *Vibrio fischeri* Autoinducer

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Sixty leave blades representing 14 different plant species in the Woods Hole, Massachusetts, area were collected for the isolation of phylloplane bacteria employing a leaf-print method on 10% King's B medium supplemented with 100 μg/ml cycloheximide. Colonies that appeared after 2-3 days were overlaid with Luria Agar (pH 7.4) containing approximately 10^9 cells/ml of *Escherichia coli* carrying *Vibrio fischeri lux* operon with a 250-base pair non-polar deletion in *luxI*. Presumptive autoinducer-producing bacteria were isolated from the spots of bioluminescens in the overlaid plates. These kinds of phylloplane bacteria represented approximately less than two percent of the total leaf-surface bacteria. Based on preliminary observation of colony morphology and limited physiological assays, they were two main groups of phylloplane bacteria belonging to either *Xanthomonas* sp. or *Pseudomonas syringae*. Accordingly, this method has the potential to be developed into a rapid and reliable diagnostic test, to study the distribution as well as relative abundance of certain strains or pathovars of phylloplane bacteria.

INTRODUCTION

Autoinduction is an environment sensing-system that allows bacteria to monitor their own population density, which is also coined as "quorum sensing" by some authors (Fuqua *et al.* 1994). This system requires a diffusible compound produced by the corresponding bacteria, which can accumulate in the surrounding environment during growth. This diffusible quorum-sensing compound is called autoinducer (Fuqua *et al.* 1994).

Vibrio fischeri autoinducer (VAI) is N-3-(oxohexanoyl) homoserine lactone which is required to activate autoinduction of bioluminescence. VAI at a concentration of 10 nM or approximately 40 molecules/cells is sufficient to activate autoinduction in this bacterium. In the light organ, *V. fischeri* can reach a very high density concentration as high as 10^{10}-10^{11} cells/ml. In seawater, however, *V. fischeri* is only found at approximately 10^2 cells/ml (Dunlap & Greenberg 1991, Ruby & McFall-Ngai 1992). There- fore, the autoinduction system might allow *V. fischeri* to discriminate between free-living (low cell-density) state and host-assosiated (high cell-density) state (Fuqua *et al.* 1994).

Previously, it was thought that the autoinducer for *V. fischeri* was species-specific, ie. other spesies of luminous bacteria neither responded to the pure molecule nor produced a compound that stimulated luminescence by *V. fischeri*. However, a large body of evidence showed that a compound similar to VAI is widely employed in many regulatory systems in Proteobacteria (Bainton *et al.* 1992, Pirhonen *et al.* 1993, Canamero & More 1993, Pearson *et al.* 1994). One implication of this study, is the understanding that certain bacterial behavior can be performed efficiently only by a sufficiently large population of bacteria.

The leaf surface is considered to be an inhospitable environment for microbial growth. Microorganisms which inhabit leaves must be able to evolve mechanisms to overcome the stressful or fluctuating environmental conditions (O'Brien & Lindow 1989). Recently, it has been demonstrated that colonization and survival of *Pseudomonas syringae* was dependent on the density of the inoculum (Wilson & Lindow 1994). In addition, virulence determinants of certain plant and animal-pathogenic bacteria have been shown to be regulated by VAI (Jones *et al.* 1993).

This study was aimed to evaluate the potential use of *lux* system of *Vibrio fischeri* for screening and characterization of specific autoinducer-producing phyloplane bacteria.

MATERIAL AND METHODS

Escherichia coli JM83 (pAK211) and JM83 (pAK-203) were obtained from Paul Dunlap, Woods Hole Oceanographic Institution, Wood Hole, Mass. Plasmid pAK211 carries all *lux* genes of *Vibrio fischeri*, except for the non-polar deletion of *luxI* (gene for autoinducer synthesis), while pAK203 carries functional intact *luxI* gene. *Pseudomonas syringae* pv. *tabaci* strain 2024, *Erwinia amylovora*, and *Xanthomonas campestris* pv. *glycines* 8Ra were obtained from Paul D. Shaw, Dept. of Plant Pathology, Univ. of Illinois, Urbana. *Xanthomonas campestris* pv. *glycines* XP202 (=NCPPB 1141), *X. campestris* pv. *phaseoli* strain XP34, XP28, and XP20 were obtained from Steven E. Lindow, Dept. of Environment Science, Policy and Management, Univ. of California, Berkeley.

Phylloplane of bacteria were isolated from surface of leaf-blades collected from around Woods Hole area. Leaves collected belonged to the following species of trees or shrubs: Rhododendron, Black Beech, Mint plant, Maple, roses, Acacia, Alnus, Yellow Lily, White Lily, Morning Glory (violet), as well as four kinds of grasses belong to Graminea.

Each individual leaf was pressed onto 10% King medium B agar (2 g Protease Peptone; 15 ml glycerol; 0.15 g K_2HPO_4; 0.15 g $MgSO_4.7H_2O$; 20 g Bacto agar; 1L distilled water; pH was adjusted to 7.4 by adding 1 N NaOH) suplemented with 100 μg/ml of cycloheximide. These leaf-print plates were incubated at 25-28°C for 2-3 days. Three leaf blades were used for each different plant species.

For the overlay method, *E. coli* JM83 (pAK211) was grown in Luria Bertani broth (pH 7.4) supplemented with 35 g/ml of chloramphenicol, at 25-28°C for 20-24 hr. Cells were harvested by centrifugation at 5000 rpm for 5 min. After washing in saline solution (0.9% NaCl), the cell pellet was resuspended in saline solution to approximately 10^{10} cells/ml. The cell suspension was mixed proportionally with liquid LA (5 g NaCl; 5 g yeast extract; 10 g tryptone; 15 g agar; 1 L distilled water; pH 7.4; 50°C) to yield a final concentration of approximately 10^9 cells/ml and a final agar concentration at 1-1.2%. This mixture (4-5 ml per plate) was quickly poured onto leaf-print plates to cover all of the collonies. Spots of bioluminescence were observed in the dark room after an 8-18 hr incubation. The putative autoinducer colonies were repurified by streaking each of them, side by side, with *E. coli* JM83 (pAK211) on LA plates.

The autoinducer-producing strains were characterized further, employing limited morphological and physiological assays, such as: characteristics of colonies, cell morphology, Gram-stain, catalase, oxidase activity, ability to grow anaerobically, as well as the ability to metabolize certain kinds of sugars (Smibert & Krieg 1994).

Scanning Electron Microscopy (SEM) was conducted as described by Behmlander & Dworkin (1991) and Watson *et al.* (1980). Fluorescence in-situ hybridization (FISH) was essentially performed as described by Braun-Howland *et al.* (1993).

RESULTS

The density of bacteria that occupied the leaf surface was determined to be approximately 22-300 cells/g of fresh leaf. All of the phylloplane isolates could be classified into 1-7 distinct groups depending on colony morphology (ie. color, appearance, elevation, margin, and texture) on 10% King's Medium B agar.

From approximately 80 individual colonies, it was found that isolates WP1 (WP = Woods Hole Phylloplane), WP18, and WP19 generated bioluminescence signals comparable to that of the positive control, ie. JM83 (pAK211), which was streaked besides JM83 (pAK203). Isolates WP8, WP14, and WP17 yielded a relatively weak signal of bioluminescence. The rest of the colonies did not show any bioluminescence when they were streaked besides JM83 (pAK211). Isolates WP20U did not produce *luxI* complementation, despite its identical colony morphology to WP1. It would be interesting to see the degree of similarity of these two isolates (WP1 and WP20U) at the genomic level.

Although the overlay assay was performed for all of the leaf-print isolates, only seven luminescence spots were observed. Reisolation and morphological characterizations showed that these seven isolated are belonged to either of two distinct groups (ie. WP1 and WP18) which were described in Table 1.

Based on colony morphology and limited physiological characteristics, isolate WP1 might be assigned in the genus *Xanthomonas*. In addition, FISH analyses also indicated that this isolate did not belong to Enterobacteriaceae, where the genus of *Erwinia* belongs to. Therefore, the identity of the WP1 isolate needs further clarification employing more rigorous physiology or genetic analyses.

Isolate WP18 seems to be a strain of *Pseudomonas syringae*, due to its obligate aerobic metabolism and its negative result in the oxidase test. It is interesting to note that from all plant pathogenic bacteria, included in this study, only *P. syringae* pv. *tabaci* yielded compound that could substitute the *V. fischeri* autoinducer. *Erwinia amylovora* yielded only a weak bioluminescence signal in this lux assay.

Isolates WP14 and WP20U showed similar colony morphology to that of WP1. These isolates were also facultative anaerobes. However, the lux assay indicated that these isolates did not produce the *V. fischeri* autoinducer. All *Bacillus* species obtained from leaf surface in this experiment were not able to complement the *V. fischeri* autoinducer.

DISCUSSION

Phylloplane bacteria, which can substitute the *V. fischeri* autoinducer (VAI), were present in low abundance on a healthy leaf surface, and these can be assigned to only two distinct groups as described above. Therefore, this method might be very useful for the development of a rapid

Table 1. Two main groups, of bacteria, obtained with the overlay method.

Isolates	Characteristics
WP1	Yellow with cupious of slime on 10% King's B, bright yellow on LA (pH 7.4), gram-negative, catalase positive, oxidase negative, rods, motile, facultative anaerobe. Scanning electron microscopy (SEM) demonstrated the presence of fibrous material (probably polysaccharide) interconnecting among the cells of this bacterium (Fig. 1). Flourescence in situ hybridization (FISH) analyses showed that this isolate did not appear to be a member of either Enterobacteriaceae, α or β - group of Proteobacteria (Woese, 1987).
WP18	White, rather mucoid on 10% King's B or LA (pH 7.4), gram negative, catalase positive, oxidase negative, rods, motile, obligate aerobe. SEM did not show fibrous materials on the cell surface as in WP1. FISH analyses showed identical results as in isolate WP1.

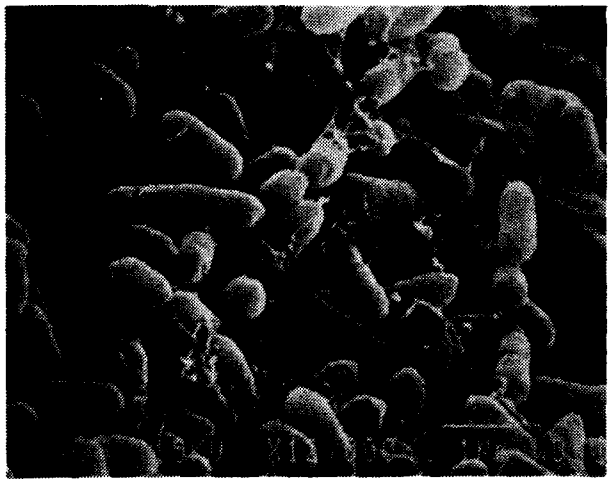

Figure 1. SEM of isolate WP1 showed the presence of extensive fibrous materials interconecting among the bacterial cells. This fibrous materials might be polysaccharide which was responsible for the excessive mucoid appearance of the collonies grown on 10% King's B medium.

strain or pathovar identification in phytobacteriology. To date, pathovar or strain identification for most plant pathogenic bacteria involve a time-consuming and laborious test, as well as a pathogenicity assay on particular plants. The availability of a rapid but reliable diagnostic test for these bacteria, will certainly facilitate both the basic study of pathogenicity and practical applications for early detection of a disease agent in agriculture.

Although this bioluminescence system is very sensitive, it should be conducted and observed in its optimal light emission. On LA (pH 7.4), bioluminescence was conveniently examined after an 8-24 hr incubation at 25-27°C. Very early examination or prolonged incubation produced false-negative results. Light production might be kept longer by increasing buffer capacity of the growth media. In addition, the overlay agar should be as thin as possible (not more than 3 mm), to allow as much oxygen supply to the culture underneath it during the luminescence assay.

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