Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2011, Article ID 701928, 10 pages
doi:10.1155/2011/701928

Research Article

Proteomics Analyses of the Opportunistic Pathogen *Burkholderia vietnamiensis* Using Protein Fractionations and Mass Spectrometry

Samanthi Wickramasekara,[1] Julie Neilson,[2] Naren Patel,[2] Linda Breci,[1] Amy Hilderbrand,[1] Raina M. Maier,[2] and Vicki Wysocki[1]

[1] *Department of Chemistry and Biochemistry, University of Arizona, 1306 E University Bolevard, Tucson, AZ 85721, USA*
[2] *Department of Soil Water and Environmental Science, University of Arizona, Tucson, AZ 85721, USA*

Correspondence should be addressed to Vicki Wysocki, vwysocki@email.arizona.edu

Received 2 June 2011; Revised 27 August 2011; Accepted 27 August 2011

Academic Editor: Isabel Sá-Correia

The main objectives of this work were to obtain a more extensive coverage of the *Burkholderia vietnamiensis* proteome than previously reported and to identify virulence factors using tandem mass spectrometry. The proteome of *B. vietnamiensis* was precipitated into four fractions to as extracellular, intracellular, cell surface and cell wall proteins. Two different approaches were used to analyze the proteins. The first was a gel-based method where 1D SDS-PAGE was used for separation of the proteins prior to reverse phase liquid chromatography tandem mass spectrometry (LC-MS/MS). The second method used MudPIT analysis (Multi dimensional Protein Identification Technique), where proteins are digested and separated using cation exchange and reversed phase separations before the MS/MS analysis (LC/LC-MS/MS). Overall, gel-based LC-MS/MS analysis resulted in more protein identifications than the MudPIT analysis. Combination of the results lead to identification of more than 1200 proteins, approximately 16% of the proteins coded from the annotated genome of *Burkholderia* species. Several virulence factors were detected including flagellin, porin, peroxiredoxin and zinc proteases.

1. Introduction

Burkholderia is a ubiquitous organism that occupies many diverse ecosystems such as soil, water, and even the human respiratory tract. The "*Burkholderia cepacia* complex (BCC)" consists of nine closely related *Burkholderia* species that have been widely studied and used for various purposes including biological control of plant pathogens, bioremediation of organic compounds, and plant growth promotion [1]. There are several pathogenic strains of *Burkholderia* that cause diseases in plants and animals. Among those that are pathogenic, *Burkholderia pseudomallei* is the causative agent for Melioidosis, and *B. mallei* is responsible for glanders disease and was used as a biological weapon in World War I; both remain a potential threat today [2]. For this study we used the opportunistic pathogen *Burkholderia vietnamiensis*, which causes diseases in immunocompromised individuals and patients suffering from cystic fibrosis, as the model organism [3]. *B. vietnamiensis* has minimal growth requirements and easily adapts to a range of nutritional conditions enabling it to survive in unfavorable conditions [4].

There are many virulence factors associated with the pathogenicity of the *Burkholderia* species. The most common factors that have been studied are the quorum-sensing molecules which help cell-to-cell communication and adherence [5, 6]. According to previous studies many proteins asociated with pathogenicity of *Burkholderia* species are expressed in very low amounts within the bacterial cell. In previous studies designed to identify these virulence factors, the proteins were overexpressed in *E. coli* and amplified before their analysis [7–9]. In this study mass spectrometry was used to identify these low abundance virulence factors without prior amplification.

Recently, Riedel et al. reported use of a 2D reference map to identify proteins from *B. cenocepacia* [10]. This involved analysis of the digested peptides using a MALDI TOF-TOF

mass spectrometer. In our study, both gel-based LC-MS/MS and gel-free multidimensional protein identification technology (MudPIT) analyses were used to identify proteins from *B. vietnamiensis*. By combining both methods, many more proteins were identified compared to the 2D gel MALDI approach.

The main objectives of the present experiments are to achieve high coverage of the opportunistic pathogen *Burkholderia vietnamiensis* G4 proteome expressed in a minimal medium and to identify the expressed proteins, including low abundance virulence factors, using mass spectrometry. Minimal media and late log phase collection were chosen to favor enhanced production of virulence factors and to provide a "baseline" for future studies where growth conditions will be varied. The proteome of the organism was divided into extracellular, cell surface, cell wall, and intracellular protein fractions. Gel-based and gel-free proteomics which have been used to identify proteins from many sources including pathogenic bacteria, cancer cells, and different tissue types were used. For the gel-based approach, a 1D gel separation and LC-MS/MS analysis was applied. By initially fractionating the sample, cutting each fraction's whole gel lane into 16 pieces, and digesting slices individually, we generated more protein identifications than spot selection on a 2D gel as indicated in the MALDI-TOF MS approach [10]. Clearly, those authors could have run MALDI on every spot, resulting in additional IDs. In addition, MudPIT was used to analyze the bacterial proteins. This method involved two types of HPLC separation, namely, strong cation exchange (SCX) followed by reverse phase separation.

2. Materials and Methods

2.1. Growth of Bacteria and Protein Assay. B. vietnamiensis G4 strain (100% homologous to AY268153 Gene Bank DNA sequence accession number [11]) was initially obtained from the Maier lab culture collection. One liter of mineral salt medium (MSM-a minimal medium) containing 2% glucose as the sole carbon and energy source was used to grow the bacterium at room temperature according to methods described in the literature [12]. The bacterial cells were harvested in the late exponential phase when the optical density of the culture reached about 1.6 at $\lambda = 600$ nm. Optical density was measured using a HITACHI U2000 spectrophotometer. Total cellular protein from the bacteria was determined by the Lowry assay using the calibration curve plotted with standard bovine serum albumin (BSA-Sigma) [13, 14].

2.2. Extraction of Extracellular Proteins. Extracellular proteins were separated from the bacterial culture using previously described methods [10, 15] with slight modifications. Briefly, the bacterial culture was centrifuged using a Beckman J2-21 centrifuge at 5000 rpm for 20 min. The cell pellet was used for the fractionation of intracellular, cell wall, and cell surface proteins. One hundred mL of the bacterial supernatant (extracellular fraction) was mixed with ice cold 10% (w/v) trichloroacetic acid (TCA-Sigma) and kept overnight at 4°C. The solution was then centrifuged at 5000 rpm for 20 min. Precipitated proteins were washed with 95% ethanol, air dried and divided into two portions. One portion was dissolved in protein sample buffer (buffer S-250 mM Tris [pH = 6.8], 30% glycerol, 8.0% sodium dodecyl sulfate (SDS), 1%β-mercaptoethanol and a trace amount of bromophenol blue as the coloring agent) to run protein gel electrophoresis. The second portion was dissolved in urea buffer (8 M urea in 50 mM ammonium bicarbonate) for MudPIT analysis.

2.3. Extraction of Intracellular and Cell Wall Proteins. The cell pellet obtained after the removal of the supernatant was washed twice with 0.9% NaCl and centrifuged again for 20 min at a speed of 5000 rpm. The pellet was then washed with Tris/HCl (pH = 7.5) and centrifuged again. One gram of the cell pellet was suspended in 10 mL of Tris/HCl buffer and sonicated using a Branson Sonifier 450 probe sonicator for 20 min. After sonication, the cell wall fraction was removed by centrifugation. To analyze the cell wall proteins, the protein pellet was dissolved in protein sample buffer (buffer S) for SDS-PAGE separation and urea buffer for MudPIT analysis. The supernatant, after removing the cell wall pellet, was used to analyze intracellular proteins. The proteins present in the supernatant were precipitated using a 3-fold volume of ice-cold acetone and kept overnight at −20°C. The precipitated proteins were separated by centrifugation at 5000 rpm for 20 min. The precipitate was washed, air dried, and dissolved in protein buffers for SDS-PAGE and MudPIT analysis.

2.4. Extraction of Cell Surface Proteins. Five grams of the bacterial cell pellet was suspended in 100 mL of 0.2 M glycine/HCl with stirring at 20°C for 1 h. In the acidic medium, all the soluble proteins present in the surface of the cell will move into the solution. The cellular debris was removed by centrifugation with the soluble proteins remaining in the supernatant. The supernatant was extracted and neutralized with 0.1 N NaOH and proteins were precipitated with ice-cold acetone (three volumes) and kept at −20°C overnight. The precipitate was washed two times with 95% ethanol and air dried. The protein pellet was used to analyze cell surface proteins using gel-based LC-MS/MS and MudPIT mass spectrometric techniques.

2.5. 1D SDS PAGE and In-Gel Digestion. For SDS-PAGE and in-gel digestion, 50 mg of protein fractions were dissolved in the protein sample buffer (Buffer S) and boiled in a water bath for 5 min. Samples were spun down and loaded into the 50 μL well in a BioRad (12% Tris-HCl) precast ready gel (BioRad, Hercules, Calif, USA). Protein gels were run by using 1x running buffer (Buffer R-259 mM Tris base, 2 M glycine, 1% (w/v) SDS diluted with distilled water) at a constant voltage of 85 V for 20 min followed by 150 V for 40 min (until the dye runs off the gel). The gels were then stained with silver stain as described previously [16]. A single lane of stained gel was cut into 16 pieces of approximately the same size and transferred into 16 wells of a 96-well plate to perform in-gel tryptic digestion.

Gel bands were destained [17] and tryptically digested using a PerkinElmer MultipPROBE II Plus Ex robotic liquid handling system according to methods indicated in the literature [18]. The digested peptides were extracted with 50% acetonitrile and 2% formic acid solution. After extraction, the peptides were transferred into 500 μL Eppendorf tubes and dried down using a Savant Speed Vac concentrator. Each concentrated peptide solution (10 μL) containing ~50 μg of protein was loaded into individual HPLC autosampler vials and analyzed by LC-MS/MS.

2.6. Preparation of Samples to Run by MudPIT. Proteins were reconstituted in 100 μL of 8 M urea in 100 mM NH_4HCO_3. Reduction of the proteins was carried out using 2 μL of 100 mM dithitheritol (DTT) with incubation for 15 min at room temperature [18]. Reduced proteins were alkylated by adding 3 μL of 100 mM iodoacetamide and incubated for 15 min in the dark at room temperature. Enzymatic digestion was carried out using 12 μL of 0.3 μg/μL endoproteinase Lys C (Princeton Separation, Freehold, NJ, USA) and incubated for 2-3 h at 37°C. The solution was diluted with 650 μL of 100 mM NH_4HCO_3, 50 μL ACN and 9 μL of 100 mM $CaCl_2$. Twelve microliters of 0.1 μg/μL Trypsin (Sigma, Saint Louis, Mo, USA) was then added to the diluted sample mixture, mixed thoroughly and incubated overnight at 37°C. Digested peptides were cleaned and desalted using Spec PT C18 solid phase cartridge (Ansys Diagnostics Lake Forest, Calif, USA). The resulting peptides were dried under vacuum and redissolved in 0.5% formic acid before the MudPIT analysis.

2.7. Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) of Digests from Gel Bands. Silica capillary columns (100 μm i.d.) were packed with methanol/Zorbax C18 stationary phase slurry, using a bomb loader, up to about 7 cm [18]. The packed column was connected with the commercial instrument according to the method established in the lab [18, 19]. A flow splitter was used to connect the capillary column to the HPLC system. The sample was injected onto the HPLC column by the Surveyor autosampler. The HPLC system was connected to the linear ion trap LTQ mass spectrometer (Thermo Fisher, San Jose, Calif, USA) to carry out peptide analysis.

The digested peptides from the gel bands were eluted in a buffer gradient consisting of buffer A (H_2O with 0.1% formic acid) and buffer B (ACN with 0.1% formic acid) at a flow rate 400 μL/min into the LTQ. The peptides were eluted with a linear gradient from 0 to 50% buffer B over 30 min intervals after the initial 10 min washing step with buffer A. The concentration of buffer B was increased from 50 to 98% over 10 min followed by a 5 min wash with 5% B. Spectra were scanned over a mass/charge range of 400–1500 mass units. Xcalibur data-dependent software was set to fragment the seven most intense peaks as described previously [19].

2.8. SCX/LC-MS/MS (Multidimensional Protein Identification Technique-MudPIT). For MudPIT analysis two types of columns were prepared. First, a silica column (100 μm i.d.) was packed with 5 μm poly-hydroxyethyl A strong cation exchange resin (PolyLC; TheNest Group, Southborough, Mass, USA) to 6 cm in length. Second, a C18 column was packed as previously described in Section 2.7. The two columns were connected through a small frit. A T-junction was used to apply voltage through a gold electrode [20]. For the MudPIT analysis, 13 different solvent/salt steps were used. A 10 μL (~50 μM) sample was injected into the sample loop using a Surveyor autosampler. The sample was loaded into the SCX column using an isocratic gradient of 95% buffer A and 5% buffer B for 70 min. To elute the nonbonded peptides a solvent gradient similar to that of reverse phase separation was applied over a period of 125 min. A 4 min pulse of 10% Buffer C (250 mM ammonium acetate, 5% ACN, 0.1% formic acid) was applied to the LC system followed by a gradient of 5–50% solution B over 65 min, 50–98% B over 5 min and 5 min wash with 98% B. The percentage of Buffer C was increased in 10% increments up to 100% so as to form a step elution gradient. The last step of the elution was 50% of buffer D (1.5 M ammonium acetate, 5% ACN, 0.1% formic acid) for 4 min followed by reverse phase gradient with extended washing time at 98% buffer B to elute strongly bound peptides. Data from all 13 steps were combined including the loading and initial washing step to get comprehensive coverage of all the peptides eluted from the column.

2.9. Data Analysis. The SEQUEST database search algorithm (Thermo Fisher, San Jose, Calif, USA; version 27) was used to interpret the MS/MS spectra obtained from the LC-MS/MS and MudPIT runs. The SEQUEST database search algorithm correlates experimental data with theoretically generated spectra from a known protein sequence in a database [21, 22]. All spectra were matched to a protein database created using all web-available sequenced *Burkholderia* protein databases and common contaminants such as trypsin and human keratin peptides. The *Burkholderia* database consists of *B. vietnamiensis, B. cenocepacia, B. thailandensis, B. cepacia, B. multivorans, B. pseudomallei, B. mallei, and B. xenovorans*. *E. coli* proteins were added into the database as distractants. Protein sequences were obtained from NCBI (http://www.ncbi.nlm.nih.gov/), Sanger Institute (http://www.sanger.ac.uk/) and Expasy (http://www.expasy.org/). In the SEQUEST database search, the following criteria were used: the minimum X-correlation factor (Xcorr) was set to 1.8, 2.5, and 3.5 for singly, doubly, and triply charged peptides, respectively. The delta correlation score (dCn) was set to 0.08 which indicates a significant difference between the best-matched peptide reported and the next best-matched peptide. Carbamidomethylation (57) of cysteine was used as a static modification and oxidation of methionine (16) was used as a dynamic modifications for the SEQUEST search [23, 24]. In addition, protein data were searched for phosphorylation as a posttranslational modification. All SEQUEST data searches were filtered using DTASelect and Contrast programs [25]. Here we solely report theproteins with two or more peptide

matches obtained from the SEQUEST database search algorithm. To confirm protein identification, all data were reanalyzed using a reverse database from the same protein sequences.

All data were also analyzed with a second algorithm using the web available X-Tandem (http://www.thegpm.org/) software [26, 27]. To run X-Tandem, first all the data containing.*dta* files were combined using a lab developed perl script "sub_append.pl and append.pl" [28]. For the X-Tandem analysis, the expectation score was set to 0.02. Parent ion mass tolerance of the peptide was set at 1.5 and fragment ion mass tolerance was set to 0.5. The same static and dynamic modifications were selected for the X-Tandem analysis as for the SEQUEST analysis.

Scaffold (version Scaffold-01_07_00, Proteome Software Inc., Portland, Ore, USA) was used to compare and validate SEQUEST and X-Tandem data. Proteins with two or more peptide identifications based on the combination of results from both database search algorithms were taken as true identifications. For the Scaffold analysis, the following probability values were selected: 95% for peptide identification calculated using PeptideProphet software [29] and 99% for protein identification with the additional requirement that protein should contain at least 2 peptides per protein. Protein probability was calculated using the ProteinProphet algorithm within Scaffold [30]. Proteins identified from the reverse database were eliminated from the total number of proteins identified in both gel-based and gel-free methods, and the false discovery rate (FDR) was ~1% of the total protein detected using the abovementioned methods. At least two biological replicates and three technical replicates were analyzed from each protein fraction. All proteins identified using these criteria are listed in the supplemental material (See supplementary material available at doi:10.1155/2011/701928).

3. Results and Discussion

3.1. Protein Identification. Proteins from *B. vietnamiensis* grown in mineral salt medium, with 2% glucose as the carbon source, were fractionated into 4 different fractions, extracellular; intracellular; cell surface, and cell wall proteins. By combining both gel-based and gel-free proteomic analysis data, total of 1258 proteins were identified with 99% protein identification probability. *E. coli* was added into the protein database as a distractor to further validate the protein identifications, and only 7 proteins were identified as *E. coli* proteins, increasing the confidence of protein identification. The much smaller number of hits from the distractors supports the identity of the proteins for the targeted organism. Phosphorylation was used as a possible posttranslational modification in the database search algorithms. For most of the data sets, only one or two phosphorylated peptides were found. This is likely due to the fact that that sample preparation did not include the enrichment of phosphorylated peptides and no MS^3 experiments were performed to identify phosphorylation [31]. Figure 1 summarizes the total number of proteins identified using both gel-based and gel-free proteomic analysis.

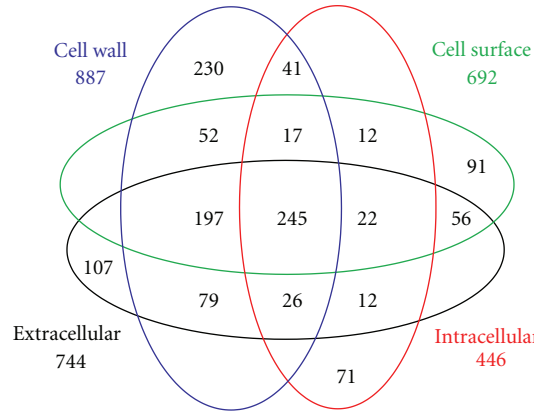


Figure 1: Venn diagram illustrating the total number of proteins identified from each fraction and the overlapping identifications using gel-based separation coupled to reverse phase LC-MS/MS and gel-free MudPIT analysis (LC/LC-MS/MS). Circles indicate the number of proteins identified with two or more peptide matches from the corresponding protein fraction with 99% or higher protein identification probability. The total number of proteins identified from each fraction is listed with the fraction name (e.g., 744 proteins were identified in the extracellular protein fraction from gel-based plus gel-free proteomic analysis of *B. vietnamiensis*).

The functionality of the proteins identified from each cellular fraction will be analyzed in the following sections according to the overlap indicated in the Venn diagram.

3.1.1. Proteins Common to All Four Cellular Fractions. There were 245 proteins common to all four cellular fractions of *B. vietnamiensis*. The functionality of these common proteins was determined by using the DBGET search function at the Kyoto University Bioinformatics Center's genome.jp website (http://www.genome.jp/dbget/). Figure 2 shows the identified protein categories of the proteins common to all four fractions. Most of them are housekeeping proteins such as ribosomal proteins, metabolic enzymes, and chaperones. Several hypothetical and uncharacterized proteins were also found in all four cellular fractions indicating the incompleteness of the gene annotation of *B. vietnamiensis* and the BCC in general. Proteins were extracted at the late exponential/early stationary phase of this bacterium. It is known that bacterial virulence factor production is controlled by quorum sensing which occurs when the cells reach a high density at a late exponential phase [32]. Extracting proteins at this stage helps us to identify more proteins associated with pathogenicity as well as proteins associated with metabolism which is clearly indicated in the data presented in the supplemental material.

Also in the early stationary phase, bacteria sense nutrient limitation and release stress related proteins. Three stress-related proteins termed universal stress-related proteins (e.g, OspA domain protein) were found common to all four cellular fractions of this bacterium. The exact functions of these proteins are not known but related studies with *E. coli* showed that they may assist cell division under nutrient-limiting conditions [33].

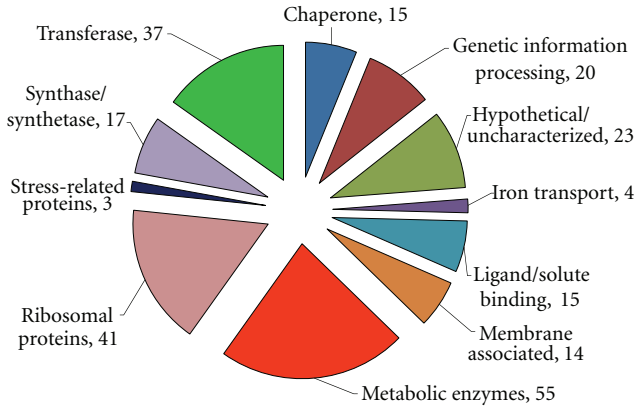


FIGURE 2: Functional categories of proteins identified common to all four cellular fractions of *B. vietnamiensis* G4. Number of proteins belonging to each category is listed along with the protein category.

One important protein found to be common to all four fractions is the protein ecotin. This is a serine protease inhibitor that can be found in many bacterial species. Ecotin binds the active site of a protease to inhibit its activity and helps bacteria survive phagocytosis within a host cell. The ecotin protein has been isolated only in *Burkholderia* species pathogenic to mammalian cells such as *B. pseudomallei and B. vietnamiensis*, but not the plant pathogen *B. fungoforum* [34, 35].

3.1.2. Protein Common to Only Three Cellular Fractions of B. vietnamiensis. There are four possible combinations in which the same identified proteins will be common to only three cellular fractions; the four possible combinations of three fractions are intracellular/extracellular/cell wall, intracellular/extracellular/surface, intracellular/surface/cell wall, and extracellular/surface/cell wall fractions. Figure 1 shows that 26 proteins were found to be common to the fractions intra/extra/cell wall, 17 proteins were found to be common to the intra/surface/cell wall fraction, 22 were found in intra/extra/surface fractions and most of these common proteins were associated with the transport system of the cell. These transport systems link the outside environment with the cell interior by spanning the cell wall. When the protein fractionation is carried out, these transport proteins can be distributed in all the fractions. There can be fraction carry over when the separation is carried out to give common proteins in the fractions. Among the proteins found to be common in these fractions is the LemA (lesion manifestation A) family of proteins which is involved in the two component secretory system of the bacteria. This is a membrane protein which has a signal sequence, usually 20–30 amino acids long and interacts with a signal recognition particle. Most bacteria have specific sequences for these signaling molecules, and in this proteomic study we found the LemA family protein in cell wall/extra/surface fractions. The sequence of the protein was BLAST searched through the nonredundant protein database using the NCBI website and it was found that the protein sequence is specific to *Burkholderia* species. The peptide containing the first 17 amino acid residues at the N-terminus is specific to *B. vietnamiensis* G4 strain. Figure 3 shows the spectrum of the doubly charged peptide DLQAQLEGTENR which belongs to the LemA protein. A series of y ions and corresponding b ions can be observed in the spectrum. The strong H_2O loss peak is consistent with the presence of a hydroxyl side chain (threonine) in the peptide. In addition to this peptide, five more unique peptides of LemA were identified in the extracellular fraction of *B. vietnamiensis* G4, giving 35% sequence coverage of the protein. The N terminus of this LemA is predicated to orient outside the bacterium and finding this in all extracellular/surface/cell wall fraction supports this prediction [36]. These LemA proteins associate with GacA (global activator A) to form the two-component sensory system that regulates the production of N-acyl homoserine lactones in bacteria. The N-acyl homoserine lactones (AHLs) are associated with bacterial cell-to-cell recognition generally known as quorum sensing [37]. Also AHLs are involved in biofilm formation on abiotic surfaces [38]. Several studies have shown that AHL-dependent quorum-sensing supports interspecies communication among the bacteria [39]. This has been observed in cystic fibrosis patients where both *Burkholderia* and *Pseudomonas* can colonize in the lungs of the patients and the production of extracellular proteases and siderophores from *Burkholderia* is stimulated by the presence of *Pseudomonas* in the biofilm [40, 41]. A general structure of AHL is indicated in Scheme 1.

The structure of these AHL differ in the length of their N-linked side chain, the nature of the substitution at the 3-carbon position and the presence or absence of one or more unsaturated bonds within the side chain [42]. All species that belong to the BCC can produce C6-AHL and C8-AHL molecules and in addition *B. vietnamiensis* can produce C10-AHLs [6]. Direct involvement of AHL molecules in the pathogenicity of the bacterium is not known; however, it has been reported that the presence of these signaling molecule is important to obtain full virulence of the bacteria. The presence of the proteins that regulate the production of these quorum-sensing molecules in the cell surface, cell wall, and extracellular protein fractions suggest that bacteria are attempting to regulate these signaling molecules to control the production of some of the extracellular proteases [38].

3.1.3. Proteins Common to Two Cellular Fractions. Most proteins found only in intracellular and extracellular, and intracellular and cell surface fractions belong to the transport proteins such as DNA-binding proteins and membrane-associated proteins like efflux pumps. These proteins can span the membrane and during the protein extraction parts of the protein can distribute in the two cellular fractions. Forty-one proteins were found to be common in both cell wall and intracellular fractions. When the cellular fractionation was carried out, sonication was applied in order to break open the bacterial cells. After centrifugation, the remaining pellet was used as the cell wall fraction. There can be many intracellular proteins associated with the cell debris, and those will appear in both intracellular and cell wall fractions.

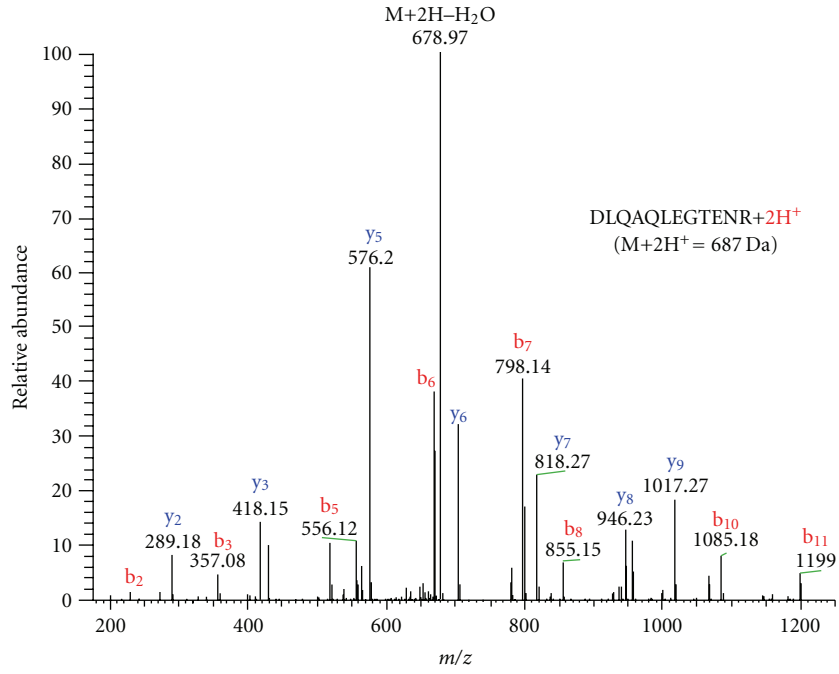
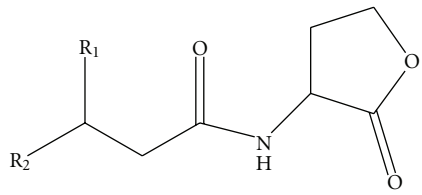


Figure 3: Fragmentation pattern of the doubly charged peptide DLQAQLEGTENR (residues 135 to146) from the protein LemA. This peptide covers 5.6% of the protein sequence and 5 additional unique peptides of this protein were found in the extra cellular fraction of *B. vietnamiensis* G4.



R_1 = H, = O, = OH

$R_2 = C_nH_{2n+1}, C_nH_{2n}$

Scheme 1: General structure of bacterial N-acyl-homoserine lactones.

Most of the proteins found to be common to intracellular and cell wall fractions are related to metabolism and few are found to associate with the membrane. *B. vietnamiensis* G4 is well known for its ability to metabolize toxic organic contaminants such as aromatic hydrocarbons.[43] Carboxymethylenebutenolidase, an enzyme responsible for biodegradation of fluorobenzoate and benzoate, was found to be common in both intracellular and cell wall protein fractions, with three unique peptide identifications that cover 28% of the protein sequence. This finding is consistent with the ability of *B. vietnamiensis* to degrade toxic molecules such as aromatic hydrocarbons.

The ability of a motile bacterium to swim toward or away from specific environmental stimuli, such as nutrients, oxygen, or light provides cells with a survival advantage, especially under nutrient-limiting conditions. This behavior is called chemotaxis and is mediated by changing the direction of the bacteria by briefly reversing the direction of rotation of the flagellar motors [44]. Chemotaxis sensory transducers are membrane proteins produced by bacteria as a sensory molecule in order to capture certain molecules. Methyl-accepting chemotaxis sensory transducer protein was found in both the cell wall and extracellular fractions. These sensory molecules are important for bacterial survival and play a role in the pathogenicity of most bacteria, especially those that are susceptible to antibiotics. Several stress-related proteins were also found in these cellular fractions indicating the nutrient limitation of the growth medium. Many hypothetical proteins and proteins with unknown functionality were also found in all cellular fractions.

3.1.4. Proteins Found in Only One Cellular Fraction. From the proteomic analysis of *B. vietnamiensis* using both gel-based and gel-free methods, many proteins were identified as specific to only one cellular fraction. Figure 4 shows the protein categories identified, specific to each cellular fraction. Many anabolic and catabolic enzymes were present in all four cellular fractions indicating the increased cellular metabolism at the late exponential phase. Proteins related to genetic information processing such as nucleotide transcription and translation were also found in high abundance. This illustrates the rapid cell multiplication with this culture medium and is represented also in the presence of several proteins involved in cell division in these cellular fractions.

Membrane or membrane-associated proteins are important in the pathogenicity and for the survival of the bacterium in the environment. Many membrane-associated proteins were identified that are specific to one of the four cellular fractions. There were ion transport/binding proteins such as ABC transporters, membrane bound porins, lipoproteins,

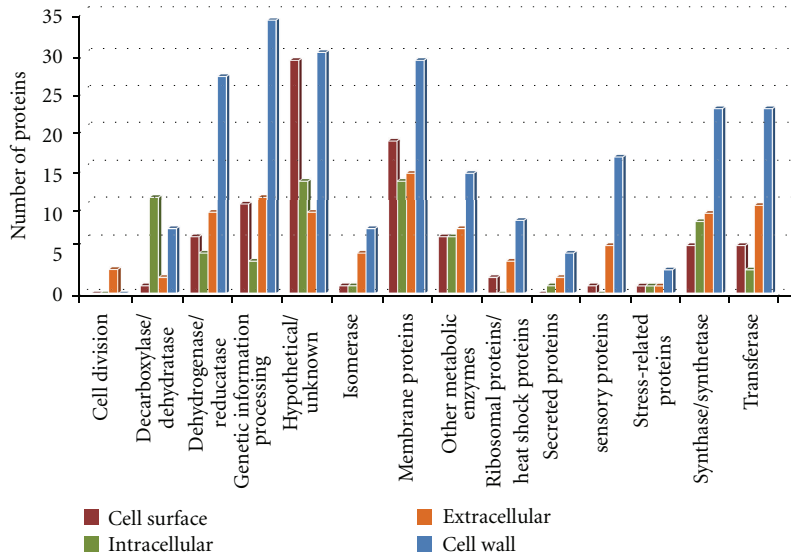


Figure 4: Distribution of proteins belonging to different functional classes found specific to only one cellular fraction of *B. vietnamiensis* G4.

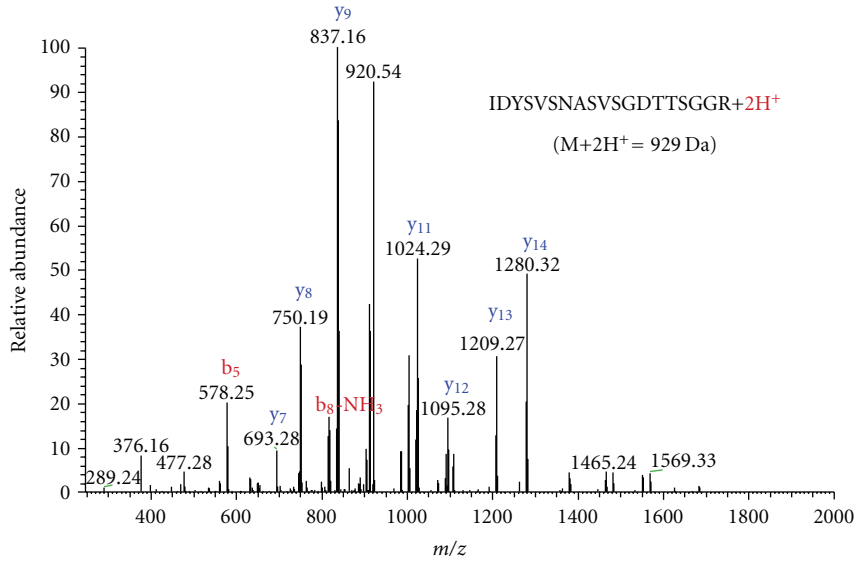


Figure 5: Fragmentation pattern of doubly charged peptide IDYSVANASVSGDTTSGGR (residues 51 to 69) from the protein arylesterase of *B. vietnamiensis* G4.

and peptidoglycan-associated proteins, sensory proteins, and some electron transport proteins. As mentioned earlier, *B. vietnamiensis* is known to degrade various organic contaminants [43, 45]. Bisphenol A is an emerging environmental contaminant, especially as a water pollutant known as an endocrine disruption chemical [46, 47]. In this proteomic analysis we found arylesterase which has the functionality to degrade bisphenol A. This protein was found specific to the cell surface fraction of *B. vietnamiensis*. Arylesterase was identified with 5 unique peptides which cover 28% of the protein sequence. Figure 5 shows the fragmentation pattern of the peptide IDYSVANASVSGDTTSGGR from the protein arylesterase. This is a doubly protonated peptide with a 2.78 Xcorr score and E value of 4.27. A predominant y ion series can be found in the spectrum. The protein sequence was BLAST searched to check for homology, and it was found that the sequence is species specific. The closest protein homologue to the *B. vietnamiensis* G4 arylesterase was found to be lysophospholipase A from *B. cenocepacia*, which has 88% sequence similarity with this protein sequence. This arylesterase can be used to distinguish *B. vietnamiensis* G4 from other *Burkholderia* species in a mixed culture system. The presence of these proteins in a cell culture illustrates the ability of *B. vietnamiensis* to degrade environmental pollutants.

Different types of stress-related proteins were found specific to all four fractions indicating the nutrient limited status of the culture medium. The cell wall fraction contained

three stress-related proteins including water stress and hypersensitive response protein. These proteins are present in most plant pathogenic bacteria and are produced when the plant defense mechanism acts against the invading bacteria. Another important class of proteins found specific to the extracellular fraction is the signaling molecules. Some are signal peptidases and some are antigens that induce the immune response in the host cells. All these categories of proteins illustrate that important cellular mechanisms of *B. vietnamiensis* are active and detectable by using mass spectrometry-based proteomic studies.

3.2. Virulence Factors Identified from the Protein Fractionation. Most of previous proteomics studies of virulence factors were carried out by expressing proteins of interest in *E. coli* and analyzing them individually. In the present study we show that mass spectrometry-based proteomic studies can be used to directly detect at least some of these low abundance virulence factors present in the pathogenic bacteria species [48–52]. Many of these identified virulence factors were found in cell surface, cell wall, or extracellular fractions of bacteria. Some of the virulence factors identified by this analysis include outer membrane lipoprotein [4], flagellin which is responsible for motility, and outer membrane porins that are responsible for many antibiotic resistance properties of bacteria [4]. Among the other virulence factors identified, phasin helps granule formation under nutrient-limiting conditions [53], superoxide dismutase, and catalase are reactive against reactive oxygen species, and adhesin helps to attach to the host [4, 54]. Several secretion proteins were identified including HlyD which is responsible for secretion and transporting hemolysin, which is embedded in the Gram-negative inner cell membrane [55]. In the present study we show that mass spectrometry-based proteomic studies can be used to detect these low abundance virulence factors present in the pathogenic bacteria species [48–52].

4. Summary and Conclusions

Proteomic studies were carried out using the opportunistic pathogen *B. vietnamiensis* G4 strain. Proteins were extracted from four different cellular fractions: extracellular, intracellular, cell wall, and cell surface proteins. Different precipitation methods were used to separate the proteins into these cellular fractions. As compared to the whole proteome analysis, fractionating the proteome into different segments allows in depth analyses and if interested, one fraction at a time can be analyzed to study a specific protein. A bottom-up proteomics approach was used to identify the proteins found in the four cellular fractions. Both gel-based LC-MS/MS and gel-free MudPIT (LC/LC-MS/MS) methods were used to analyze proteins from *B. vietnamiensis.* A total of 803 proteins were identified using the gel-based method, and a total of 775 proteins were identified using the gel-free MudPIT method.

By combining both gel-based and gel-free proteomic analysis, more than 1200 unique proteins were identified from *B. vietnamiensis* G4. This is the highest number of protein identifications reported on this genus. Previous reports on *B. cenocepacia*, which is a close relative of *B. vietnamiensis* G4, identified 390 proteins by 2D gel separated protein analysis using MALDI-TOF/TOF [10]. This indicates that the combination of both gel-based and gel-free methods provides a more comprehensive coverage of the proteome compared to either individual method. The existence of this type of knowledge on the proteins expressed at a specific stage of the bacterial life cycle will help future research work based on this *Burkholderia* species. Many proteins identified were related to specific functionality of *B. vietnamiensis*, for example, biodegrading proteins. Also, many known virulence factors were identified from this analysis, which indicates the applicability of mass spectrometry-based proteomic studies to identify possible pathogenic strains.

Acknowledgments

The authors would like to acknowledge University of Arizona proteomics facility, especially Fatimah Hickman for helping with this research. Funding for this research was provided by NIH NIAID Grant no. U54-AI065359 and in part by Grant CHE 071-4245 from the NSF collaborative research in chemistry program.

References

[1] T. Coenye and P. Vandamme, "Diversity and significance of *Burkholderia* species occupying diverse ecological niches," *Environmental Microbiology*, vol. 5, no. 9, pp. 719–729, 2003.

[2] P. Aldhous, "Melioidosis? Never heard of it," *Nature*, vol. 434, no. 7034, pp. 692–693, 2005.

[3] K. Vermis, P. A. R. Vandamme, and H. J. Nelis, "*Burkholderia cepacia* complex genomovars: utilization of carbon sources, susceptibility to antimicrobial agents and growth on selective media," *Journal of Applied Microbiology*, vol. 95, no. 6, pp. 1191–1199, 2003.

[4] J. W. Nelson, S. L. Butler, D. Krieg, and J. R. W. Govan, "Virulence factors of *Burkholderia cepacia*," *FEMS Immunology and Medical Microbiology*, vol. 8, no. 2, pp. 89–98, 1994.

[5] K. Riedel, M. Hentzer, O. Geisenberger et al., "N-acylhomoserine-lactone-mediated communication between Pseudomonas aeruginosa and *Burkholderia cepacia* in mixed biofilms," *Microbiology*, vol. 147, no. 12, pp. 3249–3262, 2001.

[6] V. Venturi, A. Friscina, I. Bertani, G. Devescovi, and C. Aguilar, "Quorum sensing in the *Burkholderia cepacia* complex," *Research in Microbiology*, vol. 155, no. 4, pp. 238–244, 2004.

[7] M. J. Knight, R. Gill, A. Ruaux et al., "Crystallization and preliminary X-ray diffraction analysis of BipD, a virulence factor from *Burkholderia pseudomallei*," *Acta Crystallographica Section F*, vol. 62, no. 8, pp. 761–764, 2006.

[8] X. Gu, M. Chen, Q. Wang, M. Zhang, B. Wang, and H. Wang, "Expression and purification of a functionally active recombinant GDP-mannosyltransferase (PimA) from Mycobacterium tuberculosis H37Rv," *Protein Expression and Purification*, vol. 42, no. 1, pp. 47–53, 2005.

[9] Y. W. Han, M. A. Uhl, S. J. Han, and W. Shi, "Expression of bvgAS of *Bordetella pertussis* represses flagellar biosynthesis of Escherichia coli," *Archives of Microbiology*, vol. 171, no. 2, pp. 127–130, 1999.

[10] K. Riedel, P. Carranza, P. Gehrig, F. Potthast, and L. Eberl, "Towards the proteome of *Burkholderia cenocepacia* H111: setting up a 2-DE reference map," *Proteomics*, vol. 6, no. 1, pp. 207–216, 2006.

[11] J. E. Gee, C. T. Sacchi, M. B. Glass et al., "Use of 16S rRNA gene sequencing for rapid identification and differentiation of *Burkholderia pseudomallei* and B. mallei," *Journal of Clinical Microbiology*, vol. 41, no. 10, pp. 4647–4654, 2003.

[12] A. A. Bodour, K. P. Drees, and R. M. Maier, "Distribution of biosurfactant-producing bacteria in undisturbed and contaminated arid southwestern soils," *Applied and Environmental Microbiology*, vol. 69, no. 6, pp. 3280–3287, 2003.

[13] G. M. Oosta, N. S. Mathewson, and G. N. Catravas, "Optimization of folin-ciocalteu reagent concentration in an automated lowry protein assay," *Analytical Biochemistry*, vol. 89, no. 1, pp. 31–34, 1978.

[14] C. V. Sapan, R. L. Lundblad, and N. C. Price, "Colorimetric protein assay techniques," *Biotechnology and Applied Biochemistry*, vol. 29, no. 2, pp. 99–108, 1999.

[15] K. Riedel, C. Arevalo-Ferro, G. Reil, A. Görg, F. Lottspeich, and L. Eberl, "Analysis of the quorum-sensing regulon of the opportunistic pathogen *Burkholderia cepacia* H111 by proteomics," *Electrophoresis*, vol. 24, no. 4, pp. 740–750, 2003.

[16] A. Shevchenko, M. Wilm, O. Vorm, and M. Mann, "Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels," *Analytical Chemistry*, vol. 68, no. 5, pp. 850–858, 1996.

[17] F. Gharahdaghi, C. R. Weinberg, D. A. Meagher, B. S. Imai, and S. M. Mische, "Mass spectrometric identification of proteins from silver-stained polyacrylamide gel: a method for the removal of silver ions to enhance sensitivity," *Electrophoresis*, vol. 20, no. 3, pp. 601–605, 1999.

[18] L. Breci, E. Hattrup, M. Keeler, J. Letarte, R. Johnson, and P. A. Haynes, "Comprehensive proteomics in yeast using chromatographic fractionation, gas phase fractionation, protein gel electrophoresis, and isoelectric focusing," *Proteomics*, vol. 5, no. 8, pp. 2018–2028, 2005.

[19] N. L. Andon, S. Hollingworth, A. Koller, A. J. Greenland, J. R. Yates, and P. A. Haynes, "Proteomic characterization of wheat amyloplasts using identification of proteins by tandem mass spectrometry," *Proteomics*, vol. 2, no. 9, pp. 1156–1168, 2002.

[20] D. A. Wolters, M. P. Washburn, and J. R. Yates, "An automated multidimensional protein identification technology for shotgun proteomics," *Analytical Chemistry*, vol. 73, no. 23, pp. 5683–5690, 2001.

[21] J. K. Eng, A. L. McCormack, and J. R. Yates, "An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database," *Journal of the American Society for Mass Spectrometry*, vol. 5, no. 11, pp. 976–989, 1994.

[22] J. R. Yates, J. K. Eng, A. L. McCormack, and D. Schieltz, "Method to correlate tandem mass spectra of modified peptides to amino acid sequences in the protein database," *Analytical Chemistry*, vol. 67, no. 8, pp. 1426–1436, 1995.

[23] K. Xiao, D. B. McClatchy, A. K. Shukla et al., "Functional specialization of β-arrestin interactions revealed by proteomic analysis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 29, pp. 12011–12016, 2007.

[24] H. Lim, J. Eng, J. R. Yates et al., "Identification of 2D-gel proteins: a comparison of MALDI/TOF peptide mass mapping to μ LC-ESI tandem mass spectrometry," *Journal of the American Society for Mass Spectrometry*, vol. 14, no. 9, pp. 957–970, 2003.

[25] D. L. Tabb, W. H. McDonald, and J. R. Yates, "DTASelect and contrast: tools for assembling and comparing protein identifications from shotgun proteomics," *Journal of Proteome Research*, vol. 1, no. 1, pp. 21–26, 2002.

[26] R. Craig and R. C. Beavis, "TANDEM: matching proteins with tandem mass spectra," *Bioinformatics*, vol. 20, no. 9, pp. 1466–1467, 2004.

[27] R. Craig and R. C. Beavis, "A method for reducing the time required to match protein sequences with tandem mass spectra," *Rapid Communications in Mass Spectrometry*, vol. 17, no. 20, pp. 2310–2316, 2003.

[28] P. A. Haynes, S. Miller, T. Radabaugh et al., "The wildcat toolbox: a set of perl script utilities for use in peptide mass spectral database searching and proteomics experiments," *Journal of Biomolecular Techniques*, vol. 17, no. 2, pp. 97–102, 2006.

[29] A. Keller, A. I. Nesvizhskii, E. Kolker, and R. Aebersold, "Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search," *Analytical Chemistry*, vol. 74, no. 20, pp. 5383–5392, 2002.

[30] A. I. Nesvizhskii, A. Keller, E. Kolker, and R. Aebersold, "A statistical model for identifying proteins by tandem mass spectrometry," *Analytical Chemistry*, vol. 75, no. 17, pp. 4646–4658, 2003.

[31] S. De La Fuente Van Bentem, D. Anrather, I. Dohnal et al., "Site-specific phosphorylation profiling of arabidopsis proteins by mass spectrometry and peptide chip analysis," *Journal of Proteome Research*, vol. 7, no. 6, pp. 2458–2470, 2008.

[32] L. Eberl and K. Riedel, "Mining quorum sensing regulated proteins—role of bacterial cell-to-cell communication in global gene regulation as assessed by proteomics," *Proteomics*, vol. 11, no. 15, pp. 3070–3085, 2011.

[33] D. Kerk, J. Bulgrien, D. W. Smith, and M. Gribskov, "Arabidopsis proteins containing similarity to the universal stress protein domain of bacteria," *Plant Physiology*, vol. 131, no. 3, pp. 1209–1219, 2003.

[34] C. T. Eggers, I. A. Murray, V. A. Delmar, A. G. Day, and C. S. Craik, "The periplasmic serine protease inhibitor ecotin protects bacteria against neutrophil elastase," *Biochemical Journal*, vol. 379, no. 1, pp. 107–118, 2004.

[35] M. G. Fain and J. D. Haddock, "Phenotypic and phylogenetic characterization of Burkholderia (Pseudomonas) sp. strain LB400," *Current Microbiology*, vol. 42, no. 4, pp. 269–275, 2001.

[36] S. E. F. D'Orazio, M. Velasquez, N. R. Roan, O. Naveiras-Torres, and M. N. Starnbach, "The listeria monocytogenes lemA gene product is not required for intracellular infection or to activate fMIGWII-specific T cells," *Infection and Immunity*, vol. 71, no. 12, pp. 6721–6727, 2003.

[37] M. Frommberger, P. Schmitt-Kopplin, F. Menzinger et al., "Analysis of *N*-acyl-L-homoserine lactones produced by *Burkholderia cepacia* with partial filling micellar electrokinetic chromatography—electrospray ionization-ion trap mass spectrometry," *Electrophoresis*, vol. 24, no. 17, pp. 3067–3074, 2003.

[38] B. Huber, K. Riedel, M. Hentzer et al., "The cep quorum-sensing system of *Burkholderia cepacia* H111 controls biofilm formation and swarming motility," *Microbiology*, vol. 147, no. 9, pp. 2517–2528, 2001.

[39] T. A. Gould, J. Herman, J. Krank, R. C. Murphy, and M. E. A. Churchill, "Specificity of acyl-homoserine lactone synthases examined by mass spectrometry," *Journal of Bacteriology*, vol. 188, no. 2, pp. 773–783, 2006.

[40] D. McKenney, K. E. Brown, and D. G. Allison, "Influence of Pseudomonas aeruginosa exoproducts on virulence factor

production in *Burkholderia cepacia*: evidence of interspecies communication," *Journal of Bacteriology*, vol. 177, no. 23, pp. 6989–6992, 1995.

[41] L. Eberl, "Quorum sensing in the genus *Burkholderia*," *International Journal of Medical Microbiology*, vol. 296, no. 2-3, pp. 103–110, 2006.

[42] A. Gotschlich, B. Huber, O. Geisenberger et al., "Synthesis of multiple N-Acylhomoserine lactones is wide-spread among the members of the *Burkholderia cepacia* complex," *Systematic and Applied Microbiology*, vol. 24, no. 1, pp. 1–14, 2001.

[43] J. L. Parke and D. Gurian-Sherman, "Diversity of the *Burkholderia cepacia* complex and implications for risk assessment of biological control strains," *Annual Review of Phytopathology*, vol. 39, pp. 225–258, 2001.

[44] M. A. Boin, M. J. Austin, and C. C. Häse, "Chemotaxis in Vibrio cholerae," *FEMS Microbiology Letters*, vol. 239, no. 1, pp. 1–8, 2004.

[45] M. L. Krumme, K. N. Timmis, and D. F. Dwyer, "Degradation of trichloroethylene by *Pseudomonas-cepacia* G4 and the constitutive mutant strain G4 5223 PR1 in aquifer microcosms," *Applied and Environmental Microbiology*, vol. 59, no. 8, pp. 2746–2749, 1993.

[46] J. H. Kang, D. Aasi, and Y. Katayama, "Bisphenol A in the aquatic environment and its endocrine-disruptive effects on aquatic organisms," *Critical Reviews in Toxicology*, vol. 37, no. 7, pp. 607–625, 2007.

[47] B. Ning, N. Graham, Y. Zhang, M. Nakonechny, and M. G. El-Din, "Degradation of endocrine disrupting chemicals by ozone/AOPs," *Ozone*, vol. 29, no. 3, pp. 153–176, 2007.

[48] Y. Liu, Q. Wei, G. L. Bao, and Q. A. Ji, "Differential proteomics of outer membrane proteins between virulent and avirulent strains of Riemerella anatipestifer," *Progress in Biochemistry and Biophysics*, vol. 35, no. 6, pp. 691–694, 2008.

[49] H. J. Wu, A. H. J. Wang, and M. P. Jennings, "Discovery of virulence factors of pathogenic bacteria," *Current Opinion in Chemical Biology*, vol. 12, no. 1, pp. 93–101, 2008.

[50] I. Pavkova, M. Reichelova, P. Larsson et al., "Comparative proteome analysis of fractions enriched for membrane-associated proteins from *Francisella tularensis* subsp. tularensis and F. tularensis subsp. holarctica strains," *Journal of Proteome Research*, vol. 5, no. 11, pp. 3125–3134, 2006.

[51] R. L. J. Graham, C. E. Pollock, S. N. O'Loughlin et al., "Multidimensional proteomic analysis of the soluble subproteome of the emerging nosocomial pathogen *Ochrobactrum anthropi*," *Journal of Proteome Research*, vol. 5, no. 11, pp. 3145–3153, 2006.

[52] S. Rupp, "Proteomics on its way to study host-pathogen interaction in *Candida albicans*," *Current Opinion in Microbiology*, vol. 7, no. 4, pp. 330–335, 2004.

[53] G. M. York, J. Stubbe, and A. J. Sinskey, "New insight into the role of the PhaP phasin of *Ralstonia eutropha* in promoting synthesis of polyhydroxybutyrate," *Journal of Bacteriology*, vol. 183, no. 7, pp. 2394–2397, 2001.

[54] E. Mahenthiralingam, T. A. Urban, and J. B. Goldberg, "The multifarious, multireplicon *Burkholderia cepacia* complex," *Nature Reviews Microbiology*, vol. 3, no. 2, pp. 144–156, 2005.

[55] R. Schulein, I. Gentschev, S. Schlor, R. Gross, and W. Goebel, "Identification and characterization of two functional domains of the hemolysin translocator protein hlyD," *Molecular and General Genetics*, vol. 245, no. 2, pp. 203–211, 1994.