Rapid Detection of Cadmium-Resistant Plant Growth Promotory Rhizobacteria: A Perspective of ELISA and QCM-Based Immunosensor

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Received: August 23, 2011 / Revised: November 17, 2011 / Accepted: February 11, 2012

Plant growth-promoting rhizobacteria (PGPR) pseudomonads have a large number of lipopolysaccharides on the cell surface, which induces immune responses. Cd-resistant PGPR prevalent at the Cd-affected sites under biophytostabilization was monitored. Transmission electron microscopy was used to the study the behavior of tolerance of PGPR to cadmium level and its effect on pseudomonad strains (Z9, S2, KN2, CRPF, and NBR1). An immunosensor was developed by immobilizing antibody (anti-Z9 or anti-S2) against selected PGPR on a piezoelectric quartz crystal microbalance (QCM). Immunosensors were found to supplement the inherent specificity of antigen–antibody reactions with the high sensitivity of a physical transducer. On comparison of the efficiency of detection with ELISA, the spectrophotometric technique, the developed immunosensor was found to be more sensitive, fast, and reliable even after regeneration for several times. Thus, the immunosensor may be used for future detection of PGPR strains after automation of the screening process.

Keywords: PGPR, ELISA, immunosensor, QCM, rhizobacteria, bioremediation

With the advent of industrialization, pollution due to heavy metals like cadmium has considerably increased. Cadmium is a heavy metal having toxicity 2–20 times higher than other heavy metals and is placed at the 7th position among the top 10 priority hazardous substance list as provided by the American Agency for Toxic Substances and Disease Registry and is the 4th most toxic metal to vascular plants. It is a by-product of the zinc smelting industry, a component of phosphatic fertilizers, a B1 human carcinogen, induces premature senescence in plants, and does not have any biological significance except for its use in storage batteries [19].

Bioremediation by fluorescent pseudomonads, the established plant growth-promoting rhizobacteria (PGPR) [20] that have cadmium resistance, emerged as a powerful technique for remediation of cadmium-affected sites [8, 11]. The cell surface of Pseudomonas bears an overall negative charge owing to the presence of various anionic structures. This gives bacteria the ability to bind metal cations. Thus, microbes have attracted attention because of the biotechnological potential of microorganisms for metal removal directly from polluted soils or the possible transfer of accumulated metals to higher plants [15]. Transmission electron microscopy is thus utilized to study cadmium uptake or metabolism by the PGPR. The surface of fluorescent Pseudomonas possesses a number of lipopolysaccharide macromolecules that can be used to induce immune responses and thus for test strains monitoring. The two possible approaches, ELISA (the serological markers) and immunosensors (an immunoassay), were used for such monitoring.

Quartz crystal microbalance (QCM)-based immunosensors are important analytical tools for monitoring antibody–antigen reactions, allowing rapid and continuous analysis of the binding event without the requirement for added reagents or separation/washing steps [3]. Immobilization of biomolecules on gold surfaces is readily achieved by making use of self-assembled monolayers (SAMs) [1]. In contrast, ELISA, the most common and widespread immunoassay technique based on spectrophotometry, despite being cost-effective, has certain demerits [6, 11]. ELISA

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employs a very complex set of steps that make it very frustrating at the commercial level. The facts that a label is not needed in a QCM-based piezoelectric immunosensor, detection is insensitive to those interferences that sometimes affect spectrophotometric measurement, and the ease of use are of course some advantages over ELISA. Furthermore, these could provide shorter analysis times and higher sensitivity, while also requiring lower sample volumes. As a result, great interest has focused on commercializing immunosensors for applications in clinical, environmental, and food areas [4, 9, 10, 18]. The detection via QCM piezoelectric crystal is based on the fact that a decrease of resonance frequency is correlated to the frequency change (ΔF) to the mass loaded (ΔM) by Saurbrey’s equation

\[
\Delta F = -2.3 \times 10^6 F^2 \Delta M/A
\]

where \(\Delta F\) = frequency change in the oscillating crystal in Hz, \(F\) = frequency of piezoelectric quartz crystal (=10 MHz), \(\Delta M\) = mass of deposited film in g, and \(A\) = area of gold-coated QC surface (=14 mm²) [21].

In this paper, we developed an effective, fast immunoosensor technique specific for Cd-resistant *Pseudomonas*, the PGPR monitor, which proved to be better than conventional ELISA for the determination of PGPR.

**MATERIAL AND METHODS**

**Materials and Bacterial Strains**

Bacterial strains (5 PGPR *Pseudomonas* strains, viz., Z9 (C1), S2 (C2), KNP2 (C3), CRPF (C4), and NBRI (C5)) used in this study were provided by Dr. Rajesh Kumar, Assist. Professor, of Department of Microbiology, GBPUAT Pantnagar, India, for this research activity. These strains were revived on King’s B medium, pH 7.0, containing 10 g/l peptone, 1.5 g/l anhydrous K₂HPO₄, 15 g/l glycerol, and 5 ml of MgSO₄ (1 M) broth by incubation at 30°C for 24 h. All chemicals are procured from HiMedia Lab. Ltd., Mumbai, India.

**Effect of Cadmium Concentration on Tolerance and Size of *Pseudomonas***

All five strains were tested at different concentrations of cadmium sulfate (0 to 500 ppm) in King’s B broth under growth conditions at 30°C for 48 h. The respective tolerance level of all the five strains in the presence of cadmium sulfate was measured spectrophotometrically at 600 nm.

The effect of cadmium sulfate on size of *Pseudomonas* strains (Z9 and S2) was studied using micrometry and TEM. The control as well as cadmium-salt-fed cultures were taken and a comparative morphological study was performed using TEM micrographs after negative staining of the strains. The isolated material (bacterial sample in the form of pellet in PBS) was mixed with glutaraldehyde and the suspension was spread on a support grid coated with plastic (film). After negative staining in uranyl acetate (c-dense metal stain), the samples were placed onto formvar carbon-coated grids for electron microscope observation. The samples were scanned and micrographs were taken using a JEM 1011 transmission electron microscope.

**Antigen Preparation and Antibody Generation**

Loopful cultures of *Pseudomonas* strain Z9 (C1) and S2 (C2) were suspended in water of condensation in Roux bottles containing King’s media, and incubated at 28°C for 48 h. A homogenous suspension was harvested using glass beads and, after filtration through a Buchner’s funnel, centrifuged at 10,000 rpm for 15 min to get a pellet, which was dissolved in a minimum amount of PBS (whole-cell antigen), sonicated at the rate of 15 cycles each of 60 s, with a time interval of 60 s after each operative consecutive cycle, extracted, and stored at 4°C.

A 6-week-old rabbit (New Zealand variety) collected from the Indian Veterinary Research Institute (IVRI), Bareilly, injected subcutaneously with 1 ml of whole-cell antigen mixed with Freund’s complete adjuvants (CA)/ incomplete adjuvants (IA) in 1:1 ratio, was injected intramuscularly (IM) and subcutaneously (SC) at multiple sites, at a time, every 7th day. For the 0th day, Freund’s CA was used. Five days after the immunization, blood was obtained from the ear of the rabbit and processed to clot, and antiserum obtained. The antibody titer was observed by serial dilution of antiserum and the agglutination test.

**Monitoring of Cultures Using ELISA**

The population of PGPR strains Z9 and S2 was monitored by ELISA. Both homologous (Z9, S2) and heterologous (KNP2, NBRI, CRPF) reactions were run as positive and negative controls. Antigen (200 µl) was poured per well and incubated at 5°C for overnight. The ELISA plate was washed with washing buffer (0.05% Tween-20 in PBS, pH 7.4) and then 200 µl of blocking solution (1% BSA) was added. It was incubated at 37°C for 1 h and then 200 µl of primary antibody [high titer homologous anti-fluorescent specific for *Pseudomonas* strain Z9 (C1) and S2 (C2)] was added. The plate was incubated at 37°C for 2–3 h and then 200 µl of alkaline-phosphatase-labeled anti-rabbit goat IgG (SRL Chemicals, Mumbai, India) was added, and incubated at 37°C for 1–2 h. Finally, 200 µl of substrate p-nitrophenyl phosphate [0.6 mg/ml in substrate buffer (9.7 ml of diethanolamine in 1.1. distilled water, pH 9.8; SRL Chemicals, Mumbai, India)] was added and then incubated at room temperature for 30 min in the dark. The reaction was stopped by addition of 50 µl of 3 M NaOH solution. The chromogenic color obtained was measured at 405 nm.

**Quartz Crystal Microbalance (QCM) and Transducer Preparation**

QCM consisted of a gold-coated AT-cut quartz crystal of 10 MHz. Two identical gold electrodes, 2,000 Å thick and 5 mm in diameter, were deposited by evaporation techniques on both sides of the crystals with a chromium underlayer supplied by Maxtek, USA Co. Ltd. The resonators were carefully cleaned by acetone and then ethanol for 2 min in an ultrasonic bath, and were dried under pure nitrogen and connected with a silver conducting paste, through wires, to BNC connectors. An oscillator attached to the frequency counter was to drive the crystal at a constant frequency controlled by a computer. All the measurements were taken at room temperature. The crystal installed in the poly (vinyl chloride) non-flowing cell was connected to the oscillation circuit. The diameter of this poly (vinyl chloride) cell well was 5 mm.

**Immobilization of Antibody on Gold Disk**

**Experimental design.** The classical “one-variable-at-a-time approach” was employed to evaluate the effect of incubation time, pH, antibody concentration, and antigen concentration on frequency shift and
hence on immobilization efficiency. Ten replicates per specimen were taken for each measurement and statistical analysis was performed using Microsoft Excel and Origin softwares.

Antibodies were immobilized on the crystal by the dip-and-dry cystamine method [9]. The crystals were first immersed in a cystamine solution (10 mmol/l cystamine, 50 mmol/l phosphate buffer, 0.15 mol/l NaCl, pH 7.0) for 1 h. The crystals were washed with distilled water and dried, until a stable frequency was reached. The cystamine-coated crystals were incubated with the antibody solutions containing different amounts of anti-Z9 or anti-S2 antibodies (20, 40, 60, 80, 100, 120, 140, 160, 180, 200 µg/ml buffer solution) at different pH values (5.2, 6.2, 7.2, 8.2, or 9.2). The buffers used were sodium acetate (pH 5.2 to 6.2), phosphate (pH 7.2 to 8.2), and glycine buffer (pH 9.2). The incubation period was also changed in the range of 2–20 h. Physically adsorbed antibody molecules were removed by washing with PBS-T (PBS with Tween-20). The frequency shifts were measured as a function of time and standardized with each immobilization step.

**PGPR detection.** The piezoelectric crystals carrying anti-Z9 or anti-S2 antibodies were incubated in the solutions containing different amounts of antigens Z9 and S2 (30, 60, 90, 120, and 150 µg/ml) for 1 h, which tend to adsorb onto the surface of the gold-coated crystal at pH 7.2 (phosphate buffer). The crystals were washed several times with PBS-T, dried, and the frequency shifts were measured in air. Ten replicates per specimen were taken for each measurement. **Statistical analysis.** For statistical analysis, a standard deviation for each experimental result was calculated using the Excel spreadsheets available in Microsoft Excel.

**RESULTS AND DISCUSSION**

**Effect of Cadmium on Pseudomonas Strains**

All the five strains were known to be cadmium resistant so we tested for this property at different concentrations of cadmium sulfate ranging from 0 to 500 ppm in King’s B broth after incubation at 30°C for 48 h (Fig. 1). Absorbance values at 600 nm were more than at zero time and hence zero time values are not shown. All the strains were found to tolerate 500 ppm of cadmium sulfate salt, with strains Z9 and S2 giving better responses than the other three Pseudomonas strains. However, with increasing concentration of cadmium salt, there was a fall in the absorbance values, indicative of the toxicity of the salt.

TEM analysis of PGPR strains Z9 and S2 showed an increase in cell size when grown on cadmium-sulfate-fed culture as compared with the control samples grown in cadmium-less medium (Table 1 and Fig. 2 and 3). This might be due to cadmium adsorption by the Cd resistance property in bacteria. The turbid structure around the bacteria in Fig. 2B and 3B seems like the bacterial cell is surrounded by the cadmium solvated in culture phase. The Cd-uptake process is apparent in these micrographs, whereas such type of phenomenon is absent in Fig. 2A and 3A where Cd salt was not given to the strains.

**Antibody Titer and ELISA Analysis**

Antibody titer was found to be 200 for Z9 and 6,400 for S2 and used for ELISA analysis. Graphical representations of ELISA as absorbance at 405 nm are shown in Fig. 4 and 5. Anti-Z9 and anti-S2 had been used against four strains, KNP2, NBRI, S2, and Z9. Strain Z9 exhibited maximum absorbance while taking anti-Z9, and S2 with anti-S2. This could be due to a homologous Ag-Ab biocomplexation showing more binding in comparison with a heterologous one (i.e., with others strains of same Cd-resistant rhizobacteria). KNP2 and Z9 showed very little reactivity with Z9 and S2, respectively, which was factual representation of some cross-reactivity, but since it was negligible it could be ignored.

**Table 1.** Effects of different concentrations of cadmium sulfate on strains cell size as studied by micrometry.

<table>
<thead>
<tr>
<th>CdSO₄ (ppm)</th>
<th>Z9</th>
<th>S2</th>
<th>KNP2</th>
<th>CRPF</th>
<th>NBRI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>length × breadth (µm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.71-1.16 × 0.71-0.74</td>
<td>0.71-1.23 × 0.69-0.70</td>
<td>0.71-1.06 × 0.68-0.71</td>
<td>0.68-1.13 × 0.66-0.71</td>
<td>0.71-1.13 × 0.65-0.71</td>
</tr>
<tr>
<td>250</td>
<td>0.71-1.42 × 0.58-0.60</td>
<td>0.68-1.42 × 0.69-0.71</td>
<td>0.71-2.84 × 0.68-0.71</td>
<td>0.58-2.22 × 0.69-0.71</td>
<td>0.71-1.42 × 0.68-0.71</td>
</tr>
<tr>
<td>500</td>
<td>0.71-2.84 × 0.68-0.70</td>
<td>0.71-2.44 × 0.0.71-0.72</td>
<td>0.71-1.42 × 0.65-0.71</td>
<td>0.71-1.44 × 0.64-0.71</td>
<td>0.71-2.84 × 0.66-0.68</td>
</tr>
</tbody>
</table>
Effects of incubation time. The antibody concentration was constant at 100 µg/ml both for anti-Z9 and anti-S2 at pH=7.2. The frequency shifts (average of 10 crystals and SD) were observed at different incubation times (Fig. 6). Sixteen hours was found to be the optimum for immobilization of both anti-Z9 and anti-S2 molecules.

Effects of pH. The change of frequency shifts was observed with the change in pH (Fig. 7). The frequency shifts with anti-Z9 and anti-S2 were maximum for the crystal coated with antibodies at pH 7.2.

Effects of antibody concentration. The optimum concentration of the ligand in the immobilization medium was 140 µg/ml for Z9 and 120 µg/ml for S2, which showed maximum shift in frequency (Fig. 8).

Immunosensing by QCM

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PGPR detection. The piezoelectric crystals carrying antibodies were tested for PGPR sensitivity. Three reactions were run,
first with BSA (control), second with homologous antigen (Z9 or S2), and third with heterologous antigen (those showing little cross-reactivity with the two antigens; i.e., KNP2 for anti Z9 and NBRI for S2). As compared with the homologous reactions, only a slight change in frequency shift was observed in the heterologous ones. The frequency shifts measured for the homologous reactions were 99/10, 150/8, 176/8, 208/7, and 208/11 Hz for Z9 (Fig. 9) and 124/12, 163/9, 200/8, 200/11, and 200/9.8 Hz for S2 (Fig. 10) at 30, 60, 90, 120, and 150 µg/ml antigen concentration, respectively.
DISCUSSION

Piezoelectric biosensor systems are very attractive systems that, in principle, may be used for a wide range of applications in the food industry, environmental monitoring, clinical diagnostics, and biotechnology. Earlier, many piezoelectric immunosensors have been developed for monitoring *Vibrio cholera* [2], *Candida albicans* [17], *L. monocytogenes* [12], *Salmonella* Typhimurium [23], porcine reproductive and respiratory syndrome virus (PRRSV) [22], and mycobacteria [13]. This exclusive report on monitoring of cadmium-resistant PGPR responsible for bioremediation, using a QCM-based piezoelectric immunosensor, is based on the same idea that if the sensor surface is coated with specific antibodies, the bacteria will bind to it when the sensor is allowed to come in contact with the bacterial solution. Because of this, the mass of the crystal increases while the resonance frequency of oscillation decreases proportionally.

The immobilization employing cystamine was found to be successful. The cystamine adsorbs to the gold electrode as its disulfide bonds are broken. Cystamine-NH binds to the C=O of the IgG molecule of anti-fluorescent *Pseudomonas* and not to the -NH of the antigen binding sites in the IgG molecule [3, 5, 14].

The low pH of the regeneration buffer could easily disrupt Ag–Ab complexes, but not disrupt Ab–SAM complexes. Even if the Abs had been partly disrupted at low pH, enough Abs were present on the SAM to react with the antigens, as there were many hydrogen bonds in Ab–SAM.

An important outcome of this work is that the sensor was reusable about 10 times without any detectable loss in activity. This is a positive aspect, as piezoelectric crystals are not cheap enough to justify disposal after a single assay. The regeneration solution, 0.1 mol/l glycine-HCl buffer (pH 2.1), was used after each measurement [3, 4]. Immunosensor systems to detect PGPR or pathogens at ports and in field situations at low cost are the demand of this area.

Acknowledgments

The senior author (Ruchi Agrawal) deeply acknowledges the assistance provided by the Department of Microbiology, GBPUAT, Pantnagar and Department of Science and Technology (DST), Government of India for providing an INSPIRE fellowship during the doctoral program.

REFERENCES


